VALIDATION-BASED INSERTIONAL MUTAGENESIS (VBIM) TECHNOLOGY IDENTIFIES ADENOMATOUS POLYPOSIS COLI (APC) LIKE PROTEIN (ALP) AS A NOVEL NEGATIVE REGULATOR OF NF-κB

Rasika S. Mundade

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Pharmacology and Toxicology,
Indiana University

January 2015

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

Master's Thesis Committee

Tao Lu, Ph.D., Chair

William J. Sullivan, Jr., Ph.D.

Ahmad R. Safa, Ph.D.

DEDICATION

This thesis work is dedicated to my husband, Sumit, for his endless support, encouragement, and love

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Dr. Tao Lu, for her consistent guidance and advice throughout my graduate training. Her efforts and confidence in me have been greatly appreciated. The wonderful opportunity for me to start my research here actually led me into the fascinating world of Pharmacology.

I would like to express my deep gratitude to my graduate committee members, Drs. Ahmad R. Safa and William J. Sullivan, Jr. I appreciate all of their time and input. Their advice and expertise greatly helped me improve my thesis.

I would like to thank Dr. Han Wei from Dr. Tao Lu's Lab, for her helpful suggestions and technical assistance during my entire experimental work.

I thank Drs. Gustavo Arrizabalaga and Jingwu Xie for allowing me to use the fluorescence microscope in their labs and for useful suggestions.

I thank Dr. Anirban Mitra from IU Bloomington, for his excellent technical assistance with the Migration experiment, and his kindly answering to my general questions.

I thank Drs. George Sandusky and Constance Temm from the Department of Pathology and Laboratory Medicine, for their help and guidance with the Immunohistochemical staining and analysis of tissue microarray.

I am grateful to the Amy Lawson and Lisa King, for assisting me in many different ways and handling the paperwork.

Thanks also to all the members of the Department of Pharmacology and Toxicology, for providing a good atmosphere in the department and for useful discussions.

Lastly, I am much appreciated to my parents for their constant support and sustaining love and to my beloved husband, for his unshakable faith in me and his willingness to endure with me through the toughest time during our lives.

Rasika S. Mundade

VALIDATION-BASED INSERTIONAL MUTAGENESIS (VBIM) TECHNOLOGY IDENTIFIES ADENOMATOUS POLYPOSIS COLI (APC) LIKE PROTEIN (ALP) AS A NOVEL NEGATIVE REGULATOR OF NF-κB

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the United States. The nuclear factor κB (NF-κB) is an important family of transcription factors whose aberrant activation has been found in many types of cancer, including CRC. Therefore, understanding the regulation of NF-κB is of ultimate importance for cancer therapy. Using a novel validation-based insertional mutagenesis (VBIM) strategy, our lab has identified the novel adenomatous polyposis coli (APC) like protein (ALP) gene as a negative regulator of NF-κB. Preliminary studies from our lab demonstrated that overexpression of ALP led to decreased NF-κB activity by κB reporter assay and electrophoresis mobility gel shift assay (EMSA). The current project aims to further evaluate the role of ALP in the regulation of NF-κB signaling in CRC cells. We found that overexpression of ALP in human CRC HT29 cells greatly reduced both the number and the size of colonies that were formed in a soft agar assay. ALP overexpression also decreased the cell growth rate and cell migration ability, while shRNA mediated knockdown of ALP showed opposite effects, confirming that ALP is a tumor suppressor in CRC HT29 cells. Overexpression of ALP led to decreased NF-κB activity by κB reporter assay and condition media assay in CRC HT29 cells. Furthermore, immunohistochemical analysis with human colon

tissues revealed that there is a gradual loss of ALP protein with tumor progression. We also found that ALP predominantly localizes in the cytoplasm, and binds to the p65 subunit of NF-κB, and might be functioning downstream of IκB kinase (IKK). In summary, in this study, we provide evidence regarding the tumor suppressor role of ALP in CRC by functioning as novel negative regulator of NF-κB. This discovery could lead to the establishment of ALP as a potential biomarker and therapeutic target in CRC.

Tao Lu, Ph.D., Chair

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LIST OF ABBREVIATIONS

Abbreviation	Description
ALP	APC like protein
AOM	Azoxymethane
APC	adenomatous polyposis coli
ARMC4	Armadillo repeat motifs containing protein 4
ATCC	American Tissue Culture Collection
BAF	B-cell activation factor
Bcl	B-cell leukemia
β-gal	β-galactosidase
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CAC	colitis-associated colon cancer
CapeOx	Capecitabine and Oxaliplatin
CBP	CREB binding protein
CD40L	CD40 ligand
CILD23	ciliary dyskinesia primary 23
CK1α	casein kinase 1α
CMV	cytomegalovirus promoter
co-IP	co-immunoprecipitation
cPPT	central polypurine tract
CRC	colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DSS	dextran sodium sulfate
Dsh	phosphoprotein Dishevelled
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FAP	familial adenomatous polyposis coli
FBS	fetal bovine serum
FBXL11	F-box and leucine-rich repeat protein 11
FU	Fluorouracil
GCV	Ganciclovir
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase 3β
HCC	hepatocellular carcinoma
HEK	human embryonic kidney

Abbreviation	Description
IBD	inflammatory bowel disease
IECs	intestinal epithelial cells
IF	immunofluorescence assay
IHC	Immunohistochemistry
IκB	inhibitor of κB
IKK	IκB kinase
IL-1	interleukin 1
IRES	internal ribosome entry sequence
LPS	Lipopolysaccharides
LRP	lipoprotein receptor-related protein
LTR	long terminal repeat
LTα1β2R	lymphotoxin α1 β2 receptor
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
NLS	nuclear localization signals
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	Paraformaldehyde
PIAS1	protein inhibitor of activated signal transducers and activators of transcription 1
qPCR	quantitative PCR
RANK	receptor activator for NF-κB
RHD	Rel homology domain
RIP	receptor interacting protein
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RT-PCR	real time PCR
SA	splice acceptor site
SD	splice donor site
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
SIN	self-inactivating LTR
SOCS6	suppressor of cytokine signaling 6
SODD	silencer of death domains
S100A3	S100 calcium binding protein A3
TCF	T-cell factor
TK	thymidine kinase
TMA	tissue microarray

Abbreviation	Description
TNFα	tumor necrosis factor α
TO	tetracycline operon
TRIM16	Tripartite motif-containing protein 16
VBIM	validation based insertional mutagenesis
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
Zeo	Zeocin

CHAPTER I INTRODUCTION

1.1 Colorectal cancer (CRC) and its therapies

CRC is an epithelial cancer of the colon and rectum, resulting from uncontrolled growth of colonocytes, the cells lining the colon and rectum. CRC starts as a growth of tissue called a polyp in the colonocytes. These polyps start to grow through the layers of colonocytes, and may undergo malignant transformation to cancer. This malignant transformation is the result of a progressive accumulation of genetic and epigenetic alterations that lead to mutation or deletion of major regulator genes, resulting first in hyperplasia moving toward adenoma to carcinoma and then metastasis (Mundade R *et al.* 2014).

CRC affects men and women of all racial and ethnic groups, and is most often found in people 50 years or older. It is the third leading cause of cancer-related deaths in the United States. There are approximately 160,000 new cases of CRC every year in the United States and approximately one-third of CRC patients die from the disease (Jemal A *et al.* 2009). The lifetime risk of developing CRC is about 1 in 20 (5%) and it is expected to cause about 50,830 deaths in 2014. (http://www.cancer.org/cancer/colon-cancer-key-statistics). With the established and available techniques, there has been considerable advancement in the management of CRC; however, mortality still remains high and unchanged with the 5-year survival rate of only 62%, which is attributable largely to complications of metastatic disease (Hawk ET *et al.* 2005).

There are three types of CRC, including hereditary, familial and sporadic diseases. A present estimate is that ~15–30% of CRCs may have a major hereditary component. Genetic mutations have been identified as the cause of inherited cancer risk in some colon cancer–prone families; these mutations are estimated to account for only 5% to 6% of CRC cases overall. About 75% of patients with CRC however have sporadic disease with no apparent evidence of having inherited the disorder (Mundade R *et al.* 2014). The development of sporadic colon cancer is thought to be influenced by diet, lifestyle, environmental factors, and acquired somatic mutations. The spectrum of somatic mutations contributing to the pathogenesis of CRC is likely to be far more extensive than previously appreciated. Thus identification of other undiscovered genes contributing to the development of CRC is essential and may also lead to potential prognostic information and targets for novel therapies.

The pace of recent advances in understanding the molecular basis of CRC and expansion in the drugs designed to treat CRC have led to substantial gains in quality and quantity of life in CRCs patients. To date, CRC, when discovered early, is highly treatable. Even if it spreads into nearby lymph nodes, surgical treatment followed by chemotherapy is highly successful. CRC is treated depending upon its stage and progression. Stage 0, Stage I and early Stage II CRCs are treated with colectomy, Stage III and some Stage II CRCs are treated with postoperative adjuvant chemotherapy. Combination chemotherapy with multi-drugs including 5-fluorouracil and leucovorin and CapeOx (capecitabine and oxaliplatin) and radiation therapy are used to treat recurrent or advanced

disease. These treatment regimens now combined with the use of genetically engineered monoclonal antibodies in treating CRCs. Although more than a million people in the US count themselves as survivors of CRC (Mundade R *et al.* 2014), CRC remains a significant public health burden in the US as well as other countries around the globe. Like most other cancer therapeutics, these treatment regimens are associated with side effects and have not yet shown significant efficacy in most instances. In the most difficult cases when the cancer has metastasized, treatment can only prolong and add to one's quality of life. Thus identification of new candidate therapeutic target genes involved in the genesis of CRC has become an urgent issue in targeted therapy of CRC.

1.2 NF-κB

1.21 Signaling pathways activating NF-κB

The transcription factor NF-κB was discovered as a nuclear factor that binds to the enhancer element of the immunoglobulin κ light-chain of activated B cells (Sen R *et al.* 1986). NF-κB plays a central role in the regulation of diverse biological processes, including immune responses, development, cell proliferation and survival. There are five proteins in the mammalian NF-κB family: RelA (p65), RelB, c-Rel, p50/p105 and p52/p100. v-REL (reticuloendotheliosis) is an oncoprotein of the REL retrovirus (REV-T). All proteins of NF-κB family share a Rel homology domain (RHD) in their N-terminus which is essential for dimerization, nuclear translocation as well as binding to cognate DNA elements and inhibitor of κB (IκBs).

The NF-κB signaling includes the canonical (classical) and the noncanonical NF-κB signaling pathways. **Figure 1,** adapted from Horie K *et al.* 2012, illustrates both the NF-κB signaling pathways. In the canonical pathway, the family of inhibitors called IκBs masks the nuclear localization signals (NLS) of NF-κB proteins and keep them sequestered in a latent, inactive, IκB-bound complex in the cytoplasm. When a cell receives any of a multitude of extracellular signals like stress, cytokines, free radicals, radiations such as lipopolysaccharides (LPS), tumor necrosis factor α (TNFα) or interleukin-1 (IL-1), it activates its receptor respectively. Through a variety of adapter proteins and signaling kinases this leads to an activation of IκB kinase (IKK), which in turn phosphorylates IκB (Leonardo MJ *et al.* 1987) and leads to its degradation by the proteasome. The NF-κB complex rapidly enters the nucleus where it can 'turn on' the expression of target genes involved in cell proliferation, cell survival, cell differentiation, and immune response.

In the noncanonical pathway, p100/RelB complexes are present in an inactive state in the cytoplasm. Receptor signaling through B-cell activation factor (BAF), CD40 ligand (CD40L), receptor activator for nuclear factor κB (RANK) or lymphotoxin α_1, β_2-receptor ($LT\alpha_1\beta_2R$), leads to activation of IKKα by the NF-κB-inducing kinase (NIK), which in turn activates IKKα complexes. The IKKα complexes phosphorylate C-terminal residues in p100, which leads to ubiquitination and proteasomal processing of p100 to p52. p52/RelB complexes then translocate to the nucleus and induce target gene expression.

1.22 The NF-κB signaling pathway and cancer

The molecular identification of p50 subunit of NF-κB as a member of the REL family provided the first evidence that linked NF-κB to cancer (Thanos D *et al.* 1995). Constitutive NF-κB activation has been noted in 95% of all cancers (Aggarwal B *et al.* 2006). Oncogenic role of NF-κB involves regulation of cell proliferation, control of apoptosis, promotion of angiogenesis, and stimulation of invasion/metastasis in cancer cells. Since NF-κB plays a very important role in immune cell function, it is frequently associated with the development of leukemia and lymphoma, cancers of the bone marrow and lymph nodes respectively. NF-κB can be constitutively activated in blood cells in response to growth factors and cytokines. Expression of certain viral oncoproteins or by chromosomal rearrangements that affect genes that encode NF-κB or IκB proteins lead to lymphomagenesis (Baud V *et al.* 2009).

Though the role of NF-κB in lymphomagenesis was somewhat anticipated, numerous studies have now documented the role of NF-κB in the development of solid malignancies. NF-κB has been shown to be involved in the development of carcinomas — cancers of epithelial origin, such as breast cancer. Constitutive NF-κB DNA-binding activity is seen in mammary carcinoma cell lines and primary breast cancer cells of human and rodent origin (Cogswell PC *et al.* 2000). NF-κB is also shown to have a complex role in another inflammation-linked cancer, hepatocellular carcinoma (HCC), the most common form of liver cancer. A study by Pikarsky E *et al.* 2004, demonstrated that inhibition of NF-κB activation in

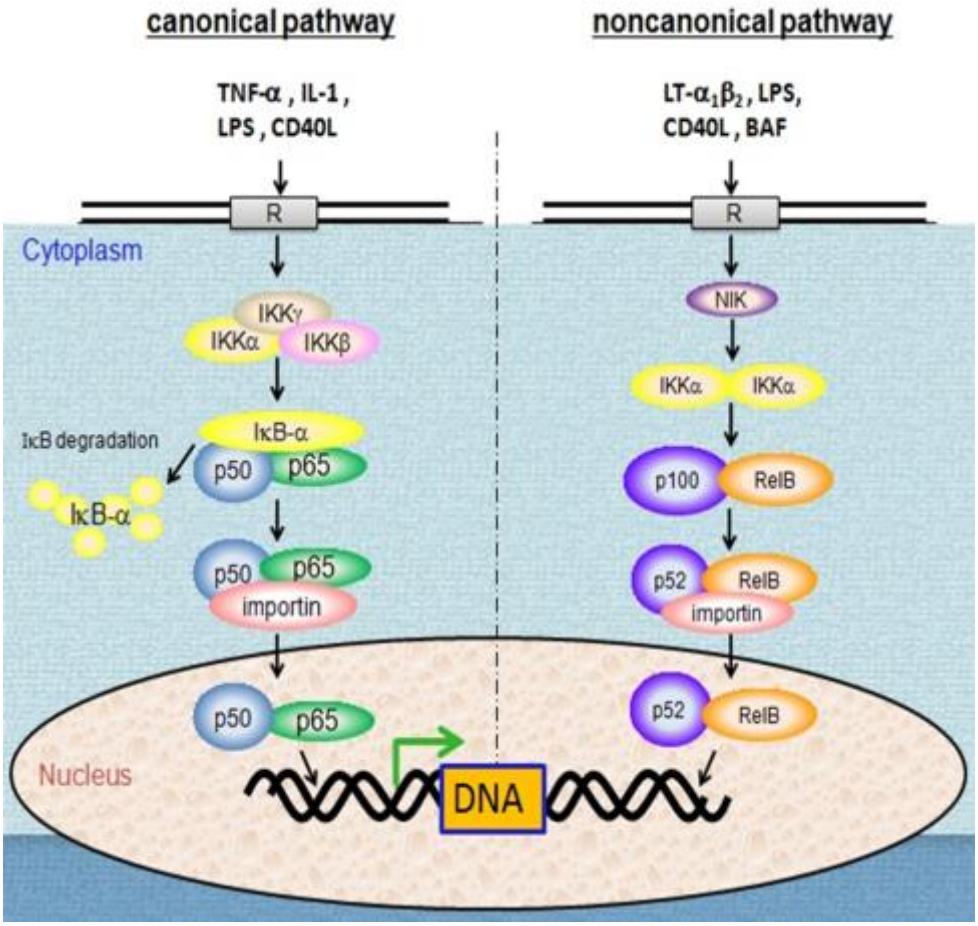

Figure 1. Canonical and noncanonical NF-κB activating pathways (Adapted from Horie K *et al.* 2012)

Abbreviations: BAF, B-cell activation factor; CD40L, CD40 ligand; DNA, deoxyribonucleic acid; IKK, IκB kinase; IL-1, interleukin-1; LPS, lipopolysaccharide; LT-$\alpha_1\beta_2$, lymphotoxin α_1, β_2-receptor; NIK, NF-κB inducing kinase; NLS, nuclear localization signal; R, receptor; TNFα, tumor necrosis factor α.

hepatocytes of mice with homozygous knockout of p-glycoprotein gene Mdr2−/− retarded and reduced HCC development via apoptosis.

Furthermore, it has been demonstrated that upregulation of the NF-κB levels is involved in both the progression and increase of metastatic potential of melanoma, the most aggressive form of skin carcinogenesis (Kashani-Sabet M *et al.* 2004). Additionally, the NF-κB transcriptional factors are also shown to be constitutively activated in the majority of pancreatic cancers and are involved in the regulation of numerous aspects of pancreatic tumor development and progression.

1.23 The role of NF-κB signaling pathway in CRC

With the given role of NF-κB in different cancer types, the signature changes in NF-κB and its involvement in CRC is not surprising. NF-κB coordinates immune host defense by regulating inflammatory response and providing a barrier against extrinsic hazard. Deregulation of this homeostasis predisposes to inflammatory bowel disease (IBD) and CRC. NF-κB has been recognized as a key player in the initiation and propagation of CRC. Human colon tumor samples and sporadic adenomatous polyps are found to have increased NF-κB activity (Hardwick JC *et al.* 2001; Lind DS *et al.* 2001). The first concrete evidence that linked NF-κB to CRC came from a study that demonstrated that down regulation of RelA expression in IL-10 deficient mice with constitutively active NF-κB significantly reduced IBD symptoms and hence the risk of CRC (Kuhn R *et al.* 1993). Studies with Azoxymethane (AOM)/Dextran Sodium Sulfate (DSS) mouse models of

colitis-associated colon cancer (CAC) and animal model carrying a conditional disruption of IKKβ have been used to show that IKKβ-driven NF-κB activation within intestinal epithelial cells (IECs) is essential for development of colonic adenomas (Okayasu I *et al.* 1996). Sakamoto K *et al.* 2009, showed that constitutive NF-κB activation in CRC plays a key role in promoting angiogenesis and tumor growth. Recently it has been demonstrated that, mutant p53 prolongs NF-κB activation and promotes chronic inflammation and CAC (Cooks T *et al.* 2013). Studies like these are only a few of several lines of evidence suggesting that NF-κB plays a crucial role in CRC development.

Consistent with this role of NF-κB, it is also observed that several NF-κB inhibitors are useful alone or in combination with cancer therapies to cause tumor cell death or growth inhibition. Advanced CRC uses chemotherapy, however most cases develop tolerance to such treatments and thus new strategies are required to replace or complement current therapies. It has been observed that some tumor cells secrete factors cause constitutive NF-κB activation and lead to resistance to chemotherapy. An interesting study by Shakibaei M *et al.* 2013, demonstrated that combination treatment regimen of 5-fluorouracil (5-FU) and curcumin in CRC cells enhances the effect of chemotherapy against CRC cells by inhibition of NF-κB. The relationship between CRC development and NF-κB is becoming extremely clear, making NF-κB the subject of much active research and represents possible novel drug targets for pharmaceutical treatments of anti-cancer therapy.

1.3 Adenomatous polyposis coli (APC) and APC like protein (ALP)

1.31 APC gene and its role in CRC

Adenomatous polyposis coli (APC) also known as deleted in polyposis 2.5 (DP2.5) is a protein that is encoded by the APC gene in humans. The human APC gene is located on the long (q) arm of chromosome 5 in band q22.2. APC is classified as a tumor suppressor gene and was identified through its association with an inherited syndrome of CRC known as familial adenomatous polyposis coli (FAP). APC mutation is one of the earliest mutation in CRC progression and mutation or loss of APC is found in more than 80% of all the colorectal adenomas and carcinomas (Goss KH *et al.* 2000). In order for cancer to develop, both copies of the APC gene must be mutated, resulting in full length loss of protein in tumor cells. This mutational inactivation of APC is seen in both familial and sporadic tumors, and must be followed by other mutations to become cancerous. In carriers of APC inactivating mutations, the risk of CRC by age 40 is almost 100% (Markowitz SD *et al.* 2009).

APC plays a critical role in governing physiological processes like cell proliferation, differentiation, metastasis and apoptosis. Dysregulation in the levels of APC alters these important processes and predisposes a person towards inherited and sporadic CRC. APC is a key player in the Wnt/β-catenin pathway and regulates the cytoplasmic concentrations of β-catenin by regulating its degradation. β-catenin is an essential component of the adhesion junction complex and also has a role in modulating gene expression. **Figure 2,** adapted

from Eisenmann DM, 2005, illustrates the canonical Wnt/β-catenin signaling pathway. The APC protein builds a complex with glycogen synthase kinase 3-beta (GSK-3β) and axin which then further binds β-catenin in the cytoplasm. In the absence of signal, β-catenin can be sequentially phosphorylated by casein kinase 1 (CK1) and GSK-3β, which is a target for ubiquitination and degradation by the proteasome. Due to the key role APC plays in leading β- catenin to degradation, in the absence of functional APC, free β-catenin will be accumulated in the cytoplasm and associated with members of T-cell factor (TCF) family of transcription factors, which then translocate to the nucleus, bind to the TCF consensus binding sites and trigger the transcription of specific target genes. The transcriptional targets of this pathway govern cell growth, migration, differentiation and apoptotic processes (Clevers H, 2006).

The altered functional role of APC in CRCs thus leads to dysregulation of the β-catenin/TCF pathway, affecting the transcriptional profile in these cells, leading to cell overgrowth, altered cell migration and chromosome instability.

1.32 Cross-talk between APC and NF-κB signaling pathway

Interestingly, crosstalk between APC and NF-κB pathway has been proposed. An interesting study by Shaked H *et al.* 2012, showed that in constitutive IKKβ $(EE)^{(IEC)}$ transgenic mouse intestinal epithelial cells (IECs), chronic epithelial NF-κB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. The results from this study suggested that, persistent NF-κB activation in IEC may increase β-catenin expression and tumor progression by

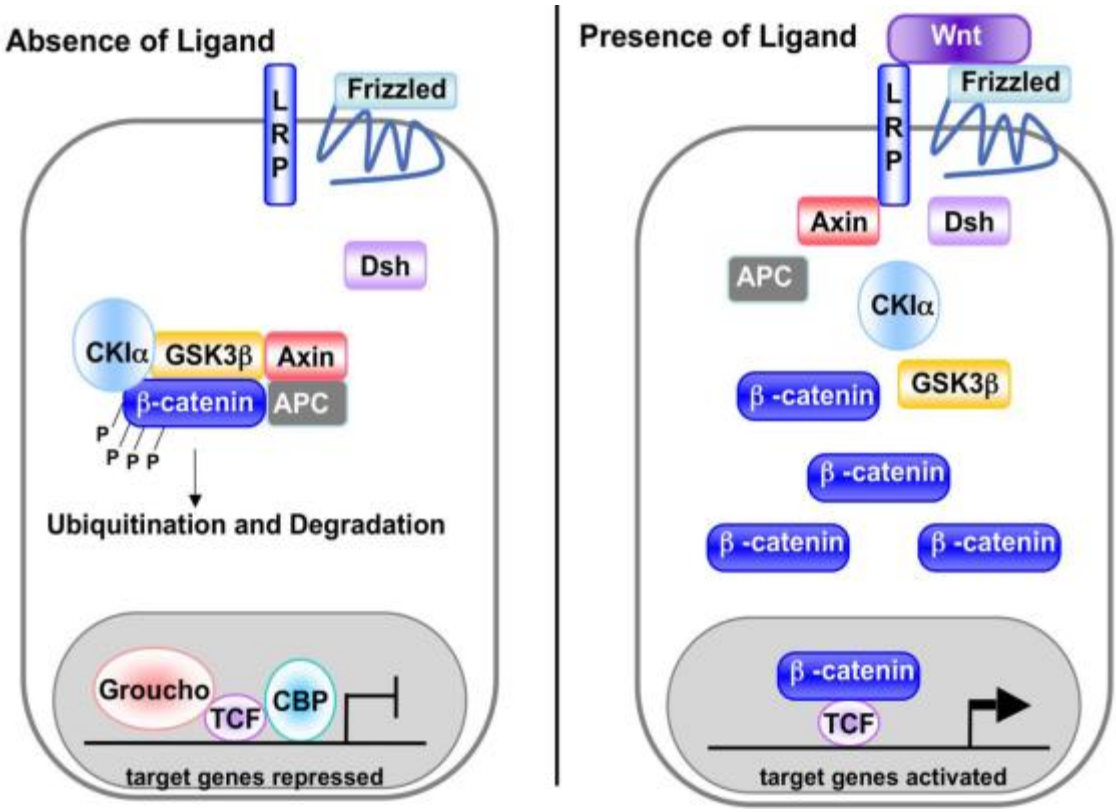

Figure 2. A canonical Wnt/β-catenin signaling pathway (Adapted from Eisenmann DM, 2005; Wormbook.org)

Abbreviations: APC, adenomatous polyposis coli; CBP, CREB binding protein; CK1α, casein kinase 1α; Dsh, phosphoproteinDishevelled; GSK-3β, glycogen synthase kinase 3β; LRP, lipoprotein receptor-related protein; TCF, T-cell factor.

accelerating APC loss. This study suggested that functional cross-regulation between APC and NF-κB signaling pathway has complex roles in the pathogenesis of certain diseases including CRC.

1.33 Similarity between ALP and APC

Using VBIM technology, we identified the novel adenomatous polyposis coli (APC) like protein (ALP) gene as a novel negative regulator of NF-κB in an unbiased fashion (*discussed later in introduction and results section*). Due to the great similarity of this novel regulator with APC and the important role APC plays in colon cancer, we name this novel regulator as APC Like Protein. ALP also has official alternative name as Armadillo Repeat-Containing Protein 4, (ARMC4) or Ciliary Dyskinesia, Primary 23 (CILD23). To date, very few studies have been devoted to this novel and interesting protein. ALP belongs to Armadillo repeat (~40 amino acids long) containing protein superfamily. Other members in this protein superfamily include APC, β-catenin and α-importin. ALP is located on chromosome 10p12.1-p11.23, contains 20 exons encoding a protein of 1,044 amino acids. ALP has 10 ARMs and one HEAT repeat. In contrast, APC is located on chromosome 5q21-q22 and encodes a protein of 2,843 amino acids. It has 6 Armadillo repeat motifs (ARMs) and 4 HEAT repeats. Although a role for ALP in cancer has not been reported, ARM proteins are known to be involved in a variety of processes including cell migration and proliferation, signal transduction, and maintenance of overall cell structure. It is also reported that tandem ARM-repeat units fold together as a super helix, forming a versatile platform for interactions with many protein partners (Tewari R *et al.* 2010). Based

on the functional significance of proteins in this superfamily and their important role in CRC regulation, it is very likely that ALP will also play an important role in regulation and development of CRC.

1.4 Validation based insertional mutagenesis (VBIM) technology

1.41 Design and novelty of VBIM technology

Given the crucial role of NF-κB in the proliferation and progression of CRC (Prasad S *et al.* 2010), manipulating NF-κB activity could be critical in the treatment of CRC. Towards this end, we used a novel VBIM strategy to identify a novel regulator of NF-κB, and further evaluate its role in regulating the NF-κB signaling in CRC cells at the molecular and biological level.

VBIM technology is a novel technique developed in our laboratory to isolate dominant mutant clones overexpressing the protein of interest. This innovative strategy distinguishes itself from other traditional approaches by several advantages. Owing to its technical simplicity, ability to infect both the dividing and non-diving cells, and robust target validation, VBIM has broad range of applications. The characteristic feature of this technique is random integration of a strong cytomegalovirus (CMV) promoter into the genomes of mammalian cells in tissue culture. **Figure 3,** adapted from Lu T *et al.* 2009, illustrate the schematic diagram of the VBIM lentiviral vectors. The VBIM vector has lentivirus backbone and splice donor sequence. A tet-operator (TO) element upstream of CMV promoter regulates its activity, following introduction of the TR-KRAB fusion protein which binds to this element in a doxycycline-dependent manner to

suppress the function of the adjacent promoter. It also has internal green fluorescent protein (GFP) reporter gene and internal ribosome entry site (IRES) downstream of GFP. The cellular protein encoded downstream of the insertion site is translated by the IRES and has a FLAG tag. The vector contains splice donor site (SD), and separate constructs for three different reading frames of FLAG-SD.

The working principle of VBIM lentiviral vectors is demonstrated in **Figure 4**, adapted from Lu T *et al.* 2009. Libraries of cells are generated using VBIM, with one or two different integrations of a strong CMV promoter randomly into the genomes of mammalian cells. VBIM vectors have LoxP sites on both ends of the vector. The promoter stimulates high expression of downstream genes generating dominant mutants. The mutant phenotype is attributed by the overexpressed protein (**Figure 4A and B**). The overwhelming advantage of this technique is the ease with which overexpressed proteins are identified. Any protein, as long as it confers a selectable phenotype can be identified with this technique.

Validation is done by using Cre. Cre is a recombinase that silences or removes the highly active CMV promoter. Reversion of mutant phenotype back to the wild type upon removal of CMV promoter provides conclusive genetic evidence that the insertion caused the mutation (**Figure 4C**). Upon excision of the vector by Cre, the gene might be left with very small footprint from the VBIM vector however, this does not affect the gene property and the cell can be reversed from mutant phenotype to wild type phenotype. The location of site of insertion is

identified by cloning and aligning it to human genome database. After gene identification, full length cDNA of the gene is expressed in normal cells and the overexpression of protein in these cells confirms whether the insertion event is the cause of the change of the cell phenotype.

1.42 Using VBIM to identify ALP as the negative regulator of NF-κB signaling

The long term goal of our lab is to understand the regulation of NF-κB pathway. Elucidating the underlying molecular mechanisms in the regulation of this pathway would lead to potential prognostic information and novel therapies targeted at inhibiting this hyper activated pathway. Previously, using VBIM technology, our lab has successfully identified and confirmed ALP gene as a novel negative regulator of NF-κB in an unbiased fashion (unpublished work).

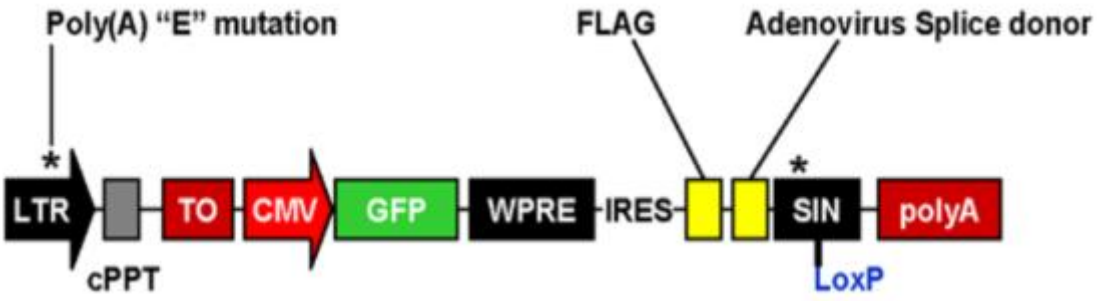

Figure 3. Schematic diagram of the VBIM lentiviral vectors (Adapted from Lu T *et al.* 2009)
Abbreviations: CMV, cytomegalovirus promoter; cPPT, central polypurine tract; GFP, green fluorescent protein; IRES, internal ribosome entry sequence; Lox P, site for Cre-mediated recombination; LTR, long terminal repeat; SA, splice acceptor site; SD, splice donor site; SIN, self-inactivating LTR; TO, tetracycline operon; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

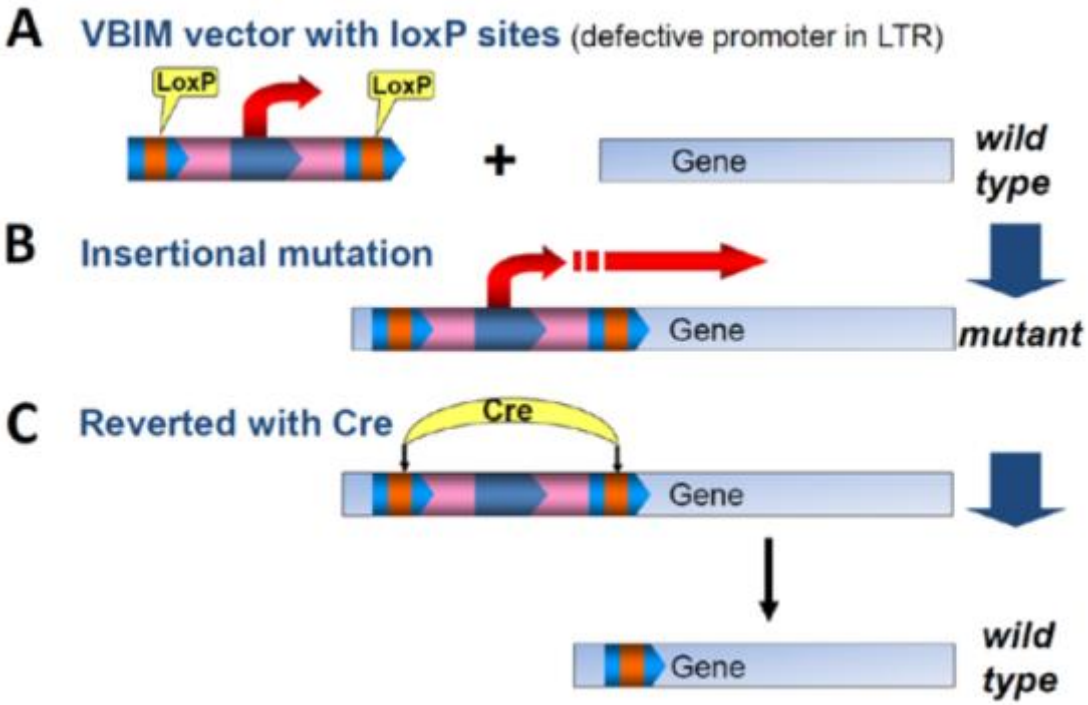

Figure 4. Working principle of the VBIM lentiviral vectors (Adapted from Lu T *et al*. 2009).
(A)A random gene in the genome of mammalian cells is represented (right). This gene attributes wild type cell phenotype. The CMV promoter is flanked on both sides with LoxP sites (left). (B)Following virus infection, the VBIM vectors randomly integrate into the gene and depending on the site of integration of CMV promoter will drive expression of different genes. This will attribute to changing cell from wild type to mutant phenotype. (C)To validate if the change in wild type to mutant phenotype is attributed by the VBIM insertion event, Cre recombinase is used to excise the vector from LoxP sites on both ends. If the cell phenotype changes back from the mutant to wild type, it proves that the insertional event was the cause of change of the cell phenotype.

To screen for negative regulators of NF-κB, Dr. Lu *et al.* used Z3 cells. Z3 cells have constitutively active NF-κB. These cells were obtained following chemical mutagenesis and selection for zeocin resistance of parental 293-thymidine kinase (TK)/zeocin resistance (Zeo) cells. The Z3TK/Zeo dual lethal selection system would allow cells with constitutive NF-κB to express the Zeo and TK genes. Active TK would then convert ganciclovir (GCV) into toxic product to kill Z3 cells, whereas expression of Zeo gene would allow cells to be resistant to Zeo treatment. Therefore, Z3 cells die in GCV and survive in Zeo (Lu T *et al.* 2004). The expression of a negative regulator of NF-κB in Z3 cells would cause the constitutive NF-κB activity to shut down, a phenotype can be selected for by requiring the cells to survive in GCV and die in Zeo (*Lu T et al. 2004*). By using this novel selection system, Dr. Lu identified ALP as a negative regulator of NF-κB (Lu T *et al.* unpublished data).

1.5 Central hypothesis and specific aims

Given the role of NF-κB signaling in CRC, the central hypothesis of this proposal is that, the ALP gene negatively regulates NF-κB signaling and functions as a tumor suppressor in CRC. My work follows to further evaluate the role of ALP in regulating the NF-κB signaling in CRC cells at the molecular and biological level by pursuing the following two specific aims:

Aim 1: Study the role of ALP in regulating NF-κB signaling at molecular level in colon cancer cells.

Rationale: Using VBIM technique, Dr. Lu has identified ALP gene as a negative

NF-κB regulator. It has long been appreciated that regulation of NF-κB signaling pathway is complex and given its role in the initiation and progression of CRC, understanding the regulation of NF-κB pathway at molecular level is of ultimate importance. Although we identified ALP as a negative regulator of NF-κB, however, the exact molecular mechanism of the regulation of complex NF-κB signaling by ALP is completely unknown. In order to provide new targets for preventive and therapeutic interventions in CRC, understanding the molecular mechanism by which ALP regulates NF-κB signaling and explore if similar results are observed in human colon cancer cells is extremely important.

Aim 2: Determine the biological effects of ALP in colon cancer cells.

Rationale: NF-κB signaling pathway is aberrantly activated in colon cancer and in Aim 1 we found that hyper activity of NF-κB can be inhibited by ALP. NF-κB transcription factors are critical in regulating genes with whole variety of functions like cell proliferation, differentiation, inflammation, and it is apparent that NF-κB has a key role in genesis of colon tumors. Using Mass Spectrometry protein identification method, Dr. Lu *et al.* identified that ALP was pulled down together with p65 (data not shown). We postulate that by associating with NF-κB, ALP will down regulate the transcription of these genes and will 'turn off' the function of these genes. The next logical step following this is to test the effect of ALP on the biological attributes of CRC cells like proliferation, colony size, metastasis and invasion ability with biochemical and genetic approaches.

CHAPTER II MATERIALS AND METHODS

2.1 Cell lines and antibodies:

The human embryonic kidney (HEK) 293 cells, also referred as 293 cells, and 293-NF-κB reporter cells (Lu T *et al,* 2004) were cultured in Dulbecco's modified Eagle's media supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS). The HT29 and DLD1 colon cancer cell line were purchased from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA) and were cultivated in RPMI1640 media with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Experiments were carried out when the cells reached 90% confluence. The following antibodies were obtained from commercial sources: anti-ALP, anti–NF-κB p65, and anti-IκBα were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin and anti-Flag were from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Construction of stable ALP overexpressing or shALP HT29 cells:

ALP protein was cloned into the lentiviral vector as a full length cDNA or shRNA pool (containing 5 different shRNA constructs) against ALP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Viruses were generated and used to infect HT29 cells. Since both the lentiviral vector and the shRNA pool carry the puromycin resistance marker, puromycin-resistant clones were selected in 1μg/ml puromycin after virus infection. Approximately 10 days after puromycin

selection, cells were pooled and tested for the best overexpression and knockdown by the Western Blot method with antibodies against ALP.

2.3 Transfections and luciferase assays:

For NF-κB luciferase assays, the κB-luciferase construct p5XIP10 κB (contains five tandem copies of the NF-κB site from the IP10gene) (Lu T *et al.* 2004) was transfected transiently into the cells and luciferase activity was assayed 48 h later. A β-galactosidase (β-gal) construct was co-transfected to normalize for transfection efficiency. The cells were then washed with cold phosphate-buffered saline (PBS), and lysed in 80µl of 5X lysis buffer from Promega Corporation (Madison, WI, USA). After incubation on ice for 15–20min, cell debris was pelleted at 15,000 g, for 5 min at 4°C. A measure of either 30 µl of luciferase assay substrate or β-gal substrate (Promega Corporation) was added to 20 µl of supernatant solution before being read in a luminometer. The relative luminescence was normalized to the optical reading of β-gal (Bio-Rad Laboratories, Hercules, CA, USA).

2.4 Western analysis:

Cells were cultured to 95% confluency, treated or untreated with IL-1β for different times, as indicated in the descriptions of individual experiments and washed with 1X PBS and pelleted at 5,000 g at 4°C for 4 min. Cell pellets were lysed with RIPA buffer [1X PBS/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% sodium dodecyl sulfate (SDS)]. Cellular debris was removed by centrifugation at 15,000 g for 10 min. The amount of protein in the supernatant

solution was determined, and samples were heat-treated in 2X SDS sample loading buffer at 100°C for 5 min. Equal amounts of samples were fractioned by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat skim milk powder in PBS for an hour and then probed with primary antibodies, which were visualized with horseradish peroxidase-coupled secondary antibodies by using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA).Human IL-1β (National Cancer Institute Biological Resources Branch Preclinical Repository) was used at 10ng/mL.

2.5 Reverse PCR, quantitative real-time PCR and genomic PCR analysis:

The total RNA was extracted with the TRIzol reagent at room temperature following the protocol provided by Invitrogen Life Technologies (Carlsbad, CA, USA). Random primers were designed using the Primer Express 3.0 software, and cDNA was made by reverse PCR from total RNA of 293 cells by using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed for each cDNA with Econo Taq PLUS Green 2X PCR Master Mix (Lucigen, Middleton, WI, USA), with gene-specific primer pairs. Samples were amplified according to standard PCR procedure. For Quantitative PCR (qPCR) experiments, cells were cultured to 80–90% confluency and were untreated or treated with IL-1β for 60 min. Samples were analyzed by using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics) qPCR reactions. The quantitative mRNA expression was calculated based on the $2^{-\Delta\Delta C}{}_{T}$ method (Kreuzer KA *et al.* 1999). GAPDH was selected as the housekeeping gene for

normalization; each gene was running along with GAPDH and difference between threshold cycles (C_T) was designated as ΔC_T. $\Delta\Delta C_T$ is the difference between their respective controls.

The genomic DNA was extracted using the QIAamp DNA mini kit from Qiagen (Valencia, CA, and USA). The genomic PCR was performed with 100 ng of genomic DNA and samples were amplified according to standard PCR procedure. All reactions were independently repeated at least three times to ensure the reproducibility of the results. GAPDH was used as a control for all target genes. Primer Express 3.0 software was used to design primers after obtaining the primer sequences from National Center for Biotechnology Information (NCBI).

2.6 Co-immunoprecipitation assay:

Cells cultured in 15-cm plates to 95% confluency were lysed in co-immunoprecipitation buffer (1%Triton X-100, 50 mMTris-HCl, pH 7.4, 150 mMNaCl, 1 mMEDTA, 1 mM sodium orthovanadate, 20 μM aprotinin, and 1 mMphenylmethanesulfonyl fluoride and pepstatin A). Prewashed immobilized protein A/G (Pierce) was premixed with anti-p65 for 1 h, and then mixed with cell lysates with equivalent amount of proteins at 4°C overnight. Gel beads were washed four times with 20 volumes of IP buffer with rotation at 4°C for 5 min each time. At the last step, the gel beads were resuspended in an equal volume of 1XSDS sample loading buffer (6% glycerol, 1% β-mercaptoethanol, 2% SDS, 50 mMTris-HCl, pH 6.7, 0.004% bromophenol blue) and boiled for 5 min.

Supernatant was then separated by SDS/PAGE. For anti-Flag-M2 antibody, EZView beads were used (Sigma-Aldrich).

2.7 Conditioned media assay:

The 293 cells were seeded into 12-well plates, cultivated to 90% confluency, and transfected with different plasmids: empty vector, WTALP, shALP. After 24 h of transfection, the media were replaced and the cells were kept for an additional 48 h. The conditioned media were collected, floating cells were pelleted at 3,000 g at 4°C, for 10min, and the supernatant was aliquoted into sterile tubes and either used immediately or stored at -80°C. The media were then used to treat 293-NF-κB reporter cells and luciferase assay was performed as previously described.

2.8 Cell growth and soft agar assays:

HT29 control cells or cells with overexpression or shALP knock down of ALP were plated in triplicates at 20,000/well in a 6-well plate with 3 ml of medium and the medium was changed every 3 days. Cell number was counted on different days using a cell counting chamber.

For soft agar assays, type VII agarose (Sigma Aldrich) was autoclaved and mixed with RPMI1640 cell growth medium. Cell culture dishes were coated with 1.2% agarose as the bottom layer. Cells were resuspended in 0.6% of soft agarose, and plated on top of the bottom layers. Cells were cultured in the semisolid medium for about 2–3 weeks. The colonies formed were checked under a microscope and measured and counted with the help of ImageJ software (ver. 1.47t).

2.9 Migration assay:

The Boyden chamber containing cell culture inserts with polycarbonate membrane at the bottom with a pore size of with 8 μM pore size and 6.5-mm diameter (Corning-Costar, Lowell, MA, USA) were used. The inserts were coated with 0.1% gelatin for 2-3 h at 37°C. HT29 control cells, or cells with the overexpression or shRNA knock down of ALP were suspended in serum-free RPMI1640 medium and plated in triplicates at 200,000/insert in the upper chambers. The lower chambers were filled with 10% FBS RPMI1640 medium. The cells were incubated at 37°Cfor 72 h. The inserts were then removed and migratory cells at the bottom of the chamber were fixed and stained in a 4% paraformaldehyde (PFA)/0.1% Crystal Violet solution, followed by washing in deionized water to remove redundant staining. Non-migrated cells remaining at the upper side of the membranes were carefully removed with cotton swabs and inserts were dried in darkness overnight. The following day stained membranes were pictured in five random non-overlapping fields and counted manually at 20X objective and 20X eyepiece on a transmitted-light microscope.

2.10 Immunofluorescence assay:

293 cells were cultured overnight at 100,000 cells/coverslip coated with 0.1% gelatin. Following treatment with IL-1β for 30 min, the cells were fixed with 4% PFA for 30 min and were permeabilized for 10 min in blocking buffer (1X PBS/0.2% Triton™ X-100/1% bovine serum albumin [BSA]). The two primary antibodies, anti-ALP antibody (diluted 1:150) and anti-p65 antibody (diluted

1:200) were added and allowed to bind for a minimum of 1 h in blocking buffer and the cells were washed three times in PBS for 10 min each to remove excess primary antibody. The two primary antibodies were visualized with an anti-rabbit IgG secondary antibody with a red fluorescent tag (Alexa Fluor 594 - Molecular Probes) and an anti-mouse IgG secondary antibody with a green fluorescent tag (Alexa Fluor 488 - Molecular Probes) (diluted 1:2000) respectively. Cells were washed three times in PBS for 10 min each and stained with DAPI (Molecular Probes) according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The coverslips were then inverted onto a glass microscope slide, mounted, sealed and viewed using a Zeiss Axiovert 40 CFL microscope (60X magnification) and digital photographs were taken.

2.11 Immunohistochemical analysis of tissue microarray (TMA):

The colon cancer TMA CO951 (30 cases/95 cores) was purchased from US Biomax, Inc. (Rockville, MD, USA). The cores are duplicated; 30 cancer and 8 of which has matched normal adjacent tissue and 10 cases of matched metastasis, with follow-up data 2 cores per case. All histochemical stains were carried out at the IUSM Immunohistochemistry (IHC) Core, by an expert pathologist Dr. Constance Temm and included standard deparaffinization in xylene, quenching in 1% hydrogen peroxide/methanol for 10 min, and rehydrated through sequentially graded ethanols. Antigen retrieval was performed by Ethylenediamine-tetraacetic acid (EDTA). Using a DAKO automated immunostrainers (DAKO, Carpinteria, CA, USA), the slides were blocked for 30 min in horse serum and incubated with ALP antibody, followed by incubation with

secondary antibody. The Universal ABC Elite kit (Vectastain, Burlingame, CA, USA) with 3,3′-diaminobenzidine development was used to visualize antibody binding, and the slides were subsequently counterstained with hematoxylin. The tissue arrays were stored at 4C and heated to 60C for 1hour before use.

2.12 Evaluation of immunohistochemical staining:

The TMA sets were scanned using Images were scanned with the Aperio Scanscope Imaging System (Leica Biosystems, Buffalo Grove, IL, USA). The TMA tissue cores were individually copied, labeled and stored in a separate folder as tiff files. The immunohistochemical staining results of individual core tissues were evaluated by an expert pathologist (Dr. George Sandusky), who was blinded to patient's clinocopathological details. The immunohistochemical staining was categorized according to a scoring method based on the staining intensity (score 0, no staining intensity; score 1, weak staining intensity; score 2, intermediate staining intensity; and score 3, strong staining intensity). In the case of heterogenous staining within the samples, the respective higher score was chosen if >50% of the cells showed higher staining intensity.

2.13 Statistical analysis

The data represent the means ± SD from at least three separate experiments performed in triplicate. The differences between groups were analyzed using Student's *t*test, and a P-value <0.05 was considered statistically significant. Statistical analyses were carried out using JMP software (ver. 7.0)

CHAPTER III RESULTS

Previously, Dr. Lu *et al.* identified and confirmed ALP as a negative regulator of NF-κB in 293 cells (*discussed earlier in introduction section*). To follow up this preliminary finding, I performed the following experiments:

3.1 Overexpression of ALP down-regulates the expression of most NF-κB inducible target genes

Dr. Lu *et al.* have found that in 293 cells, ~46.2% of NF-κB genes were down-regulated by twofold or more by the overexpression of ALP (microarray data not shown). These data demonstrated that the ALP down-regulates the expression of most of the NF-κB inducible target genes. Among these genes are chemokines, cytokines, growth factors *etc.*, most of which are known to have an important role in cancer development (data not shown). Following up on Dr. Lu T *et al's* work, to confirm the microarray data, I first performed a qPCR to confirm the expression of several candidate genes. As shown in **Figure 5**, the mRNA levels of suppressor of cytokine signaling 6 (*SOCS6),* bone morphogenetic proteins *(BMP1),* Tripartite motif-containing protein 16 *(TRIM16),* andS100 calcium binding protein A3 *(S100A3)* were strongly induced in IL-1β–treated 293 cells, but the induction was substantially less in 293 cells with ALP overexpression. These results are consistent with the idea that ALP down-regulates NF-κB inducible target gene expression.

3.2 Overexpression of ALP decreases NF-κB activity in human colon cancer HT29 cells

Since Dr. Lu *et al.* have proved that ALP is a negative regulator of NF-κB in 293 cells; we asked whether ALP could negatively regulate NF-κB in colon cancer cells, which usually have high constitutive NF-κB activity. To test the functional significance of ALP, we either overexpressed or used shRNA pool to knockdown ALP in human colon cancer HT29 cells, which has constitutively active NF-κB (Lu T *et al.* 2004). As shown in **Figure 6A**, western analysis indicated stable HT29 lines with expected level of ALP were successfully generated. HT29 with vector infection were used as normal control. Furthermore, a luciferase reporter assay in these cells demonstrated that, overexpressing ALP reduced the constitutive activity of NF-κB by approximately ten folds, while knocking ALP down enhanced this activity (**Figure 6B**). Therefore, collectively, these data suggest that ALP is a strong NF-κB inhibitor in human colon cancer HT29 cells.

3.3 ALP affects the cell growth, anchorage independent growth, and migration in colon cancer HT29 cells

NF-κB can affect all six hallmarks of cancer through the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation and suppression of apoptosis (Baud V *et al.* 2009). Since our results demonstrated that ALP inhibits the NF-κB pathway in colon cancer HT29 cells, we examined the effect of ALP on the biological attributes like cell proliferation, tumor size, and metastasis ability in these cells.

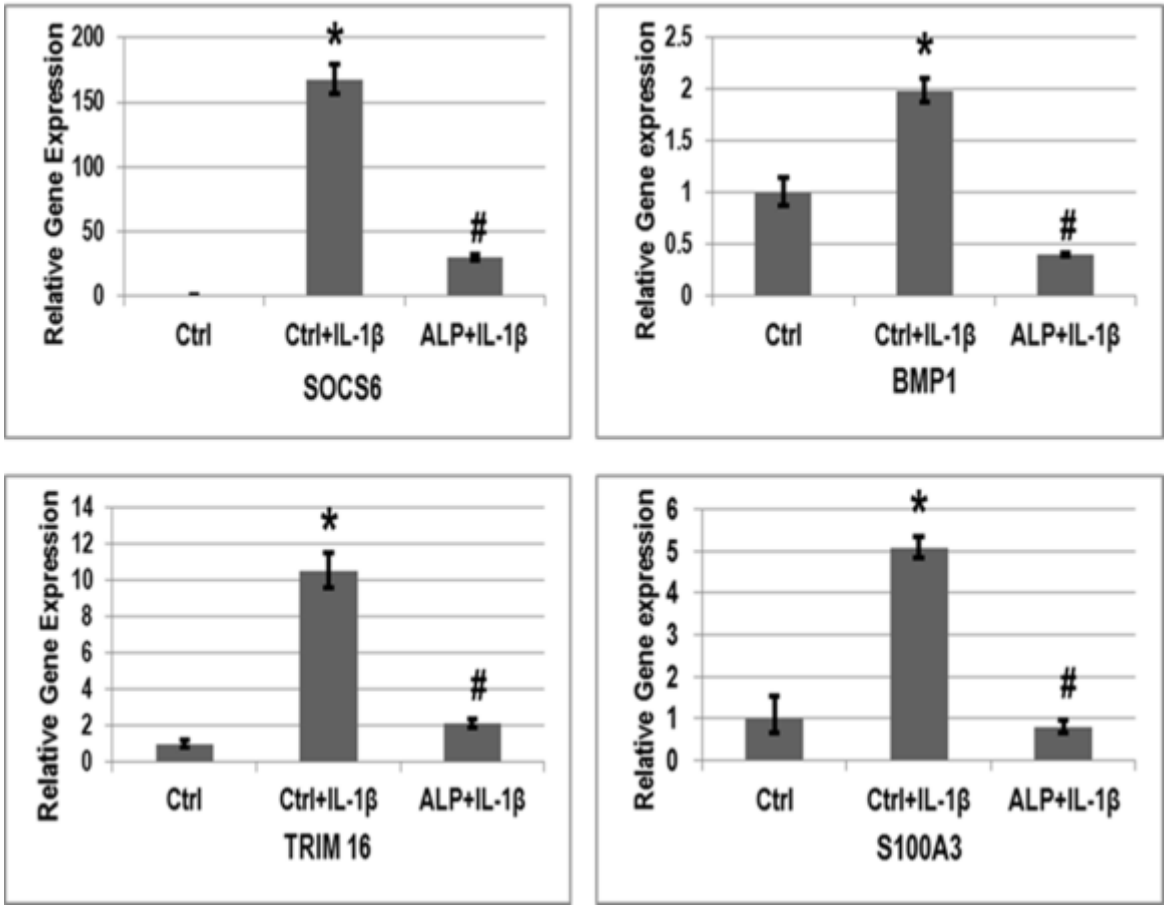

Figure 5. Regulation of NF-κB–dependent gene expression by ALP
Confirmation of Illumina array data by qPCR analysis from 293 cells overexpressing ALP protein. 293 cells were used as the control. The expression of *SOCS6, BMP1, TRIM16* and *S100A3* was tested, confirming that these genes were strongly induced upon IL-1β treatment but significantly decreased with ALP overexpression. The data represent the means ± SD from three independent experiments. *P <0.01 vs. Ctrl group; $^{\#}$P < 0.01 vs. Ctrl+IL-1β group.

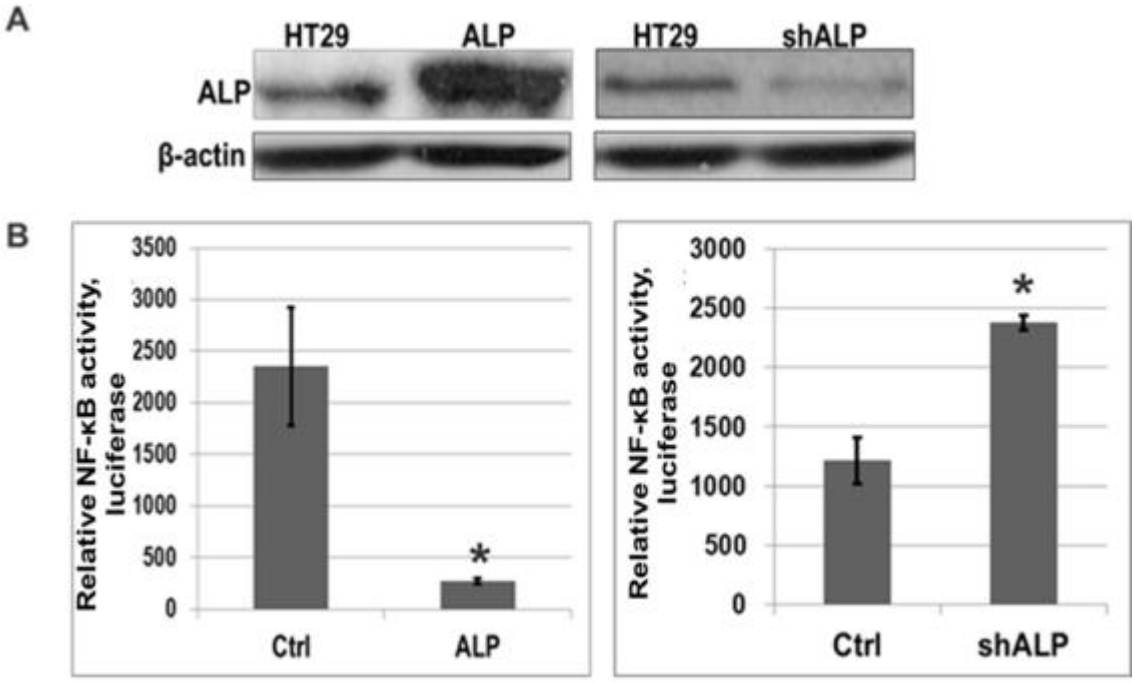

Figure 6. ALP is a negative regulator of NF-κB in colon cancer HT29 cells

(A) Western assays, showing either overexpression or shRNA-mediated knock down of ALP in colon cancer HT29 cells. (B) Luciferase assay of NF-κB in HT29 cells, showing that overexpression of ALP decreased NF-κB activation, whereas reduced ALP expression further enhanced NF-κB activation. The data represent the means ± SD for three experiments. *P < 0.05 vs. Ctrl group.

As shown in **Figure 7**, in HT29 cells, which have constitutively active NF-κB, knocking down ALP markedly increased cell growth, whereas increased expression of ALP significantly decreased cell growth. These results indicate that ALP is a potent suppressor of cell proliferation in these cells.

Furthermore, in a colony independent assay (soft agar assay), knocking down ALP significantly increased the sizes of colonies, while increased expression of ALP had opposite effect (**Figure 8A and B**), strongly suggesting that ALP is a potent tumor suppressor of colony formation in human colon cancer HT29 cells. Another important aspect for tumor progression is metastasis. In the *in vitro* experimental system, we could perform cell migration assay to examine the ability that a cell can migrate. As shown in **Figure 9**, overexpression of ALP in the HT29 cells led to decrease in migration ability as measured by Boyden chamber assays. However, the observed decrease in migration was significantly reverted when ALP was knocked down**.** These data suggest that ALP plays an important role in mediating migration functions in human colon cancer HT29 cells.

3.4 Conditioned media from HT29 cells with ALP overexpression led to decreased NF-κB inducing ability as compared to HT29 cell.

Constitutive NF-κB activation in cancer is often caused by the autocrine action of secreted cytokines (Wolf JS *et al.* 2001; Arlt A *et al.* 2002; Coward WR *et al.* 2002). To test if ALP had an effect on the secretion of activators in culture medium, we assayed conditioned media from HT29 cells, and HT29 cells with

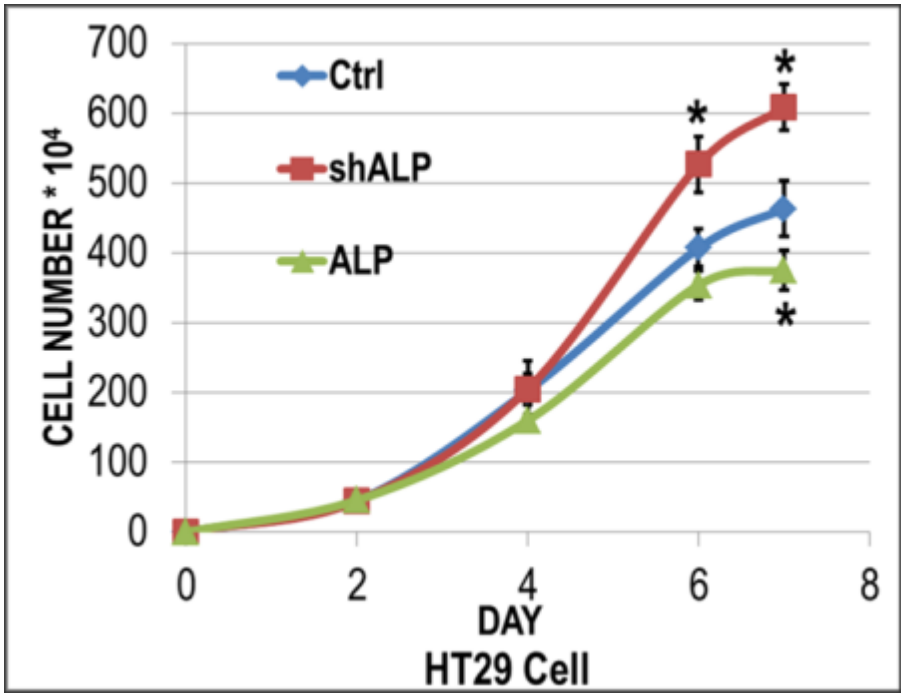

Figure 7. Effect of ALP on cell proliferation in colon cancer HT29 cells

High levels of ALP decreased cell growth, whereas decreasing ALP expression with shRNA increased cell growth in HT29 cells. The data represent the means ± SD for three experiments. *P < 0.05 vs. Ctrl group.

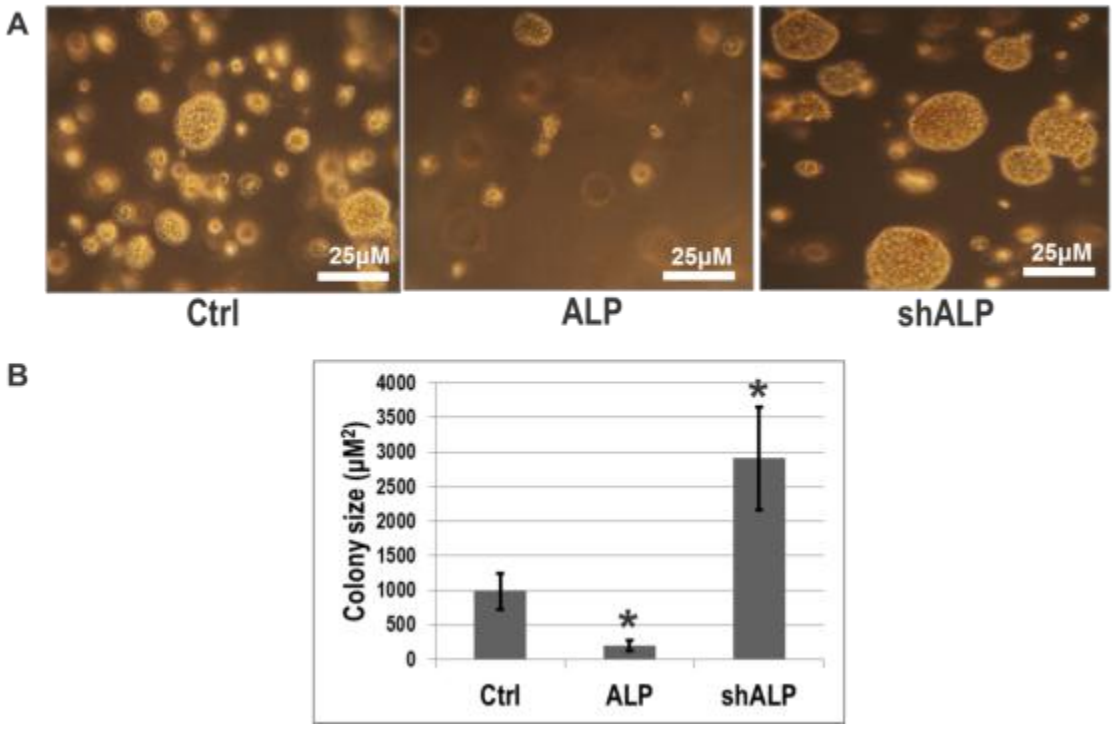

Figure 8. Effect of ALP on anchorage independent growth in colon cancer HT29 cells

(A) Images of colonies in a soft agar assay, showing that high levels of ALP decreased sizes of colonies formed, whereas decreased expression of ALP increased sizes of colonies in HT29 cells. (B) Statistical analysis of the sizes of the colonies observed. Results of triplicate assays are shown as means ± SD. *P < 0.05 vs. Ctrl group.

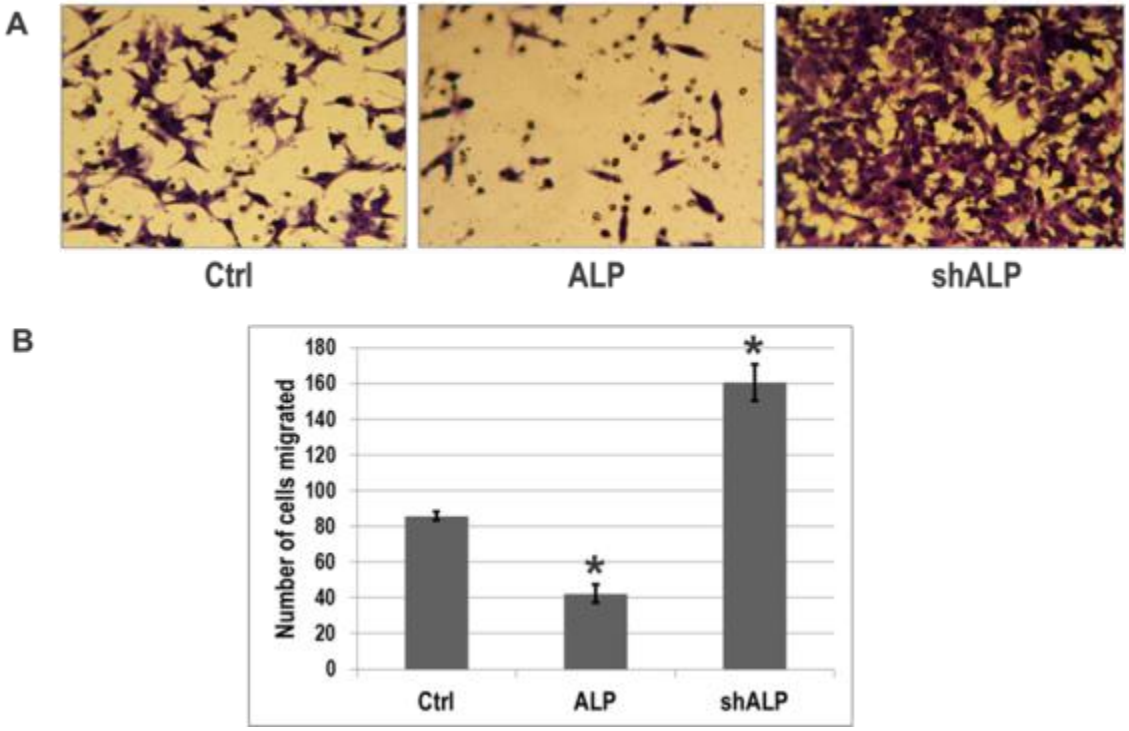

Figure 9. Effect of ALP on cell migration in colon cancer HT29 cells
(A) Images of migrated cells in a Boyden chamber assay, showing that high levels of ALP decreased the number of migrated cells, whereas decreased expression of ALP increased the number of migrated cells in HT29 cells. (B) Statistical analysis of the sizes of the colonies observed. Results of triplicate assays are shown as means ± SD. *P < 0.05 vs. Ctrl group.

both overexpression and shRNA knock down of ALP. Normal cell culture media was used as basal control. As shown in **Figure 10**, a well-established 293-κB reporter cell line (Lu T *et al.* 2004) was used; different media secreted from the above HT29 cells were used to treat this 293-κB reporter cell line. Data suggested that media from the HT29 cells with ALP overexpression had decreased NF-κB inducting ability as compared to that of HT29 cells. On the other hand, media from shRNA for ALP showed greatly increased NF-κB inducing ability, strongly suggesting that ALP negatively regulates NF-κB signaling and knocking it down leads to the activation of NF-κB, partially through the action of secreted factors.

3.5 ALP expression is reduced in different human colon cancer cell lines and is gradually lost with tumor progression in CRC

The expression of ALP was examined in human colon tissues using immunochemical staining. The combination of IHC and tissue microarray (TMA) technology allows for the simultaneous *in-situ* protein expression analysis in hundreds of tissue samples. The expression of the ALP protein was observed and scored by IHC on the TMA, which included 40 colon adenocarcinoma and 8 normal colon tissues. IHC result showed that the ALP protein was heavily stained in normal colon tissue, while as the cancer progressed, a lesser staining could also be observed (**Figure 11A**). Digital image analysis (Aperio ScanScope) revealed the percentage of Aperio positive staining for ALP protein, brown tissue nuclei corresponding to positive ALP staining. We observed that the advanced stages of CRC showed ALP negative tumor nuclei. Figure 11B, represents the

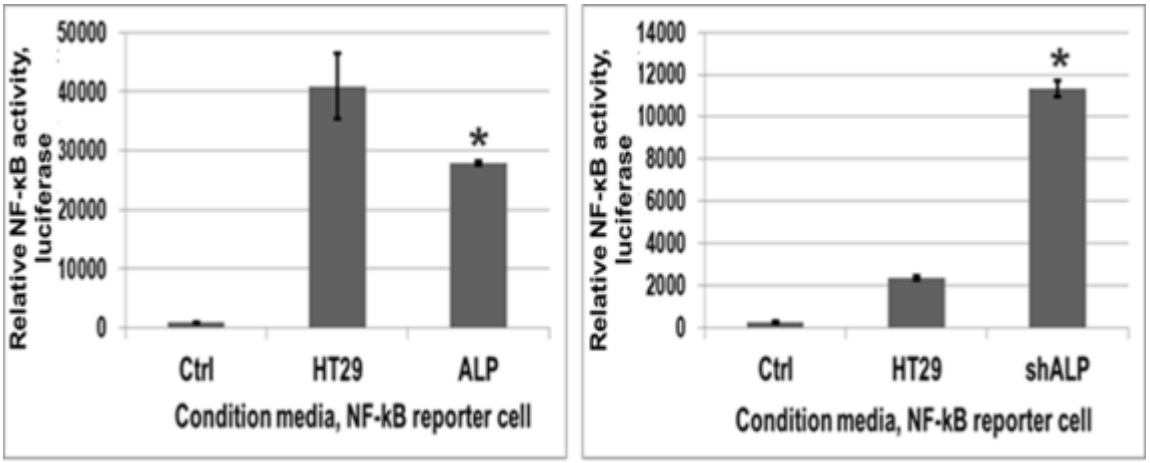

Figure 10. Assays of conditioned media

Conditioned media from colon cancer HT29 cells overexpressing ALP protein had lower NF-κB-inducing activity than media from HT29 cells, whereas conditioned media from HT29 cells with reduced ALP protein had much higher NF-κB-inducing activity than media from HT29 cells. Ctrl (control) represents the regular cell culture media. Stable 293-NF-κB reporter cells were used. The data were normalized to the total number of cells that generated the conditioned media and to the total amounts of protein. The data represent the means ± SD from three independent experiments. *P < 0.05 vs. HT29 group.

scoring results obtained from the pathologist (Dr. George Sandusky). Taken together, these data demonstrate the loss of expression of ALP with tumor progression in human colon tissue.

3.6 Initial exploration of the causes of loss of ALP in CRC

Cancer cells are characterized by increased transcription of tumor promoter genes and decreased transcription of tumor suppressor genes. Also, DNA amplifications activate oncogenes and are hallmarks of nearly all advanced tumors (Myllykangas S *et al.* 2006). Thus, in order to examine if there was any variation of the mRNA levels or DNA copy number of ALP, we used different colon cancer cells like HT29 and DLD1 to carry out both reverse transcription polymerase chain reaction (RT-PCR) and genomic PCR, respectively. RT-PCR analysis revealed that the mRNA levels of ALP were greatly reduced in different colon cancer cell lines as compared to that in 293 normal control cells (**Figure 12A**). Additionally, PCR analysis of genomic DNA extracted from these cells indicated that the ALP copy number was decreased in colon cancer cell as compared to the 293 cells (**Figure 12B**). GAPDH was used as an endogenous control as it was stable among different cell lines of interest. These data not only demonstrate that ALP expression is reduced in different groups of colon cancer cell lines but also suggest that ALP may serve as a potential marker for the diagnosis of CRC.

3.7 ALP does not affect upstream events of NF-κB signaling

In order to shed lights onto the molecular mechanism by which ALP inhibits NF-κB activation, we first examined the phosphorylation and degradation of IκBα. To

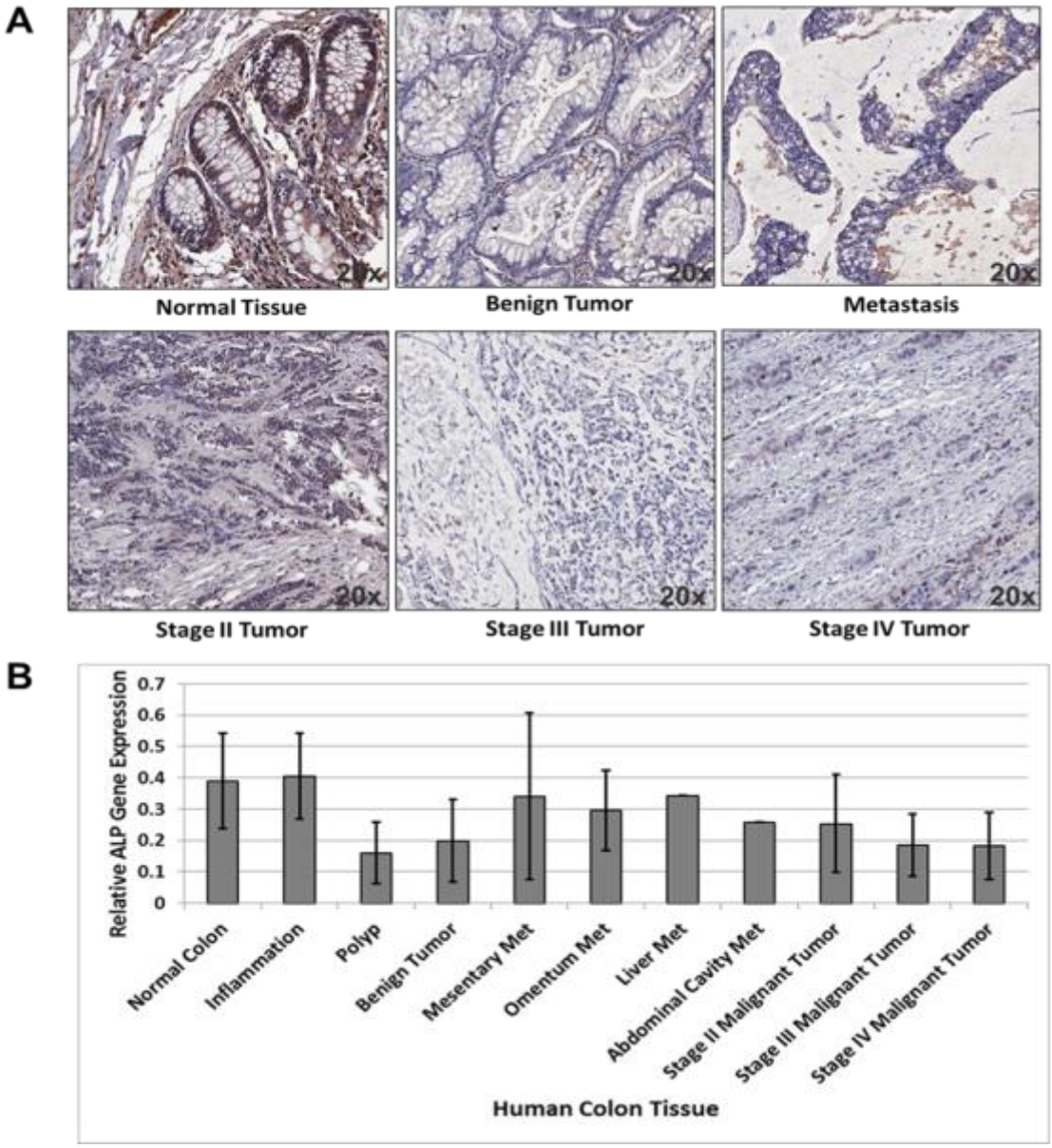

Figure 11.Loss of ALP with tumor advancement in human colon tissues

(A) Images of ALP staining pattern in major subtypes of CRC showing the gradual loss of ALP protein upon tumor progression (positive staining = brown nuclei) (20X).(B) Quantification of immunostaining performed using the automated Aperio ScanScope CS system indicated reduced expression of ALP in patient tissues was correlated with advanced clinical stage.

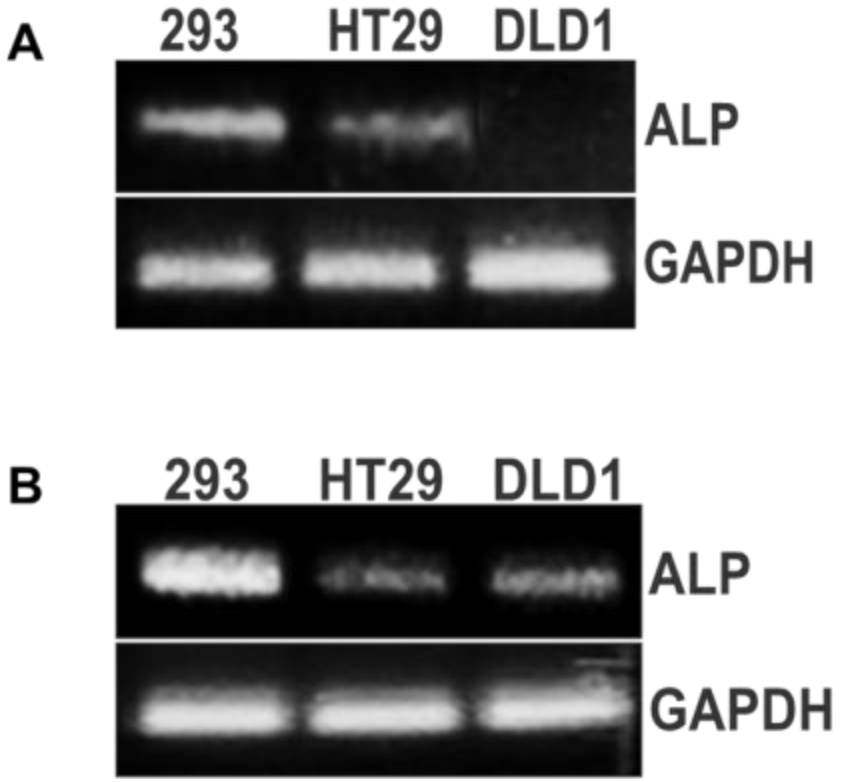

Figure 12. Reduced levels of ALP mRNA and copy number in different colon cancer cell lines
(A) An RT-PCR experiment followed by agarose gel electrophoresis and EtBr staining indicated that the mRNA levels of ALP are decreased in HT29 and DLD1 colon cancer cell lines. (B) Genomic-PCR experiment indicated that the ALP copy number is dramatically decreased in HT29 and DLD1.

test whether ALP functions upstream or downstream of the IKK complex, we performed a western analysis, comparing human colon cancer HT29 cells with the same cells stably overexpressing or knocked down ALP (**Figure 13**). Upon treatment with IL-1β, the expression of ALP did not affect either the IκBα degradation or its resynthesis significantly, indicating that ALP functions downstream of the IKK-IκBα axis.

3.8 ALP binds to the p65 subunit of NF-κB

To gain further insight into the molecular mechanisms underlying ALP mediated NF-κB inhibition, the next logical step was to see if ALP mediates this inhibition by directly binding to p65. In order to test this, we treated 293 cells and the same cells overexpressing ALP with IL-1β for 1 h. Since ALP is Flag-tagged, we used anti-Flag to pull down endogenous ALP, and further carried out western analysis to probe for p65. The result clearly shows that ALP binds to p65 in a signal-dependent manner (**Figure 14**), suggesting that ALP could perform its NF-κB negative regulator function through direct binding to NF-κB.

3.9 ALP co-localizes with p65 predominantly in the cytoplasm and translocates to the nucleus upon NF-κB activation

To date, the distribution of ALP is completely unknown. We attempted to examine the distribution of ALP by immunofluorescence (IF) assay in 293 cells (**Figure 15**), and in human colon cancer HT29 cells (**Figure 16**). Cells were viewed with blue excitation/emission settings to detect 4',6-diamidino-2-phenylindole (DAPI) staining of the nuclei, with red excitation/emission settings to

detect ALP, and with green excitation/emission settings to detect p65. We found that ALP protein was predominantly localized in the cytoplasm in 293 cells (**Figure 15**). Some fluorescence is observed in the nucleus. However, a proportion of this fluorescence can be attributed to the fluorescence seen using the secondary antibody alone, which stains the entire cell faintly. The distribution of p65 we observed is consistent with previous studies that showed that p65 is a cytoplasmic protein when it is inactive (Espinosa L *et al.* 2002).

Double-staining experiments allowed for the simultaneous detection of ALP (revealed by red fluorescence) and p65 (revealed by green fluorescence) indicated that the two proteins co-localize in the cytoplasmic compartment in the colon cancer HT29 cells. We also examined the distribution of ALP/p65 before and after IL-1β treatment. IL-1β is a pro-inflammatory cytokine that induces NF-κB activity. In normal untreated HT29 cells, ALP/p65 spares the nucleus and was predominantly cytoplasmic; however, after 30 min of IL-1β treatment and NF-κB activation, a large portion of ALP/p65 was found within the nucleus (**Figure 16**). These data indicate that ALP regulates gene expression by a general mechanism that may involve its nuclear translocation upon NF-κB activation.

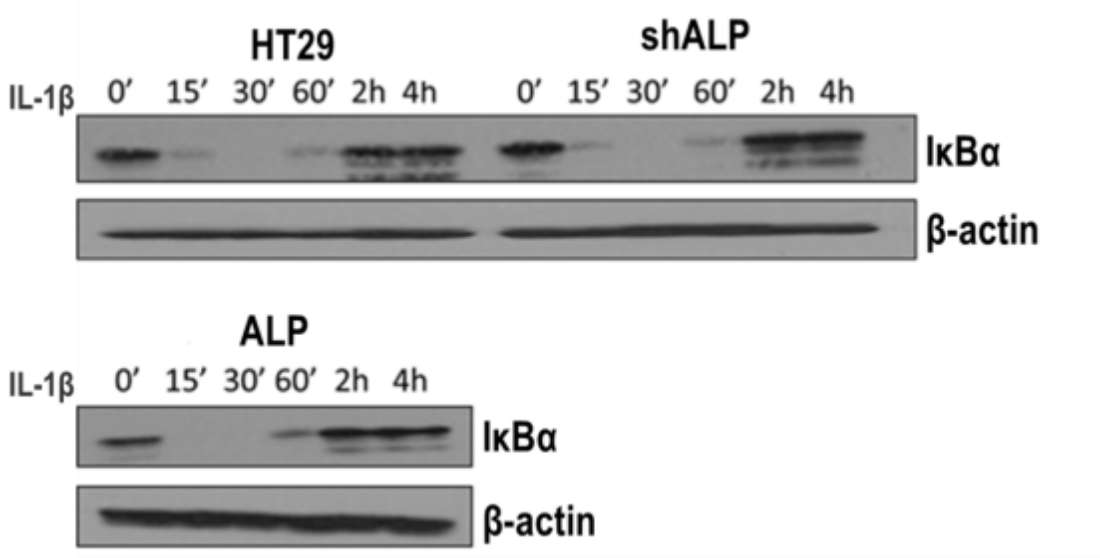

Figure 13. Expression of ALP does not affect degradation of IκBα significantly

Western analysis, showed similar pattern in the levels of total IκBα in colon cancer HT29 cells and the same cells in which ALP have been over-expressed and knocked down, all three cell lines were treated with 10 ng/mL IL-1β.

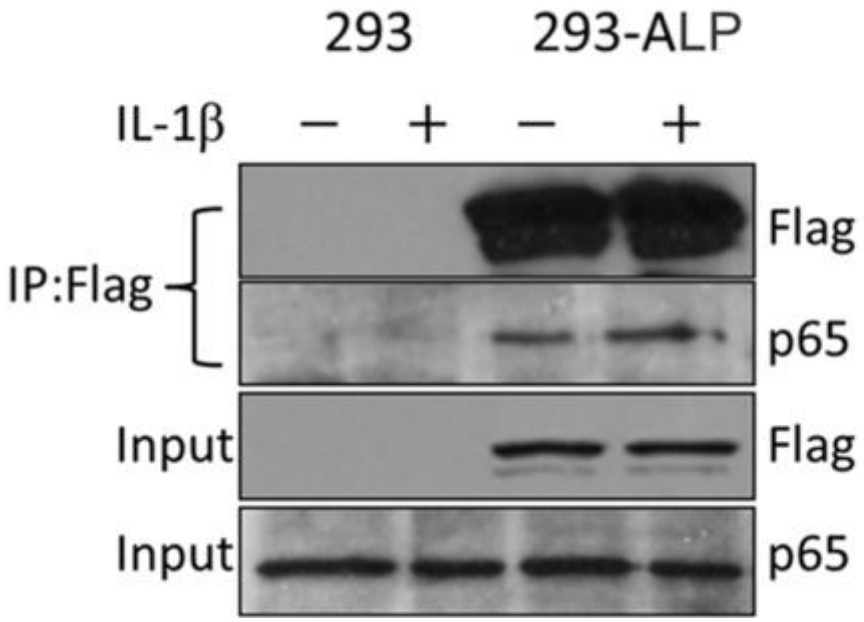

Figure 14. ALP binds to the p65 subunit of NF-κB
ALP is Flag-tagged; endogenous ALP co-immunoprecipitated with p65. 293 cells were treated with 10 ng/mL of IL-1β for 1 h or were untreated. ALP bound to p65 even when NF-κB is inactivated.

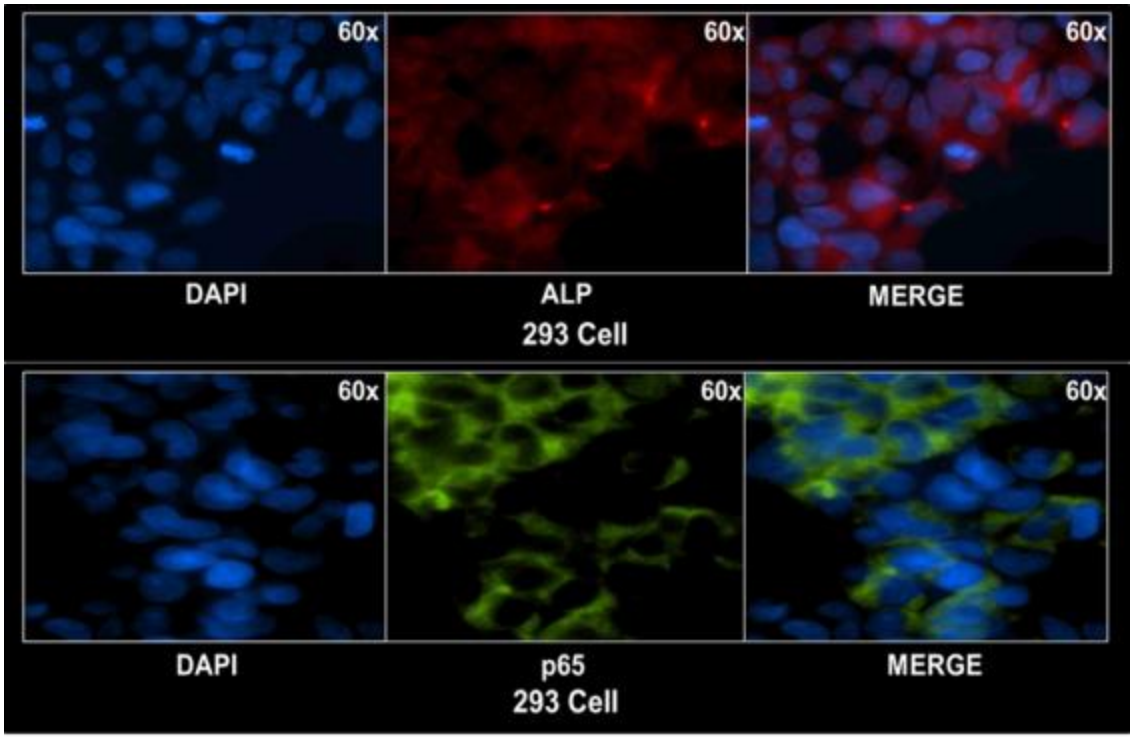

Figure 15. ALP is predominantly a cytoplasmic protein
Endogenous subcellular localization determined by Immunofluorescence staining in 293 cells using antibody for ALP (red) and antibody for p65 (green) showed that ALP and p65 are predominantly localized in the cytoplasm. Images are representative of three independent experiments, counterstained with DAPI and taken at 60X magnification.

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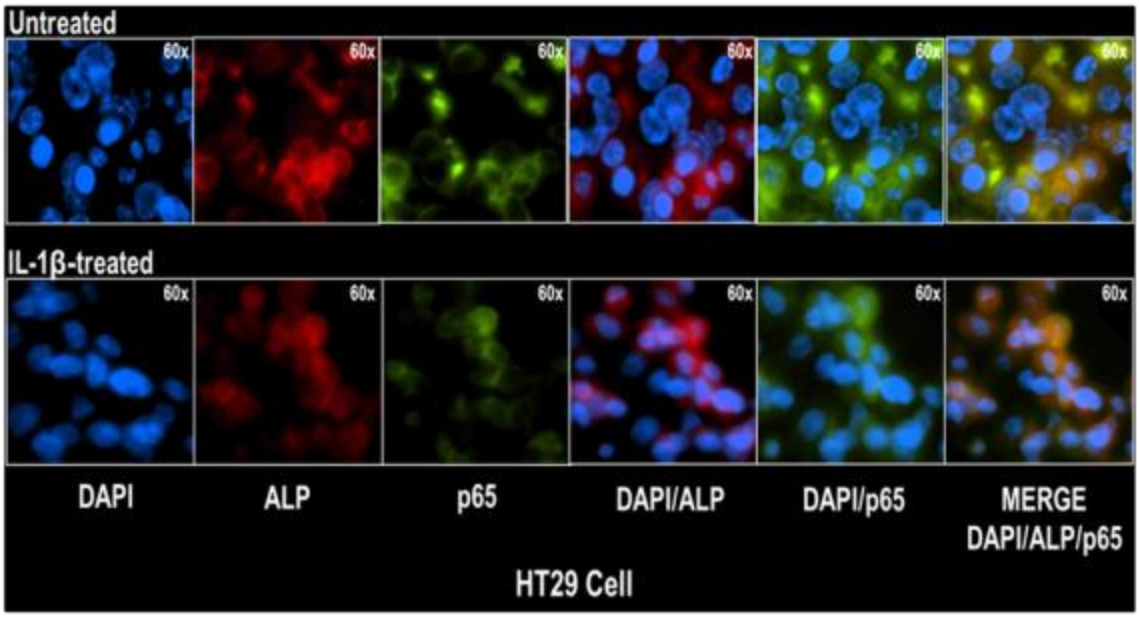

Figure 16. ALP is translocated to the nucleus upon NF-κB activation
The images (Upper panel) represent the fluorescence distribution of ALP (red) and p65 (green) in untreated colon cancer HT29 cells. Immunofluorescence staining (Lower panel) of ALP and p65 in HT29 cells after treatment with 10 ng/mL of IL-1β for 30 min. Images are representative of three independent experiments, counterstained with DAPI and taken at 60X magnification.

CHAPTER IV DISCUSSION

NF-κB is constitutively active and plays a key role in variety of human diseases (Baldwin AS, 2001). NF-κB has recently become a potential target for pharmacological intervention. Although more than 700 inhibitors of the NF-κB activation pathway have been identified, no NF-κB blocker has been approved for human use (Gupta SC *et al.* 2010). Most of the NF-κB inhibitors act as general inhibitors of NF-κB activation, while few others target specific steps in the NF-κB pathway. General inhibitors of NF-κB activation lack specificity and thus interfere with NF-κB's physiological roles in immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. Thus in order to avoid the risk of undesirable side effects, developing NF-κB inhibitors aimed to target specific pathways or cells is important. Identification of regulators of specific NF-κB activation needs deeper insight into understanding the regulation of this pathway, as it would lead to the manipulation of this pathway in the clinic and would offer promising strategies for the treatment of diseases. Though many small molecule activators associated with specific NF-κB activation pathways have been reported, but relatively very few negative regulators have been reported to date.

In this study we report that ALP, a protein with few known biological functions, is a negative regulator of NF-κB signaling. We found that ~46% of NF-κB inducible genes were significantly down-regulated by increased expression of ALP. These

genes encode cytokines, chemokines, kinases, growth factors, and cell-adhesion molecules, including SOCS6 (Lai RH *et al.* 2010), BMP1 (Hwang EY *et al.* 2014), and S100A3 (Schäfer BW *et al.* 1996), whose roles are widely accepted to be essential in tumor progression and invasion. Several members of the Armadillo repeat containing superfamily family have been shown to key players in regulating cellular signaling, most notable among which is APC, which acts as tumor suppressor in CRC (Mundade R *et al.* 2014). Our data suggested that like APC, ALP also has a suppressive role in CRC. Functional data supporting a role for ALP in colon cancer were obtained by over-expression of ALP in human colon cancer cell line, which led to marked growth suppression, colony formation and migration ability of the cancer cells. These data demonstrates the profound effect of ALP on biological activity in CRC and may suggest a tumor suppressive role of ALP in CRC. The detailed mechanism by which ALP inhibits CRC cell proliferation and invasion should be investigated in future work. Although the results of the migration analyses may in part be explained by a reduction in cell number rather than a change of cell migration ability, the analysis of mRNA levels of some cell motility-associated genes such as mitogen-activated protein kinase (MAPK), phospholipase C, gamma 2 (PLCG2) in different CRC cells can be done in future to confirm this finding.

Immunohistochemical studies with human colon tissues revealed that ALP expression is reduced in a large percentage of CRC tumors compared with non-tumor tissues and is gradually lost in high-grade tumors. This finding gives rise to the possibility that ALP functions as a tumor suppressor, in the context of

pathological conditions such as cancer development in actual human samples. Our study thus indicate that, inactivation of ALP through genetic and epigenetic alterations are common in CRCs, and may contribute to the pathogenic development of this malignancy.

There are multiple mechanisms that lead to copy number variation. The major cause of copy number variation is mis-segregation of chromosomes at mitosis or meiosis, cohesion and checkpoint defects, non-disjunction or recombination or repair events generating dicentric and acentric chromosomes (Lee H *et al.* 2014). In context of cancer cells, increasing number of experiments have revealed that copy number variations confer several advantages that may affect the behaviors of malignant cells, including cell growth, apoptosis, anticancer drug sensitivity and the invasive and metastatic potentials (Wen SL *et al.* 2013). This link between copy number and cancer cell fitness is supported by high-throughput profiling of 8,000 cancer genomes, where pan-lineage alterations have been linked to kinases and cell cycle regulators (Kim TM *et al.* 2013). In the present study, using genomic PCR analysis, we detected an alteration in the copy number of ALP in several human colon cancer cell lines as compared with normal cells. Analysis using RT-PCR revealed that, a pervasive loss of ALP gene copy number in human CRC cell lines is also associated with an impairment of ALP mRNA level in malignant cells. The positive correlation between gene loss and gene expression level indicate that ALP gene might have a transcriptional dampening effect. Importantly, these results are the first to identify that ALP is the target of frequent copy number loss in human CRC, and this genomic loss is

accompanied by a down-regulation of ALP mRNA expression, supporting a tumor suppressive role for this gene. Further studies like *in vivo* approach in the mouse to determine whether or not ALP is a haploinsufficient tumor suppressor, genome sequencing of CRC cells, RNA-Seq profiles to explore the biological consequences of copy number changes in CRC patients and downstream products, would be beneficial to understanding the function of ALP genomic DNA in malignancy.

Previous studies have shown that p65 is a cytoplasmic protein and nuclear translocation of p65 subunit of NF-κB is required for the expression of a subset of NF-κB target genes (Zhang T *et al.* 2013). In our attempts to see the sub-cellular localization of ALP, we found that ALP is predominantly a cytoplasmic protein. In the future, we will further confirm the sub-cellular localization identified in this study by separating the nuclear and cytoplasmic extracts and doing a Western analysis with antibodies to the protein. Further, from our co-immunoprecipitation and co-localization experiments, we showed that ALP directly interacts with the p65 subunit of NF-κB. Knowing the sub-cellular localization of ALP and that it interacts with p65 is a major step in determining the role of ALP in regulating the NF-κB signaling. To further examine the effect of ALP on activation of NF-κB, we treated the 293 and HT29 cells with cytokine IL-1β to induce NF-κB activity. While the non-stimulated cells retained a large portion of ALP/p65 complex, the translocation of ALP/p65 complex from cytoplasm to nucleus in cytokine-stimulated cells was striking. We also showed that ALP works downstream of IKK in the NF-κB pathway. Based on these observations we can postulate that, ALP

may function as a transcriptional repressor of NF-κB by binding to p65 and inhibiting its function. Nevertheless, how this interaction between nuclear NF-κB protein p65 and ALP is controlled and regulated is a question that remains unresolved. Detecting and characterizing proteins and protein complexes associated with ALP using mass spectrometry, meta-analysis of microarray data using a pathway-based approach, and possibly looking at different sites of action of ALP in the NF-κB pathway will be needed to extend this model further in the future.

Multiple mechanisms are implicated in the negative regulation of NF-κB activity. A study published in *Science* by Jiang Y *et al.* 1999, demonstrated that SODD (silencer of death domains) negatively regulates NF-κB signaling by suppressing the ability of tumor necrosis factor (TNF) to activate NF-κB dependent reporter gene expression. Overexpression of SODD causes it to associate with the intracellular domain of TNFR1, forming the TNFR1-SODD complex thereby blocking the association of TNF with its receptor. A study by Heyninck K *et al.* 2005, showed that a zinc finger protein A20, negatively regulates NF-κB signaling by two opposing activities: sequential de-ubiquitination and ubiquitination of the TNF receptor-interacting protein (RIP), thereby targeting RIP to proteasomal degradation. Furthermore, it was reported that binding of PIAS1 (protein inhibitor of activated signal transducers and activators of transcription, STAT-1) to p65 inhibits cytokine-induced NF-κB dependent gene activation by blocking the DNA binding activity of p65 both *in vitro* and *in vivo* (Liu B *et al.* 2005). Additionally, it has been identified that B-cell leukemia (Bcl)-3 negatively

regulates NF-κB activation in a TLR signaling dependent manner by blocking ubiquitination of p50 subunit of NF-κB. Bcl-3 stabilizes p50 complex that inhibits gene transcription (Carmody RJ *et al.* 2007). These are a few of the examples demonstrating the multiple mechanisms of NF-κB regulation, and also reiterating the complexity of the same.

Epigenetic properties of the cancer genome correlate with the development and function of the cancer cell. Negative regulation of NF-κB signaling at post-translational level was demonstrated in 2009; Phosphorylation of Serine (Ser) 536 is essential for the transactivation function of p65 and recruitment of the transcriptional co-activator p300. As a negative regulator of NF-κB, WIP1 was reported to be a direct phosphatase of Ser 536 of the p65 subunit of NF-κB (Chew J *et al.* 2009). Loss of function or transcriptional silencing via hypermethylation has been widely identified for tumor suppressor genes (Ting AH *et al.* 2006). Dr. Lu *et al.* 2009, identified F-box and leucine-rich repeat protein 11 (FBXL11) as another negative regulator of NF-κB (Lu T *et al.* 2009). This important study unfolded yet another mechanism of negative regulation of NF-κB signaling. FBXL11 consists of the JmjC domain, which encodes the histone H3K36 demethylase activity; this activity is essential for the negative regulative effect of FBXL11 on NF-κB through the methylation of the p65 subunit of NF-κB. It is also possible that the expression of ALP is gradually down-regulated in CRC via epigenetic mechanisms, such as DNA hypermethylation. In the future, we can perform the methylation-specific PCR (MSP) assay to further investigate this possibility. These analyses will not only directly verify the

implicated cancer functioning role of ALP, but also will be of particular use in tumor biospecific based diagnostics and pathway directed therapeutics.

In the present study, we provide unique insight regarding how high levels of ALP may inhibit tumorigenesis, at least in part by inhibiting NF-κB activation. These results open a brand new avenue for future study. An important question for future research is to determine the mechanism of regulation of NF-κB signaling by ALP. By motif search, we have identified six ARM-repeats in ALP. It is well known that ARM-repeats are crucial for interactions with many proteins, whether ALP function is regulated by these repeats, and improving their stability may lead to a more profound effect on ALPs function remains to be studied. Another area that remains to be explored is to understand the causes of reduced ALP expression during tumor development, as this will be of fundamental importance in understanding the important role of ALP in CRC progression. A detailed understanding of how ALP negatively regulates NF-κB signaling will further raise the possibility of focusing on ALP as a drug target and biomarker. Furthermore, future *in vivo* experiments will provide a basis for further investigating ALP as a tumor suppressor and its inactivation in the pathogenic development of CRC.

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CURRICULUM VITAE

RASIKA S. MUNDADE

EDUCATION

Indiana University **GPA 3.65** Indianapolis, USA
Master of Science in Pharmacology *January 2015*

Thapar University **GPA 3.80** Patiala, India
Masters of Science in Biotechnology *June 2010*

University of Pune **GPA 3.70** Pune, India
Bachelors of Science in Chemistry and Biotechnology *June 2007*

RESEARCH EXPERIENCE

Dr. Tao Lu Lab, IUSM *Graduate Student* *May 2013-Dec 2014*

- Studying the role of YBX1 phosphorylation in the regulation of NF-κB
- Studying the biological effects of the mutant YBX1 on colon tumors
- Investigating the molecular mechanism by which ALP regulates NF-κB signaling
- Setting up colon cancer cell lines with overexpression and knockdown of ALP and investigating the role of ALP in regulating colon tumors size and proliferation
- My thesis work involves using multiple molecular, genetic, biochemical techniques to confirm ALP as a novel negative regulator of NF-κB and a tumor suppressor in colon cancer

Dr. Ronald Wek Lab, IUSM *Rotating Student* *March 2013-May 2013*

- Cloned the BTB domain of the human IBTK gene involved in stress response
- Designed an expression vector for the BTB domain of human IBTK gene based on domain homology
- Expressed the recombinant BTB peptide for purification for future biochemical assays and antibody development
- Learnt different molecular biology techniques like transformation, ligation, digestion, gel extraction, protein purification

Dr. John Turchi Lab, IUSM *Rotating Student* *Jan 2013-March 2013*

- Studied the structure/function analysis of small molecule inhibitors (SMIs) of the human single stranded DNA binding protein, Replication protein A (RPA)
- Screened several small molecule inhibitors that inhibit RPA/DNA binding activity
- Performed EMSAs (electrophoretic mobility shift assay) to measure the inhibition of binding of purified protein to single-strand DNA
- Performed mutational analysis of RPA to examine the inhibitory activity in context of the mutants to delineate a binding interface for SMI
- This study is expected to increase cisplatin efficacy in a cellular model for cancer treatment

Dr. Murray Korc Lab, IUSM *Rotating Student* *Oct 2012-Dec 2012*

- Looked at aberrant signaling pathways in cancer cells with a focus on signaling by VEGF receptors and its downstream effectors
- Developed cultured cell lines which were used as a model system for the study
- Learnt laboratory techniques like cell culture, western blotting, PCRs, protein quantification assays, RNA extraction

Thapar University, India *Research Assistant* *July 2009-June 2010*

- Examined the potential of *Aspergillus* sp. RBD01 in esterifying fatty acids to corresponding esters
- Studied esterification using three different acids (oleic, stearic and palmitic acids) and carried out a comparative analysis of their yields
- Observed the influence of amount of biomass vis-à-vis bound enzyme on the esterification process
- Determined the optimal temperature and acid to alcohol molar ratio required for maximum esterification
- Determined optimum time interval between stepwise alcohol additions for maximum esterification
- Studied reusability and recycling of the biomass and its effect on the yield of esters
- Successfully achieved over 90% conversion of fatty acids to alkyl esters (biodiesel) by optimizing different parameters and refining the processes for Whole Cell catalyzed esterification of fatty acids
- Successfully developed a protocol for generation of **BIODIESEL**, which would result in a cost saving of more than 70% over existing processes

Maharashtra Hybrid Seeds Company, India *Summer Intern June-July 2009*

- Proactively worked with researchers and technicians in the production and commercialization of *Bt* Crops
- Worked on Colonization and Characterization of APN (amino peptidase) Gene
- Determined the mechanism and insect specificity of a particular endotoxin from *Bacillus thuringiensis*
- Cloned and purified receptors for different toxins and studied their interaction at the molecular level
- Determined the role of various factors (pH, proteases and membrane composition) that might affect the broader insect-order specificity of the main classes of toxins
- Analyzed data using bioinformatics tools such as mapping, sequence analysis, primer design and assisted senior researchers in developing crop improvement initiatives
- Independently handled multiple projects concurrently with tight deadlines

TEACHING & MENTORING EXPERIENCE

Indiana University School of Medicine, Indianapolis, IN

- Student mentor for Ph.D. student *Aug 2013-Oct 2014*
- Supervised summer student *June 2013*

Thapar University, India *Teaching Assistant June2009-July 2010*

- Taught an introductory Biochemistry course to undergraduate students to over forty students per course
- Developed new curriculum that emphasized several in-class group activities
- Conducted Biochemistry laboratory sessions for undergraduate students

HONORS & AWARDS

- Indiana University Melvin and Bren Simon Cancer Center Travel Award *March 2014*
- Paradise Travel Award, Department of Pharmacology & Toxicology, IUSM *Jan 2014*
- International Students Representative for IBMG batch of 2013 *August 2013*
- Graduated Cum Laude, M.S. Biotechnology, Thapar University, India *June 2010*
- Graduated Cum Laude, B.S. Chemistry, University of Pune, India *June 2007*
- Awarded “Merit Ranker” by the State Board, Senior year *June 2003*

- Awarded “Merit Ranker” by the State Board *June 2001*
  Sophomore year

PUBLICATIONS

- **Mundade R**, Yang M, Wang B, Liu Y, and Lu T; 2015. Role of phosphorylation of YBX1 in colon cancer. *Oncotarget.* Manuscript in submission
- Prabhu L, **Mundade R**, Korc M, Loehrer P, and Lu T; 2014. Critical role of NF-κB in pancreatic cancer (Review). *Oncotarget*. 5: 10969-10975
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- **Mundade R**, Imperiale TF, Loehrer PJ and Lu T; 2014. Genetic pathways, prevention, and treatment of sporadic colorectal cancer (Review). *Oncoscience*. 1: 400-406
- **Mundade R**, Wei H, and Lu T; 2014. PRMT5, a pivotal player in cancer (Review). *Austin Journal of Pharmacology and Therapeutics.* 2(2): id1014
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- Aulakh S, Chibbar M, **Mantri R,** Prakash R; 2011. Whole cell catalyzed esterification of fatty acids to biodiesel using *Aspergillus* sp. *Biocatalysis and Biotransformation.* 29: 354-358

POSTER & PRESENTATION ABSTRACTS

- “VBIM technology identifies APC like protein (ALP) as a novel negative regulator of NF-κB” *American Association for Cancer Research 2014. San Diego, CA*
- “VBIM technology identifies APC like protein (ALP) as a novel negative regulator of NF-κB” *Cancer Research Day 2014. Indianapolis, IN*
- “VBIM technology identifies APC like protein (ALP) as a novel negative regulator of NF-κB” *Pharm/Tox Research Day 2014. Indianapolis, IN*
- “Whole Cell Catalyzed Esterification of Fatty Acids” *Graduate Research Program Symposium 2010. Patiala, India*
- “Colony and Characterization of APN gene” *Summer Meeting of the Marathwada Research Society 2009. MAHYCO, India*

MEMBERSHIPS

American Association for Cancer Research 2013-*present*