Molecular and Epigenetic Insights into RASSF1A Regulated Pathways in Inflammatory Bowel Disease

by

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Abstract

Inflammatory Bowel Disease (IBD) is an idiopathic non-curable disease characterized by pain, chronic diarrhea, rectal bleeding with weight loss and erosion of the colon in severe cases. In many cases, IBD is a chronic relapsing inflammatory disorder of the gastrointestinal tract that usually onsets in late childhood or early adulthood leaving the patient with decades of chronic inflammation to deal with. IBD has two main sub-types, ulcerative colitis (UC) and Cohn's disease (CD). Additionally, it is a common inflammatory disorder that is associated with a greater risk of CRC [9]. The unpredictable nature of IBD exerts a burden on both the patients and governments with hospitalization, surgery, poor economic productivity and less social involvement.

The advancement in diagnostic tools as well as the emergence of the concept of personalized medicine rationalizes the importance of understanding the disease in a more specific fashion, at the level of the individual. Our current understanding of IBD outlines genetic factors including > 200 genes linked to IBD [35]. This is in synergy with external environmental variables as well as internal microenvironment conditions, mainly the microbiome of the gut. In this thesis, several genes were explored as potential drivers of disease pathogenesis of IBD that may influence different aspects of the pathogenesis of IBD. Our first molecular focus was on the tumor suppressor gene, Ras association domain family 1A (*RASSF1A*). This gene has been reported to be epigenetically silenced by promoter specific hypermethylation in IBD and in IBD related colorectal cancer

(CRC). Prior to the start of my thesis project, our group demonstrated that the loss of *RASSF1A* resulted in excessive intestinal inflammation and injury. RASSF1A was found to restrict signaling of the pathogen recognition receptors (PRRs) following receptor engagement and subsequent restriction of the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a key molecular driver of inflammation. *Rassf1a*$^{-/-}$ mice were highly susceptible to chemical induction of colitis in a murine model to suggest that RASSF1A controlled pathways are dysregulated in IBD and significantly modulate disease pathogenesis. Our research group also demonstrated that RASSF1A physically interacts with the Toll-like receptor (TLR) family of innate immunity PPRs and with the nucleotide-containing oligomerization domain protein (NOD2) family of intracellular PRRs. The modulation of NOD2 results in the failure to activate its obligate kinase, receptor interacting protein kinase 2 (RIPK2), the second molecular focus of my thesis. We thus hypothesized that epigenetic loss of *RASSF1A* would result in hyperactivation of RIPK2 to drive NFkB-directed inflammation. Connected to both epigenetic modifications and elevated inflammation in IBD patients and in our mouse models of IBD, is a metabolic disorder syndrome that results in modulation of AMP-activated kinase (AMPK), the third molecular focus of this thesis. Acute inflammation in IBD patients resulted in the loss of AMPK activity and metabolic disorder. Thus to effectively treat IBD, we need to prevent epigenetic loss of *RASSF1A* (or regain expression of RASSF1A), restore the activation levels of AMPK and inhibit RIPK2 to prevent further inflammatory damage.

The objectives of this thesis were:

(1) Exploring *RASSF1A* promoter methylation in IBD;

(2) Exploring therapeutics to targeting NFκB dysregulated pathways including the abnormal NOD2/RIPK2 pathway;

(3) Identification of molecular biomarkers for both diagnostic and prognostic purposes in IBD.

We believe that our observations will aid in better understanding the key players that control inflammation in IBD and possibly progress in IBD related CRC. The identification of these novel biomarkers will allow rational design of specific or multi-specific therapeutics to reduce inflammation and better manage IBD.

Preface

This thesis is the original work by Mohamed Salla. Animal studies compiled with the Canadian Council of Animal Care as approved by the University of Alberta Welfare Committee (Animal User Protocols 218 and 219). Human specimens were obtained with the approval of the University of Alberta Health Research Ethics Board ("The role of RASSF1A in Inflammatory Bowel Disease", ID Pro00001523). Work involving radioactive material was solely done by my supervisor.

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† **Equal Contribution :** Mohamed Salla and Natalia Volodko contributed equally to the design and performing of the real time PCR and methylation experiments, data analysis as well as manuscript editing. El-Arbi Abulghasem contributed to DNA/RNA extraction. Krista Vincent and Lynne Postovit obtained the TCGA data. Matthew G.K. Benesch, Todd P.W. McMullen and Oliver F. Bathe contributed as providing tissues for the study. Shairaz Baksh coordinated the study, drafted and edited the manuscript.

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Dedication

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Table of Contents

List of Tables **Page**

List of Figures

List of Abbreviations

ABL	c-Abl oncogene 1, non-receptor tyrosine kinase
ADA	Adalimumab
AMPK	AMP-activated protein Kinase
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
ASA	Amino-salicylic acid
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X protein
BGS	Bovine growth serum
BH3	Bcl-2-homlogy domain 3
bp	Base pair
BSA	Bovine serum albumin
CAC	Colitis Associated Cancer (IBD-related Cancer)
CD	Crohn's disease
cdc	Cell division control protein
CRC	Colorectal cancer
CSF-1	Colony Stimulating Factor 1
CXCL	Chemokine (C-X-C motif) ligand
DAB	3,3'-Diaminobenzidine
DAI	Disease activity index
DAP3	Death associated protein 3
DAPK	Death-associated protein kinase

DAPI	4'-6-Diamidino-2-phenylindole
DAXX	Death-associated protein 6
DEDD	Death effector domain-containing protein
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferases
dNTP	Deoxynucleotide triphosphate - (dATP,dCTP,dGTP,dTTP)
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbance assay
EMT	Epithelial Mesenchymal Transition
ER	Endoplasmic reticulum
GSK3	Glycogen synthase kinase
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
GWAS	Genome wide association study
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IAPs	Inhibitors of apoptosis
IB	Immunoblotting (Western blotting)
IBD	Inflammatory bowel diseases
IEC	Intestinal epithelial cell

IFX	Infliximab
IHC	Immunohistochemistry
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, inhibitor of KB
IKK kinase	Inhibitor of kappa light polypeptide gene enhancer in B-cells, epsilon, IκB kinase
IL	Interleukin
IP	Immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
IRGM	Immunity related GTPase family M protein
kDa	Kilodalton
KO	Knockout
LATS	Large tumor suppressor kinase
LKB1	Liver Kinase B1
LPS	Lipopolysaccharide
MAP	Mitogen activated kinase
MAPKKK	MAP kinase kinase kinase
MCP1	Monocyte chemoattractant protein 1
MDP	Muramyl Dipeptide
MDM2	Mouse double minute 2 homolog, E3 ubiquitin-protein ligase
MOAP-1	Modulator of apoptosis 1
mRNA	Messenger RNA
miRNA	Micro RNA
MST1/2	mammalian sterile 20-like kinase 1/2
MYC	v-Myc avian myelocytomatosis viral oncogene homolog
MyD88	Myeloid differentiation primary response gene
NaPP	Sodium pyrophasphate (Na4O7P2)

NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NOD	Nucleotide-binding Oligomerization Domain Receptors
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferati ng cell nuclear antigen
PCR	Polymerase chain reaction
PKB	Protein kinase B
PKC	Protein kinase C
PI3K	Phosphatidylinositol-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pathogen Recognition Receptor
PTK	Protein tyrosine kinase
PVDF	Phenylmethylsulfonyl fluoride
RA	Ras association domain
Raf	v-Raf-1 murine leukemia viral oncogene homolog
Ras	Rat sarcoma
RASSF1A	Ras association domain family member 1 isoform A
RIPK2	Receptor Interacting Protein Kinase 2
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNI	Reactive Nitrogen Intermediates
ROS	Reactive oxidative species
RPM	Revolutions per minute
RUNX	Runt-related transcription factor
SARAH	Salvador/RASSF/Hippo domain
SDS	Sodium dodecyle sulphate
SNP	Single-nucleotide polymorphism

STAT	Signal Transducer and Activator of Transcription
TAE	Tris-acetate-EDTA
TAK	Transforming growth factor beta-activated kinase
TAM	Tumor Associated Macrophages
Taq	Thermus aquaticus DNA polymerase
TBS	Tris buffered saline
TBX5	T-box transcription factor TBX5
TEAD	TEA domain family member
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TNFR1	TNF-α receptor 1
TP53	Tumor protein P53 (p53)
TP73	Tumor protein P73 (p73)
TRAIL	TNF-related apoptosis-inducing ligand
TRAF	TNF receptor associated factor
UC	Ulcerative colitis
UV	Ultraviolet
qPCR	Real time PCR
WNT	Wingless-type MMTV integration site family, member
WT	Wild type
YAP	YES associated protein
YES	v-Yes-1 Yamaguchi sarcoma viral oncogene homolog

CHAPTER ONE

Introduction

1.1 Inflammatory Bowel Disease

1.1.1 Types, symptoms and epidemiology

Inflammation is a complex biological process that constitutes an important defensive mechanism against biological and chemical insults. If left uncontrolled, chronic inflammation is a root cause of several cancer types. Almost one third of cancers are preceded by chronic inflammation [2]. Several transcription factors appear to be hyperactivated in inflammatory diseases and may provide important links between inflammation and cancers. For instance, NFκB is hyperactivated in inflammation and its transcriptional activity leads to the upregulation of surface receptors that results in a surge of pro-inflammatory cytokines [3]. Among the many signalling pathways that are controlled by NFκB, innate and adaptive immunity responses are tightly regulated by NFκB [4]. Dysregulated and constitutively activated NFκB has been implicated in many cancers and in inflammatory diseases [5]. The activation of NFκB can be induced by pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) or the nucleotide-binding oligomerization domain receptors (NODs) [5, 6]. This induction emphasizes a central role for NFκB in regulating the innate immune response. The classical pathway for activating NFκB involves activation of TLRs, interleukin 1 receptor (IL-1R) or tumor necrosis factor receptor 1 (TNFR1) that will recruit the adapter protein myeloid differentiation response gene 88 (MyD88) [7]. This

process results in the activation of the IκB kinase (IKK), phosphorylation of IκBα and the release of NFκB into the nucleus, to execute its transcriptional role [8]. An atypical pathway involves the activation of NFκB through MDP dependent activation of NOD2. Activated NOD2 in turn activates RIPK2, which in turn activates IKK/NEMO to free NFκB from IκBα, as described above [6].

Inflammatory bowel disease which includes the two main sub-types ulcerative colitis (UC) and Cohn's disease (CD) is a common inflammatory disorder that is associated with a greater risk of CRC [9]. Inflammatory bowel disease is a chronic relapsing inflammatory disorder of the gastrointestinal tract that usually onsets in late childhood or early adulthood leaving the patient with decades of chronic inflammation to deal with. Over 1.5 million and 2 million people suffer from a form of IBD in North America and Europe, respectively [10]. The epidemiology of IBD is shifting across the world and a general theme is an increase in the incidence of IBD in societies that are following a "western" like life style. For example, a recent systematic review of population based studies concluded that even though the prevalence rates remain high in North America and Europe, but the incidence rates are either stabilizing or decreasing in the western societies [11]. They also point out that the incidence rates are increasing on the other side of the world including Eastern Europe, Asia and Africa [11]. One possible explanation is that the incidence rates of IBD were high in North America and Europe in the last century and with the advancement of treatments and public awareness, the incidence rates stabilized but prevalence is still high.

The unpredictable nature of IBD exerts a burden on both the patients and governments with hospitalization, surgery, poor economic productivity and social involvement. Crohn's disease and ulcerative colitis are very similar when it comes to symptoms, making it hard to diagnose without invasive colonoscopies. Internally, Crohn's disease can affect any part of the gastrointestinal tract, whereas ulcerative colitis is mainly confined to the large bowel [12]. Microscopically, only the epithelial lining is affected in ulcerative colitis, whereas the whole bowel wall can be affected in Crohn's disease. In general, patients with ulcerative colitis experience pain in the lower left part of the abdomen, diarrhea, and rectal bleeding [13]. In contrast, patients with Crohn's disease complaint from pain in the lower right abdomen, with rectal bleeding not as common[13].

The gastrointestinal barrier provides an important protection against bacterial invasion of the underlying tissues and circulation. The main layers that constitute the gastrointestinal tract are the mucosa, submucosa, muscularis externa and adventitia [14]. The innermost layer which is the mucosa ensures food digestion as well as prevention of bacterial entry into the organs and blood. This is mainly attained by the tightly associated epithelial cells and the goblet cells that secrete mucin to form a mucus layer, further detaining bacteria in the lumen. In fact, several studies have described IBD as a perturbed intestinal barrier with altered mucus production leading to increased bacterial permeability and activation of inflammatory signalling [15, 16]. The exposure of the internal immune cells to the external luminal contents results in pro-inflammatory cytokine production, and in the absence of efficient repair this can lead to IBD.

1.1.2 Diagnosis and treatment

An effective treatment of disease requires proper and accurate diagnosis. Medical history plays an important role in diagnosing IBD, but imaging examinations, such as endoscopy, is usually the cornerstone to initiate a diagnostic tool towards IBD. The first step is suspecting IBD when patients present symptoms such as abdominal pain and diarrhea and it should be included as a differential diagnosis regardless of age. This ensures early detection and better management of IBD.

A critical symptom for UC is recurrent bloody stools with frequent abdominal pain [17]. Patients with more severe UC would often present symptoms such as fever, anemia, weight loss and abdominal tenderness [18]. In contrast, CD is characterized with fewer appearances of bloody stools with a higher possibility of weight loss and fever [17]. Visually, endoscopic examinations usually reveal the loss of vascular pattern, granular and easily bleeding mucosa in a continuous manner [17]. Furthermore, characteristic endoscopic observations in CD include discontinuous lesions, cobblestone appearance and lumen narrowing [17]. Reports indicate that colonoscopy can distinguish between UC and CD with an accuracy of 89% [19].

Extensive research has been done to find biomarkers that can distinguish the subtypes of IBD. A recent study by Boyd *et al* (2018) described the use of promoter and enhancer landscapes to differentiate between UC, CD and controls with an accuracy of 85% [20]. The authors utilized genome wide 5’ RNA

sequencing of capped RNAs (CAGE) to detect transcription start sites (TSSs) and promoter regions [21]. Interestingly, CAGE TSSs principal component analysis (PCA) clearly separated clusters of active IBD (UCa and CDa) and inactive IBD and controls (UCi, CDi and Ctrl) [20], indicating a distinctive genetic landscape for each group. Gene Ontology (GO) analysis identified sets of genes related to several biological processes differentially expressed within the different subgroups. Genes upregulated in IBD were those related to inflammatory responses, cytokines, remodelling of the extracellular matrix and antibacterial peptide secretion [20]. Closer analysis of GO identified a set of upregulated genes in IBD with known functions in gut epithelia barrier integrity including gap junctions (connexins *AQP5*, *GJ14-5*) and tight junctions (claudins *CLDN1, 2, 10, 14* and *18*) [20]. In contrast, genes downregulated in IBD were associated with processes involving xenobiotic response and drug/steroid processing [20]. Further clustering allowed an important identification of 337 active CD-specific and 71 active UC-specific differentially expressed TSSs [20]. Because the use CAGE and GO are still costly and not clinically relevant, 35 TSSs were suggested as micro-fluid qPCR detectable biomarkers that can distinguish active UC from active CD [20]. With further optimization, the authors propose these genetically differentiated landscapes are potential biomarkers for the different forms of IBD.

Treatment options for both UC and CD differ based on disease severity and stage. In cases of mild ulcerative colitis, 5-aminosalicylic acid (ASA) preparations are generally effective for induction and maintenance of remission in

UC [22]. The combination of oral and rectal therapies can sometimes be better and more effective than either one alone [23]. In patients with CD, even though one trial showed the efficacy of one 5-ASA formulation (Pentasa) towards small bowel CD [24], the overall consensus is that 5-ASA have little role in treating CD. In the case of mild CD, corticosteroids such as Budesonide are generally effective in inducing remission in distal ileal or right colon CD and have been used to induce remission in mild to moderate UC [25]. The long-term use of corticosteroids should be avoided and not considered as an option to maintain remission in either UC or CD because of their adverse side effects.

Moreover, immunomodulatory drugs represent an effective choice for the treatment and maintenance of remission in IBD. Thiopurines have been shown to maintain remission in both UC and CD patients [26], even though based on limited clinical trials. They are commonly used as adjuvant therapies to anti-TNF biologics and have been reported to enhance the efficacy of anti-TNF therapy either by providing additional immunomodulation or by reducing the likelihood of forming neutralizing antibodies against the biologics [25]. Thiopurines are effective in maintaining remission in UC patients, especially those dependent on steroids or unable to maintain remission by 5-ASA. Overall, there is more evidence on the use of Thiopurines in maintaining remission in CD compared to UC [27].

The use of anti-TNF agents, including infliximab (IFX) and adalimumab (ADA), has also proved to be effective for inducing remission in UC patients that are on steroids and experiencing moderate to severe UC [28, 29]. They are also

more important options when it comes to maintaining remission in UC, especially along with Thiopurines. On the other hand, anti-TNF therapies have shown efficacy in inducing remission and maintaining it as well in CD patients with active disease; the ability of ADA to prevent relapse is yet to be confirmed [28]. The use of anti-TNF is associated with possible risks and side effects including the reactivation of infections such as tuberculosis and hepatitis B [30, 31]. Therefore, it is crucial that IBD patients are tested for latent infections before administration of IFX or ADA. Although anti-TNFs have shown efficacy but 10-30% of patients who receive these therapies show no response to initial treatment and up to 46% have been reported to lose response over time [32], not to mention that they are considered expensive drugs. Hence, being able to predict non-responders is critical to ensure early management of IBD and to minimize wasted costs towards patients that will not respond. Therefore, better understanding of phenotypic indicators or biomarkers that can predict this subgroup of patients is very important moving forward with the use of anti-TNF therapies.

Other more recent immunomodulatory therapies for treatment of IBD include the biologics against integrin proteins expressed on lymphocytes. Vedolizumab is a monoclonal antibody that works in this fashion and was FDA approved in 2014 for treatment of UC and CD [25]. The blockade of integrin by Vedolizumab appears to be against gut specific ligands, which limits systemic toxicity without affecting lymphocyte trafficking to other tissues and organs [33]. Promising results have been observed for the induction of remission in both UC and CD. About 42% of UC patients who received Vedolizumab were in clinical

remission at week 52 as contrasted to only 16% of those who received the placebo [34]. Similar results were obtained for the CD study as well. As far as toxicity is concerned, Vedolizumab does not appear to have more toxicity than anti-TNFs and it could be even less [25].

1.1.3 Gene susceptibility and relevant biology

In addition to providing an effective treatment for IBD patients based on an accurate diagnosis, it is also crucial to explore and understand with high certainty the genetic and molecular alterations occurring in IBD which can in turn affect or suggest other therapeutics. The genetic and molecular alterations can be looked not only as a general overview of the disease but also at a more specific personalized manner. The advancement in diagnostic tools as well as the emergence of the concept of personalized medicine rationalizes the importance of understanding the disease in a more specific fashion, at the level of the individual. Our current understanding of IBD outlines genetic factors including at least 163 genes linked to IBD [35]. This is in synergy with external environmental variables as well as internal microenvironment conditions, mainly the microbial populations of the gut. The complex nature of interactions between these different modulators not only makes the pathogenesis of disease harder but also allows different levels of therapeutic intervention. Current research on how the innate and adaptive immune systems cooperate, as well as identifying several biomarkers of disease pathogenesis and understanding the role of genetic predisposition have all enhanced therapeutic intervention for better prognosis. Genetic loci associated with IBD include those of cytokine factors such as

interleukin 10 (IL-10) [36], cytokine receptors such as IL-17R and IL-23R, elements that are involved in autophagic signalling such autophagy related protein 16 (ATG16L) and NOD2 [37]. IL-10, for example, has been extensively studied in mice models and *IL-10*$^{-/-}$ knockout mice develop spontaneous colitis like symptoms by 8 weeks. Moreover, one study showed that polymorphisms in both ATG16L and Immunity-related GTPase family M protein (IRGM) were associated with higher risk towards CD while polymorphisms in IRGM only were associated with a higher risk towards UC [38]. This emphasizes the importance of understanding the genetic landscape and its contribution to the pathogenesis of IBD. At the molecular level, recent findings have suggested an important role of NOD2/RIPK2 pathway in promoting UC like symptoms in mice. The genetic ablation of this pathway protected TRUC (T-bet$^{-/-}$RAG2$^{-/-}$, ulcerative colitis) mice from developing inflammation in the large bowel as in contrast to wild type mice with intact NOD2/RIPK2 signalling [39]. In response to pathogenic invasion, NOD2 is stimulated and triggers the activation of its obligate kinase RIPK2 to promote NFκB driven transcription and autophagy [40, 41]. The activation of the NOD2/RIPK2 pathway can be initiated by bacterial products such MDP [42] and several studies have suggested that autophosphorylation on Y474 of RIPK2 (and possibly Y381) occurs prior to S176 phosphorylation and possibly in conjunction with K63 ubiquitination (Ub) of RIPK2 in order to promote full kinase activity [43, 44]. Once activated, RIPK2 can drive TAK1 (Transforming growth factor beta-activated kinase 1) and NFκB activation to control cellular inflammation, migration and growth of cells [45]. *Nod2*$^{-/-}$ mice are resistant to chemical induced colitis and

Nod2$^{-/-}$*/Ripk2*$^{-/-}$ mice have a dysbiotic intestinal flora resulting in altered susceptibility to intestinal inflammation [46]. Further discussion on the role of RIPK2 in driving inflammation will be discussed later in this chapter as targeting this pathway represents an important part of our findings.

1.2 Inflammation and Carcinogenesis

Inflammatory bowel disease, like many other inflammatory conditions are associated with development of cancers at a later stage in life. The contribution of inflammatory pathways to the carcinogenesis is established and inflammation has been proposed as a hallmark as cancer [47]. Tumours are not just simply composed of tumour cells but rather a heterogeneous mix of different groups of cells including immune cells, endothelial cells, fibroblasts and stem cells [48]. All of these cells interact together and even though immune cells may seem to be fighting the tumour by secreting cytokines and chemokines; however, in many cases the tumour cells benefit from this surge of inflammatory signals to further grow and progress. The link between inflammation and cancer was described back in 1863 by the famous Rudolf Virchow who noticed an infiltration of leukocytes into the neoplastic tissues [49]. Years after that observation, interest in the role of inflammation in driving the process of tumorigenesis has gained scientific interest and merit. About 90% of cancers are linked to somatic mutations, environmental and micro-environmental factors [50]; uncontrolled chronic inflammation being a more important player leading to cancer [51]. Tobacco, for example, triggers lung cancer largely due to its carcinogenic nature but also owes its tumour promoting properties to its ability to induce chronic

inflammation [52]. Similarly, obesity has been associated with the promotion of tumours in the liver [53] and pancreas [54]. Table 1 summarizes some of the human cancers that can be initiated by a pre-condition of inflammation. If we consider tracking cancer with the classic four-step carcinogenesis pathway, it is now evident that inflammation contributes to the molecular events feeding into all four steps of tumour initiation, promotion, progression and metastasis.

Table 1.1: Some cancers with inflammation as a pre-condition

Cancer type	Inflammatory Pre-condition
Colorectal cancer	Inflammatory Bowel disease
Bladder cancer	Schistosomiasis, chronic cystitis
Gastric carcinoma	*H.pylori* -induced gastritis
Hepatocellular carcinoma	Hepatitis B/C virus
Ovarian cancer	Endometriosis
Prostate carcinoma	Prostatitis
Lung carcinoma	Chronic bronchitis
Esophageal carcinoma	Chronic acid reflux
Thyroid carcinoma	Thyroiditis
Squamous cell carcinoma	Chronic osteomyelitis
F. Balkwil *et al* (2005) *Cancer Cell* 7:211-217	

The first step in committing to the carcinogenesis process is usually acquiring the first mutation that confers growth advantages and is commonly termed tumour initiation. Acquiring additional mutations (4-5 mutations) is often

required in many cancers to drive the tumour growth at a higher rate [55]. These mutations have to be transmitted to progeny cells and thus must occur in long lived stems cells or in the transient dividing cells, allowing the additional oncogenic hits or mutations to occur in the progeny cells. Differentiated cells in a rapidly renewed epithelium (13-20 days) such as that of the intestine usually do not live enough to acquire multiple oncogenic mutations. An inflammatory microenvironment with uncontrolled turnover of activated immune cells can be a major source of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) which can induce DNA damage when they interact with DNA [50]. The role of inflammation in tumour initiation is mainly by increasing the chances of mutations due to production of ROS and RNI or by activating pro-survival pathways that enhance the growth of already mutated cells. Under acute inflammatory conditions, ROS and RNI are either not sufficiently long lived enough or their levels are below the damaging threshold to induce DNA damage and mutations. Under chronic inflammatory conditions, when immune cells are constantly activated in tissues, the release of ROS and RNI by neutrophils and macrophages dramatically increases. This increase allows these highly reactive species to enter adjacent epithelial cell and cause DNA damage and induce mutations resulting in advantageous pro-survival cues [50]. Alternatively, cytokines such as Tumour necrosis factor alpha (TNF-α) produced by immune cells can also act on nearby epithelial cells to induce the accumulation of intracellular ROS and thus triggering mutations that initiate tumour formation [50]. Moreover, mutagenesis triggered by inflammation may lead to the inactivation of

mismatch repair genes, and ROS has also been reported to directly cause the oxidative inactivation of the mismatch repair enzymes [47, 56]. The above mentioned mechanisms compose some of many other mechanisms by which inflammation can trigger the initiation of the carcinogenesis process.

Inflammation plays an important role in further driving the carcinogenesis process once initiated; that is, a critical role in tumour promotion. Tumour promotion is the growth of single cells, initiated with several mutations, into a fully developed primary tumour. This step involves an unequivocal shift into increased cellular proliferation and reduced cellular death. NFκB and signal transducer and activator of transcription 3 (STAT-3) are key transcription factors that are inflammation driven, and whose role in promoting the tumour development has been well reported, by activation of genes that enhance survival, invasiveness and chemokine and cytokine production [57].

The observation that tumour growth was reduced and IL-6 production was blocked when NFκB was inactivated in myeloid cells in a mouse model of colitis associated cancer (CAC) provided early evidence on the importance of inflammatory mediators in promoting tumour formation [58]. Other reports later showed that immune cells producing cytokines such as IL-6, IL-11, and TNF-α as well as other cytokines such as IL-23 mediate the growth of tumour in the CAC mouse model [59-62]. One group highlighted the presence of elevated levels of IL-6 in mice subjected to azoxymethane (AOM) and DSS as compared to control mice [58]. IL-6 signals via the gp130 receptor that activates Janus Kinases (JAKs), which in turn recruit STAT-3 and induce its activation [63]. As a

transcription factor, STAT-3 drives the expression of several oncogenes including survivin, cyclin D1, c-myc and others [64]. Two groups that worked at the same time showed that the IL-6/STAT-3 pathway is important for the development of colorectal cancer in mice. The cell specific inactivation of STAT-3 in enterocytes reduced tumour size and decreased tumour incidence in response to AOM and DSS [60, 61]. IL-23 is yet another tumour-promoting cytokine that is mainly produced by tumour associated macrophages (TAMs) in an NFκB and STAT-3 dependent fashion [65]. Antibodies that blocked IL-23 and prevented its binding to its respective receptor greatly reduced tumour burden and growth [66].

About 90% of cancer related deaths are not due to the actual primary tumour but rather due to the metastatic outcome of the aggressive tumour in secondary tissues [50]. Metastasis usually involves a four step process in which the tumour cells have to undergo an epithelial-mesenchymal transition (EMT), intravasate into nearby blood vessels, survive and travel through circulation and eventually proliferate and divide at the target site. This much complicated process allows the immune cells to ensure surveillance and elimination of tumour cells before reaching the target site. In fact, only 1 in every 10,000 tumour cells that enter into circulation will reach a compatible tissue and metastasize [67]. In tumor cells, the loss of E-cadherin expression allows the movement of β-catenin into the nucleus to drive expression of oncogenes. Otherwise, β-catenin is sequestered in the cytoplasm by the presence of E-cadherin. Recent evidence suggests that Snail is stabilized in response to TNF-α, triggering E-cadherin loss and initiation of an EMT response for migration and metastasis [68].

Furthermore, the success of a cancer cell invasion into distinct tissues requires the proteolytic breakdown of the extracellular matrix by proteases such as metalloproteinases MMP2 and MMP9 [69]. Pro-inflammatory cytokines such IL-1, TNF-α and IL-6 have been shown to induce MMPs expression mainly through STAT-3 and NFκB controlled signalling [70]. Once in circulation, cancer cells have to survive less nutrient supply and avoid immune-surveillance. Physical attachment of cancer cells to macrophages is common and ensures survival of cancer cells in an atypical microenvironment [71]. In fact, removal of macrophages in mice by knocking out the gene encoding the macrophage growth factor CSF-1 (colony-stimulating factor 1) completely ablated metastasis in a mouse model of breast cancer [71]. The final step of metastasis involves the extravasation from blood capillaries and adhesion to target cells. Transforming growth factor beta (TGF-β) has been shown to play an important role in facilitating this process especially in the lungs [72]. Moreover, systemic inflammation driven signalling associated with tumour development can also upregulate the expression of adhesion molecules and increase chances of cancer cell attachment.

1.3 Development of Sporadic and IBD related CRC

Colorectal cancer represents the third most common malignancy and fourth most common cause of cancer related deaths in the world [73]. IBD related colorectal cancer, also known as colitis associated colorectal cancer (CAC), is a subtype of CRC that is usually hard to treat. More than 20% of IBD patients will develop CAC within 30 years of the disease and more than 50% will die from

CAC [74]. Sporadic CRC and CAC can share similarities when it comes to the stages of tumour development but differ in the sequence of molecular events governing the two forms of colorectal cancer. Colitis associated cancer usually arises from dysplastic lesions in the colon and tends to occur at an earlier age (mean age <50 yrs) in contrast to sporadic CRC which mostly arises from colonic polyps at an older age (mean age >65 yrs) [75, 76].

Mutations occur in both forms of CRC; however, to a lesser extent in the IBD related form [77]. Several studies have recently attempted to define a distinctive transcriptome profile for colorectal cancer. In one study, the authors used mRNA-Seq analysis and the found that 2538 genes were differentially expressed between tumour and normal tissues; half of which were upregulated [78]. The most prominently upregulated genes in CRC included families of peptidases, proteinases (including kallikreins (KLKs) and matrix metalloproteinases (MMPs) and chemokines/cytokines [78]. For example, *KLK6* and *MMP7* showed a 231 and 126 fold increase, respectively, in the tumour tissues in contrast to the normal tissues. The role of proteases in digesting the extracellular matrix allows for tumor expansion, metastasis and epithelial to mesenchymal transition [79, 80], but proteases can also signal through G-protein coupled receptors (known as protease activated receptors PARs) and contribute to the carcinogenesis process [81]. The chemokine genes upregulated the most in the tumor tissue were *CXCL5* (138 fold) and *CCL25* (70 fold), whereas *IL-11* and *IL-6* were upregulated by 26 fold and 17 fold, respectively [78]. Other genes that appear to upregulated in CRC include the regenerating family of genes

(*REGs*), which along with *CCL25* have been reported to be overexpressed in UC as well [82, 83]. *C-MYC* was also upregulated in the CRC tissue in the expected range (4 fold) that ensures its oncogenic role in colorectal cancer [84].

On the other side, genes controlling beta-oxidation, detoxification and differentiation pathways appear to be downregulated [78]. For example, carbonic anhydrase (convert CO_2 and water into bicarbonate and protons) genes are highly downregulated (41 fold decrease) which suggests a role for acidic environments in driving cell proliferation and division [85]. Furthermore, it appears that genes encoding membrane transporters such as bestrophin 4 (*BEST4*) and solute transporters such as *SLC30A10* are also downregulated and have been reported to correlate with the loss of epithelial function in tumor cells [86]. Interestingly, one study looked at the transcriptome profile of CRC and the matched liver metastasis tumors and reports a high concordance between both transcriptome signatures, with few distinctive transcripts linked to the primary tumor [87]. Another study, using an empirical real-time PCR approach, indicates a major upregulation of genes involving drug metabolism such as *ADH1B* [88] that can account for drug resistance in tumor cells.

As a subtype of CRC, colitis associated cancer (CAC) usually evolves through the inflammation-dysplasia-carcinoma pathway in contrast to the adenoma-carcinoma pathway that prevails in sporadic CRC [89]. Therefore, common and distinctive transcriptome expressions are expected between the two forms. It is important to note that most microarrays and databases do not state whether the source of CRC tissues is of sporadic or CAC origins and hence very

few studies describe the global transcriptome differences between the two forms. One particular study attempted to explore the differential gene expression between the two forms of CRC and found that 99.73% of 10357 genes were dysregulated in the same direction in both CRC forms [90]. This study was limited by a very small sample size (n=6) of CAC samples analyzed and thus the conclusions should be interpreted with caution. Furthermore, another study aimed at identifying potential driver genes for CAC using 90 samples and compared them to the matched normal tissue. Among the 2112 genes genetically modified in CAC, 262 were recurrently mutated and the most frequently mutated genes were *TP53*, *APC*, *KRAS* and *SMAD4* [91]. When compared with sporadic CRC, *APC* mutations were significantly less in CAC (16%) than in sporadic CRC (58%) [91]. Similarly, genetic aberrations in *KRAS* and *BRAF* were less in the CAC; however an exceptional higher frequency of mutations in *TP53* was found in CAC (66%) as compared to sporadic CRC (52%) [91]. Interestingly, the type of genetic aberration affecting the same gene can be different between the two forms of CRC. For instance, Ring finger protein 43 (*RNF43*) which is a negative regulator of the Wnt pathway is mainly modified by frameshift INDELS at codons 659 and 117 in sporadic CRC and only modified by a single mutation at codon 659 in CAC [91]. These and other findings can suggest that the mutation mechanisms are different between CAC and sporadic CRC.

Molecularly, the events occurring can differ substantially between the two forms of CRC, especially those involving activation and suppression of critical oncoproteins and tumour suppressors and the order of activation and

inactivation. Figure 1.1 summarizes the main molecular changes that occur in the two forms of CRC.

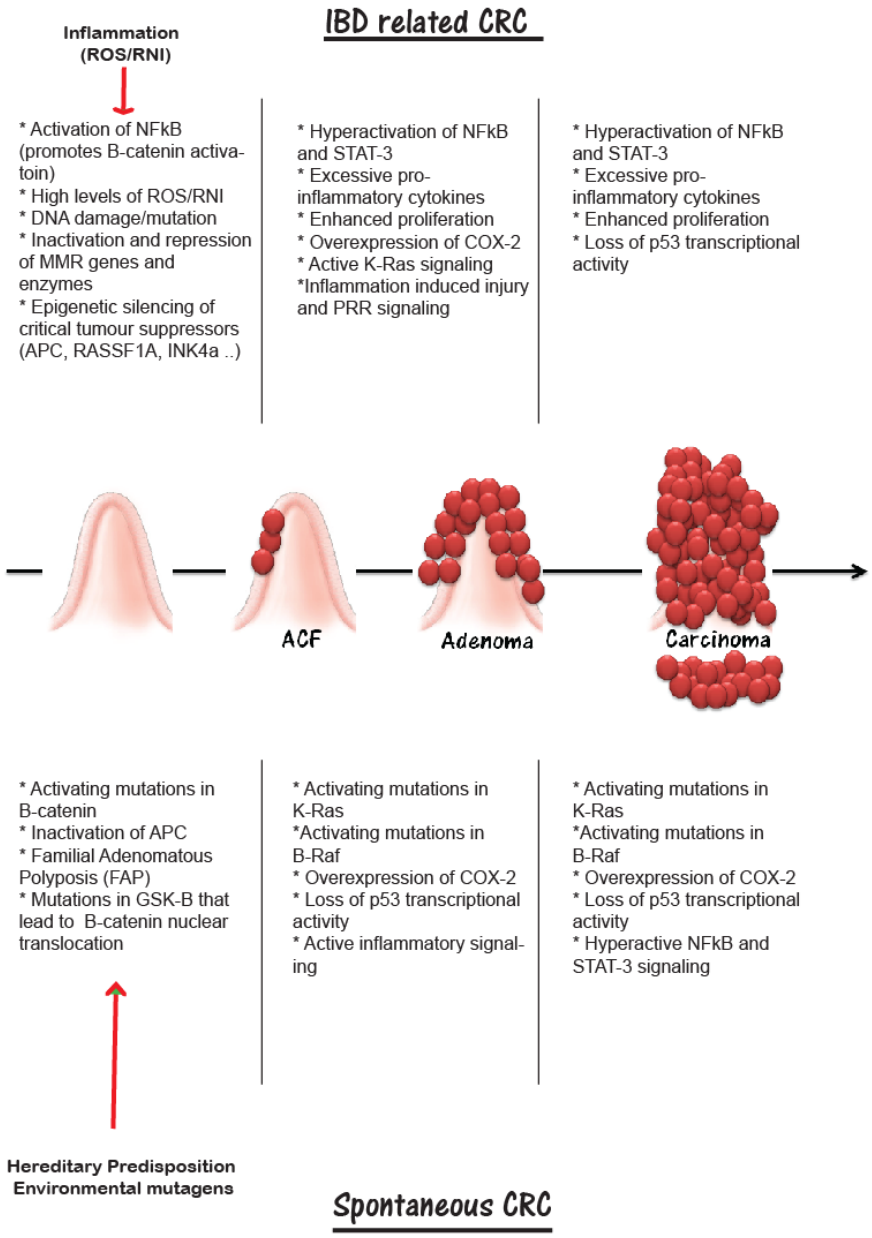


Figure 1.1: **Molecular similarities and differences between sporadic colorectal cancer and IBD related colorectal cancer**. Hereditary predisposition and environmental mutagens predispose cells to abnormal growth patterns. Sporadic CRC is caused by accumulation of mutations in oncogenes and tumour suppressor genes such as *APC* and *β-catenin* that mediate formation of adenomas. Additional mutations or abnormal signalling drives the transition into more advanced carcinomas. IBD-related CRC is usually preceded by chronic inflammation that drives mutations in critical genes and further release more pro-inflammatory cytokines to drive the carcinogenesis process.

As mentioned earlier, a critical event in the initiation of CRC is β-catenin activation and its nuclear translocation. This is usually conferred by Wnt-dependent signalling that results in the proteolytic degradation of APC (Adenomatous polyposis coli), an inhibitor of β-catenin that sequesters it in the cytoplasm [92]. Individuals with familial adenomatous polyposis carry an inactivating mutation in one the *APC* alleles and usually acquire the second mutation within 30 years of life, leaving them with multiple aggressive tumours in the colons [93]. Other mutations in glycogen synthase kinase-β (GSK-3β), β-catenin itself and members of this signalling pathway are also found in about 10% of colonic tumors [94]. Furthermore, the progression of an early adenoma into an invasive carcinoma requires additional mutations including activation mutations in oncogenes such as *KRAS* and *BRAF* as well as inactivation mutations in tumour suppressor genes such as the tumor protein 53 (*TP53*) and *BAX* [95-97]. Even though inflammation is not likely to be the main driver in the initiation of sporadic CRC, but it is an undoubted component in the later stages of sporadic CRC. Conversely, CAC is preceded by chronic inflammation that drives most of the molecular events leading to carcinogenesis. Chronic inflammation causes oxidative damage and leads to mutations and epigenetic modifications that drive a normal epithelium or stem cell to become tumorigenic. Typically, genes involved in maintaining fidelity during DNA replication or tumour suppressor genes are targeted for silencing. For instance, *APC*, *BAX*, *INK4a* and *RASSF1A* are frequently silenced in CAC, in contrast to genetic mutations in sporadic CRC [47]. Both the activity and expression of DNA methyl transferases

(DNMT), including DNMT1 and DNMT3, can be induced in response to inflammation driven pathways to further trigger gene hypermethylation and silencing [98]. Hence, one very key difference between sporadic and CAC is the role of inflammation driven processes in initiation of carcinogenesis. Whereas hereditary predisposition and environmental mutagens are the main initiators of sporadic CRC, chronic inflammation associated with IBD appears to be main driver of CAC. The later stages of both forms can share more similarities in the molecular pathogenesis as a sporadic tumour is definitely associated with an inflammatory micro-environment and the excessive inflammation in CAC will no doubt induce mutations in critical genes. In fact, IBD patients with a family history of CRC are more than 2 times likely to develop CRC than IBD patients with no family history [99]. If anything, this suggests an overlap of mechanisms between both forms of CRC, especially in the later stages of carcinogenesis.

1.4 RASSF1A: A Tumour Suppressor gene modulating NFκB and RIPK2 Activity

Given the connection between inflammation and cancer described earlier and knowing the established link between IBD and CAC, our group has been interested in deciphering the genetic and molecular link of IBD and cancer and the role of Ras association domain family member 1 isoform A (RASSF1A) in this link. There are ten members of the RASSF family of proteins named due to the presence of a Ras association domain (RA) found either in their N-terminus or their C-terminus [100]. RASSF proteins use other domains to execute diverse

biological functions including the Salvador-RASSF-Hippo (SARAH) domain that allows interaction with mammalian sterile 20-like kinases (MST1 and MST2) in order to promote apoptosis [101]. Other domains include an Ataxia-telangiectasia mutated kinase (ATM) phosphorylation site and a protein kinase C conserved region (C1). The ATM phosphorylation site has been reported to be phosphorylated upon DNA damage and would also contribute to apoptotic signalling in response to DNA damage. The C1 domain is critical for the association with microtubules and death receptor complexes such TNF-R1 and Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [102]. Figure 1.2 shows the main domains found in RASSF1A and their significance for protein interactions [102].

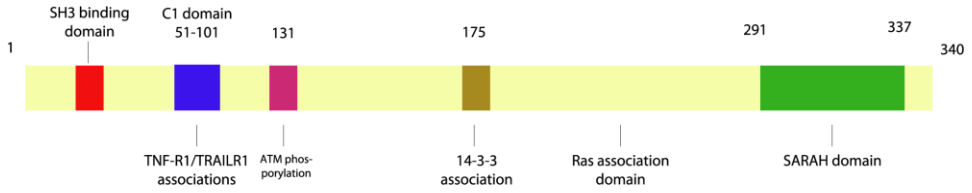


Figure 1.2: Schematic depicting the structure of RASSF1A. The SH3 binding domain allows the interaction with other SH3 containing proteins. The ATM phosphorylation site at S131 is usually phosphorylated in response to ATM sensing double stranded breaks (DSBs) in DNA. Other binding sites are also shown, including the 14-3-3 binding site and Ras association domain that allows interaction with Ras family of oncogenes. The SARAH domain modulates interactions with sterile-20 like kinases, MST1 and MST2, critical for apoptotic signalling. Adapted from El-Kalla *et al* (2005) [102].

RASSF1 has eight different transcripts termed (A-H) that arise from alternative splicing. RASSF1A and RASSF1C are the most studied isoforms of the group and are ubiquitously expressed in normal tissues [103]. RASSF1B,

also known as the minor transcript, is highly expressed in hematopoietic cells and is a much shorter protein than RASSF1A with only RA and SARAH domains [104]. RASSF1D, specifically expressed in cardiac tissues, resembles the structure of RASSF1A with just four extra amino acids. RASSF1E is expressed specifically in the pancreas and also differs from RASSF1A by only four amino acid residues. RASSF1F is translated as a truncated 92 amino acid that ends within the C1 region of RASSF1A. Similarly, RASSF1G is also truncated at 152 amino acids that terminate just before the RA domain. RASSF1H is a 75 amino acid protein that only shares the ATM domain with RASSF1A. Apart from RASSF1A and RASSF1C, the biological function of the other isoforms is not well known [105], mainly because they are unstable proteins. Two CpG islands are associated with the *RASSF1* gene: CpG island A which controls the expression of *RASSF1A, D, E, F* and *G* and CpG island C which controls the expression of *RASSF1C* [106]. The methylation of the *RASSF1A* CpG island A has received a lot of attention for its role in silencing *RASSF1A* in many cancers, whereas the *RASSF1C* CpG island C appears to be unmethylated in normal and tumor tissues [103, 106, 107]. Both RASSF1A and RASSF1C can associate with microtubules but only RASSF1A stabilizes microtubules [108]. Extensive research on the biological functions of RASSF1A has revealed various interactions modulating different outcomes including MST-kinase dependent apoptosis [109], death receptor dependent apoptosis [110], stabilization and cell cycle regulation [111], inhibition of β-catenin accumulation [112], DNA repair processes [113] and the stabilization of TP53 [114] and p73 [115]. On the other

end, RASSF1C has been reported to be an oncogene and to stimulate cell growth of cancer cells [116], attenuate apoptosis and promote cell migration [117], inhibit β-catenin degradation [112] and activate the JNK signalling pathway [118]. In other words, RASSF1A exhibits promising tumour suppressive properties in contrast to RASS1C tumour promoting properties.

RASSF1A has been shown to play an important role in stabilization of microtubules through the interaction with several microtubule associated proteins such as microtubule associated protein 1B (MAP1B) and microtubule associated protein 1S (MAP1S) [108]. This interaction appears to be critical for its tumour suppressive properties as the loss of this interaction leads to the inhibition of death receptor dependent cell death and promotion of genomic instability [119]. RASSF1A also controls cell cycle progression and apoptosis by inducing the disruption of the mouse double minute 2 homolog (MDM2)-DAXX complex causing MDM2 self-ubiquitination and TP53 stabilization [114].

As mentioned earlier, β-catenin accumulation is a critical event in the process of colorectal carcinogenesis. APC inactivation allows the nuclear accumulation of β-catenin and is associated with adenoma formation. The role of RASSF1A in the above pathway has been investigated in the APC $^{min/+}$ model of intestinal inflammation. *Rassf1a*$^{-/-}$ / *APC*$^{min/+}$ mice had significantly more adenomas in the small intestine with an increased rate of carcinogenesis [120]. Nuclear accumulation and activity of β-catenin were both higher in the mice with *Rassf1a* knocked out indicating an important role of Rassf1a in controlling β-catenin signalling and degradation. Our group has also demonstrated that

RASSF1A can associate with TNF-R1, TRAIL-R1 and modulator of apoptosis 1 (MOAP-1) to control apoptotic pathways as well [109, 121]. Moreover, its apoptotic properties can also be executed through the pro-apoptotic kinases MST1/2 and hippo-pathway signalling [101]. An important property of RASSF1A is its ability to interfere with TLR-MyD88 signalling that results in restricting NFκB signalling and unwanted excessive inflammation [122].

Furthermore, previous work in our group showed that the genetic ablation of *Rassf1a* in mice increased inflammation induced DNA damage as well as phosphorylation of Yes associated protein (YAP) at Y357 in response to DSS administration to mice [122]. The results clearly indicate a protective role of Rassf1a against inflammation induced injury and emphasize the importance of understanding the abnormal signalling patterns resulting from a dysregulated Rassf1a. In fact, *Rassf1a*$^{-/-}$ mice administered DSS were more susceptible to colitis like symptoms including increased gut permeability, increased cytokine production and increased colonic death [122].

Other groups have also looked at the importance of Rassf1a in regulating tumorigenesis in mice. After 20 months, *Rassf1a*$^{-/-}$ mice develop spontaneous tumors (32%) at a significantly higher rate than *Rassf1a*$^{+/-}$ (17%) and wild type (4%) mice [123]. Furthermore, in a chemically induced lung tumorigenesis model, tumor multiplicity was increased in the *Rassf1a* knockout mice as compared to wild type mice; however, with no significant difference between *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ knockout mice was observed [123]. Another study investigated the effect of *Rassf1a* knockout on cell cycle regulation and tumor formation and

concluded that the *Rassf1a*$^{-/-}$ mice did not exhibit a grossly impaired cell cycle progression [124], which can be explained by the fact that many other components (cyclins and cyclin dependent kinases) can make up for *Rassf1a* loss. The authors further reported that lymphocytes from knockout mice were identical to wild type when it comes to the proportions of T and B lymphocyte populations [124]. The role of Rassf1a in cardiac hypertrophy and heart failure was also investigated in *Rassf1a*$^{-/-}$ mice by utilizing a traverse aortic constriction (TAC) model. *Rassf1a*$^{-/-}$ mice experienced augmented cardiac hypertrophy and increased cardiac fibrosis, associated with an increased activation of NFκB activity after TAC [125]. These and other results reinstate the function of RASSF1A as an important player in various biological functions.

1.4.1 Regulation of RASSF1A

Regulation of RASSF1A can occur at different levels including epigenetic modifications, transcriptional targeting and post-translational alterations. Epigenetic modifications are usually in the form of histone modifications that alter chromatin remodelling or in the form of methylation of specific CG rich domains known as “CpG” islands. Normally, DNA methylation is maintained by DNMT1, whereas *de novo* methylation of non-methylated is achieved by DNMT3a and DNMT3b. *RASSF1A* is one of the most highly methylated genes in human cancers and is considered an early change in the process of carcinogenesis [126]. Methylation of *RASSF1A* occurs predominantly in the promoter region and in exon 1 to some extent. Epigenetic loss has been reported in about 30% of cervical cancer patients, 60% of breast cancer patients and as high as 99% of

prostate cancer patients [127]. The use of pyrosequencing has provided a useful tool to study individual CpG site methylation in contrast to average global methylation revealed by traditional methylation assays. Careful analysis of the promoter methylation patterns of *RASSF1A* shows high variability within and among pancreatic cancer patients [106] and breast cancer patients [128]. Hence, there is a need for accurate sequencing methodologies such as pyrosequencing to reach consensus on what is normal and “abnormal” levels of methylation.

Epigenetic silencing of *RASSF1A* has also been reported in samples from patients with ulcerative colitis (UC) [129]. As mentioned earlier, patients with IBD are at a higher risk of developing CRC than the normal population and *RASSF1A* methylation can be observed in as high as 60% of CRC patients [129], indicating that this epigenetic change can occur early on in IBD and progresses into the cancer stage. Similarly, 44% of pancreatitis patients show *RASSF1A* silencing and pancreatitis is a predisposing inflammatory condition to pancreatic cancer whose patients have up to 83% *RASSF1A* loss [130]. If anything, this indicates that epigenetic modifications act early on in diseases with chronic inflammation to drive the loss of RASS1A which promotes malignancy. Moreover, *RASSF1A* methylation has been detected in urine [131], nipple aspirates [132] and sputum [133], indicating that the methylation status of *RASSF1A* can be probably used as a diagnostic tool or prognostic indicator for inflammatory disorders and cancers if proper normalization and threshold of disease can be established.

At the transcriptional level, bioinformatics suggest that *RASSF1A* mRNA can be targeted by several micro-RNA (miRNA) including miR-326, miR-330,

miR-149 and others. miR-602, which was not predicted by bioinformatics analysis, has been empirically proven to control *RASSF1A* expression; the inhibition of miR-602 restored *RASSF1A* expression, promoted cellular apoptosis and inhibited cellular proliferation in hepatoma cells [134]. Another potential miRNA that indirectly affects *RASSF1A* expression is miR-342. A colorectal cancer study revealed that DNMT1 overexpression in CRC tissues was associated with down-regulated miR-342 and the restoration of miR-342 dramatically reduced DNMT1 levels and restored *RASSF1A* expression levels [135]. These and other studies stimulate researchers in the scientific community to further investigate the role of miRNAs in *RASSF1A* regulation.

The regulation of the RASSF1A protein expression and activity also includes post-translational mechanisms including phosphorylation and ubiquitination. A direct phosphorylation of S203 or Thr202 by Aurora-A kinase allows RASSF1A to interact with microtubules and modulate cell cycle progression [136]. Other kinases, including ATM kinase can phosphorylate RASSF1A on S131 and trigger its interaction with MST1 kinase and activation of LATS1, allowing the stabilization of p73 [137]. Negative regulation of RASSF1A can be initiated by cyclin dependent kinase 4 (CDK4) initiated phosphorylation on S203 which allows the SCF ubiquitin ligase complex to interact with RASSF1A and promote its degradation [138]. This is a critical step that facilitates the G1-S transition of the cell cycle. Furthermore, GSK-3β has also been reported to phosphorylate RASSF1A at S175, S178 and S179 and allows its interaction with the 14-3-3 complex which in turn inhibits the association of RASSF1A with death

receptors [139]. A recent study proposed an additional mechanism for translational control of RASSF1A. Pefani *et al* (2016) showed that TGF-β binding to its receptor induces the recruitment of RASSF1A to the cytoplasmic domain of the receptor and induces the degradation of RASSF1A via the E3 ligase ITCH [140]. Hence, an inflammatory setup with plenty of TGF-β not only triggers oncogenic transcriptional activity of SMADs with YAP/TAZ but also targets RASSF1A for degradation, abrogating any restriction RASSF1A may have on inflammation [140].

1.4.2 Apoptotic properties of RASSF1A

The apoptotic properties of RASSF1A rely to a great extent on its interaction with other apoptotic partners. This includes interacting with MST1 and MST2 kinases through its SARAH domain triggering the activation and nuclear translocation of these pro-apoptotic kinases [141]. In turn, MST kinases are known to phosphorylate histone H2B at S14 which possibly targets histone H2B for endonuclease attack and DNA fragmentation [142]. Yet, another apoptotic pathway influenced by the RASSF1A/MST interaction involves the transcriptional co-activator YAP. In response to MST activation, downstream large tumor suppressor (LATS) kinases are activated leading to direct phosphorylation of YAP at S127 and causing it to be retained in the cytoplasm. DNA damage signals can activate upstream Src kinases to phosphorylate YAP at Y357 and favouring its nuclear translocation and interaction with the p73 transcription factor to drive transcription of pro-apoptotic genes such as Bax [143]. Our research group has also defined a RASSF1A involved, MST independent, apoptotic pathway that

depends on the MOAP-1 protein for execution of apoptosis. In response to death receptor signals, RASSF1A associates with TNFR1/MOAP-1 and TRAIL/MOAP-1 at the membrane allowing MOAP-1 to interact with Bax and drive its localization to the mitochondrial membrane and subsequent death [121].

1.5 AMPK: Regulator of Cellular Energetics and Metabolism

Even though inflammatory bowel disease is an inflammatory disorder, but a dysregulated metabolic pathway definitely contributes to the pathogenesis of IBD. This could be deduced from the observation that metformin treatment ameliorates DSS induced colitis in mice by activating the AMP-activated protein kinase (AMPK) [144]. Highly expressed in the liver and skeletal muscle and also expressed in the intestine, AMPK plays an important role in preserving energy upon low ATP/AMP conditions. AMPK belongs to the serine/threonine family of kinases with a catalytic subunit (α) and two regulatory subunits (β and γ) [145]. Activation of AMPK is mainly by phosphorylation of its conserved threonine residue at position 172 by its main upstream kinase liver kinase B1 (LKB1) [146]. The anti-diabetic drug (metformin) and non-steroidal anti-inflammatory drugs (NSAIDS) have been reported to activate AMPK [147]. While we and others have observed AMPK activation in response to the general anti-inflammatory compound resveratrol [148], it is tempting to speculate a role of AMPK in regulating inflammation, in addition to its role in metabolic regulation. AMPK activation triggers pathways that produce ATP such as glucose uptake and fatty acid oxidation (catabolic) and inhibits anabolic pathways such as fatty acid and protein synthesis (anabolic) [149]. Moreover, people with Peutz-Jeghers

syndromes that have a germline mutation in LKB1 and defective AMPK signalling, show a higher prevalence of cancers than the normal population [150]. Hence, it is reasonable to speculate that inducing AMPK activation in tumours where rapid growth and demand for protein synthesis is high will inhibit tumour growth.

About 50% of non-small-cell lung cancers (NSCLC) have inactivating mutations in LKB1 [151]. In melanoma cells, AMPK activation exerts an anti-tumour effect by inhibiting extra-cellular signal regulated kinase (ERK) signalling, reducing cyclooxygenase 2 (COX-2) expression levels [152] and inducing apoptosis by activating the c-Jun N-terminal kinases (JNK) pathway [153]. Altogether, AMPK seems to possess a tumour suppressive property; however, in some tumours and cellular contexts further investigation is needed. For example, in prostate cancer AMPK activation and increased Calcium/calmodulin-dependent protein kinase kinase (CAMKKβ) expression were associated with cell migration and pro-growth signatures [154]. Therefore, it is very critical to understand the context of AMPK activation and the downstream targets of AMPK in the cancer of interest. In relation to IBD and CRC, it is important to raise the following questions: does AMPK confer a protective and/or chemo-preventative role against the inflammatory and/or tumorigenic components of IBD induced CRC? Could AMPK activation provide cancer cells with an advantageous arrest to escape therapeutics that target proliferating cells? Further research and investigations would shed light on above important concepts.

1.5.1 Structure and function of AMPK

As shown in figure 1.3, AMPK is multi-subunit protein that is functionally composed of one α subunit, one β subunit and one γ subunit [155]. The α subunit exerts the catalytic activity of the enzyme and has two isoforms, α1 and α2, whereas the β and γ subunits are the regulatory units and can be present in two and three isoforms, respectively [156]. The different combinations give rise to 12 possible variants implying versatile cellular functions and localization. The α subunit contains a serine/threonine kinase domain, located at the N-terminal and is conserved in both isoforms, followed by an auto-inhibitory domain (AID) that upon proper stimuli can shut off the kinase activity by inducing conformational changes [157]. At the other end of the protein is the C-terminal domain (CTD) that allows interaction with β subunit and is indispensable for the formation of the αβγ complex [158]. The two α subunits differ in their sensitivity to AMP and their cellular localization.

The main regions of the β subunits include the glycogen binding domain (GBD) and the C-terminal domain that allows interaction with the other two subunits to form a functional complex. The importance of the (GBD) domain lies in the fact that AMPK can be regulated by glycogen and oligosaccharides through the β subunit and in turn regulate carbohydrate metabolism [159]. The γ subunit of the protein is perhaps the more important unit as it is actual “sensor” subunit that harbors the AMP sensing domain known as the cystathionine β synthase domain (CBS) which is preceded by the N-terminal domain and the short sequence responsible for binding to β domain [160].

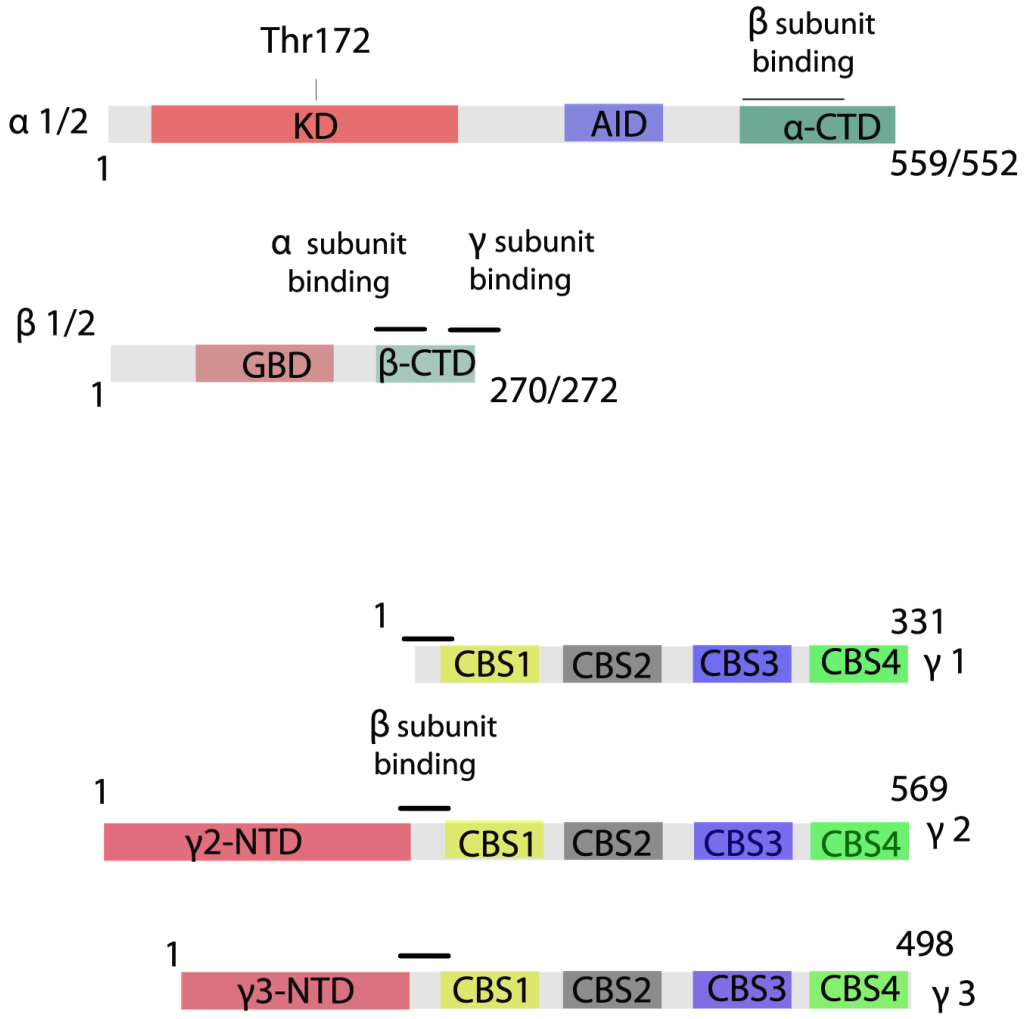


Figure 1.3: Structure and domains of the different AMPK subunits. A complete AMPK protein consists of one alpha, one beta and one gamma subunit. The alpha subunit contains a Kinase Domain (KD), Auto-inhibitory domain (AID) and a C-terminal domain (CTD) that allows binding to the beta subunit. The beta subunit contains a glycogen binding domain (GBD) and a C-terminal domain (CTD) that ensures the formation of the AMPK complex. The gamma subunit mainly functions as regulatory subunit which contains the AMP sensor domains known as cystathionine β synthase domain (CBS). Adapted from Novikova *et al* (2015) [155].

1.5.2 Activation and inactivation of AMPK

While the name of the protein indicates that AMPK is activated by AMP; however, that is not the complete story. AMPK is actually sensitive to the ratio of AMP/ATP in the cell, which is due to the fact that AMP and ATP competitively bind AMPK to allosterically regulate it [161]. Conditions that stress the cell and lower the level of ATP such as hypoxia and heat shock allow AMP to bind and activate AMPK. In addition to AMP binding there is another requirement for AMPK to be activated; there is “must” phosphorylation event that is also required. The binding of AMP represents the allosteric activation and opens up the kinase domain for phosphorylation at Thr172 by upstream kinases such LKB1, CAMKK and TAK1 [154]. As mentioned earlier, four potential adenine nucleotide binding sites are found in the γ subunit and these are known as the CBS domains. When co-crystallized in the presence of AMP, only 3 molecules of AMP were detected in the crystal structure. Site 2 appears to be always unoccupied [162] and site 4 is proposed to be permanently occupied by AMP which implies that site 1 and site 3 are the main regulatory AMP binding domains of the γ subunit. When AMP occupies one of the sites 1 or 3, allosteric activation takes place and leads to a marked conformational change [163] and renders the kinase less sensitive to phosphatases [164]. ATP binding to γ subunit causes the opposite effects and reports have indicated possible binding of ADP that does not allosterically activate the enzyme but protects it from dephosphorylation [165].

Three main kinases, referred to as AMPKK, have been identified to date that activate AMPK by upstream phosphorylation. LKB1 is considered the main

activating kinase and has been shown to phosphorylate AMPK both *in vitro* and *in vivo* [150]. The full activity of LKB1 requires the presence of two accessory proteins called STRAD and MO25, which induce the exportation of LKB1 from the nucleus and stimulate the activation of LKB1 [166]. Furthermore, knockout of LKB1 in cells was shown to drastically decrease AMPK phosphorylation but not totally abolishing it [167], emphasizing the possible role of other upstream kinases in phosphorylating AMPK. In fact, the presence of CAMKK was found to act upstream of AMPK, especially in response to increased Ca^{2+} levels [168].

Another upstream AMPKK has also been identified and represents an important link between inflammation and AMPK regulation. The TGF-β activated kinase 1 (TAK1) also directly phosphorylates AMPK at Thr172 in different tissues including skeletal muscle [169] and the heart [170]. More investigation into the physiological conditions in which TAK1 activates AMPK awaits more extensive research. The inactivation of AMPK occurs mainly through dephosphorylation of Thr172 by phosphatases 2A and 2C [171]. It is also worth noting that in addition to the activation site at Thr172, AMPK has also been reported to be phosphorylated by protein kinase A (PKA), specifically at the S485 residue, exerting an inhibitory effect [172].

1.5.3 AMPK targets and signalling

AMPK can use it kinase abilities to directly modulate both transcriptional factors for long term reprogramming of the cell and short term post translational modifications that affect several signalling pathways. PPAR gamma co-activator

1α (PGC1α) acts as a co-transcriptional activator that controls genes involved in energy metabolism and mitochondrial biogenesis [173]. AMPK is known to phosphorylate the N-terminal residues of PGC1α to trigger its activation in response to cellular stress and energy deprivation, thus enhancing oxidative capabilities of the cells as a result of enhanced mitochondrial biogenesis [174]. In the context of tumorigenesis and cancer, the somatic depletion of *Pgc1α* significantly reduced the occurrence of colorectal cancer in chemically induced mice [175], indicating a positive correlation and a possible therapeutic target for CRC. In addition to the activation of PGC1α, AMPK has also been reported to phosphorylate forkhead box O3 (FOXO3) at six different phosphorylation sites [176]. FOXO3 is a member of the FOXO family of transcription factors and is associated with tumour suppressive properties; the germline knockout *Foxo3*$^{-/-}$ mouse model results in a late onset of tumour phenotype [177]. To the contrary, several reports have indicated an oncogenic role of FOXO3 in colon cancer with a correlation between FOXO3 nuclear presence and metastatic tendency of the CRC [178]. Moreover, similar evidence in breast cancer shows that FOXO3 drives the expression metalloproteinases both *in vitro* and *in vivo* [179], making it critical to understand the context of AMPK activation of FOXO3 and whether it promotes tumour suppressive or oncogenic properties.

Another transcription factor that appears to be regulated by AMPK is sterol regulatory element binding protein (SREBP-1c), which positively controls the process of lipogenesis and controls the expression of several lipo-synthetic enzymes [180]. Elevated lipogenesis is a hallmark of cancer and with reports on

the correlation between high fat diets and several cancers; SREBP-1c has received much attention in this context. Furthermore, AMPK has been discovered as an upstream kinase that can phosphorylate SREBP-1c at S372 to reduce its nuclear translocation and transcriptional activity [181].

In addition to aforementioned regulation of several transcription factors that regulate gene expression and tumorigenesis, AMPK can target key proteins and regulatory enzymes that add to its complex role as a metabolic sensor with tumour suppressor properties. Firstly, AMPK interferes with the ability of mammalian target of rapamycin complex 1 (mTORC1) to stimulate pro-growth signals [182]. The activity of mTORC1 is greatly dependent on the presence of Raptor, an important scaffold protein that brings the complex close to its substrates. Both Raptor and mTORC1 are highly expressed in colorectal cancer and are usually associated with poorer prognosis [183]. In line with that, the knockdown of *Raptor* significantly reversed malignant abilities in ovarian cancer and its silencing in xenograft mice resulted in greater apoptosis and reduced the growth potential in hepatocellular carcinoma [184]. AMPK, among other upstream kinases, has been shown to exert a suppressive effect on the mTORC1 complex owing to its ability to phosphorylate Raptor at S722 and S729 which leads to suppressed proliferation and cell cycle arrest [185]. Closely related to above mentioned pathway, AMPK can directly phosphorylate Tuberous sclerosis complex (TSC) that also controls mTORC1 activity. Genetic mutations in *TSC1* and *TSC2* are linked to the occurrence of hamartomas in humans [186]. Phosphorylation sites on Thr1227 and S1345 of TSC2 are exploited by AMPK in

response to energy demand to protect the cells from unneeded expansion and growth under stressful conditions [182]. This phosphorylation event triggers TSC2 to suppress mTORC1 activity. In line, it has been reported that *Tsc2* knockout is linked to higher possibility of tumour formation *in vivo* [187].

The role of AMPK as a tumour suppressor extends beyond inhibiting mTORC1 activity. The tumour suppressor protein TP53 has also been described as a substrate for AMPK. TP53 is estimated to be inactivated in about 50% of human cancers [188]. Mechanistically, active AMPK can phosphorylate TP53 at S15 to drive its nuclear translocation and initiate its transcriptional activity of target genes that maintain genomic stability and energy homeostasis under destructive conditions such as DNA damage metabolic imbalance [189]. The discovery of Cyclooxygenase-2 (COX-2) has triggered research into why there are two isoforms (COX-1 and 2) that have identical activities but could play distinct roles during inflammation [190]. Recent use of genetically engineered mouse lines suggests that prostaglandin (PG) biosynthesis in platelets and kidneys requires native COX-1; *COX-2* knock-in cannot compensate for this requirement in *COX-2* deficient cells. COX-2 is well known as a pro-inflammatory enzyme whose upregulation in several cancers has been documented [191]. Most studies describe an inhibitory effect of AMPK on COX-2 and show an inverse correlation between the activation of AMPK and COX-2 inhibition in colorectal cancer [192] and melanoma [152] cell lines. A recent study in 2017 reports a novel reduction of COX-1 expression in response to AMPK activation in macrophages [193]. Further research in this regards is needed to confirm tissue

specific roles of AMPK activators on COX isoform regulation and how this can affect the future of AMPK agonists in IBD and cancer treatment.

In summary, the distinct and versatile targets of AMPK highlight promising therapeutic intervention at the level of AMPK for inflammation and inflammation related cancers. Figure 1.4 summarizes some of the proteins targeted by AMPK and their outcomes.

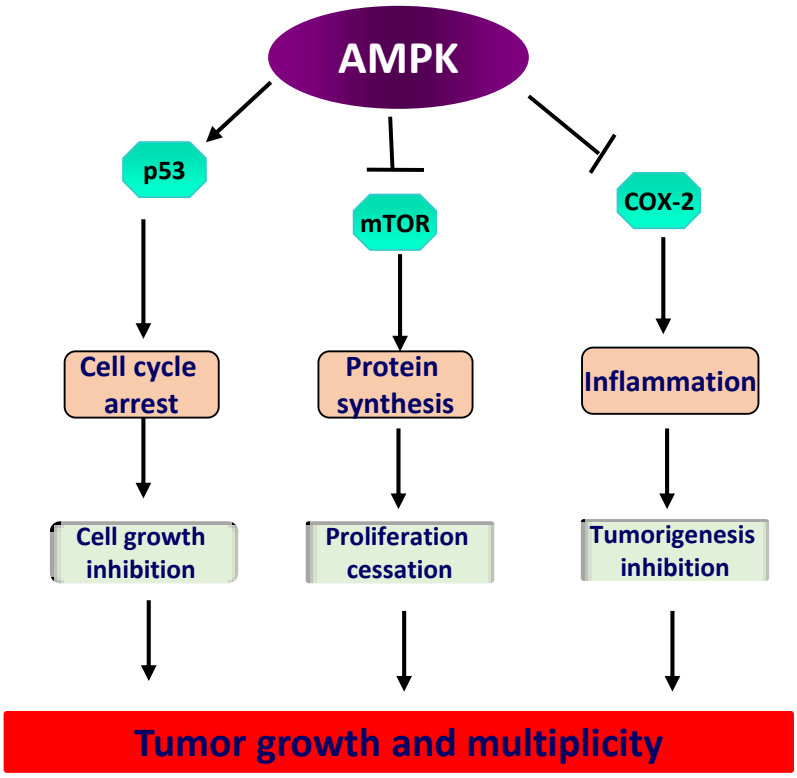


Figure 1.4: AMPK can function as tumour suppressor via control of growth and inflammation. AMPK can negatively regulate the mTOR pathway which is normally responsible for growth and proliferation. This can limit cancer growth. Active AMPK can also regulate COX-2 which if left uncontrolled can drive tumorigenesis by inducing the expression of inflammatory cytokines. TP53 is another AMPK target related to cell growth. The phosphorylation of TP53 by AMPK results in cell cycle arrest and cessation of growth in tumour cells. Overall, therapeutics involving AMPK activation could play an important in treatment of cancer and inflammation.

1.6 Resveratrol: A Polyphenol with Diverse Molecular Targets

Inflammation and inflammation associated disorders have been treated with natural compounds derived from plants for the longest times [194]. Resveratrol (3, 4', 5-trans-trihydroxystilbene), like other plant products, is part of the defensive mechanisms that have evolved in plants to deter and protect against herbivory; it is a phytoalexin present in grapes, peanuts and other plants [195]. Extensive studies have aimed at investigating the efficacy of natural compounds as anti-inflammatory and anti-tumor agents. Two important concepts are generally connected to resveratrol, the French paradox and calorie restriction. The first was introduced about 30 years ago and describes the epidemiological observation that French people have a relatively low rate of cardiovascular diseases even though their diets are rich in fats. The observation may not be fully scientifically validated but one implication has gained much interest; a component of the French diet must have protective roles against coronary complications. In fact, it was shown later that resveratrol protected against plaque formation in different animal models of atherogenesis [196] and enhanced nitric oxide production to improve vasodilatation [197]. This provided a possible explanation for the French paradox.

The concept of calorie restriction refers to the moderate intake of food and thus reduction in calorie intake without leading to malnutrition. It is one of the most reliable non-pharmacological applications that increases life span in model organisms including rats [198], insects, fish and mammals[199]. The link between resveratrol and calorie restriction effects emerged with the discovery that the

protein Sir2 (silent information regulator 2) was involved in yeast aging regulation. Sir2 overexpression increased lifespan by 30% and its genetic knockout decreased the yeast replicative lifespan by 50% [200]. In mammals, the sirtuins family consists of 7 enzymes (SIRT 1-7), with SIRT1 being the most widely investigated [201] and SIRT1 transgenic mice displaying features of calorie restriction such as increased metabolic activity and improved glucose tolerance [202]. Subsequently, resveratrol was shown to increase lifespan in lower eukaryotes in a SIRT1 dependent manner [203].

1.6.1 Molecular targets of resveratrol

As mentioned earlier, resveratrol belongs to the set of compounds with multiple rather than one molecular target. In line with that, the Sinclair research group identified resveratrol as the most potent activator of SIRT1 among a number of polyphenols expected to allosterically interact with SIRT1 [204]. As a SIRT1 activator, resveratrol affects a wide range of biological processes involving mitochondrial biogenesis, glucose and lipid homeostasis, inflammation and tumorigenesis. For example, PGC1α is deacetylated by SIRT1 to induce its activity as a co-transcriptional activator leading to the expression of genes involved in mitochondrial biogenesis [205]. In another study, Lagouge *et al* (2006) showed that the resveratrol-induced enhanced activity of PGC1α was dependent on SIRT1 as the knockdown of SIRT1 completely eliminated any PGC1α activity in the presence of resveratrol [206].

The mechanism of resveratrol induced SIRT1 activation has been investigated by many groups and it is now proposed that resveratrol and other SIRT1 activators bind to the steady-state enzyme-substrate complex rather than the isolated SIRT1 protein [207]. It was determined that the binding of resveratrol, as well as other activators, is specific to the N-terminal of SIRT1 and more specifically dependent on the presence of glutamic acid at position 230 (E230). A single point mutation to alanine or lysine largely compromised the ability of resveratrol to activate SIRT1 [207].

Even though the molecular pathways of SIRT1 and AMPK greatly overlap, some reports have indicated that AMPK can be activated within minutes of resveratrol treatment in most cell lines, a rate much faster than that required to activate SIRT1 [208]. In fact, this seems valid knowing that resveratrol and similar polyphenols can directly bind to the ATP synthase complex of the electron transport chain to inhibit ATP production [209]. Lower levels of ATP and higher levels of AMP can simply lead to the activation of AMPK. This observation is further strengthened by results indicating that resveratrol was dependent on AMPK to decrease lipid accumulation in HepG2 cells [210]. More interestingly, SIRT1 was not activated using AMPKγ knockout mice and consequently PGC1α was not acetylated in these mice [211]. These results indicate that AMPK is a critical mediator of the resveratrol effect and points out that SIRT1 is perhaps downstream of AMPK. Controversial enough, other reports clearly place SIRT1 at the apex of the AMPK signaling pathway. A SIRT1 inhibitor named splitomicin prevented resveratrol induced activation of AMPK in HepG2 cells [210], and the

use of SIRT1 specific shRNA also prevented AMPK phosphorylation in response to resveratrol [212]. SIRT1 has been reported to deacetylate LKB1, the upstream kinase of AMPK, and trigger its activation [212] to explain the importance of SIRT1 for AMPK activation in response to resveratrol.

Resveratrol has also been implicated in modulating signaling of the Toll-like receptors which generally employ adaptor proteins such MyD88 or TRIF to trigger the activation of NFκB [213, 214]. In the TRIF pathway, TANK-binding kinase (TBK1) can also activate the transcriptional regulator IRF3 (IFN regulatory factor 3), resulting in the expression of IFN-inducible genes that can drive inflammation [215]. Resveratrol has been shown to inhibit the kinase activity of TBK1 as determined in an *in vitro* kinase assay [216], preventing the phosphorylation of IRF3. It was also suggested that since both TBK1 and RIPK1 (discussed later in this chapter) associate with TRIF in response to TLR activation, resveratrol may be playing a role in the inhibition of the TRIF-TBK1-RIPK1 complex. Thus, the functional consequence of TBK1 inhibition is the down regulation of NFκB and IRF3 activities as well as their target genes COX-2, iNOS and IFN-β [216]. In fact, more than 70% of LPS-induced genes are thought to be driven by the TRIF pathway [217] and hence the resveratrol mediated inhibition of TRIF-TBK1 pathway could alleviate the exaggerated inflammatory response in chronic diseases such as IBD.

1.6.2 Cellular processes affected by resveratrol

Resveratrol has been described as a multi-target drug and hence it is expected that several pathways and processes are affected by resveratrol. Programmed cell death or apoptosis is a frequent fate for cells treated with resveratrol, especially at higher doses. Firstly, resveratrol treatment of HL-60 and T47D cells resulted in an increase in the death receptor content, particularly the Fas receptor [218]. This is indicative of a role for resveratrol in regulating death via the extrinsic pathway. Other reports revealed the involvement of mitochondrial apoptotic pathways in response to resveratrol, inducing depolarization of mitochondria [219], inhibition of the ATP synthase complex [220], modulation of the Bcl-2 pathway, as well as the upregulation of the pro-apoptotic protein Bax [221]. Furthermore, another study showed that the tumour suppressor protein TP53 is a key player in the resveratrol induced apoptosis as apoptosis was only induced in TP53 positive cells but not in TP53 deficient cells [222]. In line, another report also documented an increased expression of TP53 and a subsequent elevation in its target genes such as *p21* and *BAX* in response to resveratrol treatment [223]. The upregulation of TP53 transcriptional activity in response to resveratrol is most probably due to AMPK direct phosphorylation of TP53 at its S15 residue described earlier. Angiogenesis is a pivotal process that leads to the formation of new blood vessels for both repair processes and tumour cell nourishment [224]. The effect of resveratrol on angiogenesis has been investigated in several models. In Human Umbilical Vein Endothelial Cells (HUVEK), low doses of the polyphenol seem to transmit pro-survival signals

whereas higher doses induced growth impairment and apoptosis [225]. Closer investigation into the mechanism revealed that resveratrol interferes with vascular endothelial growth factor (VEGF) induced angiogenesis by inhibiting the phosphorylation of E-cadherin and β-catenin. More importantly, resveratrol affects both the transcriptional and translational expression of metalloproteinases (MMPs). In a study by Woo *et al* (2004), resveratrol suppressed a 23-fold increase in the promoter activity of MMP-9 in response to PMA stimulation [226] indicating a protective role of resveratrol against tumour associated angiogenesis and metastasis. In addition to inducing apoptosis and controlling angiogenesis, resveratrol is well known for its anti-inflammatory properties and which greatly contribute to its anti-tumour abilities. NFκB signalling plays an important role in mediating several biological and cellular processes, including inflammation and cancer. Even though no report so far has shown that resveratrol interacts directly with any of the subunits of the NFκB complex; however, its effect on NFκB inhibition is clear. In an unstimulated cell, NFκB subunits are sequestered in the cytoplasm as an inactive protein tethered to its inhibitory partner IκB which prevents it from nuclear translocation and transcriptional activity [227]. When stimulated with TNFα, LPS and other agents, signalling events through respective receptors lead to the activation IκB Kinase (IKK) complex that phosphorylates IκB leading to its polyubiquitination and subsequent proteosomal degradation [228]. This releases NFκB subunits, allowing them to translocate to the nucleus and bind target genes. Resveratrol prevents the activation of NFκB most probably by preventing the phosphorylation of the IκB [229].

1.7 Receptor Interacting Protein Kinase (RIPK) Signalling

Pathogen recognition receptors (PRR) are important primary sensing receptors that play an important role in stimulating the innate immune system against pathogens and microbes. Molecular structures such LPS, endotoxins, flagellin, nucleic acids and other bacterial and viral components are collectively termed pathogen associated molecular patterns (PAMPs). Different types and classes of PRRs recognize the various PAMPs and thus provide protection against the vast variety of possible molecules associated with invaders. The most common PRRs include Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [230, 231]. Upon activation, PRRs transmit the signal to a cascade of proteins leading to the activation of NFκB and the induction of a plethora of cytokines encompassing an efficient inflammatory response to attract leukocytes into the infected tissue. Long term activation of PRRs should be controlled and reversed as an exaggerated inflammatory response will drive diseases such as inflammatory bowel disease.

1.7.1 The family of RIPK kinases

The family of RIPK kinases encompasses seven members termed RIPK1 to RIPK7. As shown in figure 1.5, a common feature of all members of this family is the presence of the kinase domain (KD) which signifies the key cellular function of these proteins [232]. The molecular pathways and biological processes affected by RIPK1 to RIPK3 have been extensively studied, whereas

RIPK4 to RIPK7 are less investigated but have been reported to activate NFκB [233], drive apoptosis [234] and to be associated with Parkinson's disease [235].

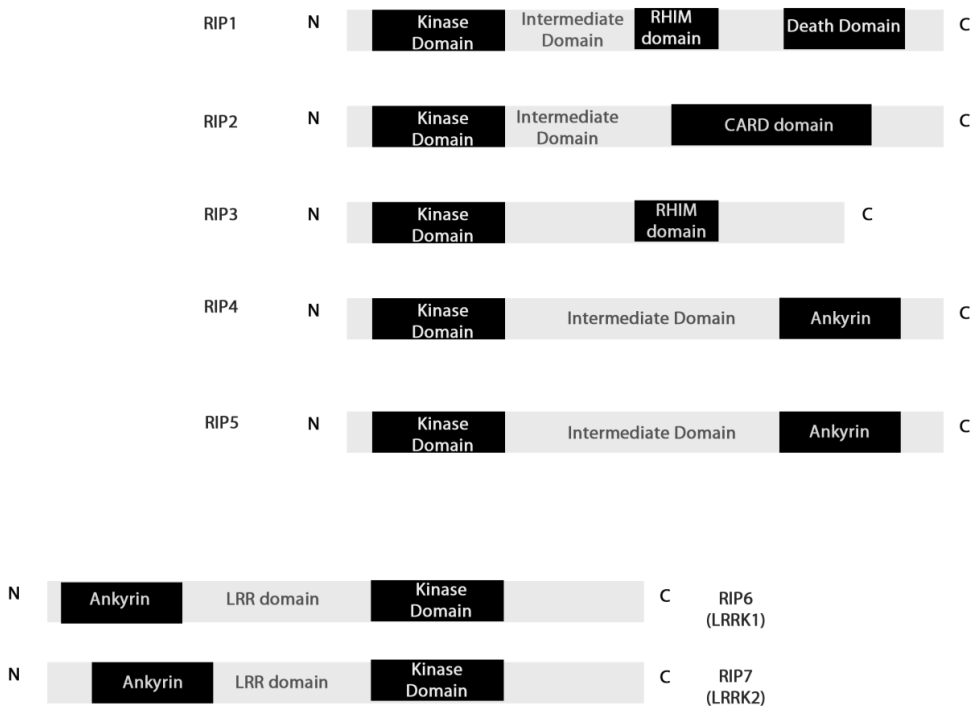


Figure 1.5: Structure and domains of the different RIPK kinases. All 7 members of the RIPK kinase family share the Kinase domain which signifies the main function of these proteins. RIPK2 kinase has a CARD domain and makes it one of most studied members of the family, and is known for the interaction with NOD2 to mediate inflammation. RIP1 is characterized by the presence of Death domain that is absent in other members. The RIP homotypic interaction motif (RHIM) is present in RIPK1 and RIPK3. RIPK4, RIPK5, RIPK6 and RIPK7 structures reveal a common Ankryin domain. The presence of leucine rich repeats (LRRs) in RIPK6 and RIPK7 also gives them common names as LRRK1 and LRRK2 respectively. Adapted from Zhang *et al* (2010) [232].

1.7.2 RIPK1 Kinase: Inflammation or apoptosis?

Among several biological processes affected by RIPK1 Kinase, inflammation and apoptosis appear in most reports as the most hits. Stimulation of cells with TNF-α and the subsequent activation of TNF-R1 leads to the interaction of TNF-R1 associated death domain protein (TRADD) and RIPK1 with

the cytoplasmic domain of TNF-R1 [236]. This, in turn, triggers the recruitment of E3 ubiquitin ligases including TNF receptor associated factor 2 (TRAF2) and cellular inhibitor of apoptosis protein (cIAP2) to the complex to change RIPK1 into its poly-ubiquitinated active form [237]. Following that is the recruitment of TAK1 and other ubiquitin binding proteins in the IKK complex [238, 239]. TAK1 can then phosphorylate IKK inducing its activation and downstream phosphorylation of IκB to release the NFκB subunits into nuclear translocation and activation.

Interestingly, NFκB drives the expression several genes including de-ubiquitinating enzymes that ensure a negative feedback loop and inactivation of RIPK1 to terminate the NFκB activation as well [240]. When in its unmodified state, RIPK1 has been reported to associate with Fas-associated death domain (FADD) and procaspase 8 to form a complex commonly named the "ripoptosome" that eventually drives the cell into the apoptotic pathway [241]. Hence, the decision to drive inflammation or cellular death in response to TNF-α is dictated by the post-translational form of RIPK1.

1.7.3 RIPK Kinases and pathogen recognition receptors

Both RIPK1 and RIPK3 play an important role in transmitting signals through TLRs, especially TLR3 and TLR5. TLR3 and TLR5 employ the TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor protein that contains the RHIM domain which allows interaction with RIPK1 and RIPK3. Even though TLR4 mainly uses the adaptor protein MyD88 to trigger activation of

NFκB, it also has been reported to activate NFκB in a MyD88 independent fashion using TRIF [242]. For example, when TLR4 is activated by LPS and TRIF is involved, RIPK1 gets recruited to the membrane complex via its RHIM domain and is then poly-ubiquitinated by the E3 ligase Pellino1 and can then pass on the signal to activate NFκB [243].

As mentioned earlier, Nod-like receptors (NLRs) constitute an important set of receptors known as the pathogen recognition receptors (PRRs) that play a crucial role as part of the innate immune response. NOD2 is the second member of NLR family of receptors to be discovered after NOD1. The two NLRs share great similarities both functionally and structurally. NOD1 and NOD2 both have leucine-rich repeat (LRR) domain, a NACHT domain and an effector domain. The effector domain contains one caspase recruitment domain (CARD) in NOD1 and two CARD domains in NOD2 [244]. Despite the fact that both NLRs share structural and functional similarities, but NOD2 has received greater scientific attention due to the fact that several single nucleotide polymorphisms (SNPs) of NOD2 have been associated with Crohn's disease [245].

Upon stimulation with the peptidoglycan fragment MDP, the cytosolic pathogen recognition receptor NOD2 is activated [246] and direct interaction between MDP and the NOD2 LRRs has been validated [247]. RIPK2 is the most well studied interacting partner of NOD2 that leads to the activation of both NFκB and mitogen-activated protein kinase (MAPK) signaling pathways [6, 248]. Both CARD domains found in NOD2 appear to be required for RIPK2 binding [249] and yet this interaction is still not sufficient to drive the downstream NOD2/RIPK2

signaling cascade. Other post-translational modifications on RIPK2 are critical for activation and these include the phosphorylation of S168, S176 in the kinase domain [250, 251], Y474 in the CARD domain [252] and other residues in the C-terminal domain of the protein. In fact, mutagenesis studies involving the Y474 residue revealed the importance of this phosphorylation event to stabilize the RIPK2/NOD2 interaction [252]. Other modifications that activate the signaling cascade of NOD2/RIPK2 are dependent on the successful ubiquitination of RIPK2 by E3 ligases such as ITCH [253] Pellino3 [254] and TRAF6 [255]. In the absence of an activating signal, RIPK2 has been shown to associate with mitogen-activated protein kinase kinase kinase 4 (MEKK4) under basal conditions to prevent the ubiquitination and activation of RIPK2 [256]. The MEKK4/RIPK2 association is broken in the presence of MDP stimulated NOD2, allowing activation of RIPK2 and the subsequent phosphorylation of downstream targets such TAK1, leading to activation of NFκB and inflammation.

The inhibition of the NOD2/RIPK2 pathway represents a promising approach to several diseases with an inflammatory component and a characteristic hyperactive RIPK2. Therapeutic targeting of this pathway is rationalized by the fact that NOD2 represents a major hub protein in control of innate immune responses to pathogenic invasion. Since RIPK2 is an obligate kinase of NOD2, RIPK2 inhibitors can provide the brakes required to shut off NOD2 signaling. Several RIPK2 inhibitors have been investigated in this regards, including the p38 inhibitor SB203580 which potently inhibited NOD2 driven NFκB activation [257]. Gefitinib, the EGFR tyrosine kinase inhibitor commonly known as

Iressa, is an FDA approved drug that inhibits RIPK2 autophosphorylation at Y474 and blocks NOD2 downstream signaling [252]. The use of SB203580 and gefitinib is comprised by the potent inhibition of their main targets p38 and EGFR, respectively [258]. Furthermore, progressive effort to target RIPK2 led to the discovery that ponatinib, another FDA approved drug that can target the inactive "DFG-out" confirmation of the kinase domain as a type II inhibitor [259]. In line with previous efforts, another group identified a potentially selective RIPK2 inhibitor termed GSK583. Even though it potently inhibited RIPK2 in the nanomolar range in rats; it was proposed to not produce a similar pharmacodynamic response in humans within an acceptable dose [260]. Further modifications of the GSK583 drug produced GSK2983559 which possessed better pharmacokinetic properties that allowed its recent integration into a phase I clinical trial for IBD in late 2017. Other RIPK2 inhibitors including WEHI-345 have been shown to delay RIPK2 ubiquitination and inhibit downstream NFκB-mediated cytokine production, resulting in the amelioration of mice encephalomyelitis [261]. Novartis Research Foundation identified, using a structure-activity relationship (SAR) approach, a novel RIPK2 inhibitor (compound 8) that inhibited MDP-driven NOD2 signaling and prevented the phosphorylation of the ribosomal protein S6 [262]. Indirect RIPK2 inhibitors include IAP antagonists [263] which block ubiquitination of RIPK2 or SMAC mimetics [264] that lead to the degradation of IAPs. Overall, targeting the NOD2/RIPK2 pathway either directly or indirectly can provide future alternatives or adjuvant therapies for inflammatory diseases including IBD.

1.8 Project Overview and Research Aims

As mentioned earlier, inflammatory bowel disease (IBD) is an idiopathic disease, with no cure completely reversing and eliminating the causes. Current treatments aim at inducing remission and maintaining it for as long as possible. The association of IBD with increased chances of developing CRC further exemplifies the established link between inflammation and cancer and emphasizes the need for effective treatments to manage the inflammatory component of IBD before it translates into a tumorigenesis precursor. Our group has recently demonstrated the importance of the tumor suppressor protein RASSF1A in restricting excessive activation of NFκB in a murine model of colitis [122]. This has allowed us to position RASSF1A as a central player with respect to the regulation of inflammation in IBD and IBD related CRC. It also prompted us to postulate that pathways controlled by RASSF1A are dysregulated in IBD. Our postulation is further strengthened by the observations that *RASSF1A* is silenced in IBD patients and it is one of the most frequently epigenetically silenced genes in a variety of cancers including CRC [107]. That mentioned, the extent of *RASSF1A* promoter methylation has been variably reported for some cancers, specifically colorectal cancer. With the above mentioned rationale in mind, we were driven into asking ourselves three main questions: 1) why is there variability in extent of *RASSF1A* promoter methylation in CRC as compared to other cancers and 2) can we compensate for the loss of *RASSF1A* in IBD by targeting the downstream dysregulated pathways 3) can we make use of the identified molecular perturbations to develop biomarkers for both diagnostic and prognostic purposes

in IBD. Our working hypothesis was that targeting pathways that are dysregulated in the absence of RASSF1A is an alternative therapeutic option for treatment of IBD. To answer our questions and test our hypothesis, we set three main aims for our study (Figure 1.6). (1) To explore a more detailed methylation analysis of the *RASSF1A* promoter in IBD and CRC and see how that molecularly and functionally links to the pathogenesis of both diseases. (2) To investigate the use of therapeutics that can target the inflammatory pathways dysregulated as a result of RASSF1A loss. (3) To translate our research observations into identifying novel biomarkers of IBD that can be used for diagnostic and prognostic purposes.

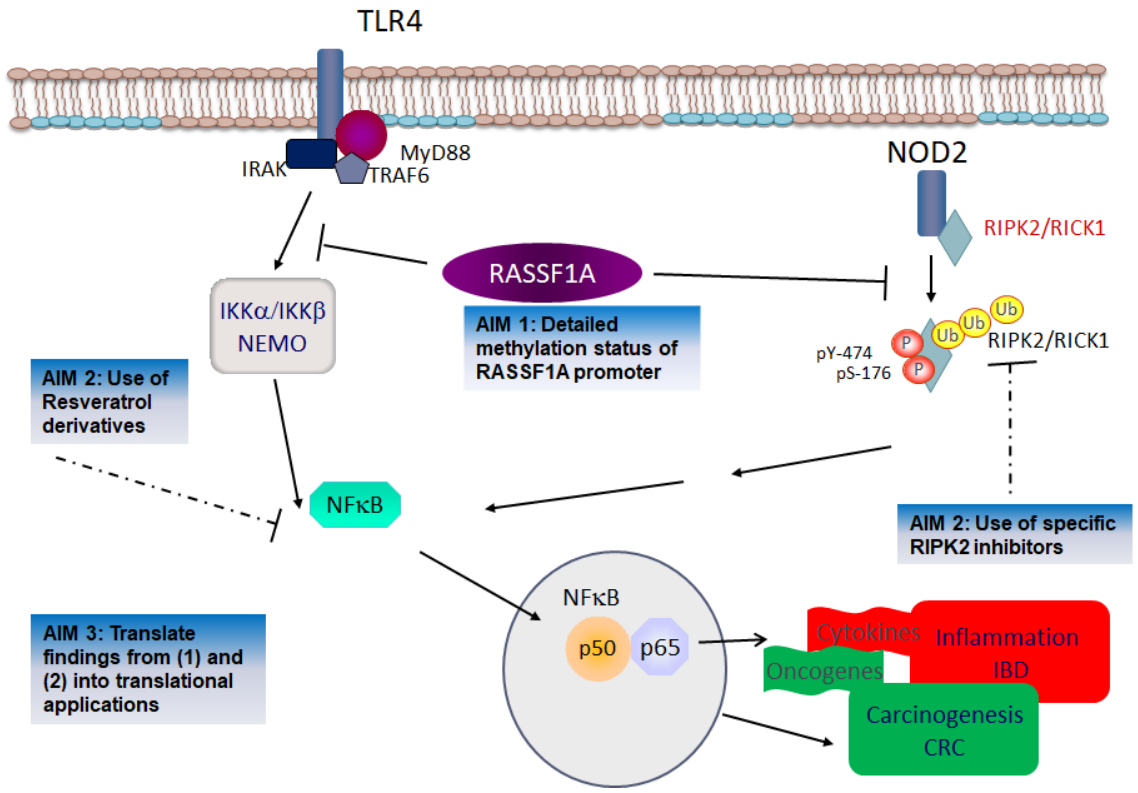


Figure 1.6: **Model for RASSF1A regulation of inflammation and description of research aims for this study.** A key role of RASSF1A is to restrict excessive unwanted inflammation delivered to NFκB through two main arms. TLR4 activation results in the recruitment of several adaptor proteins that eventually lead to the activation of TAK1 kinase that phosphorylates IκB and triggers nuclear translocation of NFκB. On the other hand, NOD2 activation by MDP results in the activation of its obligate kinase RIPK2. Upon interaction with NOD2, RIPK2 gets auto-phosphorylated at two main sites S176 and Y474 and further ubiquitination ensures maximal activation of the RIPK2 kinase. Similarly RIPK2, results in the activation of TAK1 and the nuclear translocation of NFκB. That said, the aims of this study were to (1) explore the detailed methylation status of the RASSF1A especially that reports were not consistent on the extent of RASSF1A methylation. (2) explore the use of general anti-inflammatory compound such as resveratrol derivatives as well specific RIPK2 inhibitors. (3) explore the translational application of any interesting findings of (1) and (2) in human IBD models and samples.

CHAPTER TWO

Materials and Methods

2.1 Animal Work and Mouse Lines

My work involved the use of a mouse model of colitis using 12-14 week old wild type and *Rassf1a*$^{-/-}$ mice [123] to understand the how the different therapeutics affected colitis symptoms in the context of the absence of the Rassf1a tumour suppressor protein. All the animals were on C57BL/6-129 background. Wild type C57BL/6 mice were obtained from Charles River, *Rassf1a*$^{-/-}$ mice were obtained from Louise van der Weyden (Cambridge University). The colonies were maintained at the University of Alberta and animal experiments used for this project were approved under the protocol numbers AUP00218 and AUP00219. Guidelines of the Canadian Animal Care and Use Committee and the animal ethics board at the University of Alberta were followed.

2.2 Chemicals, Buffers, Kits and Antibodies

All chemicals were used according to the manufacturer's specifications as well as in accordance with the protocols set out by the Environmental Health and Safety of the University of Alberta and work Hazardous Materials Information System (WHMIS).

2.2.1 Chemicals

Table 2.1: List of chemicals used and sources

Chemical	Source
Acrylamide	Invitrogen 15512-023
Agarose	Invitrogen 16500-500
Amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF)	Calbiochem 178281
Ammonium persulfate (APS)	BioRad 161-0700
Aprotinin	Fisher BioReagents BP2503-10
β-mercaptoethanol	Sigma Aldrich M6250
Bis-acrylamide crosslinker	BioRad 161-0201
Bovine serum albumin (BSA)	Sigma Aldrich A9647
Bradford protein assay reagent	BioRad 500-0006
Bromophenol blue	Sigma Aldrich B6131
Dextran sodium sulphate (DSS), 36,000-50,000 MW	MP Biomedicals 160110
Dithiothreitol (DTT)	Millipore 3860
DMEM	Fisher Scientific
DMSO (Dimethylsulfoxide):	Fisher Scientific
DNA ladder 100bp	Genedirect DM001-R500
dNTP mix	Qiagen 201901
Eosin	Fisher BioReagents E514-25
Ethidium bromide	BioRad 161-0433
Ethylenediaminetetraacetic acid (EDTA)	Millipore EX0539-1
Ethylene glycol tetraacetic acid (EGTA)	Calbiochem 324626
Ethanol	Commercial Alcohols P016EAAN
Glycerol	Anachemia 43567-360
Glycine	Fisher BioReagents BP3815
Harris modified method hematoxylin	Fisher BioReagents SH26-500D
Horse serum	Gibco 16050-122
Hydrochloric acid	Fisher Chemical A144C-212
Hydrogen peroxide 30% (H_2O_2)	Fisher BioReagents H323
Isopropanol	Fisher Chemical A451
Luminol	Sigma Aldrich A8511
MDP	Sigma
Methanol	Fisher Chemical A452
p-Coumaric acid	Sigma Aldrich C9008

Chemical	Source
PEI	Polysciences, USA
Permount	Fisher Chemical SP-15
PCR grade sterile water	Fisher BioReagents BP561
Phenylmethylsulfonyl fluoride (PMSF)	Thermo Scientific/ Pierce 36978
Polyvinylidene fluoride (PVDF) membrane	Millipore
Potassium acetate	Fisher Chemical 580130
Potassium chloride	Caledon Laboratories 5920-1
Potassium phosphate monobasic	Fisher Chemical P285
Precision Plus Protein Dual Color Standard	BioRad 161-0374
Protein A Sepharose	GE Healthcare
Proteinase K	Promega V3021
RNAlater ® RNA stabilization reagent	Qiagen 76106
RNAse Out	Invitrogen 1077-019
Skim milk powder	Great Value Foods
Sodium acetate	Calbiochem 567418
Sodium citrate dihydrate	Fisher Chemical S279
Sodium chloride	Fisher BioReagents BP358-212
Sodium dodecyle sulphate (SDS)	BioRad 161-0302
Sodium hyperchlorate 4%	Sigma Aldrich 239305
Sodium orthovanadate	Calbiochem 56740
Sodium phosphate dibasic heptahydrate	Fisher BioReagents BP-331
Sodium pyrophosphate	Sigma Aldrich 221368
SsoAdvanced ™ Universal SYBR Green Supermix ®	BioRad 172-5271
Taq Polymerase	New England Biolabs M0273
T-PER Lysis Reagent	Thermo Scientific/ Pierce 78510
Tris base	Invitrogen 15504-020
Triton X-100	VWR VW3929-2
Trypsin	Fisher Scientific
Tween- 20	Fisher Scientific BP337
Vectashield Mounting Medium with DAPI	Vector Laboratories H-1200
Vectastain Elite ABC Kit	Vector Laboratories PK-6100
Xylenes	Fisher Chemical X5
Z-fix	Anatech 170

2.2.2 Kits

Table 2.2: List of kits used and sources

Kit	Source
AllPrep DNA/RNA Mini Kit	Qiagen 80204
Epitect Bisulfite Kit ®	Qiagen 59104
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems 4368814
Metal-enhanced DAB Substrate Kit	Thermo Scientific 34065
PyroMark Q24 Advanced CpG Reagents ®	Qiagen 970922
PyroMark PCR Kit	Qiagen 978703
PyroMark Wash Buffer	Qiagen 979008

2.2.3 Buffers

Table 2.3: List of buffers used and compositions

Buffer	Composition
4X Separating Buffer	1.5 M Tris, 0.4% SDS, pH to 8.7 with HCl
4X Stacking Buffer	0.4 M Tris, 0.4% SDS, pH to 6.5 with HCl
5X DNA loading buffer	3% Bromophenol blue, 3% xylene cyanol FF, 30% glycerol
10X Phosphate Buffered Saline (PBS)	1.347 M NaCl, 0.027 M KCl, 0.043 M Na_2HPO_4, 0.014 M $KH_2PO_4 \cdot 7H_2O$, pH to 7.4
10X Running Buffer	0.125 M Tris, 0.96 M Glycine, 0.5% SDS, pH to 8.3
10X Tris Buffered Saline (TBS)	1.5 M NaCl, 0.5 M Tris, pH to 7.4
Sample Buffer for SDS-PAGE	26% Glycerol, 5% SDS, 0.174 M Tris, pH to 6.8 with HCl, 0.02% Bromophenol blue, add fresh 0.04% β-mercaptoethanol
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
TBS-Tween Buffer	500 mM Tris pH 7.4, 100 mM NaCl, 0.05% Tween-20
Tris-acetate-EDTA (TAE) Buffer	400 mM Tris-acetate pH 8.5, 2 mM EDTA
Wet Transfer Buffer	0.025 M Tris, 0.192 M Glycine, 20% Methanol

2.2.4 Antibodies

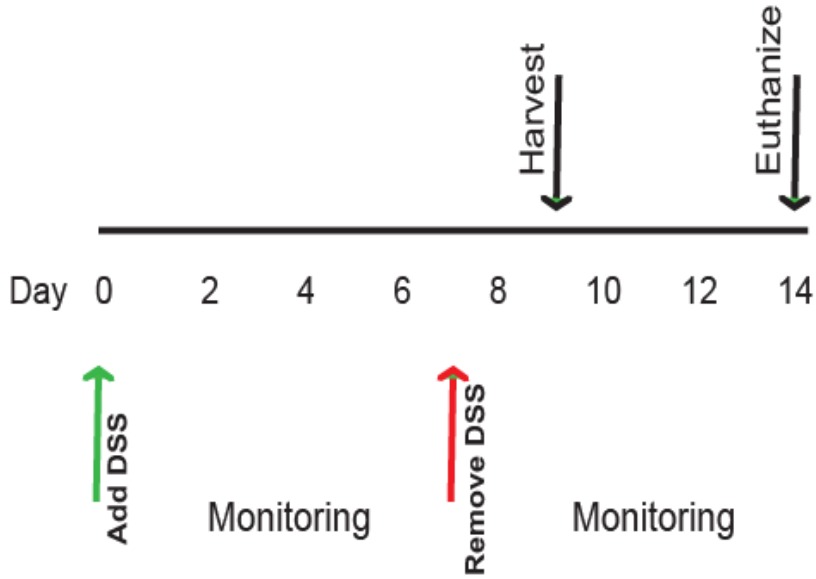
Table 2.4: List of antibodies used, sources and applications

Antibody	Source	Application
AMPK	Cell Signalling 3532	1:1000 WB
p-AMPK	Cell Signalling 2535S	1:1000 WB, 1:200 IHC
RIPK2	Santa Cruz sc-22763	1:200 WB
S176 RIPK2	Cell Signalling	1:100 WB
Y474 RIPK2	In house	1:200 WB, 1:200 IHC
RASSF1A	In house	1:50 IHC
β-tubulin	Sigma-Aldrich T5201	1:1000 WB
Anti-Mouse HRP	GE NA931V	1:7000 WB
Anti-Rabbit HRP	GE NA934V	1:7000 WB
Anti-Mouse Biotin	Jackson Labs 711065152	1:500 IHC
Anti-Rabbit Biotin	Jackson Labs 715065150	1:500 IHC

2.3 Acute Mouse Model of Colitis

Intestinal colitis can be modeled in mice chemically or genetically. We used DSS with drinking water at a 3% weight to volume (w/v). 3% DSS was given to the mice for 7 days to induce acute injury and then the mice were given 7 days for recovery. Monitoring for colitis like symptoms includes piloerection, bloatedness, tremors, lack of active movement, rectal bleeding and weight loss. All indices were given a numerical value of 1 to 5, with 5 being most severe (adapted from Madsen *et al* (2001) [265]). Animals were followed up for survival up to 14 days and for harvesting tissues up to 9 days. For weight loss a score of 1 was given for a loss of <5%, 2 for 5-10%, 3 for 10-15%, 4 for 15-20% and 5 for >20%. Figure 2.1 shows the approach of our acute inflammation model. We also measured colon length at time of harvest as an indication of colon health and reflection of inflammatory damage. For experiments involving resveratrol,

resveratrol was mixed with the mice diet at a concentration of 0.1%. Mice were fed the resveratrol diet for two weeks prior to DSS and continued to feed on resveratrol throughout the experiment. An approximate human equivalent dose (HED) was estimated to be about 800 mg per day.



Disease activity parameter	Score
Piloerection	0 (none)-5 (severe)
Bloatedness	0 (none)-5 (severe)
Tremors	0 (none)-5 (severe)
Body weight loss	1-5%: score of 1
	5-10%: score of 2
	10-15%: score of 3
	15-20%: score of 4
	More than 20%: score of 5
Rectal Bleeding	0 (none)-5 (severe)
Movement	0 (none)-5 (severe)
Diarhhea	0 (none)-5 (severe)
Sum of all parameters gives the disease activity index DAI	

Figure 2.1: Model demonstrating the acute colitis model: showing parameters and scoring used to obtain a disease activity index (DAI)

2.4 Tissue Handling

Different tissues including colon, kidney and liver samples were collected for both genetic and molecular analysis. Samples were either submerged in Z-fix for histological processing or in RNA later for nucleic acid analyses. RNA and DNA were isolated using the Qiagen AllPrep DNA/RNA spin column nucleic acid extraction kit according to the manufacturer's protocol. Nucleic acid concentration was determined using the Thermo Scientific Nanodrop. Samples intended for protein analysis were flushed with 1X PBS to clear fecal matter and immediately placed in ice-cold T-PER lysis buffer with fresh aprotinin (0.1%), phenylmethylsulfonyl fluoride (PMSF) (0.2%), sodium pyrophosphate (NaPP) (0.1%), sodium orthovanadate (1%), SL protease inhibitor cocktail (0.2%). Samples were then homogenized using a Fisher PowerGen Handheld homogenizer and centrifuged at 4ºC at max speed for 10 min. The supernatant containing proteins were stored at -80ºC for further use. Protein concentration was determined using the Bradford protein assay.

2.5 Tissue Histology and immunohistochemistry

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining were done using standard techniques. Formalin fixed, paraffin embedded sections were de-paraffinized and re-hydrated. Antigen retrieval was done by boiling in sodium citrate buffer. Endogenous peroxidase activity was quenched with 3% H_2O_2. Sections were blocked in 2% BSA + 2% donkey serum for 1 hr at room temperature, and incubated in primary antibody as indicated overnight at

4°C. Sections were incubated in 1:500 biotinylated secondary antibody for 1 hr at room temperature and signal amplification and detection was done using the VECTASTAIN Elite ABC Kit and the Metal Enhanced DAB Substrate Kit. Counterstaining was done using Harris' modified hematoxylin.

2.6 Immunoblotting

For protein analysis and quantification, discontinuous denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins from protein samples. Generally, a 7.5-10% polyacrylamide separating gel with a 5% stacking gel was used for most proteins; 40 µg of total protein was loaded per lane. Samples were electrophoresed using vertical electrophoresis using the CBS Scientific system, generally at 90 V till the loading dye crossed the stacking gel and then at 130 V for about 1.5 hrs. Proteins were transferred to methanol activated polyvinyl difluoride (PVDF) or nitrocellulose (mainly for p-AMPK and AMPK proteins) membrane using a wet transfer for large proteins (100 V for 1.5 hrs). Membranes were rinsed in ddH_2O, blocked in 5% milk in 0.01% TBS-Tween for 40 min at room temperature and transferred to primary antibody in 5% milk in 0.01%TBS-Tween at 4°C overnight with rocking. After primary antibody incubation, membranes were rinsed in 0.01% TBS-Tween for 5 min three times, and transferred to secondary antibody in 5% milk in 0.01% TBS-Tween for 2 hrs. Membranes were then rinsed again for 5min three times before incubation with enhanced chemiluminescence (ECL) detection reagent (in house). Signal detection was captured using X-ray film or the Alpha Innotech imaging system. Quantification was done using ImageJ software and

normalization was done first against housekeeping protein and then against non-treated samples or controls. Stripping and re-probing of membranes was done when needed using stripping buffer and fresh BME at 50°C for 30 min, then washed 3X with wash buffer. Blocking and antibody incubation was the same as before. For antibodies against tyrosine phosphorylated proteins 5% bovine serum albumin in 0.01% TBS-Tween was used in substitution primary antibodies only.

2.7 PCR and RFLP Analysis

For the Polymerase Chain Reaction (PCR) reaction for *TP53* analysis, each PCR reaction contained 1 x One*Taq* Standard Reaction Buffer (NEB), 0.3 µmol/L each primer, 3 µmol/L of each dNTP, 100 ng genomic DNA, 0.1U OneTaq Hot Start DNA Polymerase (NEB) in a final volume of 8 µL. The PCR conditions were as follows: denaturation at 94℃ for 4 min, followed by amplification for 40 cycles at 94℃ for 30 s, at 56℃ for 15 s, 68℃ for 40 s, with final extension at 68℃ for 10 min. The PCR products were checked on an agarose gel first 3 times and PCR appeared to work robustly, so for the rest of the analysis gel electrophoresis of PCR products was omitted. For Restriction Fragment Length Polymorphism (RFLP) analysis, 8 µL of PCR product was digested with 2 U of *Bstu*I at 60℃ for at least 4 hrs. DNA fragments were run on a 2% agarose gel stained with ethidium bromide. The Arg allele was cleaved by *Bst*UI, yielding two fragments 213 and 140 bp. The Pro allele was not cleaved by *Bst*UI and had a single 353 bp band. Heterozygotes contained all three bands.

2.8 Real time Polymerase Chain Reactions (qPCR)

Total RNA was isolated from biopsied using the QIAGEN Allprep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and quantified with a NanoDrop ND-1000 (PeqLab, Erlangen, Germany). 2 µL of RNA was treated with DNAse I (Amplification Grade, Invitrogen) at 37^{o}C for 30 min to eliminate possible DNA contamination and reverse transcribed by using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). cDNA was diluted 1:10 with nuclease-free water. Quantitative analysis of specific mRNA expression was performed by real-time PCR on LightCycler® 96 System (Roche). The 20 µL reaction mix contained 1.5 µL of 5 µM of each primer and 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad), 3 µL of nuclease-free water, and 4 µL of diluted cDNA. No-template controls were run on each plate to test for the contamination of any assay reagents. The thermocycling conditions were initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s and extension at 60°C for 30 s. Melting curve analysis was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product. Assays were done in duplicates. The amount of target was calculated by the formula, $2^{-(C_t Gene- C_t-GAPDH)}$×1,000, in which C_t is the threshold cycle value. All qPCR results were repeated 3 times for accuracy.

2.9 Pyrosequencing and Methylation Analysis

The methylation experiments were done using the Qiagen Pyromark Advanced kit according to the manufacturer's instructions. Briefly, genomic DNA isolated various tissues and was bisulfite modified using the Qiagen Epitect Bisulfite Conversion kit, using the instructions for "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA from Low-Concentration Solutions" according to the manufacturer's instructions. It is important to note that the samples analyzed for tumour and normal tissues were not matched from the same patient. Normal tissue was obtained from patients undergoing breast reduction surgery. The resulting bisulfite modified DNA was then used as a template to amplify the region of interest using a biotinylated primer set from Qiagen. A small amount of DNA was run on an agarose gel to confirm product amplification, and the 15 µL of PCR product was used in the Pyromark Advanced system. Amplified product was loaded into the Pyromark machine along with the appropriate binding buffers, reagents (Pyromark Advanced Kit), and a sequencing primer, and the Pyromark Q24 was used to analyze the nucleotide sequence and compare it against the expected sequence of the region of interest. Bisulfite modification converts cytosine nucleotide to uracil; however, methylated cytosine residues are protected from this effect. Following bisulfite cleanup and PCR amplification, any uracils will have been converted to thymine, while methylated cytosines will remain cytosine. The Pyromark system measure whether a cytosine or a thymine residue is present at each predicted CpG island and generate a percentage of methylation based on the product ratios. A positive control includes a

synthetically methylated genomic mouse DNA sample and a negative control contains no template DNA.

The Assay1 covers 11 CpGs in promoter and 1 CpG in exon1 of the *RASSF1A*. The Assay2 covers 20 CpG located right upstream of the 12 CpGs covered by the Assay1 (Figure 1). The PCR was performed using PyroMark PCR Kit (Qiagen) in a volume of 25 µL containing 12.5 µL of 2x PyroMark PCR Master Mix, 1.25 µL of each PCR primer (5 µM), 2.5 µL of 10x Coral Load Concentrate, 6.5 µL high purity water and 1 µL of bisulfite-treated template DNA. The PCR cycling programme for both primer sets was composed of an initial Taq activation/DNA denaturation step at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s. The program was finished by a final elongation step at 72°C for 10 min. 7 µL of PCR products were visualized by gel electrophoresis, and 10 µL were subjected to the sample preparation process for pyrosequencing. DNA was mixed with streptavidin-coated sepharose beads, followed by strand separation and washing utilizing the vacuum prep tool (Qiagen). The single-stranded DNA bound to the sepharose beads was mixed with 20 µL of 0.375 µM sequencing primer in annealing buffer and heated to 80°C for 5 min. For the sequencing reaction PyroMark advanced reagents were used (Qiagen). The sequencing results were analyzed using the Advanced PyroMark software (Qiagen). A control PCR reaction without template DNA (non-template control) was included in the assay. Pyromark assays were done 2 times for accuracy.

2.10 Collection and Handling of Human Samples

We collected blood samples and approximately biopsy samples from pediatric and adult IBD and control patients attending regular clinic appointments at the University of Alberta. The human handling of samples was approved under protocol No. Pro00001523 (study No. RES1598). We have also collaborated with researchers in the Centre for Excellence in Gastrointestinal Inflammation and Immunity Research (CEGIIR) at the University of Alberta, the Alberta IBD Consortium, and the Alberta Health Services pathology bank to obtain additional patient biopsy samples as necessary. Samples were collected from patients with active disease as well as those in remission from areas of active inflammation as well as from areas showing no gross inflammation when available. The non-IBD group consists of healthy blood donors with no prior history of inflammatory bowel syndrome, IBD or cancer. For DNA extraction from blood samples, genomic DNA was extracted from peripheral blood collected by venous puncture. Blood was collected into PAXgene Blood DNA Tubes (QIAGEN, Country of Origin, Germany) and kept in a fridge at 4℃ until DNA extraction (1-2 days). DNA was extracted using PAXgene Blood DNA Kit (QIAGEN, Country of Origin, Germany) according to the manufacturer's instructions.

The breast cancer tissues were obtained from the Alberta Cancer Research BioBank; colorectal cancer samples were obtained from the University of Calgary Gastrointestinal/Hepatobiliary Tumor and thyroid cancer samples from Dr. Todd McMullen. All tissues were snap frozen for 30 min to preserve the RNA.

2.11 Cell lines and Cell Culture

The ModeK normal mouse intestinal cell line (grown in Gibco DMEM high glucose supplement with 5% FBS, 1% Non-essential amino acids and 7.5 mL 1 M HEPES) was generously provided by Karen Madsen (University of Alberta). The colon cancer cell line HCT116 (obtained from Dr. Bert Vogelstein), was maintained in RPMI 10% BGS. The colon cancer cell line SW480 was maintained in DMEM supplemented with 10% BGS. All cells were maintained in a 37^{o}C/5% CO_2. Transfections were performed using the linear 25 kDa polymer, polyethyleneimine (PEI) obtained from Polysciences, USA (Catalog #23966-2). PEI transfections were done generally by mixing PEI/DNA in a ratio of 4 µL PEI/1 µg DNA in serum-free media to achieve efficiencies of 30-50%. For the methylation status experiments: T84 and HT29 (Dr. Eytan Wine), HemaLP, A2070s, A2780cp, A2058, WM793 and WM35 (Dr. Sujata Persad, University of Alberta), PNTIA, Hs578Bst125, HepG2, Hep3B, DU145, PC3, Hep3B (CRUK, London), SH-SY5Y, SKNAS, Nub7, Be(2)C, A2780cp, LAN-1, ImR-32, GoTO, KAN (Dr. Roseline Godbout, University of Alberta), PANC1 and CAPAN,, MDAMB-468, SKRB3, HTB26, MCF10A, hTERT HME, MDAMB453, MDAMB361, BT20, ZR75-1, HTB25, T47D (Dr. Mary Hitt/Dr. David Murray/Bonnie.Andrais, University of Alberta and Cross Cancer Institute, OVCAR3, SKOV3, Es-2 2c (Dr. Fu, University of Alberta), MDAMB231, IBC-3 (Dr. Woodward, MD Anderson Cancer Center) and DLD-1.

2.12 Cell Viability: MTT Assay

Cell viability assay was determined using the MTT colorimetric assay. Briefly, cells were seeded in 96-well plates at a density of 3 x 10^4 per well, and were allowed to attach for 24 hrs. Drugs were added to the corresponding 96-well plate containing 100 µL of media, and then were incubated for 24 h at 37 ºC. After that time, the media was removed (aspirated out) and the wells were washed one time with PBS before the addition of the MTT solution in PBS. The plate was then incubated for 4 hrs at 37 ºC. Finally, the insoluble formazan crystals were dissolved in a solution of DMSO, and the absorbance of each well was read at 560 nm. The percentage of cell viability was normalized to cells with no treatment, which were set to a value of 100 % viability. Each compound was assayed at least in triplicates and the experiment was performed at least three times, and the results are expressed as mean ± SD.

2.13 Gene Reporter Assay for NFκB activity

Dual-Luciferase Reporter Assay System (DLR assay system, Promega, E1910) was used to perform dual-reporter assays on NFκB Luciferase and Renilla Luciferase (internal control). Briefly, cells were equally seeded at a density of 3 x 10^4 in 6-well plates and allowed to attach for 24 hrs. Prior to transfection, cells were washed with serum free media 3 times. Dual transfection was done using PEI adding 3 µg of NFκB Luciferase construct and 60 ng of Renilla Luciferase construct as an internal transfection control. 24 hrs after transfection, cells were treated with the different drugs for 24 hrs as well and

stimulated with LPS for 4 hrs in serum free media. Cells were then lysed using the passive lysis buffer provided by the kit for 30 mins on ice. 20 µL of cell lysate were transferred in 96-well plate. Luciferase assays were analyzed based on ratio of Firefly/Renilla to normalize cell number and transfection efficiency.

2.14 Cell Cycle Analysis

For cell cycle analysis, cells were seeded in 6-well plates, allowed to grow to 60% confluency and treated with DMSO or the resveratrol derivatives for 24 hrs. Detached cells in the supernatants in addition to the attached cells were collected by centrifugation at 500 g. The pellets were washed with phosphate-buffered saline (PBS) (Gibco), fixed with 70 % ice-cold ethanol and stored at -20ºC overnight. Cells were then washed twice with PBS and incubated with RNAse A (Sigma) for 1.5 hrs at 37ºC before staining with propidium iodide (PI) (Molecular Probes, OR, USA) for 30 min. Steps involving measuring the fluorescence using FACS were done with the guidance of Vrajesh Pandya from Dr. Ing Swie Goping's group. The fluorescence intensity was measured by flow cytometry using a Fluorescence Activated Cell Sorter (FACS) flow cytometer (FL-2 channel). Cell cycle analysis was performed by Vrajesh Pandya using the Cell Quest program. Experiments were done three times for conformation.

2.15 Interleukin/Chemokine Analysis

Chemokine/Cytokine ELISA array performed by Eve Technologies, Calgary, AB. Briefly, cells were seeded equally in 6 well plates and allowed to adhere for 24 hrs then treated with the different concentrations of resveratrol and

derivatives for 24 hrs and 500 µL of supernatant was collected and sent on dry ice to Eve Technologies for analysis.

2.16 Mitochondrial Membrane Potential Assay (TMRE)

Cells are grown at a concentration of 10^6/mL in 6-well plates and treated with the drugs at 100 µM for 24 hrs. Cells were harvested and pelleted and then mixed well before adding 200 µL of the TMRE dye. Samples were placed in 96-well plates. CCCP was used as a positive control and added at 20 µM for 10 mins. Cells were then stained with 200 nM TMRE in PBS for 30 min at 37ºC. Samples were centrifuged and washed once with PBS and then suspended in 0.2% BSA in PBS. Cells were filtered with membrane and analysis was done using the FL-2 channel for measuring TMRE. 20,000 cells per sample were collected. Steps involving the use of FACS machine were done by members of Dr. Ing Swie Goping's group. Experiments were done three times for conformation.

2.17 Assay of SIRT1 activity:

The BIOMOL (Enzo Life Sciences) assay was performed as previously described [207] in collaboration with Dr. Basil Hubbard. Briefly, 0.5 U of recombinant SIRT1 was used for each reaction, and the β-NAD and FdL-p53 peptide concentrations used were 200 µM and 20 µM, respectively. 0.5 µL of vehicle or test compound was added to each 50 µL reaction. Fluorescence values corresponding to SIRT1 activity were calculated by subtracting parallel

reactions in the absence of β-NAD from those in the presence of β-NAD ($F_{corrected} = F_{+NAD} – F_{-NAD}$).

2.18 RIPK2 *In Vitro* Kinase Assay:

For this protocol, samples were lysed in 1X RIPK2 lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, with freshly added 1 mM β-glycerophosphate and protease inhibitors [PMSF and aprotinin]) and immunoprecipitated overnight using 1.5 µg rabbit anti-RIPK2 antibody. The next day, protein G sepharose was used to immunoprecipitate the RIPK2 protein complex IP for 1.5 hrs. Following this incubation, samples were washed once in 1 X kinase wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Trition X-100, 1 mM EDTA) followed by 2 washes with 1 X Kinase buffer (30 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$). After last wash, 20 µL water was added to beads, followed by 1 X Kinase buffer and ^{32}P-γ-ATP and kinase reaction was allowed to proceed for 45-60 mins at 30^{o}C. Protein loading dye was added to the mix and samples were boiled and separated on an SDS-PAGE gel. The gel was dried and then exposed to X-ray film to capture the autophosphorylation of RIPK2.

2.19 TCGA Database Analysis

Level 3 TCGA RNAseq V2 gene expression data were obtained from the TCGA Data Portal in August 2014 for breast cancer (BRCA), colorectal cancer (COAD), lung adenocarcinoma (LUAD) and pancreatic adenocarcinoma (PAAD). Correlations were tested between the genes (*DNMT1, DNMT3B, MOAP-1*) and the isoforms (*RASSF1A*: uc003dea; and *RASSF1C*: uc003dab.1) expression

values (log_2 [RSEM normalized values+1]). A value of 1 was added to RSEM normalized values to avoid infinite values in log calculations. All analyses were conducted and visualized in the RStudio programming environment (v0.98.501). R/Bioconductor packages ggplot2 and plyr were used where appropriate. Analysis and plotting was done by Krista Vincent and Lynne Postovit.

2.20 Statistical Analysis

Statistical analyses were performed using Students t-test (two-tailed), as indicated using the GraphPad Prism 5 software. Regression and correlation analysis of average methylation percentage versus individual CpG methylation percentage was performed using GraphPad Prizm 5 software as well. Survival analysis was done using Kaplan-Meier curves. Chi-square test was done for frequency analysis in case control reports. Statistical analyses for chapter four were reviewed by Sung Hyun Kang from Biostatistics Service Core of Women and Children's Health Research Institute at the University of Alberta. Results were considered significant if the p-value is <0.05. Error bars in all graphs represent the standard error. Statistics for individual experiments are indicated with the graph or in figure legends. The inhibitory concentration (IC_{50}) calculated in chapters 5 and 6 represents the relative 50% inhibitory concentration from the maximum as there was no true 0% inhibition in the graphs.

CHAPTER THREE

RASSF1A Site Specific Methylation Patterns and Correlation with *MOAP*-1 and *RASSF1C* Expression

Abstract

Epigenetic silencing of *RASSF1A* is frequently observed in numerous cancers and has been previously reported. The promoter region of *RASSF1A* has been documented to have 75 CpG sites and very few studies demonstrate how the methylation of these sites affects expression. In addition, the expression relationship between *RASSF1A* and its downstream target, Modulator of apoptosis 1 (MOAP-1), is poorly understood. In this study, our aim was to explore the mRNA expression of *RASSF1A, MOAP-1* and the well characterized splice variant of RASSF1, *RASSF1C,* in cancer cell lines and primary tumors. We hypothesized that *RASSF1A* methylation in IBD and cancers would be high across the CpG sites investigated and this would correlate with an increased expression of *RASSF1C* and a decreased expression of *MOAP-1*. We confirmed that the *RASSF1A* promoter is robustly methylated in most solid tumors, however contrary to our expectation a distinct pattern of methylation in colorectal cancer was observed. Interestingly, *MOAP-1* mRNA expression positively correlated with *RASSF1A* expression in numerous cancers whereas *RASSF1C* expression remained the same or was increased in cell lines or tissues with epigenetic loss of *RASSF1A*. We speculate that MOAP-1 and RASSF1A may be more intimately connected than originally thought and expression of both are warranted in

experimental designs exploring the biology of the RASSF1A/MOAP-1 molecular pathway. Furthermore, *RASSF1C* mRNA levels were elevated in colorectal cancer and confirmed previous data of an oncogenic role for RASSF1C.

3.1 Introduction

We have discussed in earlier chapters the importance of RASSF1A as a critical regulator of intestinal inflammation through restricting the activation of NFκB in response to pathogenic signals. Its role as a tumour suppressor and as restrictor of inflammation has attracted a lot of interest to understand how it is regulated and what drives its expression loss. The family of RASSF proteins contains 10 members with various functions and structures. RASSF1A and RASSF1C are the most commonly studied members and their functional correlation and relationship has acquired wide interest recently. *RASSF1A* and *RASSF1C* are located on the same chromosome and originate by alternate splicing and using different promoters. The methylation status of the *RASSF1A* promoter has been extensively investigated and methylation induced silencing is very common in solid tumours, less common in hematological cancers. Expression of RASSF1A can be regulated by epigenetic mechanisms as well as post-translational events, and a recent report found that TP53-directed DNMT1 methylation is also involved in the loss of *RASSF1A* expression [266]. The detection of methylated *RASSF1A* promoter is not limited to solid tissues but has also been detected leukocytes [267], urine and nipple aspirates [107]. This highlights the importance of using non-invasive mechanisms to detect *RASSF1A* promoter methylation and its usefulness as a biomarker of disease. RASSF1A is

well characterized for its tumor suppressive properties in association with TNF-R1, TRAIL or Fas activation [127]. Our group has also showed the importance of RASSF1A in restricting NFκB activation and uncontrolled inflammation in intestinal cells [122]. The role of RASSF1A in various biological systems and models is further investigated by the use of heterozygous (*Rassf1a*$^{+/-}$) and homozygous knockout (*Rassf1a*$^{-/-}$) mice that are viable and fertile. *Rassf1a*$^{-/-}$ mice experience a higher incidence of tumours by 12-16 months and are more sensitive to developing tumours in response to chemical carcinogens [123, 124]; we have noticed spontaneous colitis symptoms in these mice after 6 months. In addition to the ability of *RASSF1A* to promote extrinsic cell death via TNF-R1, TRAIL and Fas, it also modulates apoptosis via its downstream effector, MOAP-1. MOAP-1 can also promote intrinsic cell death [121, 268], activation of BH3-containig proteins and is regulated in cancer [269] by ubiquitin-dependent degradation. MOAP-1 has also been reported to associate with anti-apoptotic proteins such as Bcl-2 and Bcl-X_L [270]. The knockdown of MOAP-1 in cells results in reduced sensitivity to apoptotic stimuli including staurosporine, serum withdrawal, UV radiation, and TNF-α [109]. Our group has previously reported that RASSF1A and MOAP-1 are both recruited to TNF-R1 receptors to drive the apoptotic pathway, and the knockdown of *RASSF1A* by small interfering RNA (siRNA) resulted in a significant loss of death receptor mediated apoptosis [109]. Furthermore, the ectopic expression of MOAP-1 causes a significant reduction of foci growth on soft agar and reduced the proliferative capacity of H1299 cells [269]. Although the CpG island of *MOAP-1* is 954 bp long containing about 110

CpG sites within the promoter region, it does not appear to be regulated by promotor specific methylation in cancers. Since RASSF1A is involved in cell death [121], cell cycle control [271, 272] and regulation of NFκB [122], the biology of RASSF1A appears to suggest that MOAP-1 and RASSF1A maybe more linked than originally thought to suggest overlap of function. In this study, we wanted to explore detailed CpG methylation of *RASSF1A* and link it to *RASSF1C* and *MOAP-1* expression.

3.2 Results:

3.2.1 Correlation between methylation of individual CpG sites and overall methylation in cancer cell lines and cancer patients

To better understand the methylation status of *RASSF1A* and whether that follows a similar pattern in different cancer cell lines and how this translates into patient tissues, we utilized the technique of pyrosequencing which overcomes the limitations of other techniques such as methylation specific PCR (MSP) or combined bisulfite modification restriction enzyme analysis (COBRA). Two assays covering 32 CpG sites in the promoter region of *RASSF1A* were developed (Figure 3.1) to analyze site specific methylation of these CpGs. When the methylation percentage of individual CpG sites was plotted against overall methylation average in that cell line, the methylation at individual CpG sites correlated with the average methylation with some variation in the methylation percentage of each CpG (Figure 3.2). Similarly, methylation results from breast tumour tissues from various patients obtained from the Alberta Cancer Research BioBank showed that methylation status of individual CpGs correlated with

overall average methylation (Figure 3.2). Moreover, analysis of thyroid tumour samples showed a relatively high level of methylation through most CpG sites studied (Figure 3.3). In contrast, colorectal cancer patients showed a distinctive methylation pattern especially for CpG sites 1-7, which apparently contributed most to the average methylation of the 32 CpGs in CRC tissues (Figure 3.4).

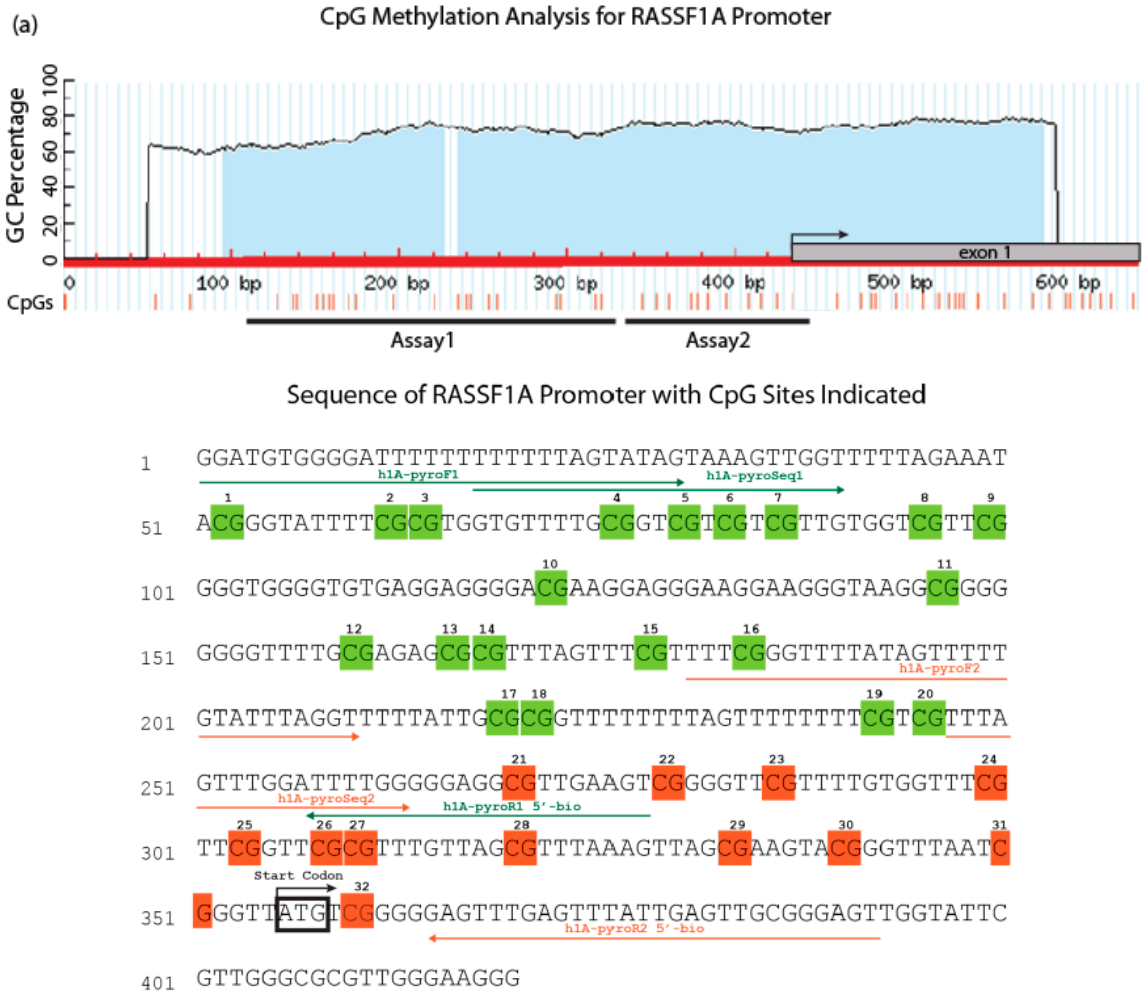


Figure 3.1: *RASSF1A* CpG island map and the pyromark assays. **(a)** Part of *RASSF1A* CpG island, as predicted by Methprimer [1]. CpG sites are indicated by red strikes. Pyrosequencing assays coverage is shown as black lines. Start codon is indicated by an arrow.

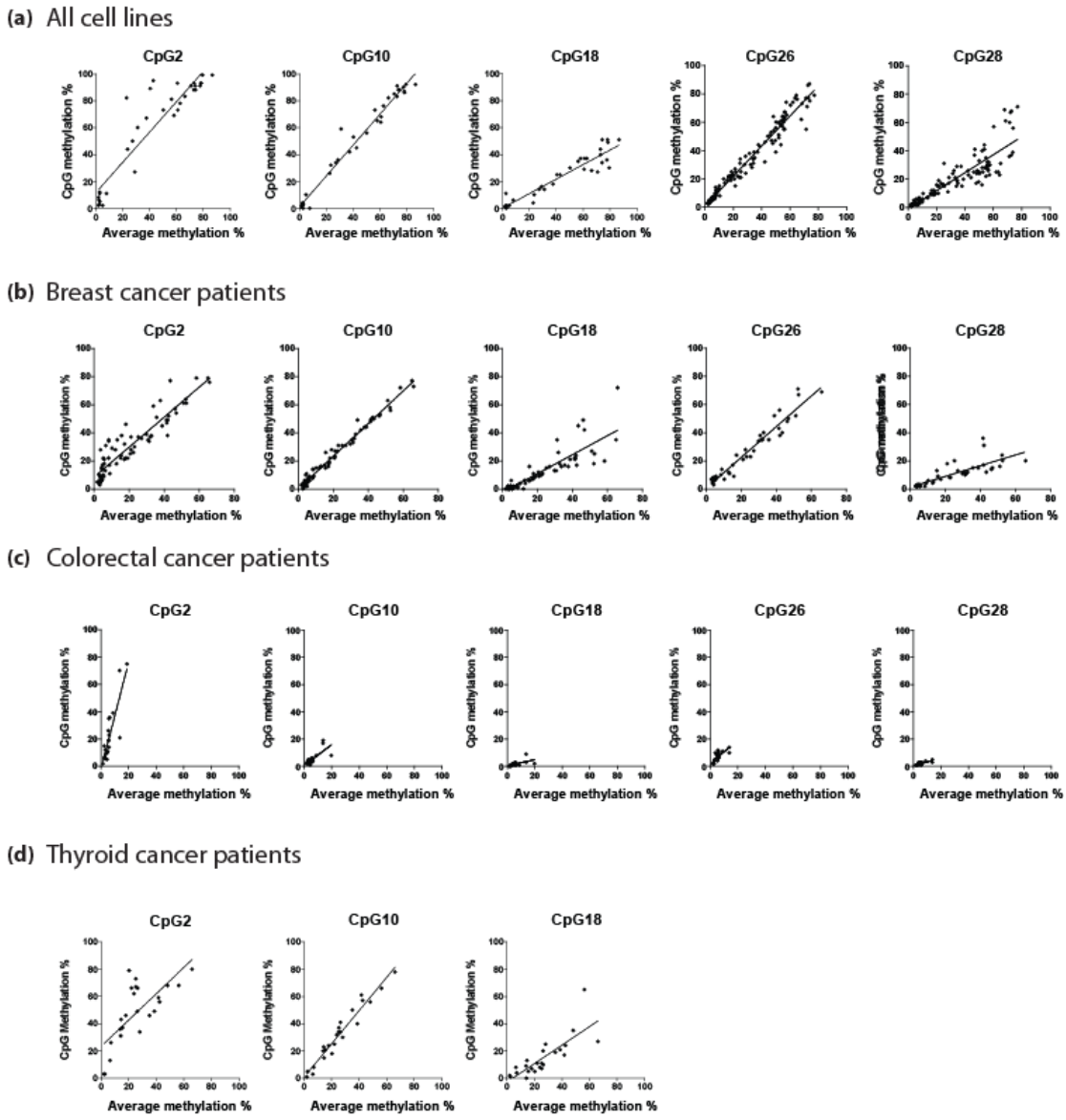


Figure 3.2: Correlation analysis of *RASSF1A* promoter methylation at the individual CpG site with average methylation % of 32 CpGs in (a) cell lines (b) and breast cancer (c) colorectal cancer and (d) thyroid cancer patients. The x-axis indicates average methylation of 32 CpGs in samples. The y-axis indicates individual CpG methylation percentage measured by pyrosequencing. Methylation of all CpGs correlated well with average methylation (r2 values ranged between 0.7926 for CpG28 and 0.9939 for CpG9 for cell lines; and between 0.5777 for CpG27 and 0.9886 for CpG7 for breast cancer patients; all p values are <0.0001).

When the first 7 CpG sites were not accounted for in the results from CRC tissues, the average methylation was much lower, and not indicative of the overall methylation. This had led us to defining the CpG sites 1-7 as potential hotspots of methylation in inflammatory bowel disease and colorectal cancer.

3.2.2 Methylation status of individual CpG sites in cancer patient tissues

The methylation of *RASSF1A* has been previously investigated for thyroid cancer using MSP and a recent meta-analysis of 11 studies revealed that *RASSF1A* methylation and loss may contribute to the risk of papillary thyroid cancer [273]. Our detailed site specific pyrosequencing of the *RASSF1A* promoter in 5 thyroid cancer patients confirmed a difference and much higher methylation in primary tumours as compared to normal thyroid tissues (Figure 3.3). Conversely, the methylation of *RASSF1A* has been extensively studied in colorectal cancer using methods that were not site specific and thus reports varied on percent of methylation from 0% [274] to up to 80% in other studies [275]. A possible underlying explanation is that the regions chosen for amplification and studying could have missed important susceptible regions with higher methylation. Based on our results, a unique pattern of methylation was evident for colorectal cancer in contrast to the other cancers investigated. A hot spot of methylation appeared around the CpG sites 1-7 (Figure 3.4). Interestingly, a similar pattern of methylation for *RASSF1A* was observed in tissues from IBD patients (Figure 3.4).

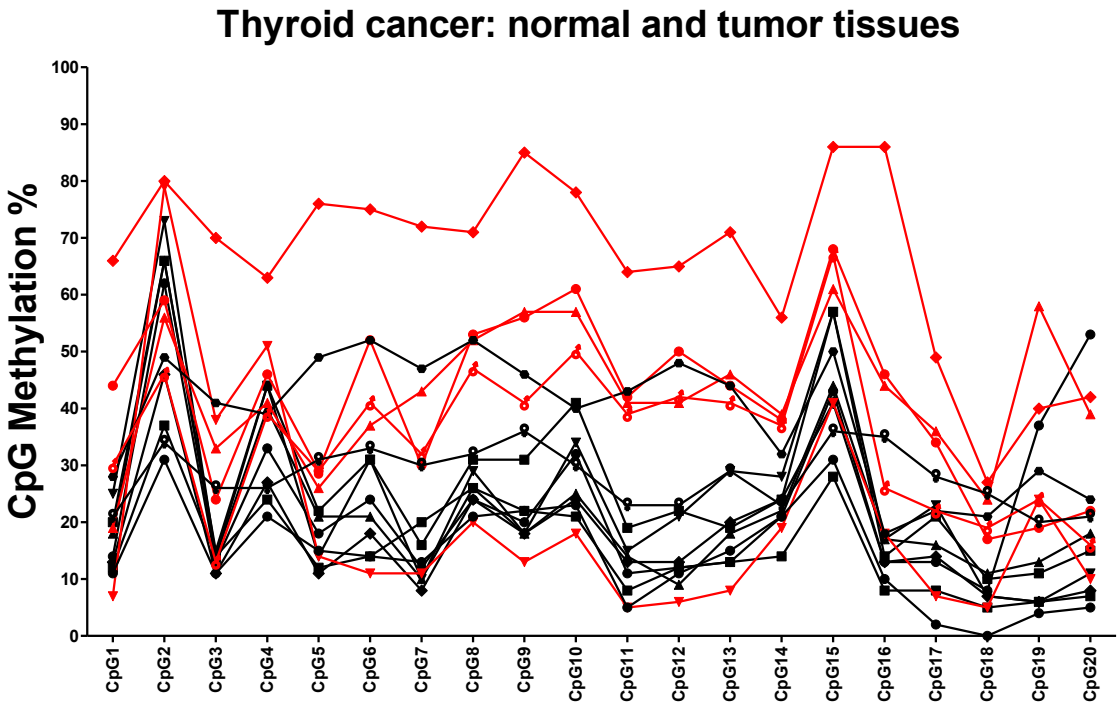


Figure 3.3: Methylation percentage of the individual CpGs in *RASSF1A* promoter in thyroid cancer patients. Red lines represent the methylation patterns in the primary tumour tissue (n=5) and black lines represent methylation patterns in normal thyroid tissues (n=9).

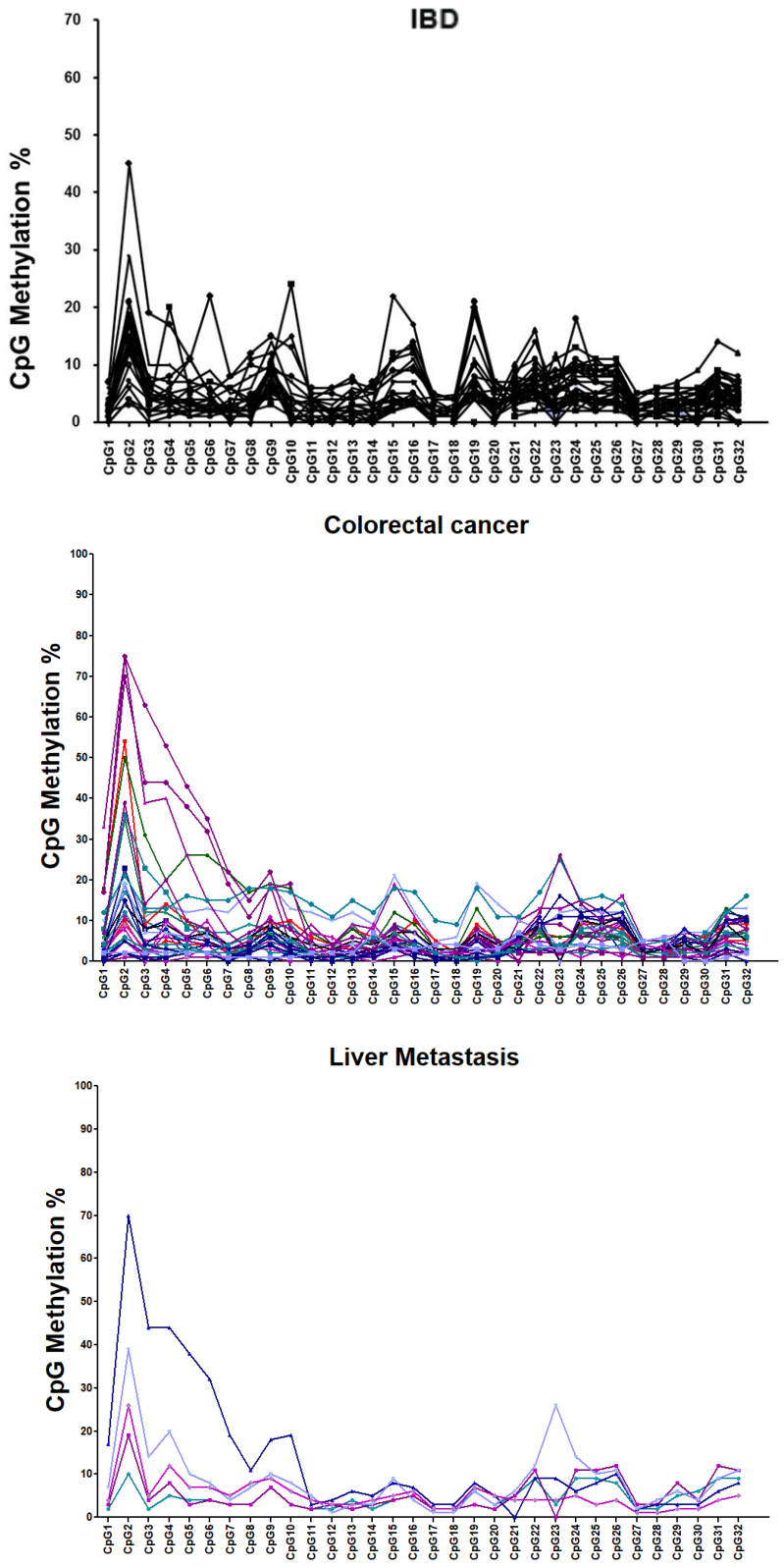


Figure 3.4: Methylation percentage of the individual CpGs in *RASSF1A* promoter in IBD patients (n=45) colorectal cancer patients (n=24) and colorectal cancer liver metastasis (n=5).

The importance of the hotspot of methylation is also observed in the metastatic lesions in the liver in CRC patients (Figure 3.4), which suggests that the metastatic tumours resemble the primary CRC tumour when it comes to the methylation status of *RASSF1A*. Intriguingly, the hotspot of methylation in colorectal cancer is potentially one explanation for the variable results found in the literature. Analysis of more CRC tissues samples is still required to completely understand why there is such a localized hotspot and whether this is enough to drive expression loss or not. Additionally, more samples from different metastatic tissues need to be explored to reach definitive conclusions.

3.2.3 *RASSF1A* promoter methylation in colorectal cancer cell lines

The methylation status of colorectal cancer cell lines revealed variable patterns and extent of methylation between the tested cell lines. For instance, DLD-1 cells showed extremely high levels of methylation throughout the 32 CpG sites studied (Figure 3.5). Caco-2 cells also showed relatively high methylation for most CpG sites. The T84 cell line (derived from a lung metastatic site of colon origin) revealed a similar pattern of methylation to the CRC liver lesions of colon origin (Figures 3.4, 3.5) with a clearly higher importance for CpG sites 1 to 7 methylation in contrast to the other sites which showed lower levels of methylation. If anything, these results clearly indicate the importance of knowing the methylation status of *RASSF1A* of cell lines before utilizing them, especially when it comes to cancer research as higher methylation of *RASSF1A* is most likely associated with its loss. It is also highly possible that cell lines with high promoter methylation of *RASSF1A* are likely to have high methylation for other

susceptible genes as well such as p16, Death-associated protein kinase (DAPK), and caspase 8 [276].

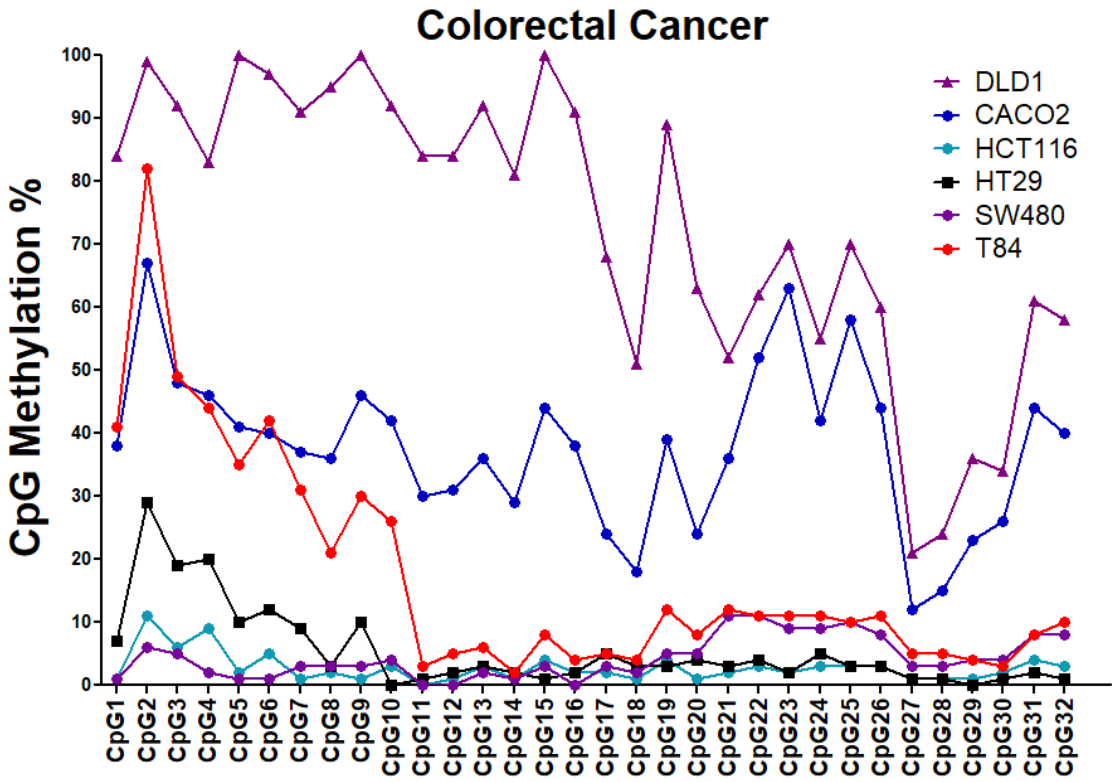


Figure 3.5: *RASSF1A* promoter methylation percentage of individual CpGs sites in colorectal cancer cell lines. The individual CpG methylation percentage indicates what percentage of DNA molecules is methylated at this site in the sample.

The methylation status of several cell lines was then investigated and plotted against the expression level of *RASSF1A* mRNA. The results suggest that greater 10% of *RASSF1A* methylation may result in significant loss of expression (Figure 3.6).

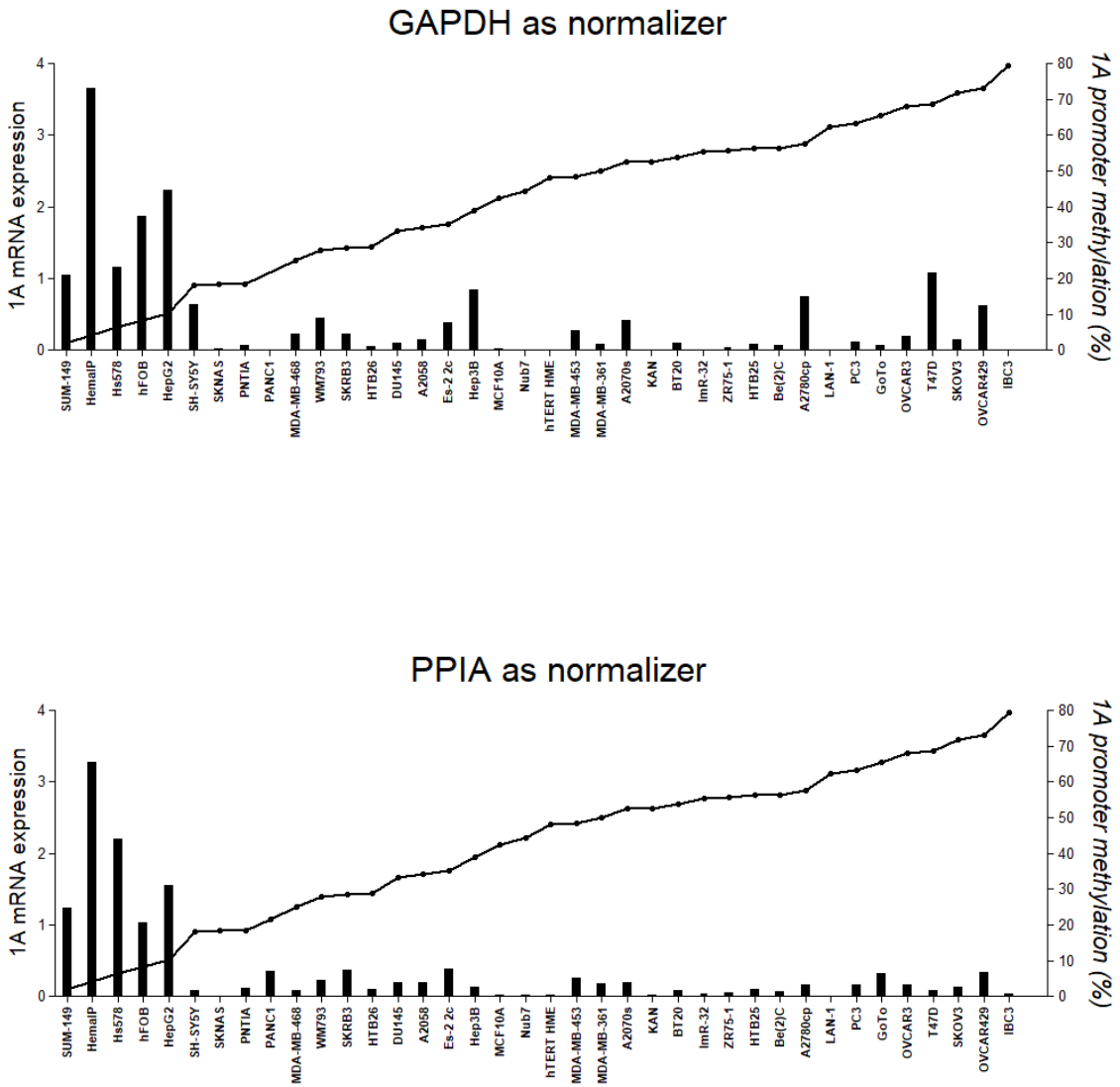


Figure 3.6: *RASSF1A* expression and methylation in cell lines. *RASSF1A* methylation results in its mRNA expression silencing in cell lines. GAPDH mRNA expression was used to normalize *RASSF1A* expression (upper). Similar results obtained if using peptidylprolyl isomerase A as a reference gene (lower)

Similarly, a subgroup of colon cancer cell lines supported the results in Figure 3.6, suggesting that probably 20% methylation is enough to drive expression loss of *RASSF1A* (Figure 3.7).

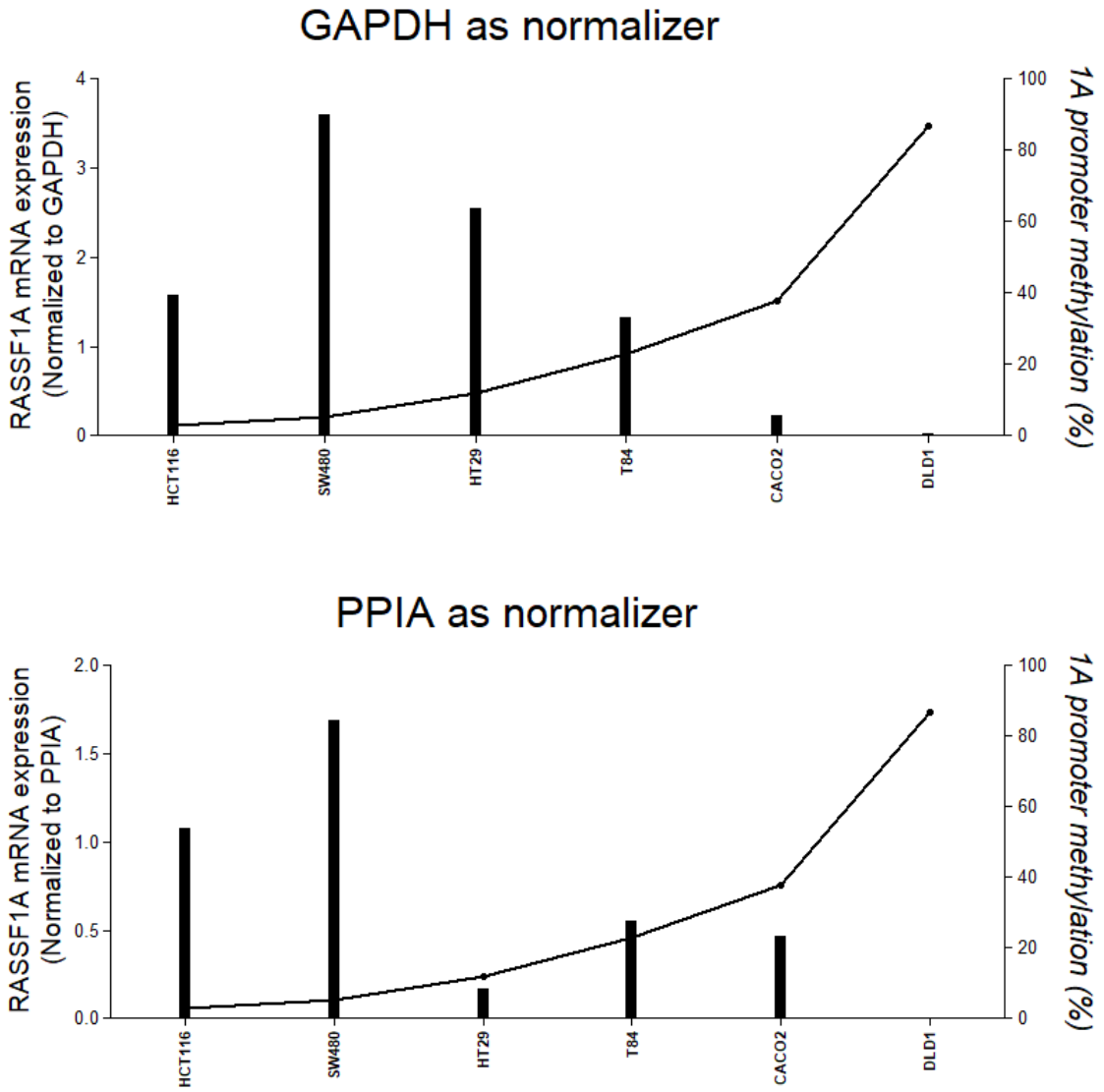


Figure 3.7: *RASSF1A* expression and methylation in colorectal cancer cell lines. *RASSF1A* methylation results in its mRNA expression silencing in cell lines. GAPDH (upper) and peptidylprolyl isomerase A (lower) mRNA expression were used independently to normalize *RASSF1A* expression.

Moreover, when the TP53 context of the cell line was taken into consideration the results showed an interesting bias of higher *RASSF1A* overall methylation in TP53 mutant (48%) and TP53 null cells (45%) versus the cells with wild type TP53 (31%) (Figure 3.8). It is believed that *RASSF1A* epigenetic loss is

an early event during tumorigenesis that may represent an early driver of malignancy. Loss of TP53 may follow the loss of *RASSF1A* in numerous cancers. Although this is a limited number of cells, it does illustrate that the TP53 status may be important to document in the context of the loss of *RASSF1A*.

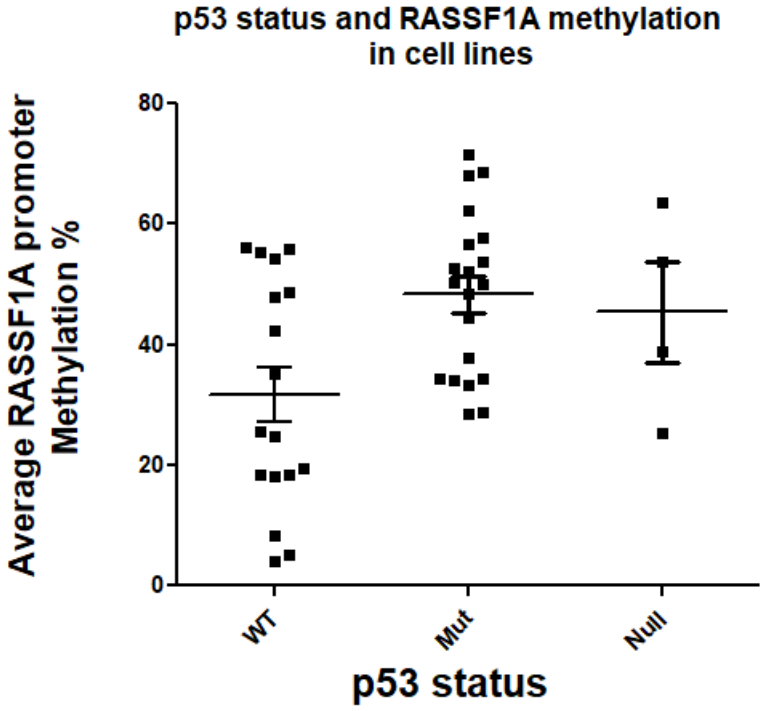


Figure 3.8: TP53 status and *RASSF1A* methylation in cell lines. Cell lines were separated to groups according their TP53 status and average *RASSF1A* methylation plotted (n=17 for WT, n=20 for mutant and n=4 for null).

3.2.4 Correlation of *DNMT1*, *DNMT3B* with *RASSF1A* mRNA expression

Evidence throughout the literature is strong on epigenetic silencing of *RASSF1A* with reports on the role of DNMT1/3B in regulating the methylation of *RASSF1A* promoter [266], Thus, the expression status of *DNMT1* and *DNMT3B* versus *RASSF1A* was explored using data that was extracted from The Cancer

Genome Atlas (TCGA). Surprisingly, no strong correlation was found in any of the cancers investigated (Figure 3.9) indicating that in many cancers the enzymatic activity changes could be more important than expression changes to drive expression loss of several genes.

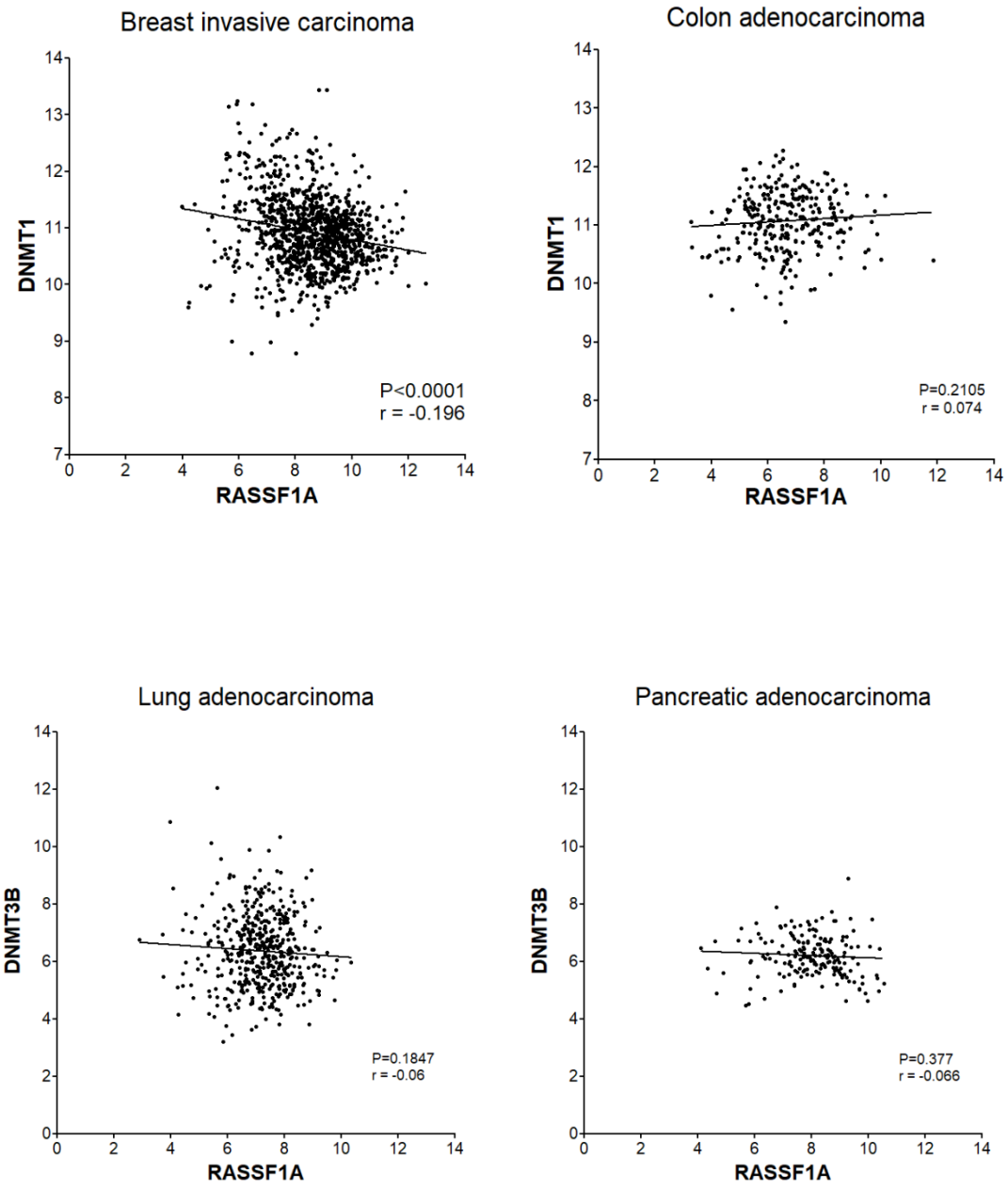


Figure 3.9: Correlation of *RASSF1A* expression with DNMT1 and DNMT3B expression in patient tumors from different cancers. Scatterplot of the expression value of *RASSF1A* (horizontal) and DNMT1 or DNMT3B (vertical) for primary tumors from TCGA (The Cancer Genome Atlas) breast cancer (n = 1062), colorectal adenocarcinoma (n = 286), lung adenocarcinoma (n = 488) and pancreatic adenocarcinoma (n = 178) patient samples. Lines indicate linear regression; Correlation coefficients and associated p-values are displayed in the bottom right corners

3.2.5 Correlation of *MOAP-1* with *RASSF1A* mRNA expression

Our group has recently published on the importance of MOAP-1 as a downstream effector for executing apoptotic functions of RASSF1A [269]. We analyzed data also obtained from TCGA for expression levels of *MOAP-1* in various cancers. The results indicated a reduction of *MOAP-1* expression in most cancers analyzed (Figure 3.10). MOAP-1 is mainly thought to be regulated by post-translational mechanisms involving ubiquitination, with a half-life of less than 30 mins [277]. These results open eyes on the importance of understanding epigenetic and transcriptional regulation of *MOAP-1*. The *MOAP-1* promoter has about 120 CpG sites that can potentially be methylated. Our group is investigating the methylation status of the *MOAP-1* promoter and preliminary data reveals very low methylation (less than 5%), at least in the 19 CpGs tested. Hence, if regulation is executed by methylation it should be in the remaining 100 CpGs or by non-DNA methylation transcriptional regulation by NFκB and/or STAT1 [269].

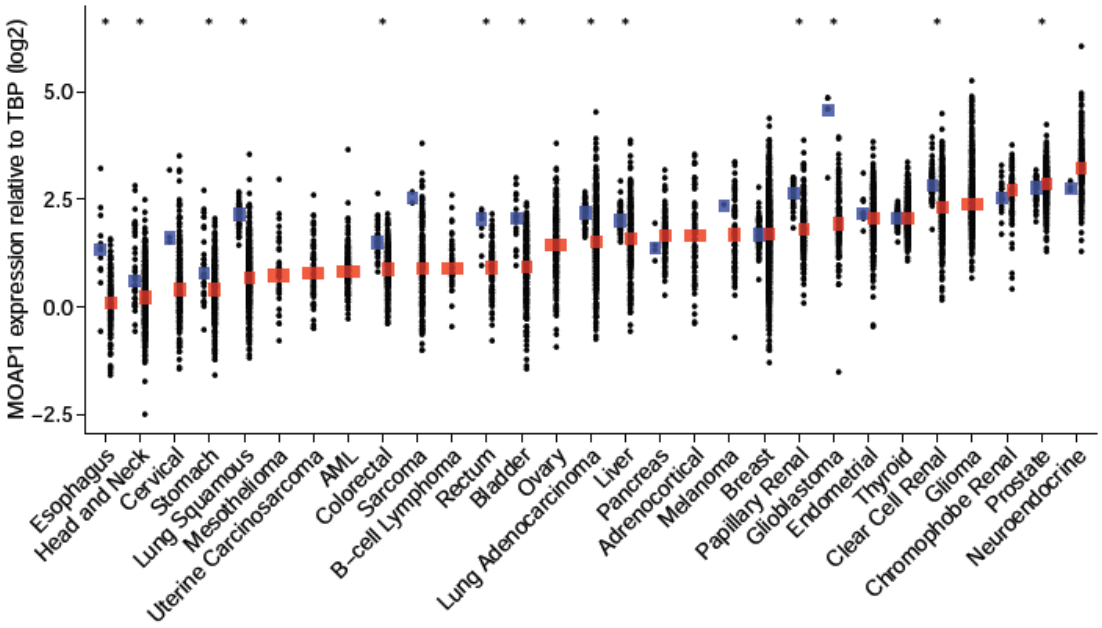


Figure 3.10: *MOAP-1* expression in various cancers as obtained from TCGA. Red squares represent the cancer value as compared to normal expression levels in blue. TBP: TATA-binding protein mRNA was used for normalization.

Furthermore, several microRNAs have been predicted to target *MOAP-1* and can explain the reduction in the expression of *MOAP-1*. It has been demonstrated that mir-1228 [278] and mir-25 [279] regulate *MOAP-1* expression in lung cancer. Therefore, investigating the role of microRNAs in other cancers shown in Figure 3.10 would unveil important information on how the expression level of *MOAP-1* is reduced in these cancers.

The mRNA expression of both *RASSF1A* and *MOAP-1* was also explored in different cancers. Correlation plots in empirical analysis using qRT-PCR in cancer tissues revealed a strong correlation in CRC and breast cancer and a moderate correlation for thyroid cancer (Figure 3.11). Hence, it is important to know both the MOAP-1 and RASSF1A status of cell lines/tissues utilized to

explore the biology of RASSF1A or any other RASSF family member associated with MOAP-1.

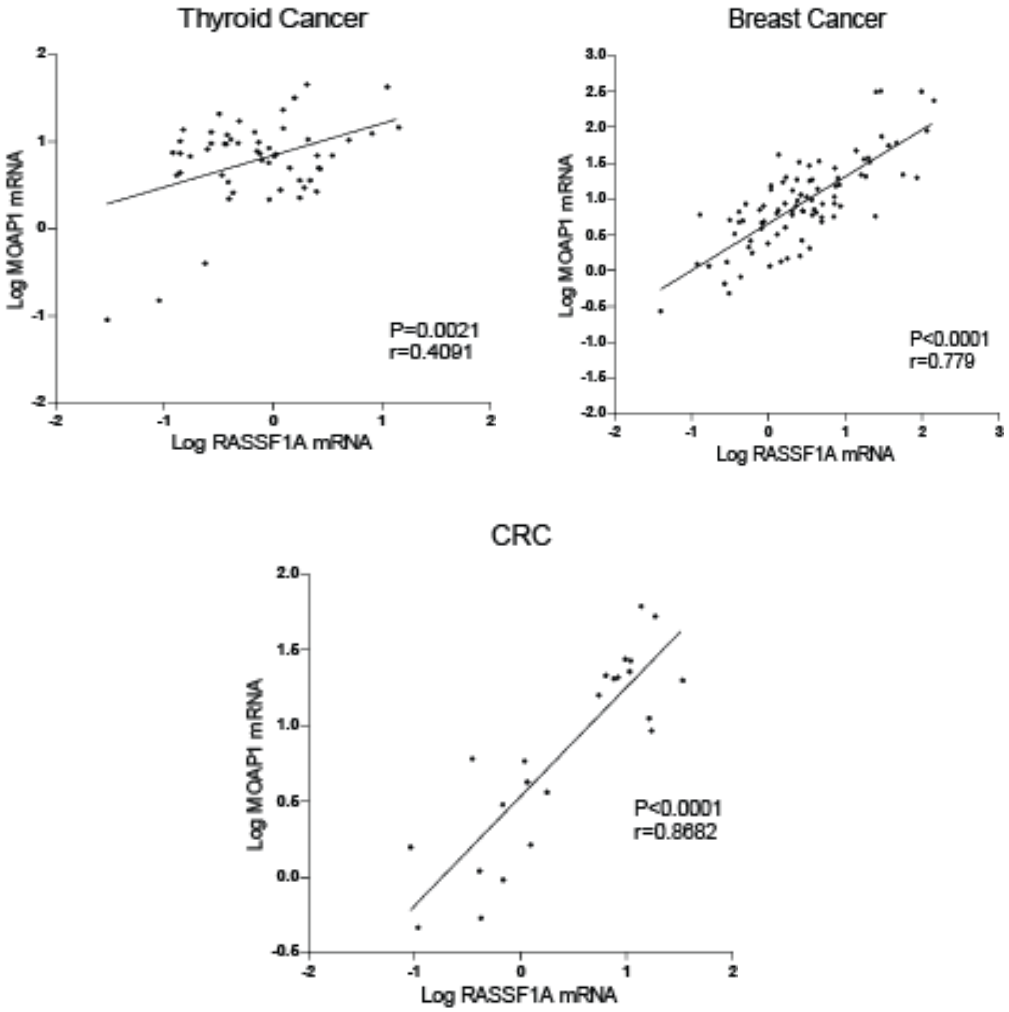


Figure 3.11: *MOAP-1* expression and its correlation with *RASSF1A* expression in different cancers. Scatterplot of the expression values (normalized to GAPDH) of *RASSF1A* and *MOAP-1* for thyroid (n = 55), breast (n = 77) and colorectal cancer (n = 31) patients. Lines indicate linear regression; correlation coefficients and associated p-values are displayed in the bottom right corners

3.2.6 Correlation of *RASSF1C* with *RASSF1A* mRNA expression

As mentioned earlier, among the RASSF1 subtypes, RASSF1A and RASSF1C are the most extensively studied members that have been

demonstrated to be localized to microtubules and involved in growth control. Even though the two isoforms share a 60% amino acid identity, but still RASSF1A and RASSF1C have opposite biological properties [127]. Many of the RASSF1 family members can homodimerize and heterodimerize with each other, including a RASSF1A/1C complex. Recently, an intriguing mechanism was proposed to suggest that the loss of RASSF1A would trigger either the release of RASSF1C from a RASSF1A/1C complex or promote upregulation of mRNA for *RASSF1C*. The result of either mechanism would be an increased pool of unbound RASSF1C to result in the activation of Src kinases and transcriptional activation of the YES associated protein (YAP) to modulate proliferation. A study by Vlahov *et al* (2015) demonstrated that protein-protein interactions of both RASSF1A and RASSF1C revealed binding of both isoforms to the tyrosine kinases c-Src, FYN and YES, but a unique association of RASSF1A to C-terminal Src kinase (CSK), a SRC inhibitory kinase that phosphorylates SRC kinases at Y527. The loss of RASSF1A releases CSK and allows RASSF1C-induced activation of SRC kinases [280].

Given the functional relationship between RASSF1A and RASSF1C we aimed at looking at the mRNA levels of *RASSF1A* and *RASSF1C* by qRT-PCR in colorectal cancer patients. Results revealed that expression changes of *RASSF1C* can occur as a result of the loss of *RASSF1A*. It can be seen that *RASSF1A* expression decreases and *RASSF1C* increases in cancers compared to normal tissues (Figure 3.12).

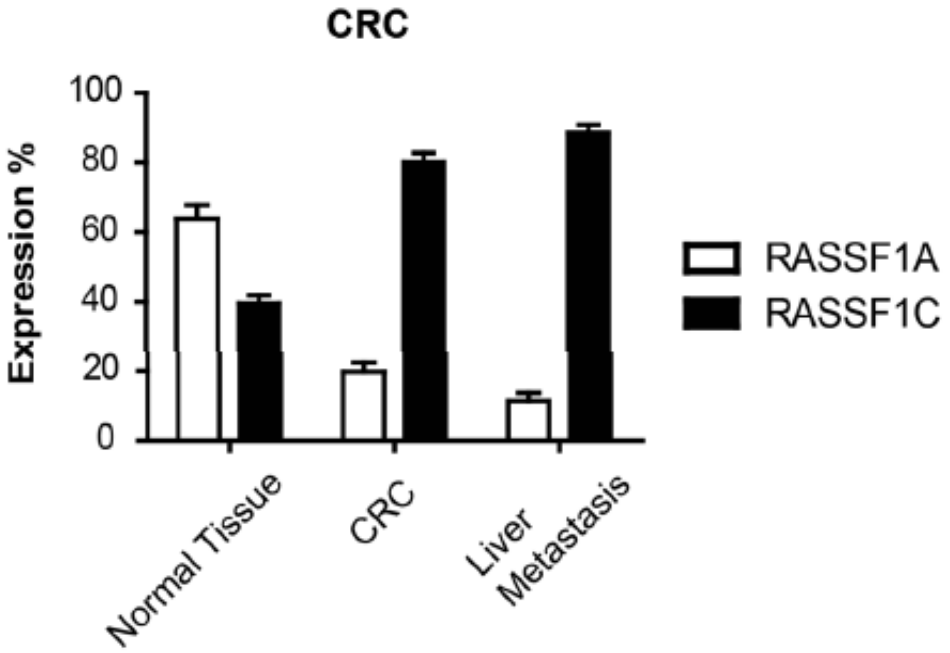


Figure 3.12: Percent expression pool of *RASSF1A* and *RASSF1C* in colorectal cancer. *RASSF1A* and *RASSF1C* expressions were summed and percentage of *RASSF1A* and *RASSF1C* expression calculated for each patient. Mean ± SD percentages are presented. CRC n=26 and for liver metastasis n=5. Normal epithelial tissue was obtained from breast reduction surgery n=13.

3.3 Discussion

Understanding the methylation status of *RASSF1A* can help uncover the importance of RASSF1A and emphasize therapeutic options for many cancers. Methodologies such as MSP and COBRA have been commonly used to explore methylation of *RASSF1A* and the conclusion is that some CpGs in the *RASSF1A* promoter are more relevant biologically for transcriptional regulation. For instance, in lung cancer it was found that there are 8 CpGs in the promoter area and 6 in the first exon that influence the expression of *RASSF1A* more than the

others [281]. That said, the extent of methylation at individual CpG sites remains largely unknown. We utilized pyrosequencing to explore the methylation signature of 32 of 75 potential CpGs in the promoter of the *RASSF1A* gene in both cell lines and tumor tissues. This analysis revealed CpG methylation hotspots within the *RASSF1A* promoter in both patient tumor tissues and cancer cell lines. Although, *RASSF1A* is heavily methylated, hope is there that it can be reversed with the DNA methyltransferase inhibitors, 5-azacytidine or 5-aza-2'-deoxycytidine (Decitabine) [282]. Active research into efficient delivery of these inhibitors to tumor sites will enhance the usefulness of DNA methyltransferase inhibitors. Furthermore, understanding how the methylation of the CpG sites 1-7 can influence transcription factor binding could provide valuable information on the expression loss of RASSF1A. GeneCards predicts more than 250 transcription factors can bind to this respective region of the CpG sites 1-7 which corresponds to approximately 300-500 base pairs upstream exon 1. The list includes mostly transcription factors with oncogenic properties such as TEADs 1, 3 and 4, SMADs 1,2 and 5, MYC, HDACs 1 and 2, SP1, STAT-1, IRF-2, JUN and others. One possible explanation could be that the hypermethylation of CpG sites 1-7 of *RASSF1A* is preventing the expression of RASSF1A as a negative feedback control by blocking access of these oncogenic transcription factors to the promoter of *RASSF1A*.

As discussed earlier, in addition to other mechanisms, *RASSF1A* expression can be regulated by epigenetic silencing directly by DNMT and indirectly by TP53/death-associated protein 6 (DAXX) biology [283]. It was shown

that TP53 binding to the *RASSF1A* promoter resulted in the recruitment of DAXX and DNMT1, DNA methylation and inactivation of the *RASSF1A* promoter. Interestingly, it was the DAXX expression levels, not TP53 levels that affected the rates of *RASSF1A* methylation [283]. Results in Figure 3.8 may suggest some influence of TP53 on the methylation status of *RASSF1A*, but further population-based analysis may be needed to confirm the influence of TP53 mutational status on *RASSF1A* promoter-specific methylation.

Discrepancies between *RASSF1A* promoter methylation and its mRNA expression level in some samples could be due to micro-RNA involvement in *RASSF1A* expression. *RASSF1A* mRNA can be targeted by at least fifteen miRNAs (miR-326, -330, -149, -16, -497, -504, -410, -99a, -99b, -100, -124, -193, -193b, -182, -181a,b,c,d) [283]. Recent empirical study proved that miR-181b targets and regulates the expression of *RASSF1A* in colorectal cancer [284]. mir-602 has also been shown to change *RASSF1A* mRNA expression in hepatoma cells and hepatocellular carcinoma [285]. Therefore, one or more of the above mechanisms may regulate *RASSF1A* and further studies and detailed investigations in cancer and other diseases that have expression loss of *RASSF1A* are required.

CHAPTER FOUR

TP53 Codon 72 Arg/Arg Polymorphism is Associated with a Higher Risk of Inflammatory Bowel Disease

Abstract

The causes of inflammatory bowel diseases are not fully known, but several genetic, epigenetic and environmental factors are thought to be at the root of IBD development. Pathogenic mutations of TP53 have been reported in 50% of colorectal cancer patients and a single nucleotide polymorphism (SNP) in the TP53 gene resulting in the presence of either arginine (Arg) or proline (Pro) or both at codon 72 was shown to alter TP53 tumor-suppressor properties. This SNP has been investigated as a risk factor for numerous cancers, including CRC. The aim of this study was to investigate the association between tumour suppressor gene *TP53* codon 72 polymorphisms and the risk of developing inflammatory bowel disease. Our hypothesis was that, like colorectal cancer, the presence of the Arg allele is associated with increased susceptibility to inflammatory bowel diseases. For that purpose, we analyzed 461 patient samples with IBD, 181 primary sclerosing cholangitis patients and 62 healthy controls for their codon 72 status. Genotyping of TP53 was performed by sequencing and restriction fragment length polymorphism analysis of genomic DNA extracted from peripheral blood. Our results reveal an interesting link between the Arg/Arg genotype and a higher risk of inflammatory bowel disease development as 54%-64% of the IBD category were genotyped as Arg/Arg in

contrast to only 32% of the non-IBD control subgroup. Arg/Pro was the most prevalent genotype in non-IBD controls (53%) and less common in patients (31%-40%). Pro/Pro frequency was not significantly different between controls and IBD patients.

4.1 Introduction

As evident in the previous chapter on *RASSF1A* promoter methylation, epigenetics plays an in important role in the pathogenesis of IBD. In fact, some studies have described distinctive epigenetic landscapes in the promoter and enhancer regions of IBD patients in contrast to normal human biopsies [20]. Moreover, the role of genetics in inflammatory bowel disease (IBD) has gained scientific interest and progress has been done in the recent years. IBD is definitely associated with geographic and ethnic differences throughout the globe and the observation that Jews are at a higher risk of CD regardless of their geographic location gives this theory higher merit and credibility [286]. Several studies have looked into the familial nature of IBD and interesting statistics have been drawn. A Finnish study including 436 patients with UC and 257 patients with CD stated that 13.8% and 15.6% of the first degree relatives of the UC and CD patients, respectively, had a form of IBD [287]. Similarly, a Canadian study of 1000 CD patients indicated that 8.7% of their first degree relatives also had CD [288]. Overall, 5-23% of IBD patients have first degree cousins with IBD [35] and families with multiple affected individuals, called multiplex families, further highlight the role of genetics in IBD susceptibility. Twin studies on monozygotic and dizygotic twins clearly confirm above studies and highly emphasize the

importance of genetic factors influencing IBD. In a Swedish study of 80 IBD twin pairs with CD revealed that concordance occurred in 62.5% of monozygotic twins and only in 3.8% of dizygotic twins [289]. The concordance rates for ulcerative colitis was lower with 6.6% and 0% in monozygotic and dizygotic twins, respectively [289]. These and similar observations have been the major triggers to push the application of molecular and genetic approaches to understand IBD. IBD, like many other diseases, does not follow a simple monogenetic Mandelian pattern of inheritance but rather involves many genes along with non-genetic factors favouring the arising of IBD. Presently, about 163 susceptibility genes are associated with higher risk of IBD [290].

According to the Canadian Cancer Society, CRC is the second most commonly diagnosed cancer in Canada with 26800 Canadians diagnosed with colorectal cancer in 2017 and as high as 9400 died from it in 2017. In the four step pathway to CRC tumorigenesis, TP53 inactivation usually occurs at a later stage leading to the transition into a carcinoma and represents a major driver event in CRC [291]. The *TP53* tumor suppressor gene, located at 17p13, encodes a transcription factor that regulates expression of genes involved in numerous biological processes, such as cell cycle control, DNA repair and apoptosis [292]. Mutations in the TP53 gene have been reported in 50% of CRC cases that mainly lead to the loss of transcriptional control [293]. More than 200 SNPs have been identified in the TP53 gene [294]. Codon 72 polymorphism encoding either arginine (CGC, 72Arg) or proline (CCC, 72Pro) is the most investigated SNP. Evolutionarily, Arg has never been found in primates other

than human [295] and hence Pro is considered the ancestral allele. As a result of this SNP, the translated protein has different biological and biochemical properties (summarized in Table 4.1), such as subcellular localization [296, 297], ability to induce apoptosis [298, 299], ability to suppress transformed cell growth[300], and transcriptional activation [301, 302]. Furthermore, the frequency of either allele (Arg or Pro) is highly affected by ethnicity. One study reported that the frequency of the proline allele increased from 17% in Swedish Saamis to 63% in African Nigerians [303]. If anything, this shows the importance of comparing patients and controls of the same ethnic group in codon 72 polymorphism association studies, when permissible. The association between the 72 codon polymorphism and cancer risk has been studied extensively for many cancers, including CRC [304]. Two reports studied the association of 72 codon polymorphism and UC development [305, 306]; however, there have been no studies to associate codon 72 polymorphism with CD and PSC susceptibility. In this study we aimed to investigate a possible link between the 72 codon polymorphism and the risks of developing IBD and thus IBD-induced colorectal cancer.

Table 4.1: Differences in biological and biochemical properties of Arg and Pro at codon 72 in TP53

Property	Description	References
Subcellular localization	Arg tends to be cytoplasmic whereas Pro tends to be nuclear.	[296-298]
Apoptosis	Arg is a stronger apoptotic inducer	[298]
Transcriptional activation	Arg associates and inactivates p73 more efficiently	[307]
Electrophoretic mobility	Arg migrates faster on SDS	[307]

4.2 Results

To investigate the possible link between genotype and allelic frequencies with either form of the diseases, we used blood samples collected from the University of Alberta Gastroenterology Clinics and at the University of Calgary. Our analysis was done against a set of 62 non-IBD control patient group with no prior history of IBS, IBD or cancer. Genomic DNA was extracted from blood as described in methods, and primers designed to amplify the region of interest were obtained based on the protocol suggested by International Agency for Research on Cancer (IARC) [308]. Figure 4.1 shows the product of the PCR amplicon obtained at the expected size of 353 bp. We then used Restriction Fragment Length Polymorphism (RFLP) to analyze the presence of an Arginine or Proline coding codon for amino acid 72. The arginine allele is usually cut by the *Bst*UI enzyme, whereas the proline allele is not digested by this enzyme. Figure 4.1 also shows representative products as a result of the restriction reaction.

Our results indicate that the allelic distribution was significantly different between patients and controls. In particular, the frequencies of Arg and Pro alleles were 75.9% and 24.1% in IBD patients while representing 58.9% and 41.1% in healthy controls respectively (Table 4.2). This suggests that the presence of Arginine in TP53 codon 72 polymorphism may serve as a predisposing factor for IBD development.

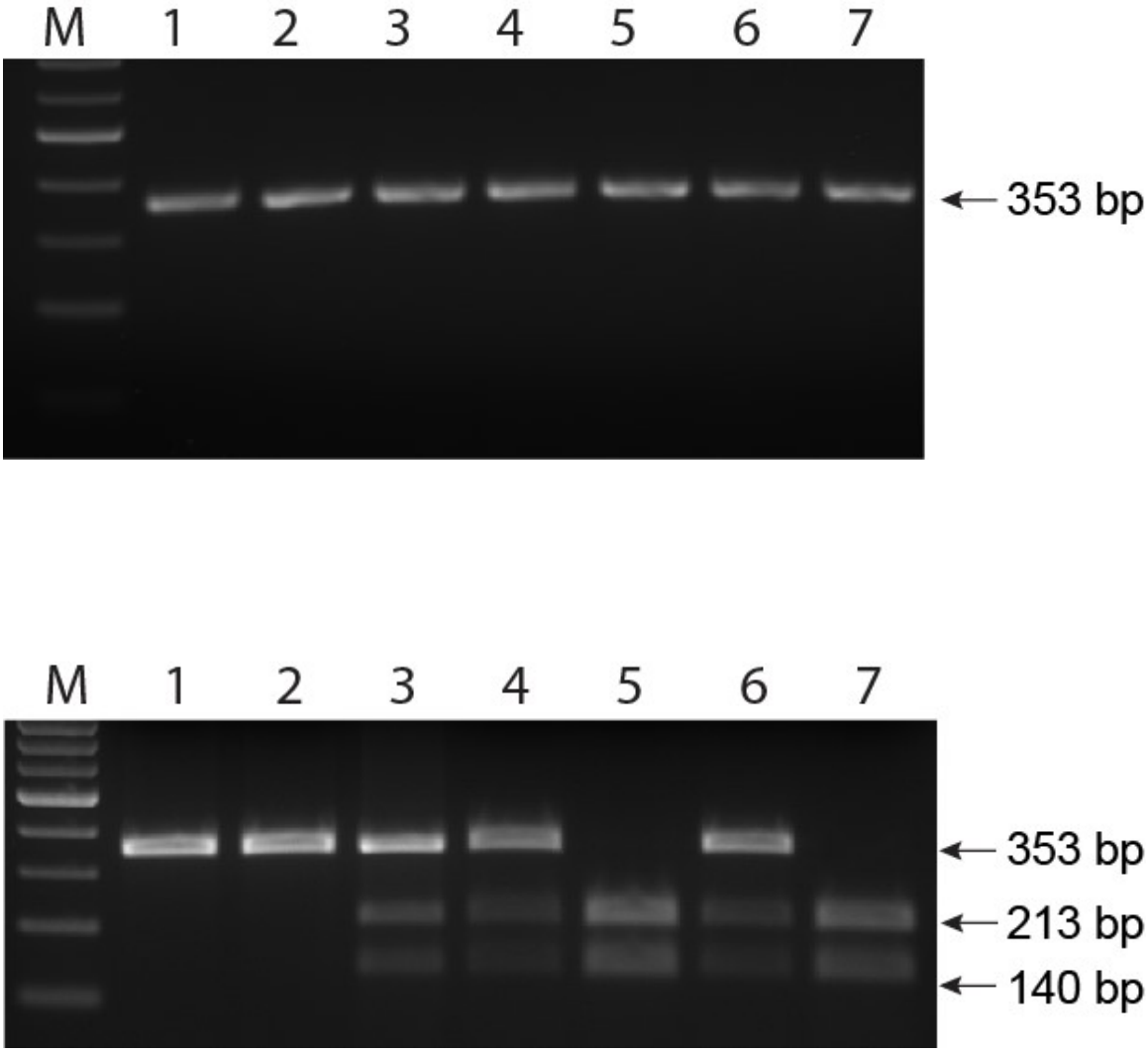


Figure 4.1: Polymerase chain reaction-restriction fragment length polymorphisms analysis. (Upper) Representative analysis of the polymerase chain reaction (PCR) product. The product corresponded to TP53 with a single band at 353; (Lower) The PCR-restriction fragment length polymorphisms (PCR-RFLP)**,** Lanes 1 and 2 correspond to Pro/Pro homozygous samples, Lanes 3, 4 and 6 correspond to Arg/Pro heterozygous samples and Lanes 5 and 7 correspond to Arg/Arg homozygous samples.

Table 4.2: Allelic frequencies of Arg and Pro in the patients and controls			
Patients/Controls	*Allele, % (n)*		*P value**
	Arg, n (%)	*Pro, n (%)*	
Cases (n=642):	**974 (75.9)**	**310 (24.1)**	<0.0001
UC Adult (n=151)	222 (73.5)	80 (26.5)	0.0029
CD Adult (n=138)	210 (76.1)	66 (23.9)	0.0005
UC Pediatric (n=78)	118 (75.6)	38 (24.4)	0.0028
CD Pediatric (n=94)	145 (77.1)	43 (22.9)	0.0006
PSC (n=42)	67 (79.8)	17 (20.2)	0.0016
PSC+UC (n=139)	212 (76.3)	66 (23.7)	0.0004
Controls (n=62)	**73 (58.9)**	**51 (41.1)**	

Consequently, the genotype for TP53 72 Arg/Arg was found to be significantly higher among all the patient groups as compared to the control group. In the control samples utilized in this study, the genotype distribution for TP53 polymorphism revealed 32.3%, 53.2% and 14.5% for Arg/Arg, Arg/Pro and Pro/Pro genotypes, respectively. This was in contrast to 56.9%, 38.0% and 5.1% in patient groups combined, respectively (Table 4.3, Figure 4.2). Pediatric and adult IBD patients were not statistically significant different for the presence of either genotype. To our knowledge 72 SNP distribution has never been studied in PSC patients. Our results indicate that PSC had a higher Arg/Arg percentage compared to IBD groups, but it may be due to a small sample size effect (n = 42).

Table 4.3: Genotypic frequencies of the TP53 72 SNP in the patients and controls

Patients/Controls	Genotype, % (n)			OR	95% CI	P value
	Arg/Arg	Arg/Pro	Pro/Pro			
Cases (n=642):	**56.9 (365)**	**38.0 (244)**	**5.1 (33)**	2.77	1.59-4.82	0.0001
UC Adult (n=151)	54.3 (82)	38.4 (58)	7.3 (11)	2.50	1.34-4.65	0.0102
CD Adult (n=138)	55.8 (77)	40.6 (56)	3.6 (5)	2.65	1.41-4.98	0.0011
UC Pediatric (n=78)	55.1 (43)	41.0 (32)	3.8 (3)	2.58	1.29-5.17	0.0078
CD Pediatric (n=94)	59.6 (56)	35.1 (33)	5.3 (5)	3.09	1.58-6.07	0.0023
PSC (n=42)	64.3 (27)	31.0 (13)	4.8 (2)	3.78	1.66-8.63	0.0046
PSC+UC (n=139)	57.6 (80)	37.4 (52)	5.0 (7)	2.85	1.52-5.35	0.0016
Controls (n=62)	**32.3 (20)**	**53.2 (33)**	**14.5 (9)**			

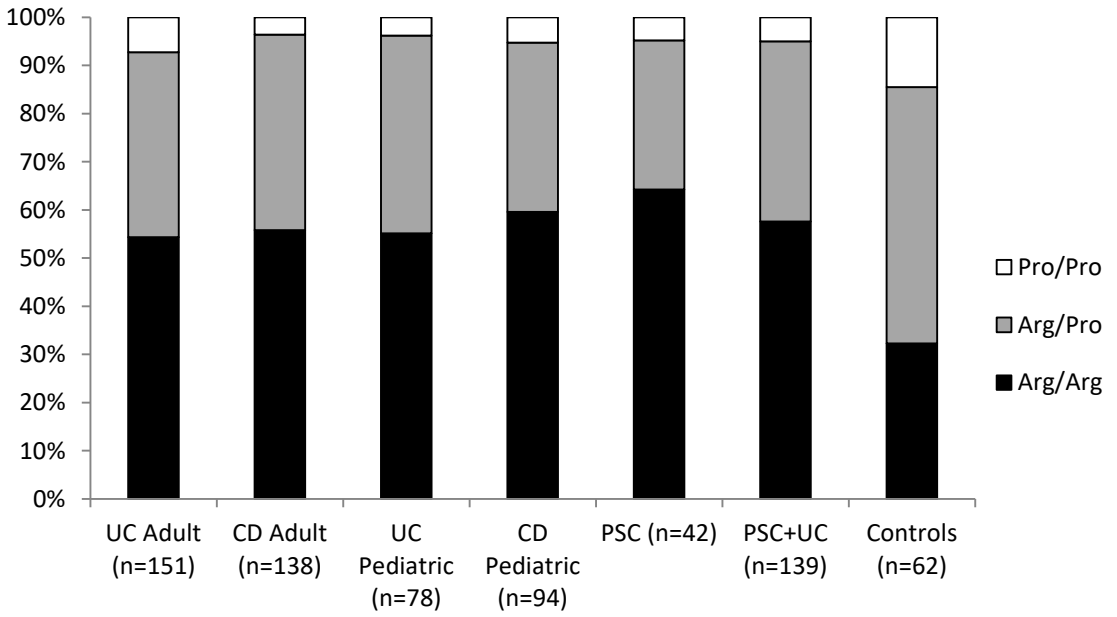


Figure 4.2: TP53 72Arg/Pro polymorphism distribution in adult and pediatric IBD, PSC, PSC/UC and healthy non-IBD controls. TP53 72Arg homozygosity is strongly associated with the development of IBD. Please find P values and ORs in Table 4.3.

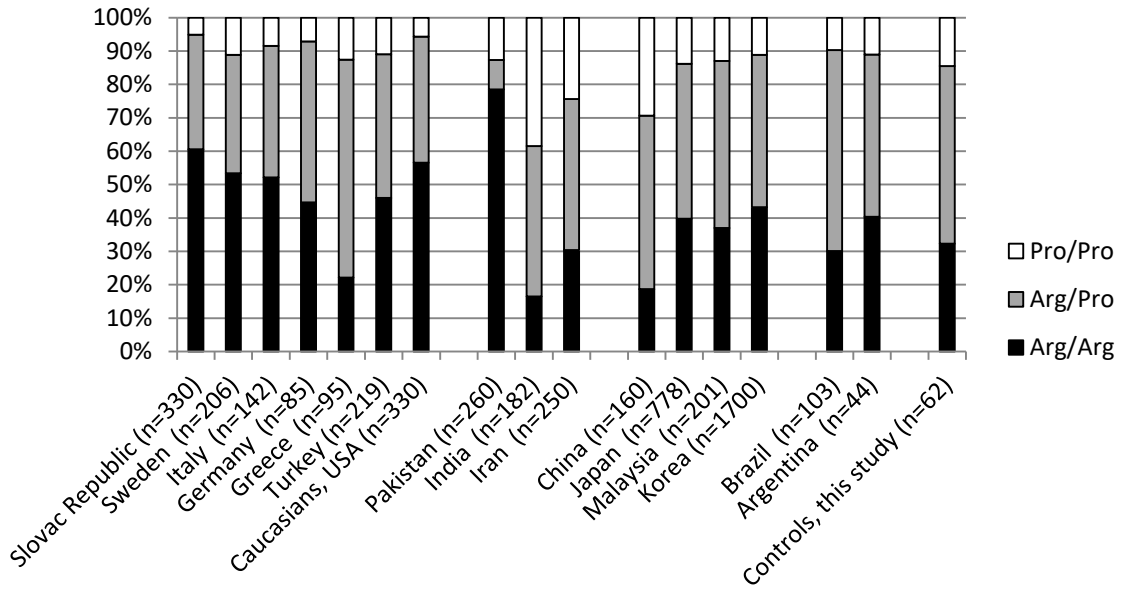


Figure 4.3: TP53 72 Arg/Pro polymorphism distributions in non-IBD controls in different countries and comparison to controls utilized in this study (mainly of Eastern European descent).

4.3 Discussion

Our results indicate that IBD and PSC patients have significantly different TP53 codon 72 polymorphism distribution when compared to the non-disease controls, suggesting that the 72 Arg allele may predispose individuals to IBD development. This could be partially explained due to increased levels of apoptosis in the colonic area since the Arg allele has been reported to be a better apoptosis inducer (Table 4.1). In fact, apoptotic degradation of the intestinal epithelial cell can lead to the impairment of gut barrier which is a characteristic feature in IBD patients [309]. Moreover, it was shown that 72 Arg genotype carriers have increased risk of TP53 mutations potentially leading to further imbalances and cancer development [310]. Thus, if differences between 72 Arg

and 72 Pro carriers progress through IBD to cancer development are confirmed in long-term studies, it could be useful for IBD related cancer development prediction and hence better screening of 72 Arg carriers would be useful.

The 72 Arg/Pro SNP being investigated in this study lies within the proline rich domain of TP53 (amino acids 64 to 92) [311]. This domain consists of five PXXP repeats that form a left handed polyproline type II helix, creating a binding site for SH3 domains [312]. The presence of an Arginine at codon 72 not only affects the apoptotic properties of TP53 [313], but can perhaps affect the structural organization of the polyproline helix and binding of TP53 to SH3 containing proteins. In fact, an important finding is that the proline rich domain exhibits an elevated level of stiffness, as determined by structural analysis which favors the type II helix structure. Furthermore, the polyproline type II helix facilitates the interaction of the transactivation domain (TAD) with SH3 domains found in kinases [314]. It is critical to further elucidate the structural functional relationships between TP53 polymorphisms and TP53 functions as a genome “gate keeper”.

The healthy non-disease controls used in this study were mainly through direct reference and were mainly composed of Caucasian individuals of Eastern European origin from the Edmonton area and Northern Alberta. Surprisingly, the TP53 codon 72 genotype distribution found in our healthy controls was different from the genotype distribution observed in controls in Europe (Figure 4.3) (Slovak Republic [315], Sweden [303], Italy [306], Germany [310]. Our controls mostly resemble the controls from Greece [316] when it comes to the genotypic

distribution of the 72 polymorphism. Other countries such as Turkey [305], United States [317], Pakistan [318], India [319] and China [320] showed a different distribution pattern as compared to our controls (Figure 4.3). The frequencies of genotypes in controls from Iran [321], Japan [322], Malaysia [323], South Korea [324] and Brazil [325] were somewhat similar to our controls. It is thus important to carefully choose controls that represent the population and derive conclusions with caution when analyzing differences between diseased and non-diseased cases.

CHAPTER FIVE

Resveratrol and Derivatives: Inhibitors of NFκB Activity and Potential Anti-Tumour Drugs

Abstract

Resveratrol is a naturally occurring polyphenol that exhibits beneficial pleiotropic health effects. It is one of the more promising natural molecules in the prevention and treatment of chronic diseases and autoimmune disorders. One of the limitations of the use of resveratrol is its extensive metabolic processing and the consequent formation of various metabolites. To possibly alleviate the extensive metabolic processing, our collaborators have worked on derivatizing resveratrol with the aim of enhancing its biological activities. In turn, we aimed at characterizing the anti-proliferative, anti-inflammatory and AMPK activating properties of these derivatives. We hypothesized that the resveratrol derivatives are more potent anti-proliferative, anti-inflammatory and anti-tumour drugs, both *in vitro* and *in vivo* as, a result of adding a salicylate pharmacophore to resveratrol. Using an MTT proliferation assay, a dual luciferase gene reporter assay, TMRE analysis and a murine DSS- model of colitis we provide analysis of how resveratrol and its derivatives can inhibit NFκB activation, cytokine production, and growth of cancer cells and alleviate intestinal inflammation *in vivo*. We identified resveratrol derivatives C3 and C11 as potential future therapeutics, closely preserving resveratrol bioactivities of growth inhibition and inhibition of NFκB activation. Interestingly, C3 shows more death inducing

properties and better AMPK activating potential. We speculate that these derivatives would be more metabolically stable to result in increased efficacy for treating immune disorders such as IBD and as anti-cancer agents. Further characterization and pharmacokinetics would confirm the *in vivo* stability and efficacy of these novel derivatives.

5.1 Introduction

Given the functional importance of RASSF1A in restricting the activation of NFκB that we have discussed in earlier chapters, this has led us to postulate that general anti-inflammatory compounds should be able to compensate for the loss of RASSF1A. Resveratrol (3,4',5-trans-trihydroxystilbene), is a naturally occurring phytoalexin present in grapes, peanuts and other plants [195] . Having a simple structure and low molecular weight, resveratrol can target key proteins such as cyclooxygenase enzymes and NFκB, two factors strongly associated with inflammation and carcinogenesis [326]. Resveratrol can also regulate targets such as PI3-kinases, SIRT1, DNMTs [327] and others. Resveratrol and other multi-target drugs are gaining interest over mono-therapies as many diseases, including cancer, involve a diversity of pathways leading to the "abnormal" state. It is also relatively non-toxic as compared to other chemotherapeutic drugs. Despite its interesting biological properties, resveratrol is required in high doses to induce apoptosis of cancer cells and its biological activity is limited by its photosensitivity and metabolic instability [328]. Resveratrol is also limited by its low bioavailability owing to its metabolism in the liver, with a serum half-life of about 14 minutes [329]. Extensive research has been done to decrease these

limitations by producing resveratrol derivatives that might have more potent biological activities at lower doses and with a higher bioavailability.

Non-steroidal anti-inflammatory drugs (NSAIDs) have recently gained wide scientific interest for promising chemo-preventative properties. Aspirin, one of the more common NSAIDs, is of particular interest in this regards. It is currently recommended for cancer prevention in patients with low risk of bleeding [330]. Aspirin can also acetylate and regulate several other molecules such as TNF-α [331], resolvins [332], PI3K [333], NFκB [334], caspase-3 [335], 6-phosphofructo-1-kinase [336] and others to add to the possible mechanisms through which it exerts its cancer protective properties.

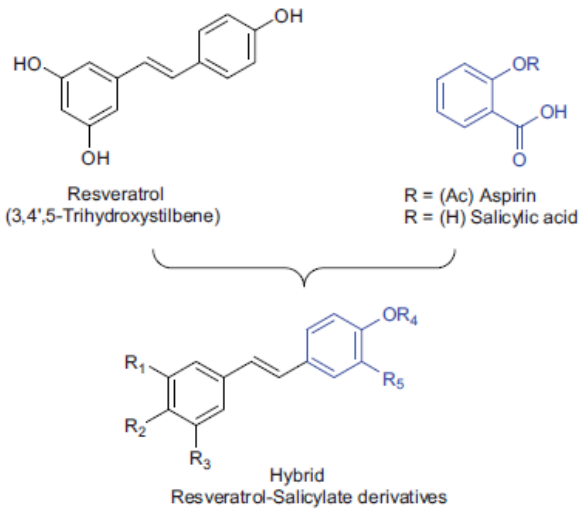
Diseases such as inflammatory bowel disease (IBD) that are characterized by a state of chronic inflammation are usually associated with higher risks of cancer. Therefore, targeting the inflammatory component of these diseases using NSAIDs and NSAID-like molecules is of great preventative value to reduce cancer incidence. The precise mechanisms in this regards remain to be investigated to determine the stage of intervention during the carcinogenesis process and the molecular targets affected. As mentioned earlier, the use of aspirin as a prophylactic drug remains to be cautious due to its adverse effects as the risk of bleeding. With that in mind, we aimed in this study to explore the effectiveness of the salicylate-like resveratrol derivatives obtained from our collaborators as described here [337], as both prophylactic and possible therapeutic drugs against cancer. Salicylic acid is known to bind to serum albumin [338], and most of our derivatives are attached to a salicylate

pharmacophore [337] which should increase binding to serum albumin and increase their solubility and bioavailability.

5.2 Results

5.2.1 Resveratrol derivatives maintain the anti-proliferative properties of resveratrol

The chemical structure of the resveratrol derivatives and how they relate to aspirin is shown in figure 5.1 as described here [337].



Compound	R1	R2	R3	R4	R5
C3	H	OCH3	H	CH3	COOCH3
C10	OH	H	OH	H	COOCH3
C11	H	OAc	H	Ac	COOH
C12	OAc	H	OAc	Ac	COOH

Figure 5.1: The chemical structure of the resveratrol derivatives most relevant to this study. The derivatives were synthesized by Fahad *et al* (2015) via the Wittig reaction [337].

The effect of these derivatives on cellular viability was investigated in several cancer cell lines using the simple MTT assay. Whereas other derivatives showed some promising anti-proliferative effects in some cell lines, C3 and C11 more consistently inhibited most of the cell lines tested (Figure 5.2) with relative IC_{50} values of 115 µM and 105 µM, respectively, in HCT-116 colon cancer cells (Figure 5.3).

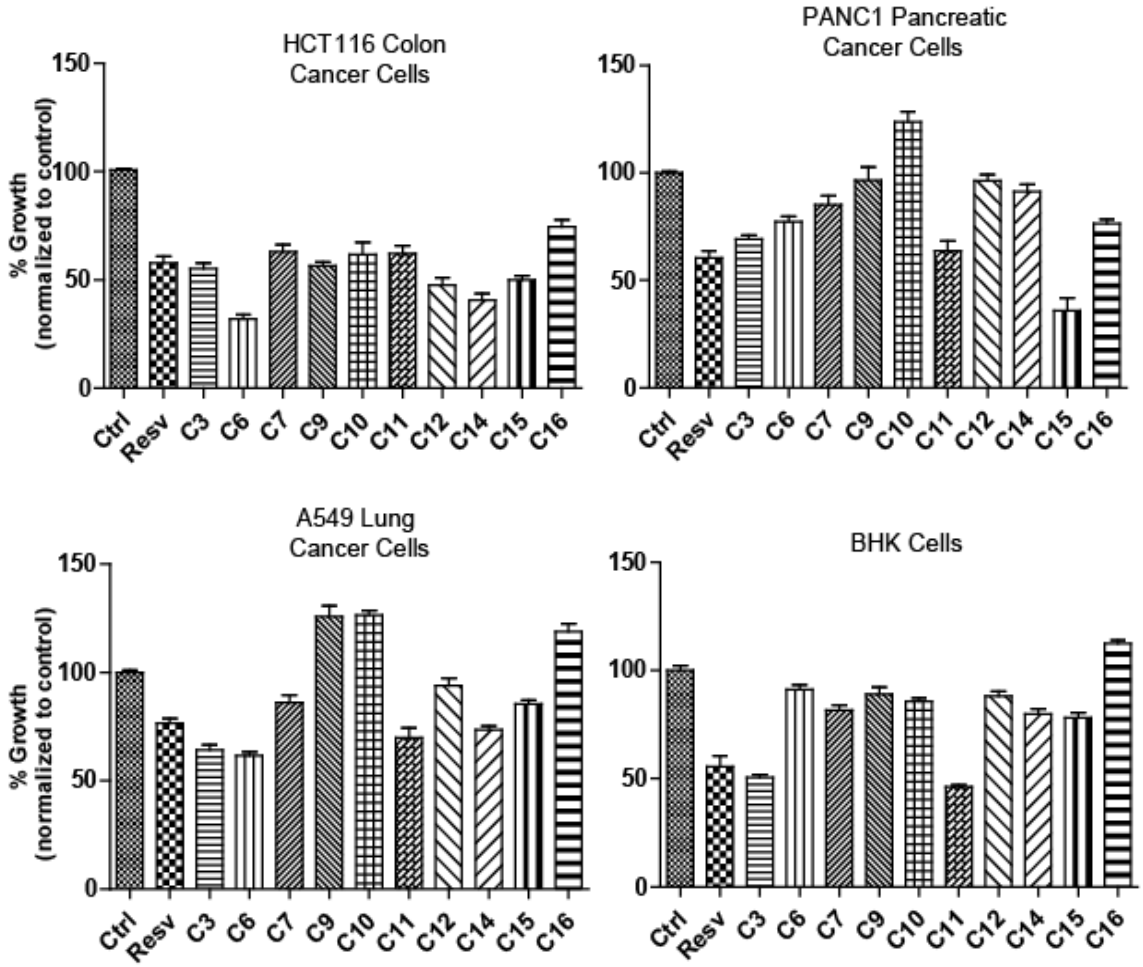


Figure 5.2: Analysis of cell viability with resveratrol and derivatives at 100 µM in colon cancer (HCT-116), pancreatic cancer (PANC1), lung cancer (A549) and normal kidney fibroblasts (BHK) cell lines. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay was performed in the indicated cells and compounds. MTT measures respiring cells and is directly correlative to the number of cells present. Normalization was done against cells not treated with drug (100% viable).

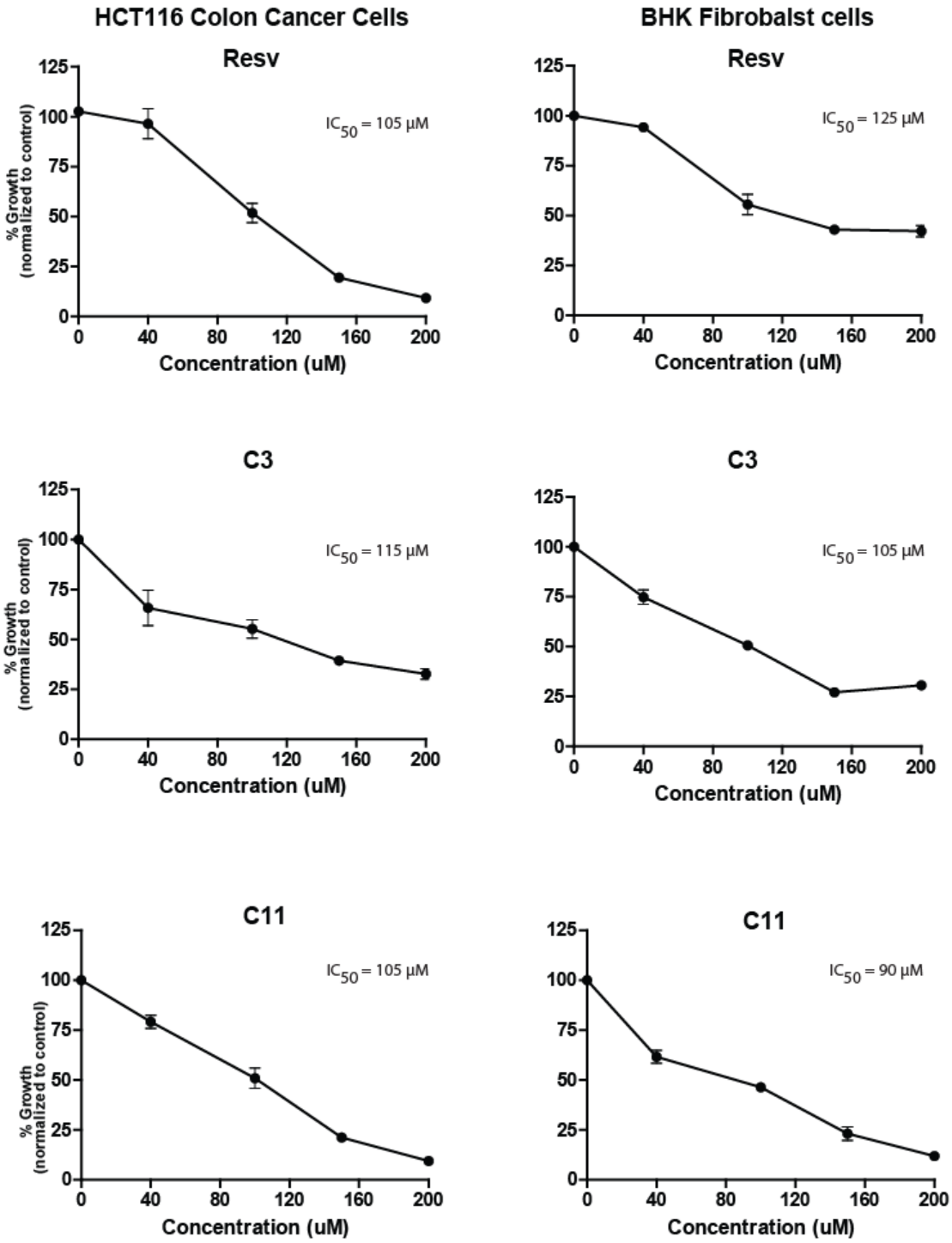


Figure 5.3: Relative IC_{50} curves for resveratrol and C3 and C11 derivatives and their effect on cell viability using MTT assay with concentrations between 0 and 200 µM to generate the curves in HCT-116 colon cancer cells and in BHK (Baby Hamster Kidney) fibroblast cells. Values were normalized against values with no drug addition and plotted. n = 3 for each concentration

5.2.2 Resveratrol derivatives inhibit NFκB activity in colon cancer and normal cell lines

Uncontrolled chronic inflammation is highly associated with various cancers including IBD driven CRC. NFκB signaling is hyperactivated in both IBD and CRC providing an important mechanistic link between the two diseases [339]. Therefore, we aimed to assess the efficacy of the resveratrol derivatives in inhibiting NFκB activity by utilizing a dual luciferase gene reporter assay for NFκB target gene (IL-6) in HCT-116 cancer cells. Cells were stimulated with the TLR4 activator, LPS, and the effectiveness of the drugs was measured by their ability to reverse the observed LPS induced activation of NFκB. Based on our screening, most derivatives maintain the anti-inflammatory properties of resveratrol, and compounds C3, C10, C11 and C12 showed the most potent inhibitory effects on NFκB activation (Figure 5.4). Given the anti-proliferative and anti-inflammatory results, we decided to move forward with characterizing the derivatives C3, C10, C11 and C12 for further assays.

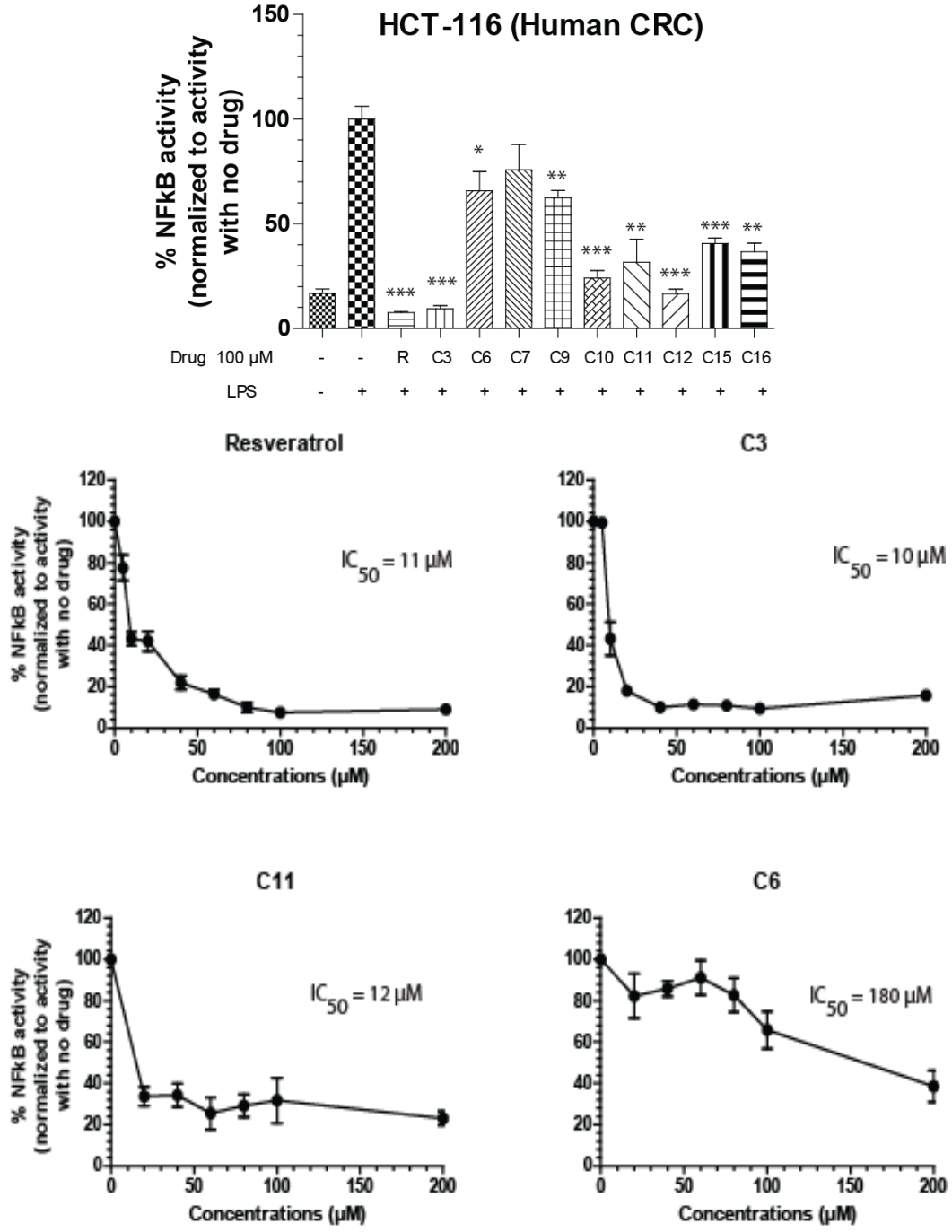


Figure 5.4: NFκB activity was determined in HCT-116 cells by a dual luciferase gene reporter assay with firefly luciferase under the control of NFκB target sequence on IL-6 promoter and Renilla-Luciferase used as an internal control. Cells were treated with drugs for 24 hrs before being stimulated with LPS (1.5 mg/mL) for 4 hrs to ensure NFκB activation. Normalization was done against LPS stimulated cells (100% activity). NFκB activity was determined by the ratio of Firefly-Luc/Renilla-Luc. The concentrations used for relative IC_{50} calculations ranged from 5 µM to 200 µM. The bar graphs show comparison of effectiveness of the different drugs at 100 µM. n = 3 per concentration. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

5.2.3 Resveratrol derivatives inhibit NFκB induced cytokine production in colon cancer cell lines

NFκB is a pivotal player in inflammation and IBD. The use of steroids in treatment of IBD is mainly directed towards inhibition of NFκB activation [339]. Uncontrolled inflammation results in unregulated NFκB signaling leading to elevated cytokines production of IL-1β, IL-2, IL-6, IL-8, IL-12 and TNF-α to mention a few [340]. Therefore we measured the levels of several cytokines thought to be involved in IBD pathogenesis in the presence of resveratrol and the derivatives upon LPS stimulation. IL-6 is a pro-inflammatory cytokine that is involved in maintaining a state of chronic inflammation by promoting the accumulation of T cells resistant to apoptosis and is associated with increased production of intracellular adhesion molecule-1 (ICAM-1) [341]. Levels of IL-6 were reduced by the derivatives tested, with C3 and C12 showing slightly greater reduction (Figure 5.5).

Other cytokines levels including IL-10 and IL-23 were also reversed upon resveratrol and derivative addition (Figure 5.5). IL-23 has been reported to be highly up-regulated in CD [342] and plays an important role in stimulating the pro-inflammatory response [343]. IL-12, is produced by macrophages and dendritic cells and enhances natural killer mediated cytotoxicity [342] and along with IL-18 triggers interferon gamma (IFN-γ) production [344]. C3 derivative showed superior inhibition of IL-12, with resveratrol and C12 also being effective as well (Figure 5.5). Taken together, the tested resveratrol derivatives can reduce cytokine production by greater than 50% in HCT-116 colorectal cancer cell lines

to suggest that modification of the parent resveratrol structure in C3, C10, C11 and C12 does not alter their ability to interfere with NFkB activity as assayed using a gene reporter or cytokine production protocols.

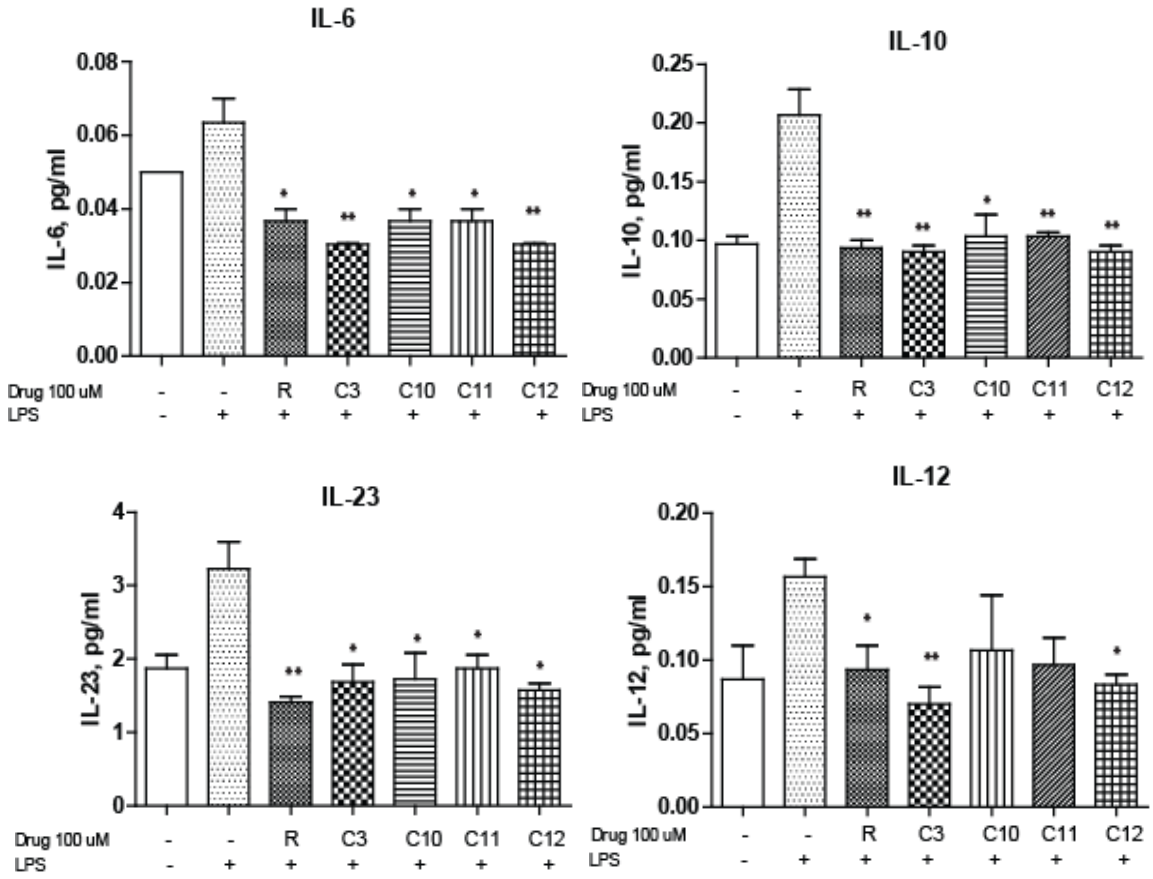


Figure 5.5: Cytokine and chemokine levels were differentially reduced with resveratrol and derivatives. Briefly, HCT-116 cells were allowed to grow in 6 well plates to 60% confluency, treated with drugs (100 µM) for 24 hrs and then stimulated with LPS (1.5 mg/mL) for 4 hrs. 500 µL of supernatant was collected and sent to Eve technologies. (https://www.evetechnologies.com/technology.php). n = 3. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

5.2.4 C3 disrupts mitochondrial membrane potential and induces more death than resveratrol in HCT-116 colorectal cancer cell line

Our results so far were not showing superiority to resveratrol but rather maintaining efficacy somewhat similar to resveratrol in the assays tested. Some studies reported that resveratrol exerts its apoptotic effects through disruption of the mitochondrial membrane potential and release of reactive oxygen species (ROS), specifically in colorectal cancer cell lines [345]. We thus assessed the effect of the derivatives on mitochondrial membrane potential and cell death by utilizing tetramethylrhodamine ethyl ester (TMRE) staining followed by flow cytometry. TMRE usually accumulates in live cells that exhibit a red shift in both their absorption and fluorescence [346], and the loss of fluorescence is indicative of dead cells. We can clearly see that among the four tested derivatives, C3 derivative significantly induced accumulation of dead cells in HCT-116 cells; about two fold more than resveratrol (Figure 5.6). C11 and other derivatives showed no significant effect on mitochondrial membrane potential and cell death (Figure 5.6).

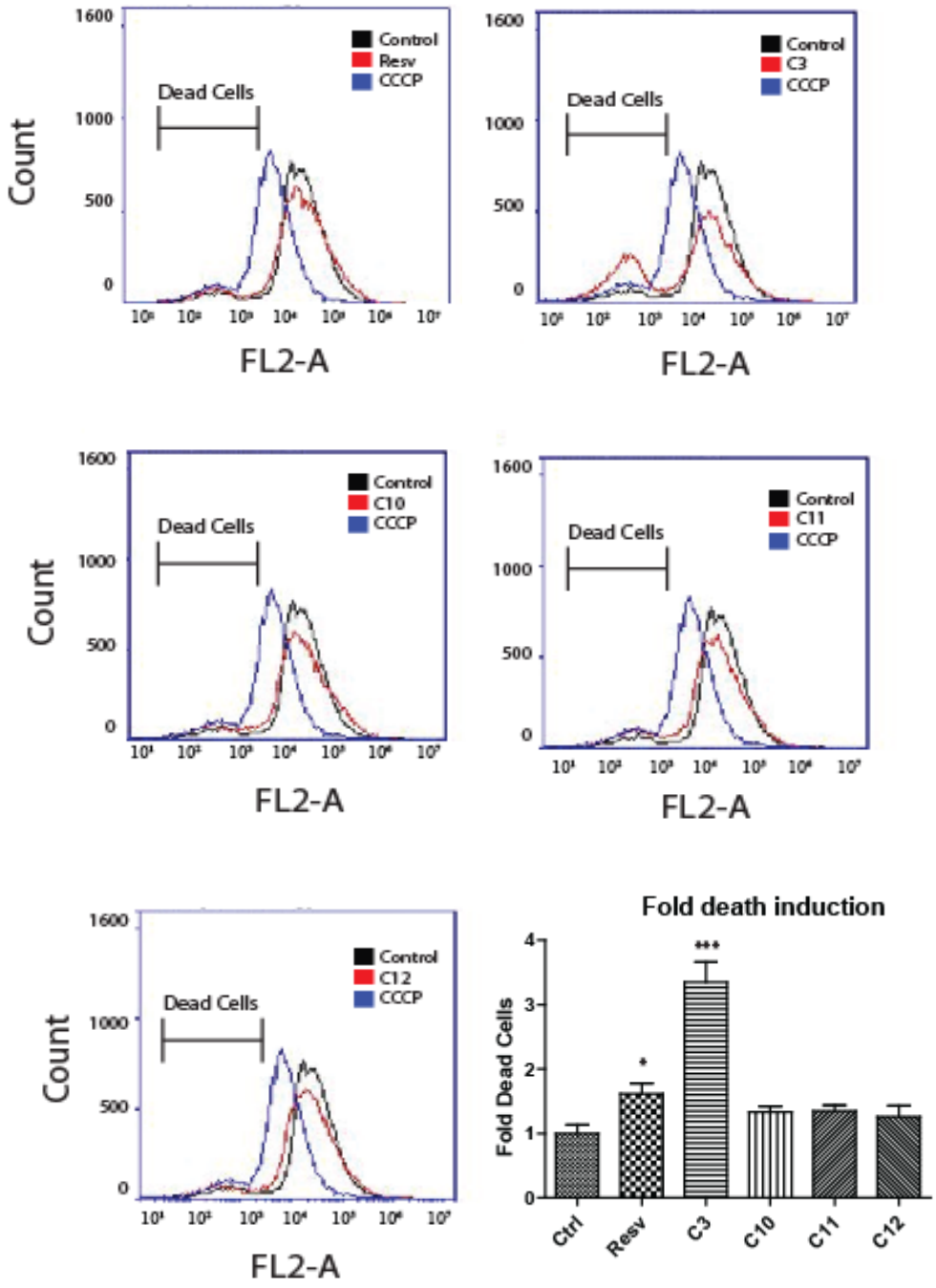


Figure 5.6: Effect of resveratrol and derivatives on mitochondrial membrane potential and cell death in HCT-116 cells: Mitochondrial membrane potential assessment was done using TMRE dye and flow cytometry. In HCT-116 colon cancer cell line, C3 (100 µM) appears to induce mitochondrial dysfunction and cell death more than resveratrol. Bar graph represents quantification of FL-2 graphs. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) is a mitochondrial membrane disruptor and use as a positive control. n = 3. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

Another important hallmark of cancer is the dysregulation of the cell cycle components, including the well-known cyclins and associated cyclin dependent kinases (CDKs). Several CDKs inhibitors have reached clinical trials recently including Flavopiridol (blocks CDK9 transcriptional activity) [347] and palbociclib that can block CDK4/6 [348, 349]. Combination therapy of CDK inhibitors with some of the established cytotoxic agents has also gained interest as well [350], adding to a possible promising role of anti-tumor drugs that target the cell cycle components. We hence, aimed to analyze the cell cycle profiling in response to these resveratrol derivatives. Interestingly, only C3 was able to significantly induce a sub-G1 (6-fold) and a G2/M (2-fold) arrest in HCT-116 cells (Figure 5.7), indicative of possible cell death pathways being activated and/or ROS generation. Moreover, when looking at the G2/M arrest resveratrol also actively arrested the cells at this checkpoint (Figure 5.7) Notably, resveratrol amongst all tested compounds shifted the cells into an S-phase arrest as well (Figure 5.7).

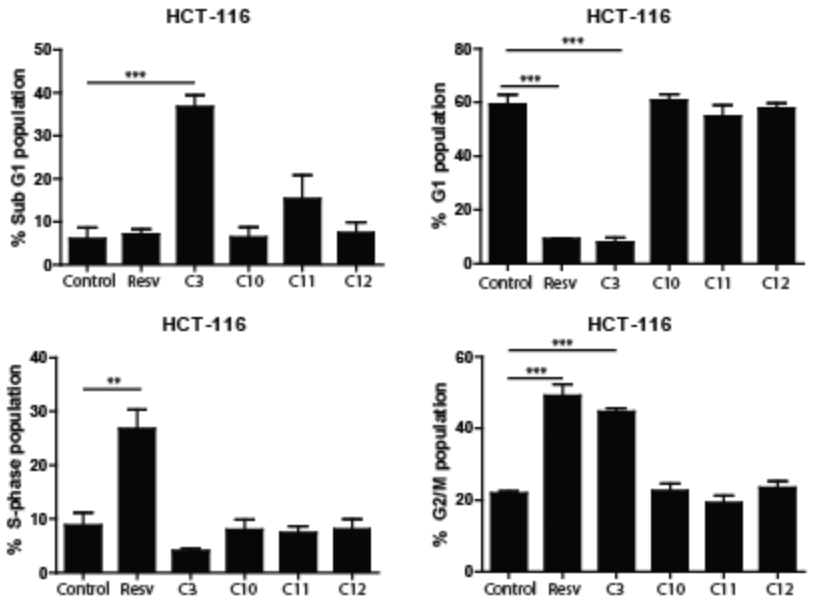


Figure 5.7: Effect of resveratrol and derivatives on cell cycle regulation in HCT-116 cells: Cell cycle analysis in HCT-116 cells using PI staining followed by FACS analysis. In HCT-116 colon cancer cell line, resveratrol (100 µM) appears to induce S-phase and G2/M arrest whereas C3 (100 µM) significantly induces cell death and a G2/M arrest. Shown here is the quantification of the cells in each phase. n=3 "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

5.2.5 Sirtuins are activated by Resveratrol and C11 but not the other derivatives

Resveratrol has been suggested to have broad clinical applications ranging from anti-aging properties, cardioprotective abilities, metabolic regulator and anti-inflammatory properties [208, 351, 352]. Several targets have been identified including the sirtuin family of proteins, primarily SIRT1[208]. Sirtuins function as lysine deacetylases to mediate deacetylation reactions coupled to NAD hydrolysis coupled to NAD hydrolysis [353]. This provides a direct link to the energy status of the cell and hence links to AMPK. It appears as though SIRT1

activation by low doses of resveratrol is the key target of resveratrol that is subsequently responsible for AMPK activation [354]. Evidence from cell culture studies suggests that overexpression of SIRT1 reduces the acetylation of LKB1, and increases the activity of LKB1 (and hence activation of AMPK), while knock-down of SIRT1 expression increases LKB1 acetylation and reduces LKB1 (and AMPK) activity. [212, 355] Thus it is important to determine effect of resveratrol derivatives on sirtuin and AMPK activities. Analysis of sirtuin activity using the β-NAD peptide assay revealed that parent resveratrol and the C11 derivative can activate SIRT1 whereas all the resveratrol derivatives cannot (Figure 5.8). This suggests an important difference between parent resveratrol and its derivatives (mainly C3) and negates modulation of sirtuin activity as primary cause of any biological property seen in response to C3.

We next investigated activation of AMPK in HCT116 cells using parent resveratrol and its derivatives. Interestingly, whereas the C11 showed similar AMPK activating properties to resveratrol, the C3 compound revealed a substantial 3.2 fold induction of AMPK activity as indicated by the expression level of its active form (p-AMPK Thr172) (Figure 5.8). Taken together, it appears that C11, similar to resveratrol, activates AMPK by activating SIRT1 whereas the C3 derivative is activating AMPK independent of SIRT1.

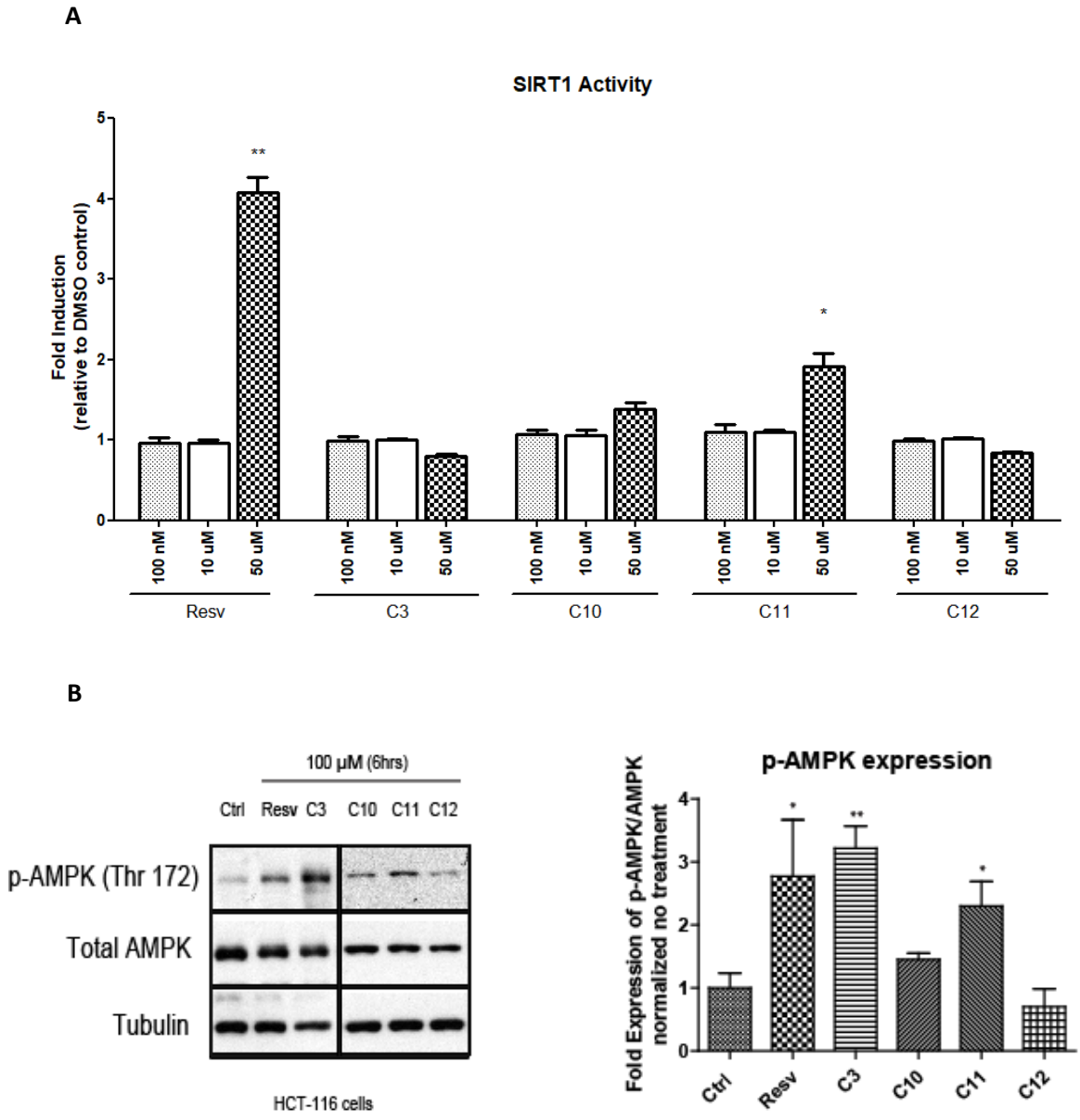
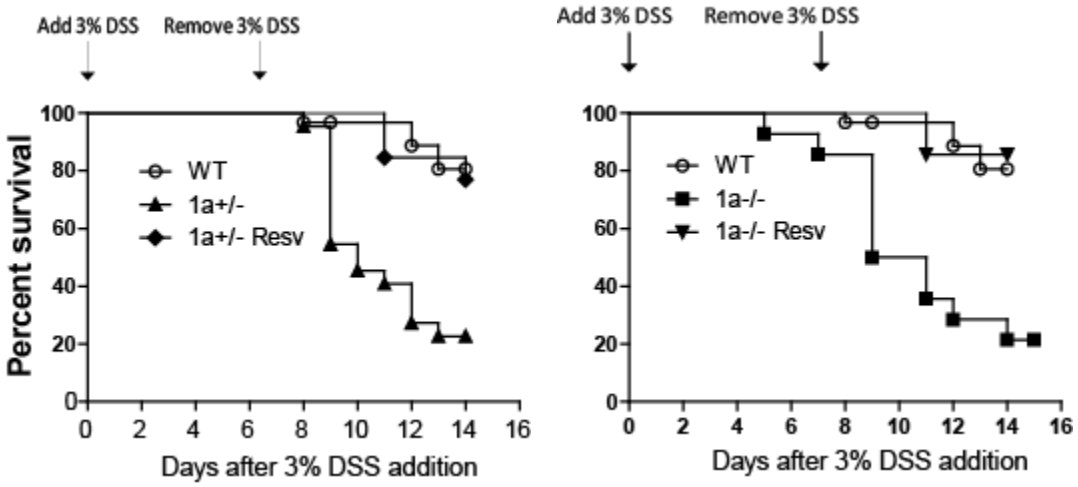


Figure 5.8: Analysis of sirtuin activity and AMPK activation using various aspirin-resveratrol derivatives. (A) SIRT1 assay is described in methods in details**. (B)** For westerns, HCT116 colon cancer cells were treated with the drugs for 6 hrs and then harvested and blotted for p-AMPK (Thr172), total AMPK and tubulin. Expression was first normalized to total AMPK expression and then to control cells (no drug added) to get fold induction. Right panel is a quantification of the left panel. n = 3 "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

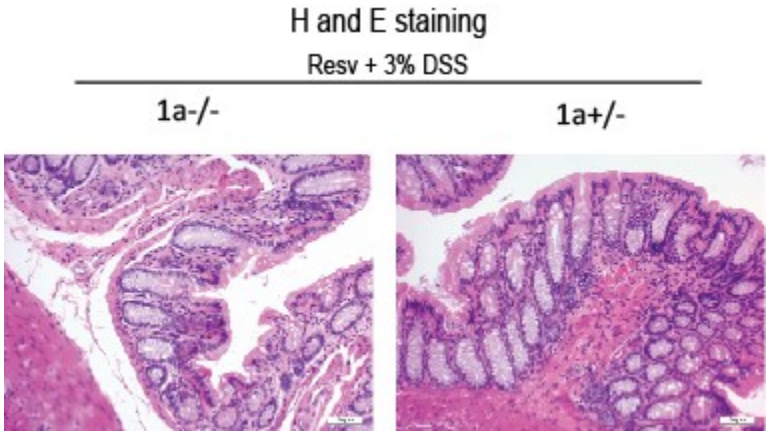
5.2.6 Resveratrol can alleviate the DSS induced acute intestinal inflammation injury

Our group has previously published on the sensitivity of the *Rassf1a*$^{-/-}$ animal in response to DSS-induced colitis-like model in rodents [122]. Hence we aimed to assess the effectiveness of treating the homozygous *Rassf1a*$^{-/-}$ and heterozygous *Rassf1a*$^{+/-}$ mice with resveratrol. Our attempts clearly indicate that resveratrol can alleviate the effect of DSS-induced intestinal inflammation with the use of diet-fed resveratrol to promote about 80% survival recovery in mice, as well as significantly reducing colitis like symptoms in these mice (Figure 5.9). Histological staining confirms efficient recovery from inflammation injury to reveal an intact and healthy crypt structure (Figure 5.9). Colon length, crypt depth and PCNA staining, as indications of a healthy repaired colon, were also significantly restored in the resveratrol fed animals (Fig 5.9). As mentioned earlier, resveratrol is limited by its bioavailability and metabolism *in vivo*. This was counteracted by providing the mice with the resveratrol on a daily basis with food at a concentration of 1g/kg of diet throughout the experiment. Further experiments with the derivatives would require optimizing the dosage, frequency and route of administration. Our speculation is that a lower dosage and less frequent intravenous injections would suffice and replace resveratrol.

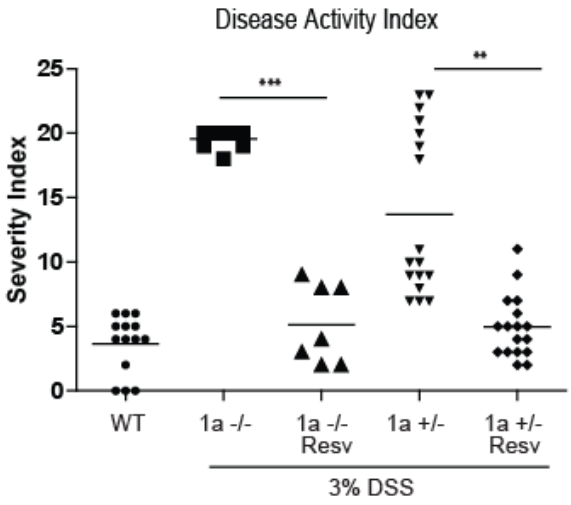
A)


Add 3% DSS
Remove 3% DSS
Percent survival
WT
1a+/-
1a+/- Resv
Days after 3% DSS addition
Add 3% DSS
Remove 3% DSS
WT
1a-/-
1a-/- Resv
Days after 3% DSS addition

B)


H and E staining
Resv + 3% DSS
1a-/-
1a+/-

C)


Disease Activity Index
Severity Index
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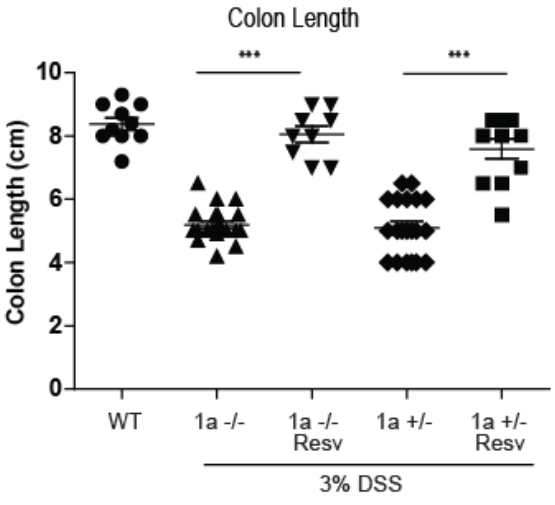
WT
1a -/-
1a -/- Resv
1a +/-
1a +/- Resv
3% DSS

D)


Colon Length
Colon Length (cm)
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WT
1a -/-
1a -/- Resv
1a +/-
1a +/- Resv
3% DSS

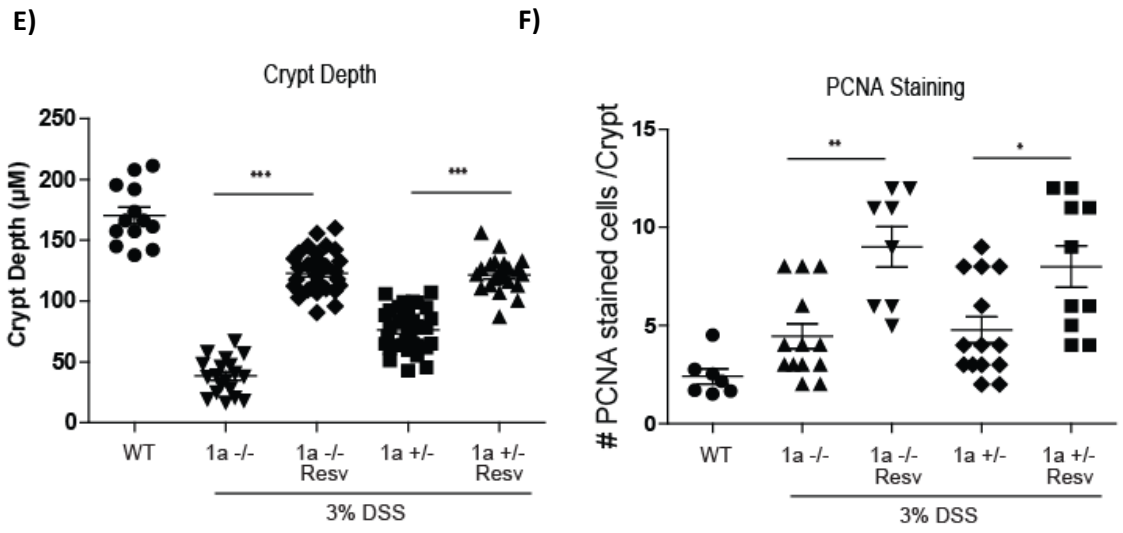

Figure 5.9: Resveratrol is protective *in vivo* against DSS-induced colitis in *Rassf1a* knockout mice. A). Kaplan-Meier curve monitoring % survival following DSS treatment. Resveratrol fed mice showed survival rates comparable to wild type when given DSS in drinking water. For both the *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice on resveratrol and DSS, p-value < 0.0001 when compared to counterpart on regular diet and DSS. **B)** Representative figures of the descending colon stained with H&E for knockout mice fed with resveratrol reveal a well-defined crypt structure. **C)** Resveratrol fed *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ knockout mice showed less susceptibility to disease when given DSS in drinking water. **D)** Colon length as measured at day 8.5 of DSS treatment. Resveratrol maintained "normal" (wild type) lengths indicative of non-injured healthy colon. **E)** Crypt depth as determined by ImageJ analysis of crypts at day 8.5. Resveratrol fed animals showed both a more intact and deeper crypt in both knockout genotypes. **F)** Resveratrol fed animals showed higher PCNA staining indicative of enhanced tissue repair and wound healing. For all experiments at least 7 animals were used. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

5.3 Discussion

Resveratrol has emerged into a multi-purpose plant-derived polyphenol that regulates diverse cellular processes ranging from energy metabolism, inflammation, cardiovascular protection and aging. For some of these, the biological target of resveratrol has been determined but for most it is still unknown. Molecular targets of resveratrol are still being uncovered [208, 351,

356] and modulation of multiple targets may explain the beneficial effects of resveratrol when used to ameliorate disease. Although having a robust *in vivo* effect, resveratrol suffers from it rapid and extensive metabolization into its glucuronide and sulfate conjugates as well as to the corresponding reduced products. In an attempt to obtain *in vivo* rapid metabolization while maintaining or enhancing biological specificity several groups have attempted to derivatize resveratrol including several monoalkoxy, dialkoxy and hydroxy analogs of resveratrol that were more effective in activation of protein kinase C (PKCa) [357, 358], mono, di, and tri-acetoxy resveratrol were more effective at *in vitro* inhibition of Cytochrome P450 3A4 (CYP3A4) [359].

In this study, we further demonstrate the effectiveness of some of these derivatives on inhibition of growth, NFκB activity, sirtuins and AMPK activities, with C3 derivative showing a greater potential in inducing cell death and activating AMPK. Interestingly, AMPK activation in response to C3 was independent of SIRT1 (Figure 5.8), thus further distinguishing this derivative from the parent resveratrol compound. Furthermore, we can demonstrate significant alleviation of intestinal inflammation with the parent compound resveratrol (Figure 5.9). We have been able to confirm the previous observations of Singh *et al* (2010) that demonstrated the beneficial effect of resveratrol on DSS-induced colitis [360]. Because of the effectiveness of several resveratrol derivatives towards both growth and NFκB inhibition (such as C3 and C11), these resveratrol derivatives could be potent inhibitors of inflammation driven malignant transformation. An analysis of oral bioavailability, glucuronide and

sulfate conjugation of these aspirin-resveratrol derivatives should be further investigated, but we speculate that the derivatives are more stable than parent resveratrol, as a result of protected hydroxyl groups, to result in increased biological activities. Although some resveratrol derivatives reveal similar relative IC_{50} values for growth and NFκB inhibition, we can start to observe differences in how they can inhibit growth. Derivative C3 can clearly promote cell death while C11 induced a slight increase the subG1 population (Fig 5.7). Parent resveratrol and C11 appreciably activated SIRT1, unlike C3, to suggest a SIRT1 independent pathway in the C3 mediated responses.

In general, preclinical studies have yielded promising results about the benefits of resveratrol for the management of a variety of disease related to inflammation [361] as well as for cancer [362]. It is important to know that treatment and/or prevention of most diseases in animals do not always translate to human studies, so care should be taken in our interpretation of the effectiveness of resveratrol in treating humans. Indeed, a prospective cohort study demonstrated that total urinary resveratrol metabolite concentration was not associated with inflammatory markers, cancer or cardiovascular disease; or predictive of all-cause mortality [363], suggesting that there is no benefit to resveratrol supplementation in humans. However, that study investigated these outcomes in older community-dwelling adults who consume resveratrol as part of their regular diet and the amount of resveratrol consumed from the dietary sources is much lower than the therapeutic doses of resveratrol used in clinical trials. Since most of the effects of resveratrol are dose-dependent [364], the

negative findings from the observational study [363] is probably not representative of the effects of resveratrol when administered as a nutraceutical. Therefore, it is important to address the challenges that face the translation of the very promising preclinical results to real-world clinical benefits of resveratrol.

Perhaps one of the largest challenges associated with resveratrol is its low bioavailability [365-367]. As mentioned earlier, resveratrol is highly absorbed when given orally, it has a very low bioavailability due to rapid metabolism to its glucuronide and sulfate conjugates [368]. Administration of approximately 25 mg resveratrol results in plasma concentrations that are between 1 and 5 ng/mL [369], and administration of even larger amounts, up to 5 g, still only yield 500 ng/mL [370]. This has resulted in the concept of the “resveratrol paradox”, which describes a molecule with a very low plasma concentration that has multiple biological effects [371]. To explain this, a number of theories have been proposed ranging from resveratrol concentrating in certain organs [372] to the evidence that shows the conversion of sulfated conjugates of resveratrol back to the parent compound in certain cell types [373]. Although this latter concept presents a possible explanation for the *in vivo* results observed for resveratrol for the treatment of disease in humans, it needs to be investigated whether the appropriate target cells also have the ability to transport the sulfated conjugates of resveratrol into the cell to be metabolized back to the parent compound [373].

Due to the poor bioavailability of resveratrol, there remains a question as to what dose should be used for clinical studies? This issue of dose is particularly important as several resveratrol effects are dose-dependent. Furthermore, since

resveratrol may exhibit a hormetic dose-response, this further complicates a dose selection for clinical studies [374]. Another important and related question is what method of administration should be chosen. Although resveratrol is poorly soluble in aqueous solutions [375], some preclinical studies administer resveratrol in the drinking water, which may create dose variability issues. How this hurdle will be overcome in clinical trials has yet to be addressed but the poor solubility of resveratrol may be enhanced by increasing its aqueous solubility via microparticulate systems, cyclodextrin complexes, nanocarrier systems, or even vesicular systems (reviewed in [367]). Whether or not this will improve the effectiveness of resveratrol in clinical studies has yet to be tested. Because of the aforementioned reasons, the bioavailability of our resveratrol derivatives will need to be fully investigated in order to be useful clinically. Our study provides *in vitro* and *in vivo* insights into these resveratrol derivatives but further usefulness will require extensive pharmacokinetic analysis and bioavailability determination before human clinical trials are attempted.

CHAPTER SIX

Identification and Characterization of Novel NFκB and Receptor Interacting Protein Kinase 2 Inhibitors

Abstract

It has been demonstrated that RASSF1A can restrict the activity of NOD2/RIPK2 and thus NFκB-directed inflammation. Our hypothesis was that since RIPK2 is an obligate kinase for NOD2 and linked to NFκB activation and inflammatory responses, RIPK2 inhibition should resolve the NFκB mediated inflammation, both *in vitro* and *in vivo*. For that purpose, our collaborators utilized the RIPK2/ponatinib crystal structure as a guiding interaction, and were able to identify several small molecules that can potentially inhibit RIPK2. We aimed at biochemically characterizing these inhibitors to assess their potential use for IBD treatment. As compared to other known RIPK2 inhibitors such as ponatinib, our novel inhibitors appear to be more selective towards RIPK2 with less off target inhibition. More importantly, when tested in the *in vivo* mouse model of intestinal inflammation, the identified inhibitors resolved the symptoms associated with DSS induced colitis and enhanced the survival of mice more efficiently than the well-known RIPK2 inhibitor, gefitinib. With further pharmacokinetic analysis and characterization, the identified novel small molecule RIPK2 inhibitors can be options for the therapeutic control of inflammation in diseases such as inflammatory bowel disease.

6.1 Introduction

The promising results in the previous chapter on using the general anti-inflammatory compound resveratrol in the DSS model of colitis encouraged us to investigate the use of a more specific and targeted approach. Signalling through pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) represents a major pathway by which NFκB is activated [230, 231]. NOD2 is an intracellular PRR that is mainly stimulated by the bacterial product MDP found in both Gram negative and positive bacteria. RIPK2 activation has been shown to be closely associated with NOD2 downstream signalling leading to the activation of inflammatory signals as well as autophagic pathways. Evidence from knockout mice with a genetic manipulation affecting the *Nod2/Ripk2* arm of signalling reveals a dysbiotic shift in the composition of the intestinal flora favouring an inflammatory environment [39]. The activation of the NOD2/RIPK2 pathway appears to also play an important role in inflammation driven pro-survival and oncogenic signals. Several reports have highlighted the role of RIPK2 in cellular migration and metastasis; the knockdown of RIPK2 resulted in decreased mRNA levels of E-cadherin and vimentin which are critical regulators of epithelial to mesenchymal transition (EMT) [376]. Given the aforementioned and other evidence, intervention at the level of RIPK2 inhibition can provide an important therapeutic alternative for both abnormal inflammation and the inflammation related carcinogenesis process.

Many groups have aimed at targeting RIPK2 activation in an attempt to block NFκB activation. That said, several RIPK2 inhibitors have been designed

with the hope to shut off the NOD/RIPK2 pathway and prevent unwanted excessive inflammation. Most RIPK2 inhibitors are small molecules of the type I inhibitor class, competing with ATP at the kinase ATP pocket. SB203580 is mainly a p38 inhibitor that has been shown to have an off target effect of inhibiting RIPK2 by acting as a type I inhibitor [257]. Gefitinib, which is known for its inhibitory effects on the epidermal growth factor receptor (EGFR), has also been reported to inhibit RIPK2 in a similar fashion to SB203580 [377]. Other novel inhibitors (OD36 and OD38) described by Tigno-Aranjuez *et al* (2010) show great efficacy towards inhibiting RIPK2 at 100 nM and can also alleviate inflammation in a rodent model of inflammatory bowel disease [252]. Still, like many other RIPK2 inhibitors, more than 80% inhibition of Fyn, activin receptor-like kinase-2 (ALK-2) and Lck was observed at the effective 100 nM concentration. Hence, selectivity towards RIPK2 with minimal off target inhibition appears to be a major limitation of the current RIPK2 inhibitors.

Recently, Canning *et al* (2015) demonstrated that RIPK2 can also be inhibited to a better extent by the FDA approved drugs ponatinib and regorafenib that act as type II inhibitors to target the inactive conformation of the kinase domain of RIPK2 [259]. This interesting finding revealed a critical allosteric site of RIPK2 suitable for future design of selective RIPK2 inhibitors. In fact, our approach to identify possible RIPK2 inhibitors relied on carefully analyzing the crystal structure of RIPK2 in association with ponatinib. Molecular docking and cheminformatics analyses were performed by our collaborators Rodrigo Aguayo-Ortiz and Carlos A. Velazquez-Martinez. Chemical databases were searched for

scaffolds having significant binding interactions similar or stronger to that of ponatinib with RIPK2 by comparing their free energies when docked inside the RIPK2 binding site. In addition, the exclusion criteria included molecules with high binding interactions with the EGFR and c-Abl binding sites, two off-targets for previously identified RIPK2 inhibitors. Table 6.1 shows some of the hits, most of which have unknown biological features. For the purpose of this study, the focus will be on two inhibitors Molport-016-359-762 (RIPK2 Inh-1) and PC_44716361 (RIPK2 Inh-2). Structures of the inhibitors cannot be shown due to pending patent processes.

Table 6.1: Calculated binding free energies of potential RIPK2 inhibitors.

Search method	Compound	ΔG_{bind} (kcal/mol)	Compound	ΔG_{bind} (kcal/mol)
Pharmacophore	Ponatinib	-11.9	MolPort-015-604-588	-10.6
	MolPort-016-359-762	-11.5	MolPort-016-412-727	-10.6
	MolPort-015-752-252	-10.8	ZINC02739307	-10.1
Structure similarity	PC_57410628	-12.0	PC_58945682	-11.5
	PC_44716361	-11.8	PC_58945685	-11.5
	PC_24826801	-11.8	PC_68349611	-11.5
	PC_57405602	-11.6	PC_58945669	-11.3
	PC_58945635	-11.6	PC_40780119	-11.1

Table 6.1: MolPort-016-359-762 is RIPK2 inhibitor 1 and PC_44716361 is RIPK2 inhibitor 2. The pharmacophore and structure similarity search as well as the molecular docking and free energy calculation were done by Rodrigo Aguayo-Ortiz and Carlos A. Velazquez-Martinez from the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta.

6.2 Results

6.2.1 Identified RIPK2 Inhibitors show minimal off target inhibition

As discussed earlier, off target inhibition represents a major limitation of many RIPK2 inhibitors especially those acting as type I inhibitors. To address the selectivity and possible off targets targeted by our identified inhibitors, we outsourced this to the DiscoverX company for utilization of their *scan*EDGE assay panel of 97 kinases. Based on previous optimization for other assays, the most biologically relevant concentration was 100 nM for both RIPK2 inhibitors 1 and 2. The kinome analysis of the 97 kinases tested revealed relatively low off target inhibition at the indicated concentration with 20-28% inhibition of mutated ABL1 (E255K), Aurora kinase B and ERBB2 for RIPK2 inhibitor 1. Similarly, RIPK2 inhibitor 2 appears to inhibit the activity of SNARK by 38% and 27% of the activity of FGFR2, GSK3-β, JNK1, CSNK1G2 (Caesin kinase 1), and MET tyrosine kinase. (Please see [378] for full list of kinases). More careful analysis is critical to assess how these inhibitors are affecting the aforementioned kinases empirically. Some off target inhibition will be expected as it is hard to obtain a one target one inhibitor equation. In fact, some off target inhibition could add or be part of the beneficial outcomes later observed with the use these inhibitors in the colitis murine model. Since c-ABL is a common off target for other RIPK2 inhibitors, we aimed at testing the effect of our inhibitors directly on activity of c-ABL using a commercial *in vitro* kinase approach with purified kinases at MRC Protein Phosphorylation and Ubiquitination Unit in Dundee, Scotland.

Both RIPK2 small molecule inhibitors do not seem to empirically inhibit c-ABL at concentrations up to 100 µM (Figure 6.1). Given this observation, we hope that any other off target effects will also be in a vastly different concentration range of 100 nM.

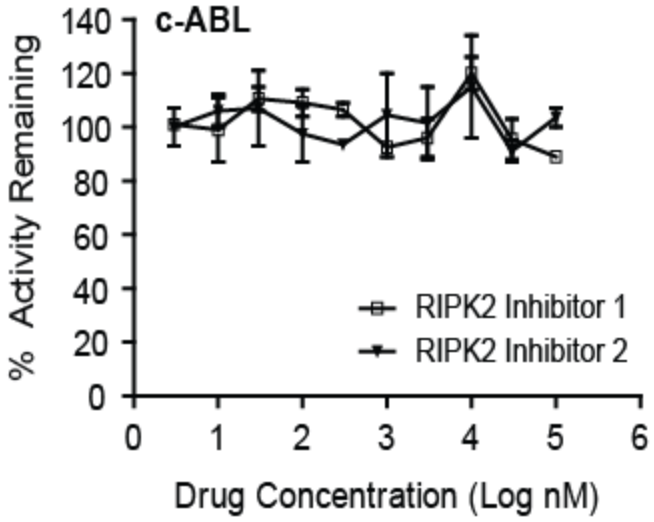

Figure 6.1: *In vitro* kinase activity for c-ABL was tested in the presence of inhibitor 1 and 2. Relative IC_{50} for c-ABL for both inhibitors is > 100 µM. Analysis was done using purified c-ABL and the substrate peptide, EAIYAAPFAKKK, in association with the MRC Unit on Phosphorylation and Ubiquitination, University of Dundee, Scotland.

6.2.2 Identified RIPK2 Inhibitors effectively prevents RIPK2 activation and inhibits subsequent NFκB activation.

An important aspect of RIPK2 activation is its phosphorylation at tyrosine 474 (Y474). Utilizing an *in vitro* kinase assay in HCT-116 stimulated with MDP and treated with either inhibitor 1 or 2, we can clearly see a reverse of the MDP triggered autophosphorylation in the presence of the inhibitors. Furthermore, immunodepletion using an anti-RIPK2 antibody confirmed specificity of detection of the autophosphorylated RIPK2 band (Figure 6.2).

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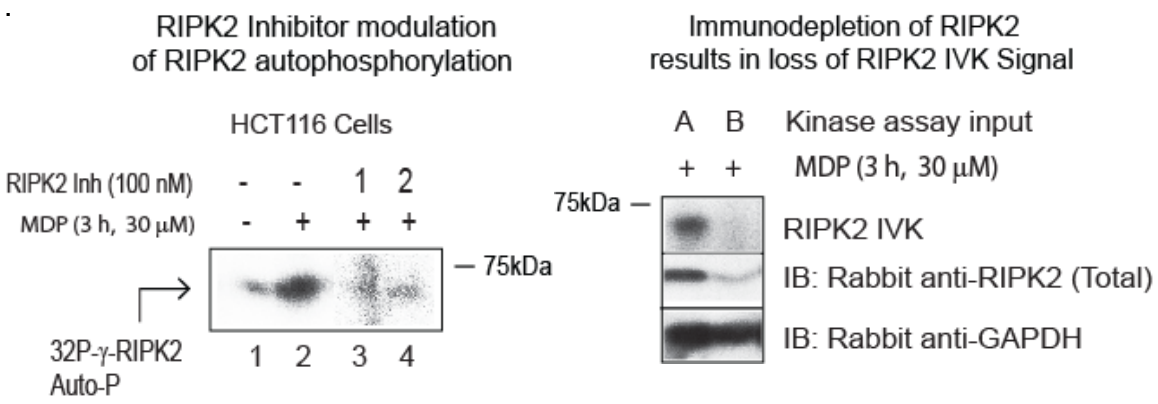


Figure 6.2: RIPK2 autophosphorylation at site Y474 is shown. Inhibition was performed on cells followed by lysis and immunoprecipitation (IP) with rabbit anti-RIPK2 overnight. Following protein G pull down and wash, ^{32}P-γ-ATP is added and kinase reaction allowed to proceed for 30 minutes at 30 C. Following kinase reaction, SDS-PAGE was used to separate out the proteins, gel was then dried and exposed to film. RIPK2 in HCT116 cells was IP with an anti-RIPK2 antibody (A) and the supernatant after IP in (A) was IP in (B) with the same RIPK2 antibody and *in vitro* kinase (IVK) assay was done for both samples.

When exploring the effect of the inhibitors across different cell lines, it was interesting to observe that the Hodgkin's Lymphoma cell lines HDMYZ and L428 constitutively show active RIPK2 (both Y474 and S176). Hence, these cell lines are useful models to assess the ability of the inhibitors to reverse this endogenous constitutively active form of RIPK2. Indeed, the use of the inhibitors as well as the known RIPK2 inhibitor, regorafenib, inhibited this signal as determined by the phospho-specific antibodies (Y474 and S176) (Figure 6.3).

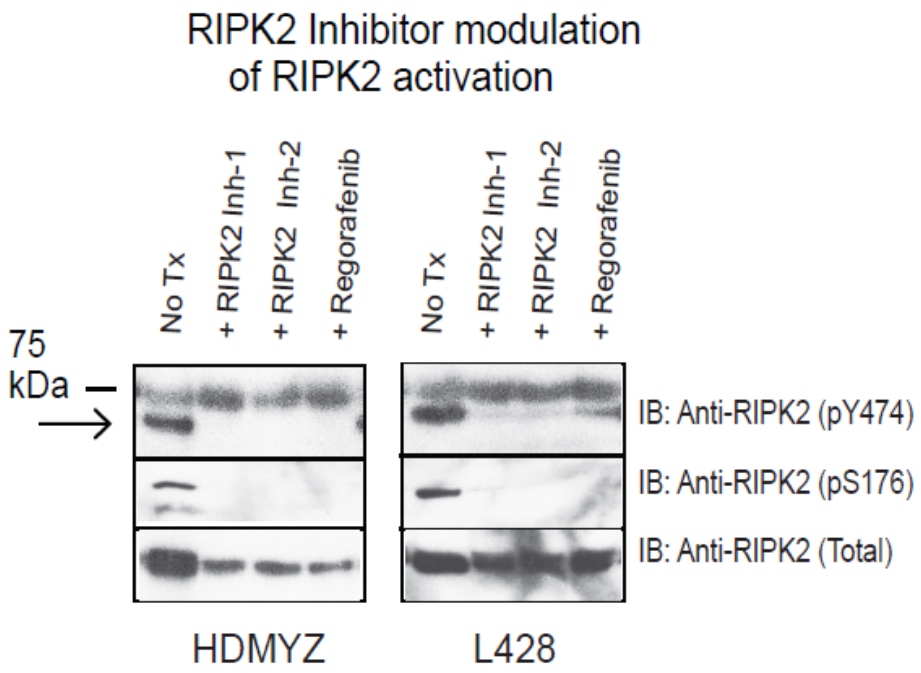
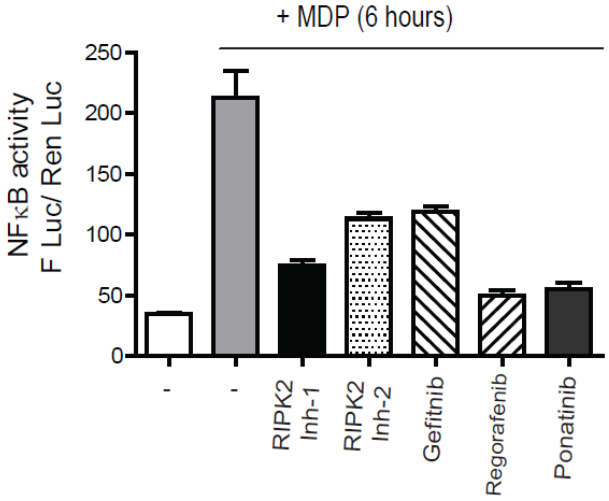
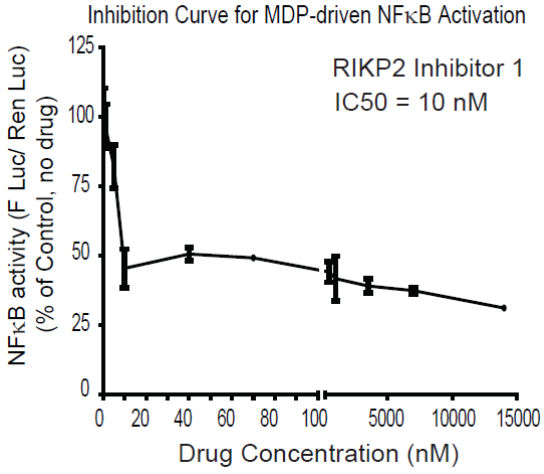


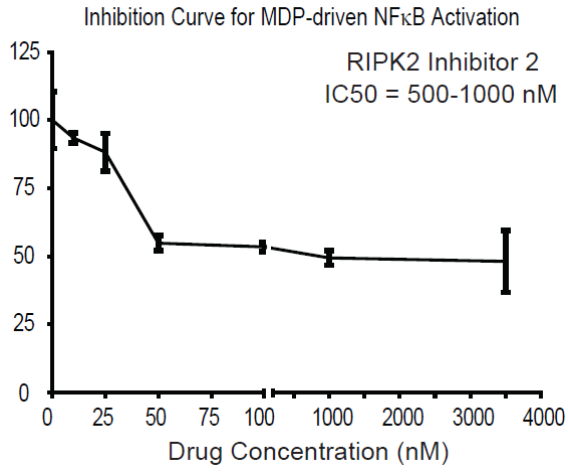
Figure 6.3: RIPK2 inhibitors inhibit the constitutively active RIPK2 in HDMYZ and L428 cells. A comparison with a known RIPK2 inhibitor is shown (regorafenib). All inhibitor concentrations were 100 nM

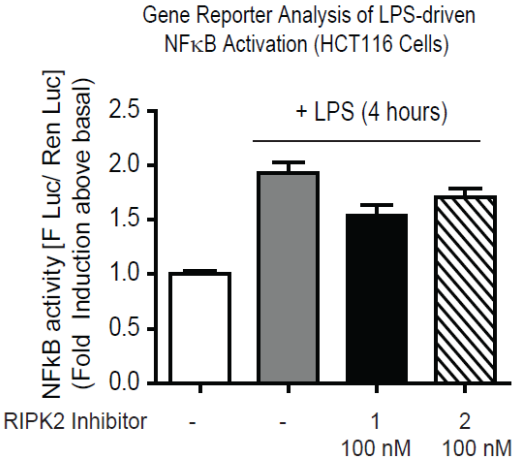
The NOD/RIPK2 pathway eventually leads to the flux of pro-inflammatory signalling through activation of NFκB. We thus investigated the functional consequence of inhibiting RIPK2 with our lead identified inhibitors 1 and 2. Clear enough, both inhibitors effectively reversed MDP-driven NFκB activation; RIPK2 inhibitor 1 being slightly more effective (Figure 6.4). The relative IC_{50} for NFκB inhibition was determined to be approximately 10 nM for RIPK2 inhibitor 1 and 0.5 to 1 μM for RIPK2 inhibitor 2 (Figure 6.4). Specificity towards the MDP-NOD2-RIPK2 driven pathway was confirmed by stimulating HCT-116 cells with LPS and applying the inhibitors. No significant reduction of LPS stimulated NFκB activation was observed with either inhibitor at 100 nM, and with concentrations as high as 20 μM more than 60% of activity was still observed (Figure 6.4).

These observations validate the specificity of our RIPK2 inhibitor for NOD/RIPK2 biology and usefulness as potential *in vivo* inhibitors of MDP-driven inflammation.









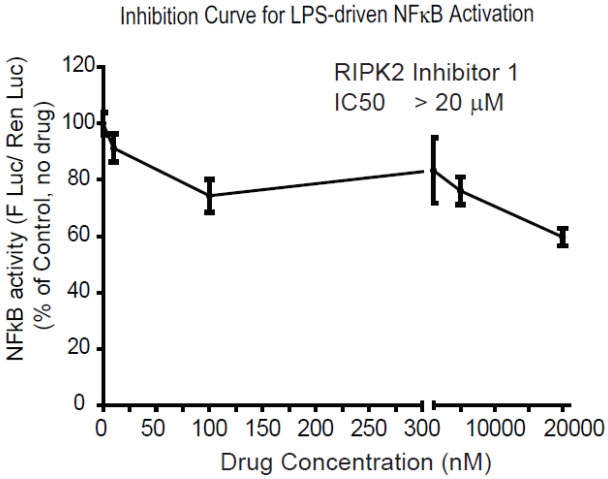


Figure 6.4: RIPK2 Inhibition of NFκB Activity. NFκB gene reporter assay determination of inhibition of MDP stimulated NFκB activity with RIPK2 inhibitors and relative IC_{50} determination for inhibition of MDP driven-NFκB activation using RIPK2 inhibitors 1 and 2. All drugs in first panel were utilized at 100 nM. P values for RIPK inhibitor treated vs MDP (no drug) = < 0.0001 (inhibitor 1), 0.06 for inhibitor 2, < 0.006 (Gefitinib), 0.0002 (Regorafenib), and 0.0008 (Ponatinib). For all n = 4-10 and relative IC_{50} curves are shown in second and third panels. Fourth panel shows NFκB gene reporter assay determination of inhibition of LPS stimulated NFκB activity with RIPK2 inhibitors (concentration as indicated) (n = 4-6). Last panel, relative IC_{50} determination for inhibition of LPS-NFκB driven inflammation using RIPK2 inhibitor 1.

6.2.3 RIPK2 Inhibitors Resolve Intestinal Inflammation in the DSS induced Model of Colitis

The use of DSS to model IBD like pathology, mainly ulcerative colitis is widely reported. DSS functions to irritate the colonic mucosa allowing bacterial invasion and promoting localized inflammation [379]. Our group has previously demonstrated the importance of RASSF1A in controlling the pathogenesis of colitis symptoms in mice [122]. The *Rassf1a* knockout mice are very susceptible to DSS-induced inflammation mainly due to excessive signalling of PRRs (TLR4 and NOD2) through NFκB [122]. We therefore injected the animals intraperitoneally with 1 µg/g body weight of RIPK2 inhibitor 1 or 2 on days 5, 7, and 9 to offset the pre-inflammation damage stage, peak inflammation damage stage and post-inflammation damage/restitution phase. Using this treatment scheme, we observed a robust difference on day 9 in the disease activity indices of wild type and *Rassf1a* knockout mice that was significantly inhibited with the use of the RIPK2 inhibitors, especially RIPK2 inhibitor 1 (Figure 6.5). Interestingly, a newly characterized RIPK2 inhibitor, gefitinib, can also inhibit

intestinal inflammation injury but only promote a 47% survival vs 73% survival with RIPK2 inhibitor 1 (Figure 6.5). Since both gefitinib and our RIPK2 inhibitors can inhibit RIPK2, either our drug has more affinity for RIPK2 or the off target effects of RIPK2 inhibitor 1 are beneficial to aid in recovery from inflammation injury using the DSS model and enhance better survival.

RIPK2 Inhibitor	Treatment and Genotype	Percent Survival
No Drug	DSS-treated *Rassf1a*$^{-/-}$	7/30 = 22%
1 (MolPort-016-359-762)	DSS-treated *Rassf1a*$^{-/-}$	8/11 = 73 %
2 (MolPort-001-746-327)	DSS-treated *Rassf1a*$^{-/-}$	5/9 = 56 %
Gefitinib	DSS-treated *Rassf1a*$^{-/-}$	7/15 = 47 %

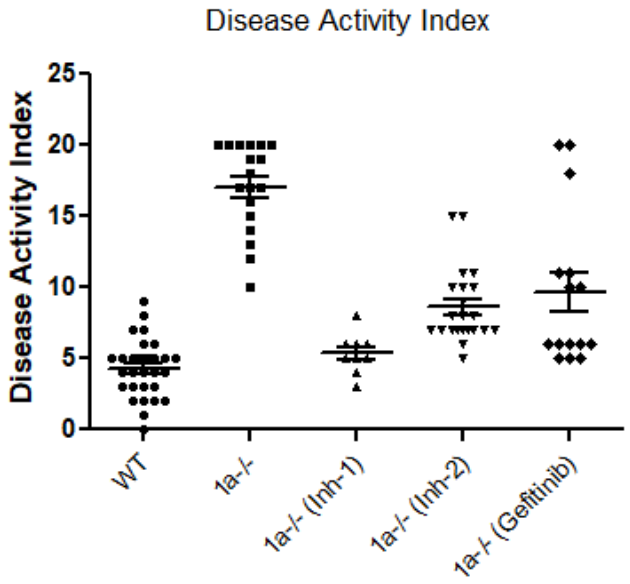


Figure 6.5: Survival of *Rassf1a*$^{-/-}$ animals during DSS-induced inflammation. The presence of RIPK2 inhibitors reduced acute intestinal inflammation and enhanced overall survival of the mice. Intestinal inflammation injury was induced using the dextran sodium sulfate (DSS) model. DSS is taken up in the drinking water and migrates to the colon to cause irritation and localized inflammation. *Rassf1a*$^{-/-}$ mice are extremely sensitive to this model with < 25% survival following a 3% DSS insult for 7 days followed by water for 7 days. Most *Rassf1a*$^{-/-}$ mice require euthanasia by day 7-9. Disease activity is scored based on rectal bleeding, piloerection, movement and body weight changes. N = 10 – 25 and p value of *Rassf1a*$^{-/-}$ (DSS) vs WT (DSS) was < 0.0001; *Rassf1a*$^{-/-}$ (DSS) vs *1a*$^{-/-}$ (DSS + inhibitor 1 or 2 or gefitinib) was < 0.0001. Animal data produced here were done with the grateful help of Ahmad Said.

6.3 Discussion

Therapies controlling excessive inflammation are critical to prevent the much unwanted arise of inflammation related cancers. Colorectal cancer is only one example that is highly related to inflammatory bowel disease (IBD). It is now known that individuals with IBD, concurrent with primary sclerosing cholangitis (PSC) are at much higher risk of developing CRC and require very close management of their disease [380]. The NOD2/RIPK2 pathway is at the root of several inflammatory disorders including IBD, arthritis [381] and significantly contributes to the severity of the experimental colitis model [382]. RIPK2 is described as an obligate kinase for NOD2 signalling and is shown to be involved in the activation of NFκB as well as the metastatic behaviour of some cancers [258]. Hence, targeting RIPK2 to resolve inflammation is a promising approach to prevent inflammation related disorders and cancer.

In an attempt to intervene with the above mentioned NOD2/RIPK2 pathway, we collaborated with Rodrigo Aguayo-Ortiz and Dr. Carlos A. Velázquez-Martínez to identify potential novel RIPK2 inhibitors which led to the identification of several possible hits, two of which were explored in this study. When compared to the commonly known RIPK2 inhibitor, gefitinib, our identified RIPK2 inhibitors appear to be superior in resolving *in vivo* intestinal inflammation and enhance survival of DSS treated *Rassf1a*$^{-/-}$ mice by an additional 25% (Figure 6.5). This is despite similar *in vitro* results with gefitinib and regorafenib, another common RIPK2 inhibitor. This could be partially due to “favourable” off target effects of our novel inhibitors not targeted by other available inhibitors. A

broad spectrum inhibitor that targets NOD2/RIPK2, TLRs and possibly some PTKs could be an effective solution to resolve inflammation [383]. Our RIPK2 inhibitors 1 and 2 slightly inhibit the LPS stimulated TLR4 mediated activation of NFκB (Figure 6.4) at 100 nM and could possibly present one explanation for better survival results observed (Figure 6.5). Even though our kinome analysis shows some 27% inhibition of the mutated form ABL1 (E255K) by RIPK2 inhibitor 1, but the empirical *in vitro* kinase assay using purified peptides shows the converse (Figure 6.1); no significant inhibition of c-ABL with concentrations up to 100 μM of inhibitors 1 and 2. Analysis of colonic tissues from animals treated with the inhibitors will be key to understand what off targets are being inhibited *in vivo* and whether or not they contribute to the inflammatory resolving abilities of our novel inhibitors or not. In fact, a previous publication from our group describes the importance of c-ABL in driving inflammation in the acute DSS model of colitis [122]. Hence, if our RIPK2 inhibitors do interfere with the kinase ability of c-ABL *in vivo*, it might be a “favourable” off target effect after all. Many of the kinases that appeared to be targeted in the kinome analysis are involved in growth related pathways, tumorigenesis and cell cycle control events such as Aurora Kinase B [384]. These off target effects may actually be beneficial in treating IBD, PSC and related CRC that are characterized by a plethora of abnormal signaling involving inflammation, oxidative damage, DNA damage and apoptotic abnormalities. Thus, eliminating all of these abnormalities using a single small molecule can possibly restore homeostasis and reverse disease physiology.

According to databases from GeneCards [385], Proteomics databases [386] and The Human Protein Atlas [387], the distribution of RIPK2 is subject to tissue specific regulation and expression. It is relatively expressed at lower ranges in oral mucosa, stomach, liver, skeletal muscle, smooth muscle, adrenal gland, cerebral cortex, hippocampus, appendix and spleen. Moderately higher expression is found in thyroid, lymph node, tonsil, heart muscle, lung, pancreas, colon, kidney and skin tissues. The highest expression in humans is observed in the gallbladder, breast and placenta. Even though some mRNA has been detected in the bone marrow, the RIPK2 protein was undetectable when stained for in the bone marrow tissue. That said, the use of the RIPK2 inhibitors should not affect the hematopoietic properties of the bone marrow unless a critical off target kinase is substantially inhibited.

CHAPTER SEVEN

Translational Applications of Biomarkers in Tissues and Leukocytes of IBD Patients

Abstract

Inflammatory bowel disease (IBD) requires precise distinctive diagnosis of its two main subtypes UC and CD to ensure that patients receive the right treatment and care. To date, no “golden” biomarker has been found to provide clinicians with such tools to diagnose IBD and assess disease severity or predict response to therapy. Serological markers such as C reactive protein (CRP) can be misleading if not accompanied with an internal microscopic examination and the non-invasive faecal calprotectin biomarker cannot differentiate between UC and CD. In line with that, we aimed at translating our findings from previous murine studies into identifying potential biomarkers for IBD. Since resveratrol (AMPK activator) and RIPK2 inhibitors ameliorated intestinal inflammation in the DSS model of colitis, we hypothesized a reduced AMPK and an increased RIPK2 activity in IBD patients will be good indicators of therapeutic intervention and biomarkers of disease severity. We utilized a dual approach looking at human colonic biopsies and whole blood leukocytes to assess the correlation of AMPK and RIPK2 activity with IBD severity. Our results herein provide insight into using AMPK and RIPK2 as IBD biomarkers and a potential role for AMPK activators and RIPK inhibitors in the treatment and management of IBD.

7.1 Introduction

Our results so far describe the importance of RASSF1A in the pathogenesis of IBD and the usefulness of resveratrol and RIPK2 inhibitors in resolving intestinal inflammation associated with inflammatory bowel disease. Some interesting questions that arise from the previous results are: What is the status of AMPK which is known to be activated by resveratrol, in a human model of IBD? If RIPK2 inhibition appears to be effective in alleviating the symptoms of DSS induced colitis, can we expect abnormal levels of active RIPK2 in a human model of IBD? Can we propose the use of these proteins as biomarkers for IBD which can, in ideal cases, replace the need for invasive assessment of disease?

The NIH defined a biomarker as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention” [388]. An ideal biomarker should be simple, easy to perform, minimally invasive, cheap and reproducible between labs and individuals [389]. Adding to that, biomarkers should preferably be disease specific, correlate with disease severity monitor the effect of treatment. If the above mentioned qualities were found in an IBD marker, it would ease the work of physicians and gastroenterologists to better manage IBD. To date, no ideal biomarker for IBD has been proven to possess all aforementioned requirements [389]. Several serum-based biomarkers have been studied and applied with limitations in IBD patients. For example, ASCA (anti-*Saccharomyces cerevisiae* antibody) often shows positive results with CD but not as positive with UC [390]. The use of ASCA as a

diagnostic tool is highly limited due to the fact that about 16% of controls tested positive for this biomarker [391]. C Reactive Protein (CRP) is another biomarker that is used as an indication of acute inflammation and is produced by hepatocytes in response to IL-6 and TNF-α stimulation. CRP use is also limited due to its short half-life (19 hrs) and can be elevated in response to viral and bacterial infections. Other fecal biomarkers have also been utilized for monitoring IBD, but not for diagnosis. Calprotectin is highly found in neutrophil granulocytes at inflamed sites but it cannot distinguish CD or UC and increased levels can be found in neoplasms, infections and polyps [392]. Lactoferrin is also another glycoprotein expressed by neutrophils as well and even though, like calprotectin, it cannot differentiate between CD and UC but it has been shown to be useful to distinguish active from inactive IBD [393]. In summary, the need of histological examination of the colonic biopsies is still a must to diagnose, suggest therapies and monitor IBD. Continuous effort and research is needed to identify usable and reliable biomarkers.

That said, and given the interesting and relevant findings in the previous chapters, we aimed at validating our results in human IBD patients using both colonic biopsies as well as leukocytes isolated from whole blood. We believe that therapeutic intervention in treating IBD can greatly benefit from the identification of novel disease biomarkers, especially if personalized medicine becomes a more applicable option for IBD treatment.

7.2 Results

7.2.1 Colonic tissues from IBD patients show loss of RASSF1A expression, reduced expression of active AMPK and enhanced expression of active RIPK2

To validate our results that we have observed both *in vitro* and *in vivo*, we aimed at looking at the expression levels of the key proteins we have investigated so far. As mentioned earlier, *RASSF1A* promoter is hypermethylated in several cancers and our data in chapter 3 highlight a hot spot of methylation found in IBD and CRC patients. The immunostaining of human colonic biopsies obtained from non-IBD and IBD patients confirms the loss of RASSF1A expression in IBD patients, both in UC and CD patients with an approximately 70% reduction in expression as compared to non-IBD "controls" (Figure 7.1). Consequently, in the absence of RASSF1A to restrict NOD2 signalling, RIPK2 appears hyperactive in IBD patients, supporting our conclusions in chapter six on the importance of the design and application of RIPK2 inhibitors to manage IBD (Figure 7.2).

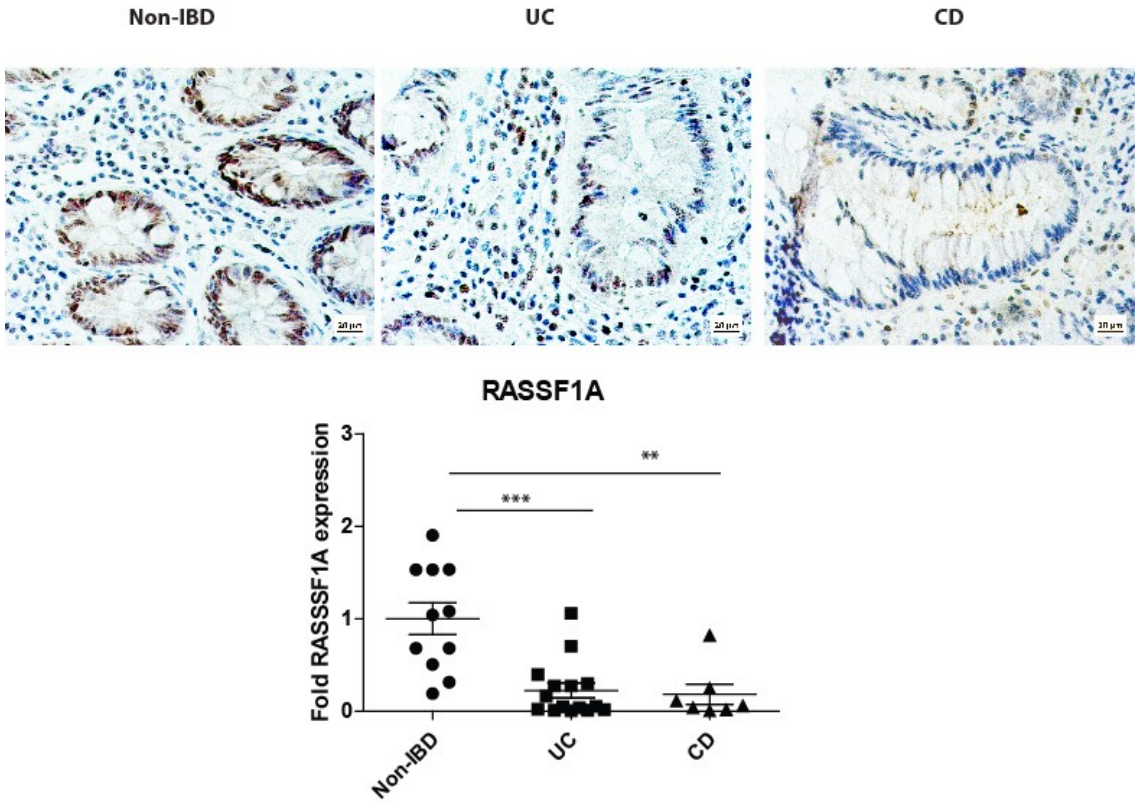


Figure 7.1: Immunostaining of colonic biopsies from IBD patients confirms loss of RASSF1A expression. Non-IBD patient biopsies or mounted sections were obtained from CRC screening individuals at the University of Alberta Gastroenterology Clinics. UC and CD patients were recruited as part of their regular follow up and endoscopy procedures. Shown here are representative figures (20x) from each category including 11 Non-IBD, 15 UC patients and 7 CD patients. Lower panel is quantification of the immunostaining done using ImageJ modified with a plugin to measure the intensity of DAB (Brown) staining. Fold change was obtained by normalization against Non-IBD staining. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

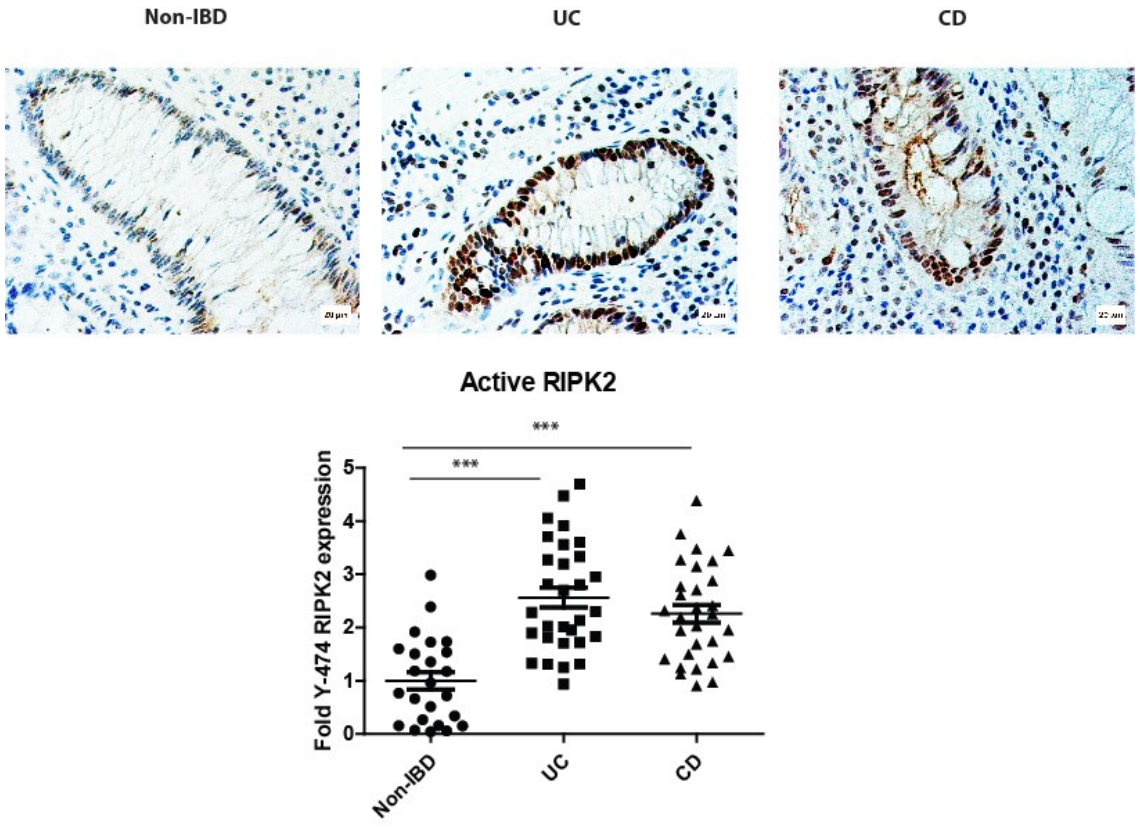


Figure 7.2: Immunostaining of colonic biopsies from IBD patients reveals hyperactive RIPK2. Non-IBD patient biopsies or mounted sections were obtained from CRC screening individuals at the University of Alberta Gastroenterology Clinics. UC and CD patients were recruited as part of their regular follow up and endoscopy procedures. Shown here are representative figures (20x) from each category including 24 Non-IBD, 30 UC patients and 30 CD patients stained with a Y474-RIPK2 antibody (active RIPK2). The quantification of the immunostaining done using ImageJ modified with a plugin to measure the intensity of DAB (Brown) staining. Fold change was obtained by normalization against Non-IBD staining. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

Furthermore, we also investigated the expression and modulation of AMPK in the colonic sections of IBD patients in contrast to non-IBD controls. AMPK is a key metabolic sensor and has recently been described as a tumour suppressor. The link between AMPK and inflammation is confirmed by studies showing a correlation between AMPK activation and COX-2 inhibition [192]. AMPK can also inhibit NFκB indirectly through the SIRT1 dependent

deacetylation of the p65 subunit NFκB [394]. IBD patients show lower expression of active AMPK (AMPK Thr172) as determined by immunostaining (Figure 7.3). This is suggestive that IBD treatments not only should address the inflammatory component but also should consider a disease with metabolic dysfunction. Given the protective role of resveratrol (AMPK activator) that we observed in the mouse model of colitis in chapter five and with the results herein, it is tempting to speculate that FDA approved drugs such as metformin (AMPK activators) will be promising for the treatment of inflammatory bowel disease.

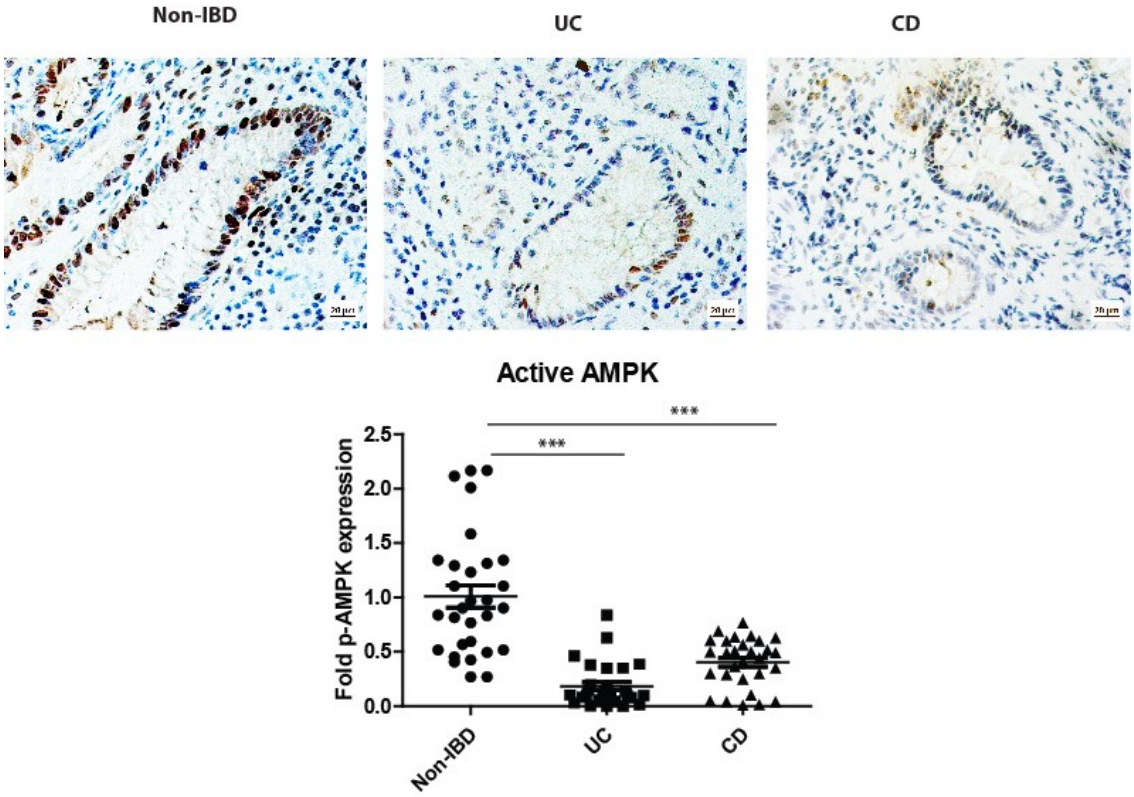


Figure 7.3: Immunostaining of colonic biopsies from IBD patients reveals a loss of p-AMPK (Thr172) expression. Non-IBD patient biopsies or mounted sections were obtained from CRC screening individuals at the University of Alberta Gastroenterology Clinics. UC and CD patients were recruited as part of their regular follow up and endoscopy procedures. Shown here are representative figures (20x) from each category including 24 Non-IBD, 30 UC patients and 30 CD patients. Lower quantification of the immunostaining was done using ImageJ modified with a plugin to measure the intensity of DAB (Brown) staining. Fold change was obtained by normalization against Non-IBD staining. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

7.2.2 Leukocyte analysis from IBD patients show reduced expression of active AMPK and enhanced expression of active RIPK2

Leukocytes are continuously recruited to inflammatory sites and play an important role in the immune response; our investigation of several biomarkers in leukocytes roots from the hope that any interesting findings can translate into an alternative or complementary tool to endoscopy and other biomarkers to assess disease progression and/or response to therapies. Similar to immunohistochemically stained colonic tissues; western blots analysis of active AMPK (p-AMPK Thr172) reveal an overall loss in both UC and CD patients (Figure 7.4).

Since acquiring blood is relatively simple and non-invasive compared to endoscopy, we attempted more detailed analysis of our results. Looking back at individual patient expression levels of active AMPK (Thr172), there appears to be some variation among the patients we investigated for both UC and CD patients (Figure 7.5).

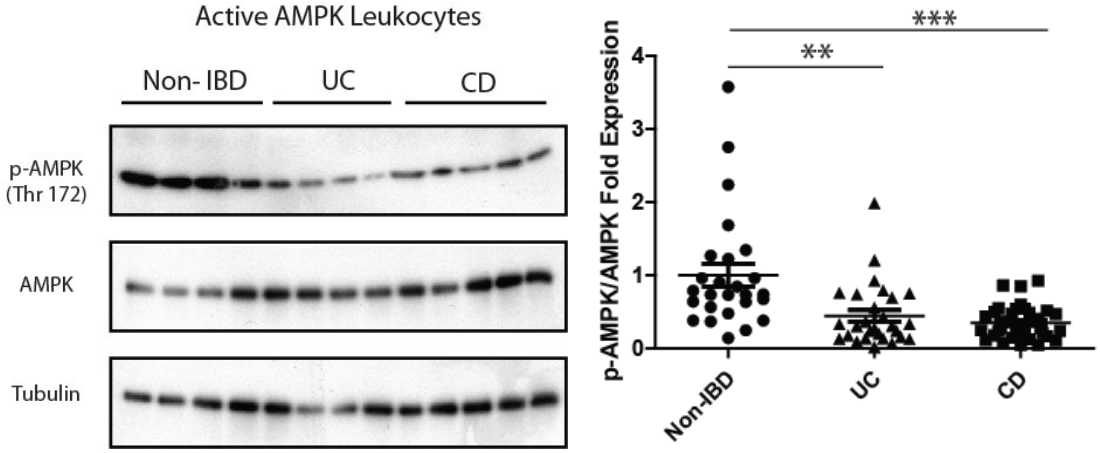


Figure 7.4: Western Blot analysis in leukocytes from IBD patients reveals a loss of p-AMPK (Thr172) expression. Whole blood was obtained from CRC screening individuals or healthy volunteers at the University of Alberta. UC and CD patients were recruited as part of their regular follow up and endoscopy procedures. Shown here is a western blot from each category including 12 Non-IBD, 26 UC patients and 26 CD patients. Quantification of the p-AMPK band was done after normalization against total AMPK values as determined using ImageJ. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

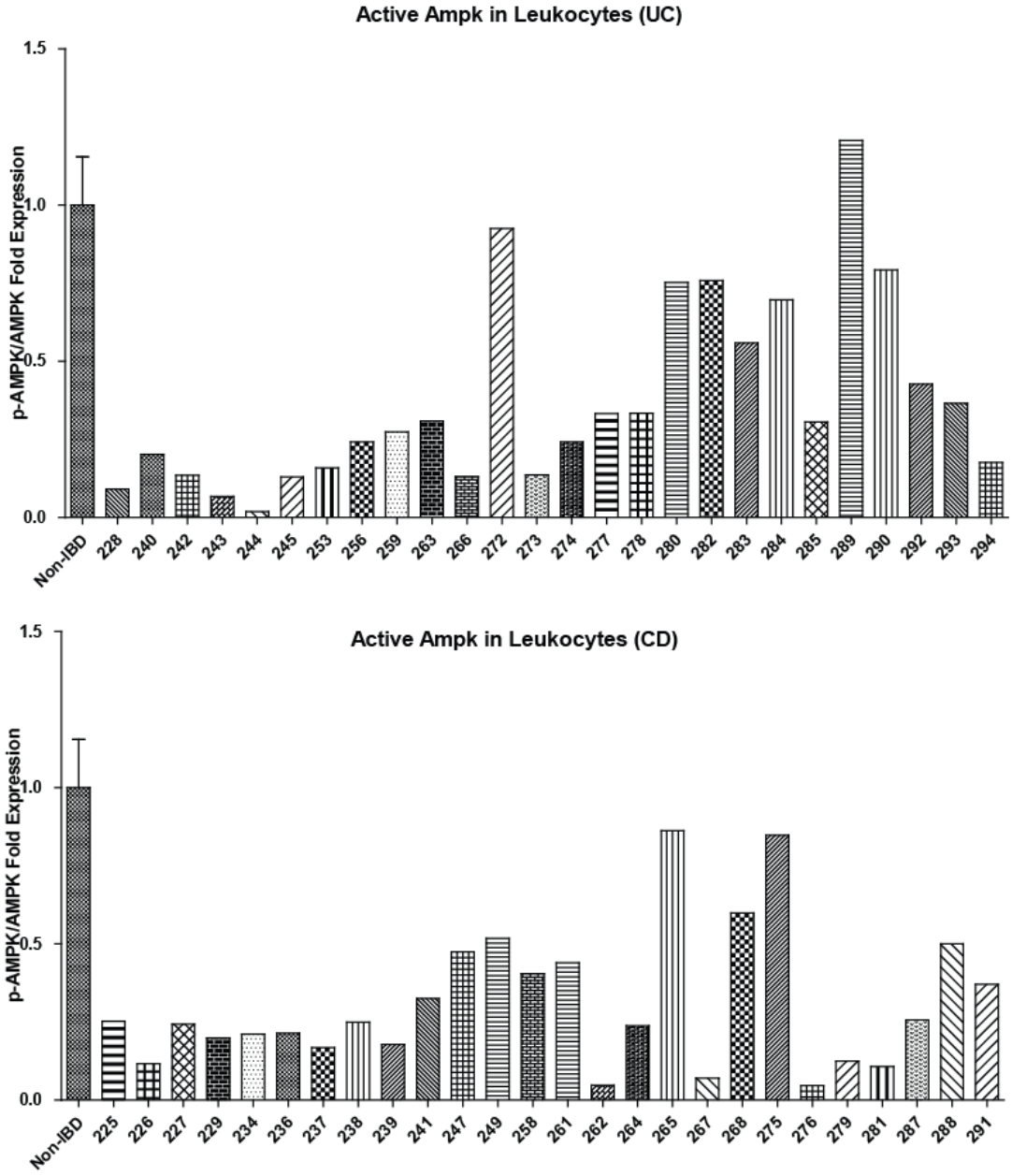


Figure 7.5: Western Blot analysis in leukocytes from IBD patients reveals some variation in active AMPK (Thr 172). Results from (Figure 7.4) were re-plotted individually.

The data from individual patients can be interesting if personalized medicine is to be considered. UC patients (272, 280, 282, 289, 290) and CD patients (265, 275) will most probably not benefit from a proposed AMPK agonistic therapy. On the other end, patients with extremely low p-AMPK levels are more likely to benefit from therapies that can restore AMPK activity and reset its metabolic function. To further decipher this variation, we tried to group the patients from each group based on number of years since diagnosis. Because we have a limited sample size, we sub-grouped our patient groups into UC and CD with less or more than 10 years of disease. This simple categorization shows that p-AMPK (Thr172) expression level in leukocytes may not be feasible to distinguish the sub-groups of UC and CD based on time with disease (Figure 7.6). Moreover, a larger sample size and more analysis would be needed to reach strong conclusions in this regards.

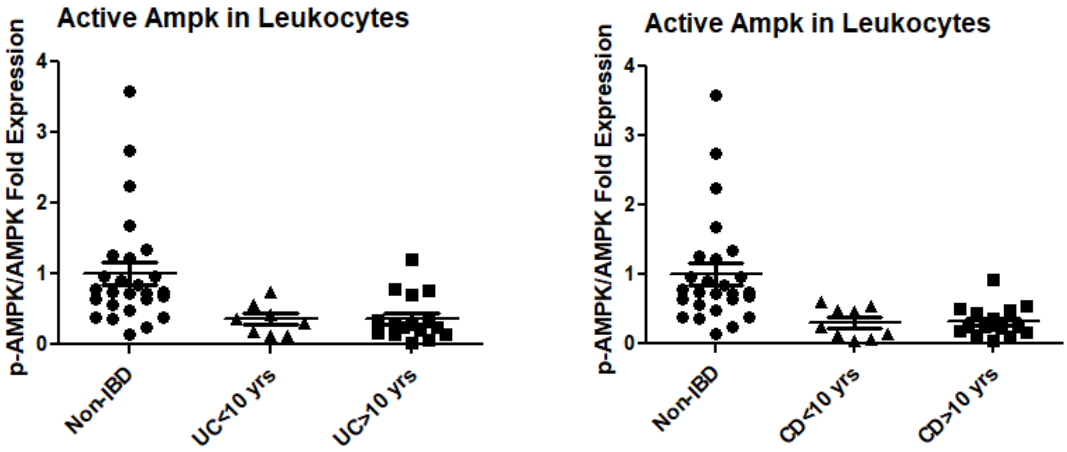


Figure 7.6: Western Blot analysis in leukocytes from IBD patients sub-grouped based on number of years with disease. Results from (Figure 7.5) were re-plotted based on the known date of diagnosis**.**

Furthermore, we reviewed whether disease was active or not on the day of endoscopy (day of biopsy/blood collection) for each patient and further classified the IBD patients with either having active inflammation or inactive inflammation, based on endoscopic signs of inflammation and whether the patient reported feeling well or if there was a recent flare up. Interestingly, in both UC and CD the status of p-AMPK (Thr172) expression was significantly different between active and inactive disease (Figure 7.7). It is also important to mention that the sample size needs to be increased for stronger conclusions. As more patients are recruited and analyzed, the significance of our results can be further exploited.

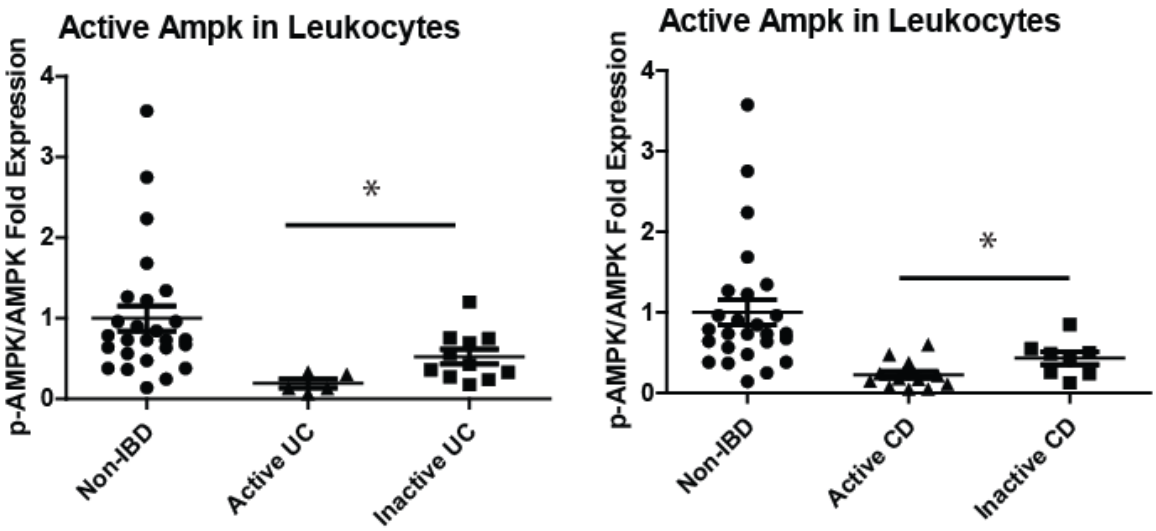


Figure 7.7: Western Blot analysis in leukocytes from IBD patients sub-grouped based on whether disease was active or inactive on day of biopsy collection. Results from (Figure 7.5) were re-plotted based on the status of disease. p-value for Active UC vs Inactive UC (0.036) and p-value for Active CD vs Inactive CD (0.021).

In addition to p-AMPK analysis in leukocytes, we also investigated the activity status of RIPK2 in leukocytes derived from the patients. RIPK2 activity was assessed using a specific phosphor-specific RIPK2 antibody (S176) to detect expression levels. Interestingly, similar to immunostaining results of colonic biopsies, RIPK2 is hyperactive in the leukocytes of UC and CD patients (Figure 7.8).

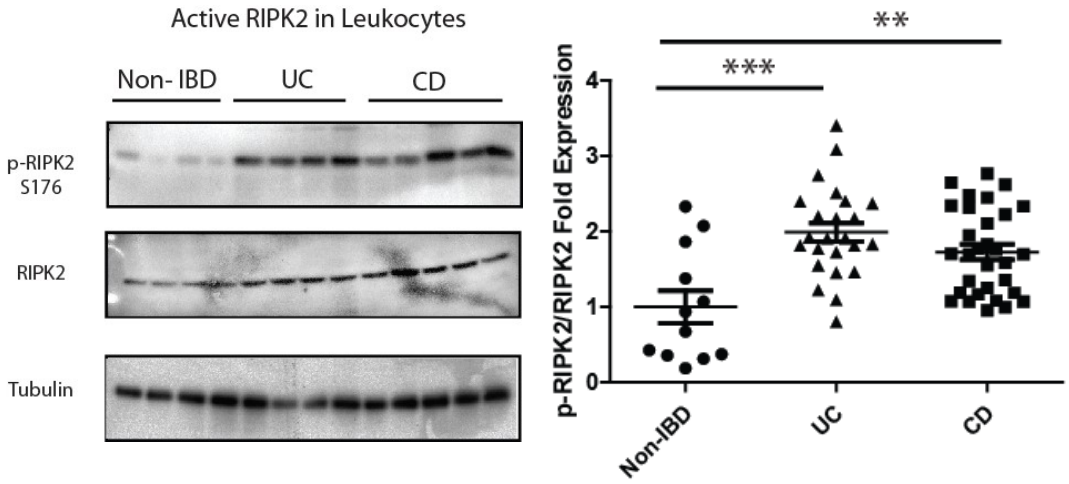


Figure 7.8: Western Blot analysis in leukocytes from IBD patients reveals hyperactivity of RIPK2. Whole blood was obtained from CRC screening individuals or healthy volunteers at the University of Alberta. UC and CD patients were recruited as part of their regular follow up and endoscopy procedures. Shown here is a western blot from each category including 12 Non-IBD, 24 UC patients and 30 CD patients. Quantification of the p-RIPK2 band was done after normalization against total RIPK2 values as determined using ImageJ. "*" p-value < 0.05, "**" p-value < 0.01, and "***" p-value < 0.001. If not stated, p-value > 0.05

Further analysis of the expression level of active RIPK2 was done and plotted per patient of UC and CD. Interestingly, only 1 patient of the 24 UC and 1 patient of the 30 CD patients showed relative values of S176-RIPK2 less than controls (Figure 7.9). All other patients showed higher levels of S176-RIPK2,

indicative of hyperactivation of RIPK2 that can be measured in leukocyte derived from IBD patients.

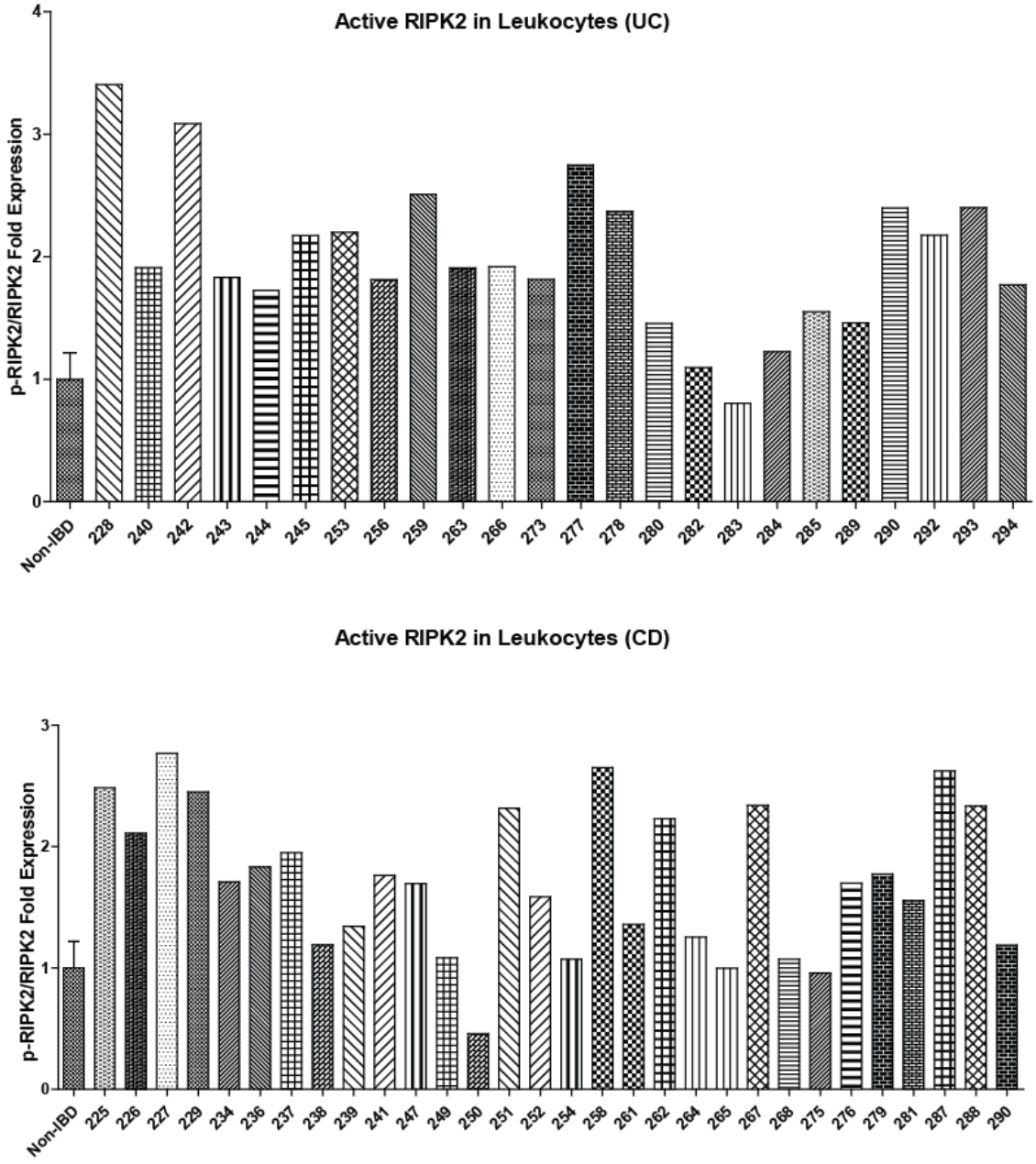


Figure 7.9: Western Blot analysis in leukocytes from IBD patients reveals hyperactivation of RIPK2 in IBD patients. Results from (Figure 7.8) were re-plotted individually.

When sub-grouped based on years with disease or disease activity, there appears to be no difference between patients diagnosed since less than 10 years or more than 10 years or based on disease severity (Figures 7.10 and 7.11).

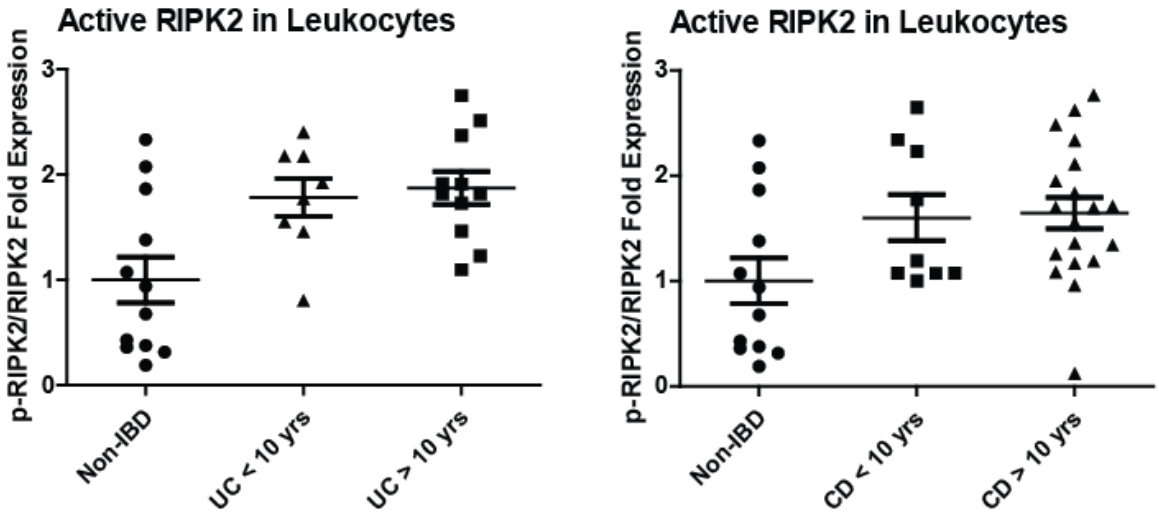


Figure 7.10: Western Blot analysis in leukocytes from IBD patients sub-grouped based on number of years with disease. Results from (Figure 7.8) were re-plotted based on the known date of diagnosis**.**

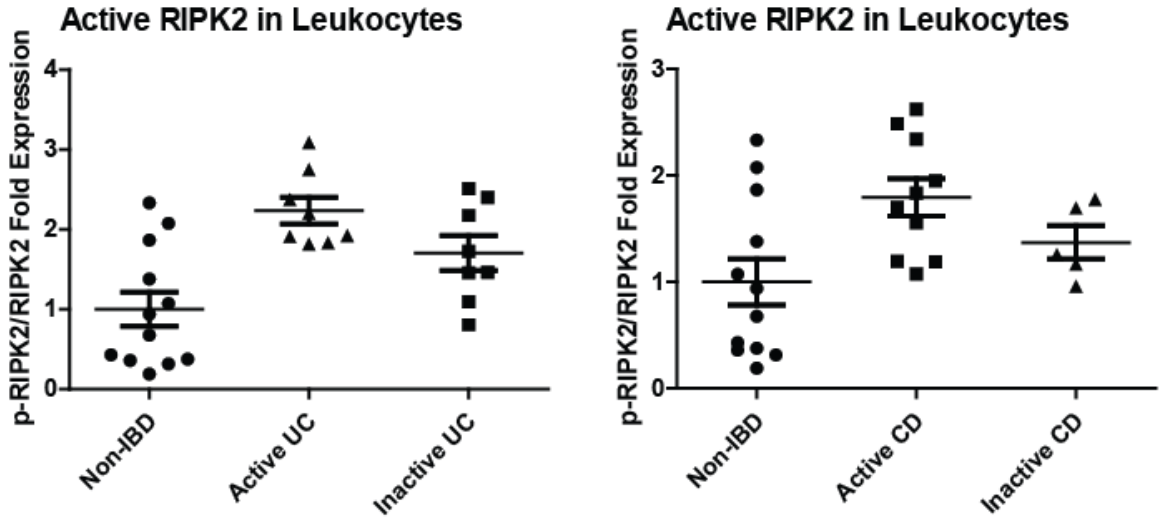


Figure 7.11: Western Blot analysis in leukocytes from IBD patients sub-grouped based on whether disease was active or inactive on day of biopsy collection. Results from (Figure 7.8) were re-plotted based on the status of disease.

7.3 Discussion

Extensive research and efforts have been put into investigating reliable non-invasive assessments of gut inflammation, such as detection of fecal white blood cells [395]. Calprotectin and lactoferrin have been well described as fecal biomarkers of IBD, while other less established fecal biomarkers include M2-pyruvate kinase, defensins, matrix metalloproteinases and human nucleic acid [396]. Most potential biomarkers have only been assessed in single cohorts and require further investigation with larger cohorts. A reliable biomarker is a product that can be measured precisely and its analysis can indicate the presence or absence of disease or “abnormal” physiology and/or severity of a disease. Moreover, disease specificity and correlation with disease severity differentiates a reliable biomarker from other less reliable ones. The distinctive diagnosis between UC and CD remains a challenge to clinicians, especially at early stages of disease. In this regards, protein biomarkers could be of great aid to improve both precision of diagnosis and suggest better therapies. Moreover, well-established biomarkers could help in predicting disease outcome or response to certain therapeutics. Overall, a well laid out scheme of personalized medicine appears to be a promising tool for better IBD management. Understanding disease mechanism and assessing as many potential biomarkers and pathways as possible will definitely push in that direction, to provide better therapeutics and management of diseases such as IBD.

In concurrence with our previous observations in the previous chapters, we assessed the expression level of some potential biomarkers in human colonic

tissues and leukocytes derived from IBD patients. In chapter one, we have shown that *RASSF1A* promoter is hypermethylated in IBD patients, CRC and other cancer patients. It is likely that methylation starts early on in the inflammatory phase of IBD and continues into tumour phase of CRC. In this chapter, we confirm loss of expression of RASSF1A in the colonic tissues of IBD patients as compared to non-IBD colonic tissues (Figure 7.1). The absence of RASSF1A will upsurge the inflammatory signals already associated with IBD leading to a dysregulation in several molecular processes including DNA damage, oxidative damage and apoptosis [122]. Our attempt to measure expression levels of RASSF1A in leukocytes was unsuccessful, most probably due to technical issues with the use of the antibody for western blot purposes. The functional consequence of RASSF1A loss is the unrestricted signaling through TLR4 and NOD2. Signaling through NOD2 can lead to hyperactivation of RIPK2 and we show the usefulness of targeting RIPK2 in the mice model of colitis in chapter six. Here, in this chapter, we show that RIPK2 is indeed highly active in colonic tissues of both UC and CD patients (Figure 7.2). This hyperactivation is translated into patient derived leukocytes as well (Figure 7.8) with a high degree of sensitivity as only 2 out of 54 patients showed less active RIPK2 than non-IBD patients (Figure 7.9). This is strongly suggestive that RIPK2 inhibitors could be successful therapies in the future management of IBD. On another level, we provide evidence that the general anti-inflammatory compound, resveratrol, protects against IBD-like symptoms in mice in chapter five. Resveratrol is well known to inhibit NFκB mainly by activation of AMPK and subsequent SIRT1

deacetylation of the NFκB complex. The assumption that IBD may also involve a metabolic dysfunction and loss of AMPK anti-inflammatory properties was confirmed with the loss of expression of p-AMPK (Thr172) in IBD patients. Both UC and CD patients experience a lower level of active AMPK in their colonic tissues (Figure 7.3) and which is also translated into lower expression levels in leukocytes (Figure 7.4). Unlike active RIPK2, there was some considerable variation in the expression level of p-AMPK (Thr172) among individual IBD patients (Figure 7.5). Such variation could allow us to suggest that AMPK agonists would be beneficial, but not to all IBD patients; perhaps in a more personalized medicine fashion. Interestingly, the level of expression of active AMPK (p-AMPK Thr172) was significantly different between patients that had active inflammation an inactive inflammation on day of endoscopy (Figure 7.7). Even though our sample size is small at this point, but these results can provide insight into the use of p-AMPK (Thr172) as a biomarker to aid in assessment of disease severity. One limitation of this analysis is that the immunostaining and leukocyte samples are not completely matched since we acquired them at different time intervals. Even though there are about 50% common samples analyzed by both methods but yet some exclusive unmatched samples exist. We are hoping that as we move on with the analyses we can expand our matched samples in both methods. Overall, we have shown that our research has the potential translational application with the usage of RASSF1A, active p-RIPK2 (S176) and active p-AMPK (Thr172). We have recently optimized and established the use of 3D intestinal organoids which can resemble to a great

extent the physiology of tissue organization and to some extent, organ functionality [397]. Intestinal organoids are grown from adult somatic stem cells (ASC) embedded in the proper extracellular matrix and given the needed molecular stimulants. The use of organoids has greatly advanced our knowledge of disease progression, in a model that is not limited by interspecies differences as is the case with animal models. These 3D structures can be grown and passaged indefinitely *in* vitro [397] and thus represent valuable tools as preclinical models. In fact, organoid bio-banking has been initiated from tumour biopsies and are termed “tumouroids”. More importantly, these tumouroids maintained the genomic landscape of the parental tumour even at later passages [397]. This suggests that 3D organoids are a trustworthy and can be used as an expandable source that will allow medium to high-throughput drug screening. Our ultimate goal is to confirm our findings in the 3D intestinal organoids and work towards optimizing this model as a model for testing therapeutics, perhaps in a fashion that makes personalized medicine a much more useful approach for IBD management. Once our findings are validated in this human model, we will be better positioned to translate our findings of basic research into diagnostic and therapeutic options.

CHAPTER EIGHT

Final Summary and Future Directions

In this study we have attempted to provide a better understanding of the epigenetic and inflammatory drivers of inflammatory bowel disease and the development of novel biomarkers that could be potentially used for diagnostic and prognostic purposes. We have identified an interesting hot spot of methylation in the *RASSF1A* promoter in both IBD and CRC patients, corresponding to CpGs 1-7, that we believe could be more influential in driving the expression loss of *RASSF1A*. If *RASSF1A* methylation designs do not take this region into account, an overall methylation does not seem to reflect actual methylation percentage and thus can be misleading. We believe our study provided valuable information towards better understanding the epigenetic regulation of *RASSF1A*. The correlation between *RASSF1A* methylation and tumour development has been reported for many cancers. For example, the methylation status of *RASSF1A* was inversely correlated to the mutation status of K-ras and B-raf in cervical cancer [398]. Furthermore, *RASSF1A* promoter methylation correlated positively with a higher tumour grade [126]. In this study, we have analyzed the first 32 CpG sites upstream exon one. Further analysis of the remaining 40 CpG sites is essential, at least in colorectal cancer, to fully evaluate the importance of our proposed hot spot of methylation at CpG sites 1 to 7. Higher levels of methylation in the remaining CpG sites can negate our proposition of this hot spot of methylation and perhaps reveal other important

susceptible areas of hypermethylation. This is also not to underestimate the importance of looking at other mechanisms of epigenetic silencing of *RASSF1A*. In fact, Strunnikova *et al* (2005) reported on the importance of chromatin inactivation by histone modifications as a prerequisite requirement to drive the expression loss of *RASSF1A* in normal human mammary epithelial cells (HMEC) [399].

As mentioned earlier, the loss of RASSF1A results in the unrestricted activation of NFκB. We aimed at investigating some therapeutics that could counteract the effects of this loss. We show evidence that salicylate-like derivatives of resveratrol, obtained in collaboration with Dr. Carlos Velazquez Martinez could be potential options for management and treatment of IBD. We demonstrate that at least some of these derivatives maintain effectiveness against inhibition of growth and inhibition of NFκB activity. We identified one derivative (termed C3) that has distinctive biological properties than the parent compound resveratrol. The C3 derivative showed higher death inducing properties and greater AMPK activating abilities than resveratrol in colon cancer cells. We also confirmed that resveratrol is protective against DSS-induced colitis in *Rassf1a*$^{-/-}$. Resveratrol has been reported to be tightly associated with the regulation of epigenetic modifications that control gene expression across the genome [400]. DNA methyl transferases (DNMTs) are aberrantly over expressed and more active in several diseases including colorectal cancer [401] and inflammatory bowel disease [402]. We have been able to isolate RNA from 3D organoids grown from IBD patients and DNA isolation is quite similar and has

been reported in at least one other study [403]. It would be interesting to make use of the 3D organoid cultures grown from IBD patients to explore the effectiveness of resveratrol derivatives on preventing or reversing DNA methylation of *RASSF1A* promoter and how that correlates with the rescue of expression of RASSF1A. A lower methylation of *RASSF1A* promoter and a reduced expression of NFκB driven cytokines such as IL-8 and MCP-1 in organoids can provide insight into the usefulness of these drugs as adjuvant therapies for IBD.

The resveratrol derivatives we acquired have been modified from parent resveratrol compound to protect the three hydroxyl groups from potential *in vivo* metabolism and interactions that render it inactive. These modifications are expected to induce more bioactivity and enhance the bio-availability of the derivatives. That said, *in vivo* analysis on the effectiveness of these derivatives in our DSS model of colitis using *Rassf1a*$^{-/-}$ mice will allow an evaluation of usefulness as compared to the resveratrol. Since we hypothesize a protective role of these derivatives (specifically C3) through activation of AMPK; the use of *Ampk* knockout animals will also provide valuable information on the mechanistic inclinations of the effective derivatives. If protection is through AMPK, then investigating the upstream activating kinase, LKB1, CAMKK1 and/or TAK1 will provide valuable information on the context of physiological AMPK activity in IBD. Whether or not differential activities of AMPK exist between intestinal epithelial cells and immune cells would also be an important question to answer as well.

The further use of these derivatives in other animal models of colitis associated colorectal cancer (AOM/DSS) or their use as potential therapeutics for IBD requires detailed pharmacokinetic and pharmacodynamics analyses. Assessment of the pharmacological properties is critical to understand the absorption, distribution, metabolism and excretion (ADME) of proposed therapeutics. ADME and pharmacokinetic analysis are being continuously modified and advanced to ensure proper translation into clinical trials. Before doing *in vivo* pharmacokinetic analysis of the derivatives, the cost effective *in vitro* approach of ADME can provide indicators on the *in vivo* fate of these derivatives. In other words, if the derivatives are determined by ADME to be rapidly absorbed, well distributed, minimally metabolically degraded, not rapidly eliminated and not being toxic; they are more likely to show promising pharmacokinetic properties *in vivo* [404]. Upon determining that the derivatives could potentially exhibit favourable *in vivo* pharmacokinetic profiles, a rapid assessment of compound exposure (R.A.C.E) would further provide valuable evaluation on the half-life of the tested derivatives as compared to the short half-life of resveratrol. It is worth noting that prebiotics are gaining more interest as adjuvant options for treatment of IBD. The definition of a prebiotic, “a non-digestible compound that through metabolization by micro-organisms in the gut, modulates composition and/or activity of gut microbiota, thus conferring a beneficial physiological effect on the host”, has dramatically evolved to include polyphenols [405], such as resveratrol. That said, and given the reported beneficial effects of resveratrol, it will not be surprising to see resveratrol or its

derivatives as adjuvant therapies to infliximab or other approved IBD drugs. On a similar note, the FDA approved type 2 diabetes drug metformin, has been shown to exert protective effects in the experimental colitis model by activating AMPK [406]. We have also seen a similar role of metformin in the DSS treated *Rassf1a*$^{-/-}$ and *IL10*$^{-/-}$ mice (unpublished data). It is thus tempting to speculate that among the national population of type 2 diabetics that are being treated with metformin, the incidence rate of IBD is lower than the general population. A case control population based study comparing both groups would be interesting to pursue, and if type 2 diabetes patients have lower incidence of IBD then metformin can be suggested to enter phase II trials for treatment of inflammatory bowel disease.

We also investigated the possible targeting of another RASSF1A regulated pathway that involves the activation of RIPK2 kinase via the intracellular PRR, NOD2. This was also done in collaboration with Dr. Carlos Velazquez Martinez who used molecular docking and cheminformatics analysis to identify potential RIPK2 inhibitors. We demonstrated that both inhibitors tested in this study were better than gefitinib (common RIPK2 inhibitor) in resolving intestinal inflammation. RIPK2 Inhibitor 1 enhanced the survival of *Rassf1a*$^{-/-}$ mice on DSS with an additional 26% as compared to gefitinib. We report no significant empirical inhibition of c-ABL, a common off target for other common RIPK2 inhibitors; at concentrations up to 100 μM. Further assessing off target effects using colonic tissues from the treated mice will provide insight into the selectivity of the inhibitors and/or any other favourable/unfavourable effects. Several studies have investigated the importance of the NOD2/RIPK2 pathway in

diseases such as inflammatory bowel disease, multiple sclerosis and arthritis [381]. Even though several single nucleotide polymorphisms have been reported for NOD2, some of which are associated with CD [407], few studies have associated *RIPK2* genetic variations with clinical susceptibility [408]. Thus, given the relevance of the NOD2/RIPK2 pathway to the pathogenesis of IBD, further investigation on whether or not known RIPK2 polymorphisms or mutations contribute to IBD is indispensable.

A recent study by Jurynec *et al* (2018) reported that a RIPK2 variant (rs200818100) was associated with early onset of osteoarthritis [409]. The variant resulted in missense substitution of Asp for Asn at position 104 of the protein and which corresponds to an amino acid in the kinase domain, rendering RIPK2 hyperactive and contributing to early onset of osteoarthritis [409]. Similar to our TP53 analysis of codon 72 and its association with IBD, it would be interesting to explore the association of *RIPK2* codon 104 variant and its association with UC, CD and PSC or more interestingly with an early pediatric onset of IBD. Restriction fragment length polymorphism (RFLP) can be utilized too since the 104 codon sequence is susceptible to digestion by *MssI* restriction enzyme that recognizes GTTT^AAAC sites. Moreover, other studies have recently came to a conclusion that another *RIPK2* variant (rs16900627) was strongly associated with systemic lupus erythematosus (SLE) [410] and gastric cancer (inflammation related) susceptibility [408]. Exploring the association of the above mentioned *RIPK2* polymorphisms (rs16900627 and rs200818100) with inflammatory diseases such as IBD will not only predict populations or individuals that are at

higher risk developing IBD, but will also provide insight into molecular mechanisms or interactions (that could be affected by these variants) exploited by RIPK2 to drive the pathogenesis of IBD.

Since our evaluation of the RIPK2 inhibitors showed promising results *in vivo*, further analysis in a human model would definitely strengthen our conclusions of the novelty and effectiveness of the inhibitors. Organoids can provide an advanced human model for drug discovery. They are stem cell derived and self-organizing 3D structures that resemble the cellular composition, architecture and to a certain extent the functionality of the tissue of origin [397]. The advantage of using organoids over animals in drug discovery lies in the fact that the analysis is not limited by unexpected interspecies differences and responses. Even though the intestinal organoids lack the immune cell component that is very important for evaluating a new lead in drug discovery; however, co-culture of organoids with intraepithelial lymphocytes and immune cells [411, 412] has been done and could overcome some of the limitations of the organoid model. The so-called co-cultures can provide us with indispensable tools to investigate the effectiveness of the identified RIPK2 inhibitors. The proposition is to grow the organoids from IBD patients up to 30 days and then apply the small molecule RIPK2 inhibitors. Initially, monitoring pro-inflammatory cytokine (IL-8, MCP-1) production would provide an idea on whether RIPK2 driven NFκB activation is inhibited or not. Immunoblotting for active nuclear NFκB (p65) would also be an interesting and applicable approach. IBD patients undergoing biological treatments such as infliximab or immunosuppressive treatments such

as Thiopurines are prone to losing response and relapsing. Investigating whether or not our RIPK2 inhibitors can prevent NFκB activation in those relapsing or non-responding patients, using the corresponding grown organoids, would be a cornerstone and strong evidence to suggest our RIPK2 inhibitors as an alternative therapy.

A quick look at the interacting partners of RIPK2 in “PubMed” reveals few types of interacting partners including NOD1, NOD2, B cell lymphoma 10 (Bcl10), E3 ligases such as TRAF6 and the transcription factor SMAD4. Most interactome maps of protein-protein interactions only account for direct binary and usually strong protein-protein interactions [413]. A better understanding on how RIPK2 interactions can contribute to pathogenesis of inflammation related diseases such as IBD is required. The use of proximity-dependent labelling by modifying enzymes, which overcomes the limitation of requiring strong and stable protein-protein interactions, is a novel approach to detect both transient as well as stable protein interactions. The BioID provides an interesting platform that has already made a great impact on understanding cell structure and function since its introduction in 2012 [414]. This system relies on fusing the gene of interest (*RIPK2* in our case) with an *E. coli* biotin ligase (BirA) that ensures the biotinylation of proximal proteins [415]. Biotinylated proteins are then purified with streptavidin and identified by mass spectrometry. A comparative analysis (with and without the RIPK2 inhibitors) using the above BioID technique not only can identify novel interactors of RIPK2 but can also reveal if the RIPK2 inhibitors are

affecting the kinase activity of RIPK2 and/or impairing upstream and downstream protein interactions, independent of the kinase activity of RIPK2.

Furthermore, our results indicate promising avenues to be further explored with respect to the identified RIPK2 inhibitors. Our unpublished ADME results predict that 100% of the RIPK2 inhibitor 1 will be absorbed (87.82% for FDA approved Lapatinib) and 86.76% will be bioavailable (56.5% for Lapatinib) in 24 hrs after a dosing level of 1 mg/day. Similar to Lapatinib, the RIPK2 inhibitor does not appear to be a major target of the cytochrome P450 (CYPs) family of proteins. The overall ADME risk was 3.95 for RIPK2 inhibitor 1 in contrast to 10.78 for Lapatinib. Analysis of pharmacokinetic properties of RIPK2 inhibitor 1 *in vivo* revealed measurable concentrations of the inhibitor in the serum of mice when dosed with a intraperitoneal concentration of 15 mg/kg. The mean C_{max} was 114 ng/mL after one hour of dosing, and the area under the serum concentration versus time curve was 573 ng x h/mL, with a terminal phase half-life of 1.9 hours [378]. With further bio-distribution and more extensive pharmacokinetic analyses, we believe that our inhibitors have high chances of getting into phase I clinical trials.

Finally, we have shown that our results have a translational application towards inflammatory bowel disease. Patient samples confirmed the loss of RASSF1A expression in the colonic tissues. We reported the hyperactivation of RIPK2 in both colonic tissues and leukocytes from IBD patients which also validates the use of RIPK2 inhibitors as proposed therapeutic for IBD. Inversely, AMPK is less active in both colonic tissues and leukocytes of IBD patients which

also strengthens our findings on the potential use of resveratrol and derivatives for management of IBD. Future analysis in this regards should aim at increasing the sample sizes of each, especially after stratification into subgroups (active vs inactive; < 10yrs vs > 10 yrs) which diminishes the number of samples divided into each group. An interesting follow up on the use of these biomarkers (p-AMPK, p-RIPK2) would be correlating their expression with the levels of faecal calprotectin in the corresponding patients. The level of calprotectin reflects the number of participating neutrophils in inflammation and is correlated the severity of the inflammation [416]. A positive correlation between our proposed biomarkers and calprotectin would further validate the significance of these biomarkers as indicators of disease severity. Furthermore, a prospective follow-up analysis using the expression levels of these biomarkers at the time of visit would provide information on how accurately p-AMPK and p-RIPK2 levels can predict the 5 year disease outcome, time to relapse, response to therapies or development of other IBD related complications such as colorectal cancer. The prediction would be that hyperactive RIPK2 and less active AMPK are positively correlated with severe disease outcome, shorter time to relapse, reduced response to therapies and perhaps higher chances of developing CRC.

Personalized medicine is relatively a new concept that can potentially optimize efficacy, decrease the risk of adverse drugs and reduce the cost of health care by detecting non-responders early on [417]. Since IBD is an idiopathic disease and many variable factors contribute to the disease, a universal cure is unlikely in the near future. Patients diagnosed with either form of

IBD are individually assessed for the most suitable treatment whether that being 5-ASA, corticosteroids, immunosuppressive drugs or biologics. In other words, personalized medicine is somewhat already integrated into the management of IBD. Since we have already performed immunostaining of colonic biopsies as well as immunoblotting in leukocytes of IBD and non-IBD patients, an interesting approach that further integrates the concept of personalized medicine would be creating a triad of molecular analytics that includes colonic biopsies, leukocytes and organoids from the same patient as described in Figure 8.1.

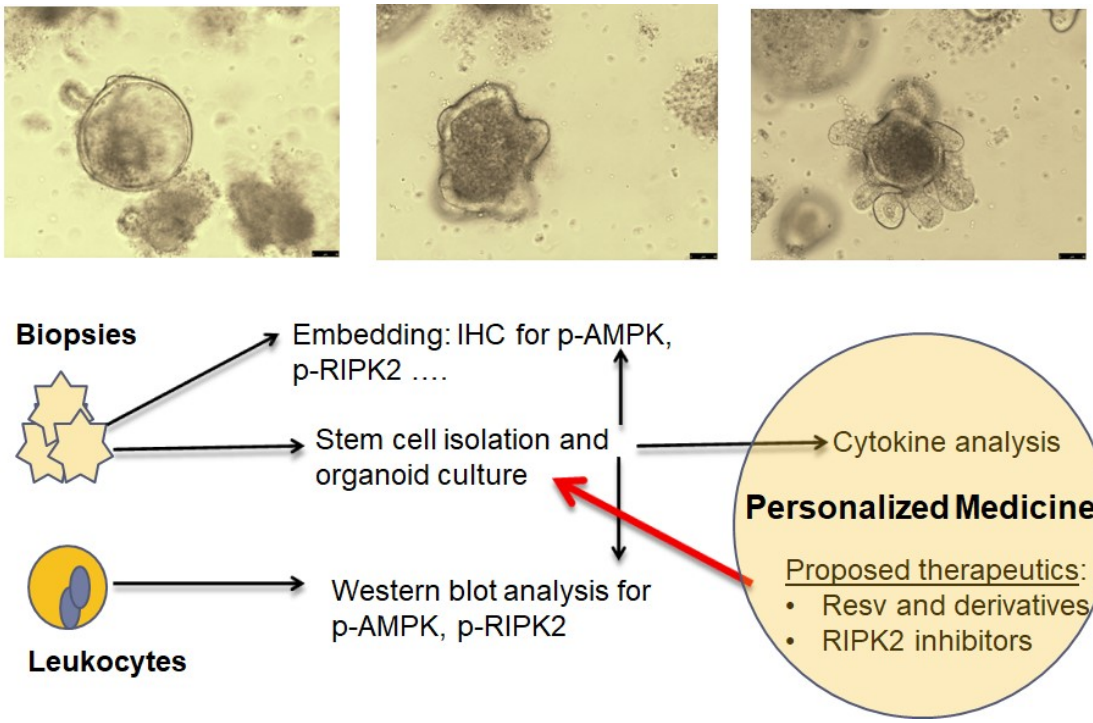


Figure 8.1: Model describing the proposition of a "Triad" of molecular analytics. Biopsies taken from the IBD patient are used for paraffin embedding and subsequent immunostaining for the proposed biomarkers. Organoids are also grown from biopsies taken from the same site and subsequent immunostaining and immunoblotting for the same biomarkers is done. Leukocytes isolated from whole blood are used to immunoblot for the biomarkers. **The first phase** of experiments would aim at investigating whether the organoids reflect and match the molecular changes in the colonic tissues and leukocytes. A positive correlation would drive the project into the **second phase** of only utilizing the organoids for biomarker analysis and medium throughput drug discovery. The **final phase** would be creating biobanks from these organoids for further molecular and therapeutic analysis.

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