

GENETIC APPROACH TO DISCOVER ARMC4 AS A NOVEL NF- $\kappa$ B NEGATIVE  
REGULATOR AND TUMOR SUPPRESSOR IN COLORECTAL CANCER

Matthew Peter Martin

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Doctoral Committee

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Tao Lu, Ph. D., Chair

---

Ahmad Safa, Ph. D.

May 24, 2019

---

Tim Corson, Ph. D.

---

Travis Jerde, Ph. D.

---

Karen Pollok, Ph. D.

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## DEDICATION

This dissertation is dedicated to mother and father. Without your love, support, and encouragement I would not be where I am today.

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Matthew Peter Martin

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The nuclear factor  $\kappa$ B (NF- $\kappa$ B) plays pivotal roles in inflammatory and immune responses and in cancer. Therefore, understanding its regulation holds great promise for disease therapy. Using validation-based insertional mutagenesis (VBIM), a powerful technique established by us, we discovered armadillo repeat containing protein 4 (ARMC4) as a novel negative regulator of NF- $\kappa$ B in colorectal cancer (CRC). ARMC4 is a rarely studied protein only known to date for its role in primary ciliary dyskinesia (PCD) and mouse spermatogenesis. Thus, my work reveals a completely new facet of ARMC4 function that has never been reported before. We showed that ARMC4 overexpression downregulated the expression of NF- $\kappa$ B-dependent genes, many of which are related to cancer. Additionally, compared to the vector control group, overexpression of ARMC4 in HEK293 cells or CRC HT29, DLD1, and HCT116 cells dramatically reduced NF- $\kappa$ B activity, cellular proliferation, anchorage-independent growth, and migratory ability *in vitro*, and unsurprisingly, significantly decreased xenograft tumor growth *in vivo*. In contrast, shARMC4 knockdown cells showed quite opposite effect. Furthermore, co-immunoprecipitation (Co-IP) experiment confirmed that ARMC4 may form a complex with the p65 subunit of NF- $\kappa$ B. Importantly, immunohistochemistry (IHC) data exhibited much lower ARMC4 expression level in CRC patient tumor tissues compared to normal tissues, indicating that ARMC4 may function as a tumor suppressor in CRC. To conclude, my important findings for the first time uncovered the negative

regulatory function of ARMC4 in NF- $\kappa$ B signaling, and present ARMC4 as an innovative therapeutic target in CRC treatment.

Tao Lu, Ph.D., Chair

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## LIST OF ABBREVIATIONS

AOM	azoxymethane
APC	adenomatous polyposis small homeotic discs 2- like
ARMC4	armadillo repeat-containing protein 4
ASA	Aspirin
ATCC	American Tissue Culture Collection
BAX	Bcl-2 associated X gene
BRAF	murine sarcoma viral oncogene homolog B
CAFs	cancer-associated fibroblasts
CapeOx	combination of capecitabine and oxaliplatin
CIN	chromosomal instability
CpG islands	(5'—Cytosine—phosphate—Guanine—3')
CRC	colorectal cancer
DSS	dextran sodium sulfate
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FOLFIRI	leucovorin, fluorouracil and irinotecan

FOLFOX	leucovorin (also called folinic acid), fluorouracil, and oxaliplatin
GCV	ganciclovir
HNPCC	hereditary nonpolyposis colorectal cancer
IBD	inflammatory bowel disease
IgG4	immunoglobulin G4
IKK	I $\kappa$ B kinase
I $\kappa$ B $\alpha$	inhibitor of $\kappa$ B alpha
IL-1 $\beta$	interleukin 1 beta
Indels	insertion and deletion mutants
IPA	ingenuity pathway analysis
KRAS	V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog
LOH	loss of heterozygosity
MAPK15	Mitogen-Activated Protein Kinase 15
MEK	mitogen-activated protein kinase kinase
MET	Metformin
MLH1	MutL homolog 1
MMR	mismatch repair
MMP	matrix metalloproteinases
MSH2	MutS protein homolog 2
MSI	microsatellite instability
MSI-H	MSI high

MSI-L	MSI low
MSS	microsatellite stable
MTOR	mammalian target of rapamycin
NF- $\kappa$ B	nuclear factor kappa b
PCD	primary ciliary dyskinesia
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PVDF	polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute medium
R.T.	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SMAD2	Mothers against decapentaplegic homolog 2
SMAD4	Mothers against decapentaplegic homolog 4
TGF $\beta$ RII	transforming growth factor $\beta$ receptor II
TMA	tissue microarray
TNF $\alpha$	tumor necrosis factor alpha
TP53	tumor suppressor p53
VBIM	validation based insertional mutagenesis

VCAM1

vascular cell adhesion molecule 1

VEGFA

vascular endothelial growth factor A

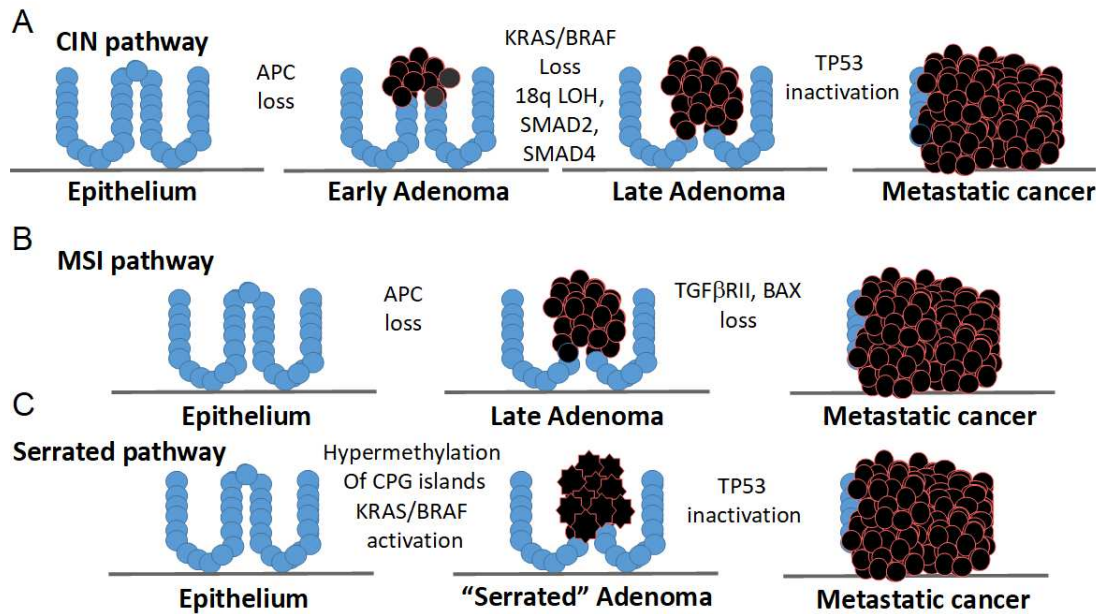
## CHAPTER 1. INTRODUCTION

### 1.1 Colorectal Cancer

#### 1.1.1 Risk factors, development and genetic mutations of CRC

Colorectal cancer (CRC) is a gastrointestinal cancer and therefore a malignant cancer of the colon and is one of the most challenging cancer types to treat (Siegel, 2013). CRC, is a serious and lethal disease with about 155,000 new cases expected in 2019 in the United States (Alteri, 2019). Roughly, one third of those diagnosed will die from this disease (Alteri, 2019). Disease recurrence is common in CRC patients with over 40% of patients expected to develop disease recurrence following initial therapy. Furthermore, CRC has an overall 5-year relative survival rate of only 11% (Siegel, 2017). This is generally regarded as due to metastatic CRC (mCRC) being particularly invasive and having very heterogeneous tumors (Punt, 2016).

CRC develops due to multiple stepwise genetic and epigenetic changes in colon epithelial cells wherein there is development of significant mutations in critical regulatory molecules (Punt, 2016). The staging of CRC can be seen in **Figure 1**. CRC originates in the colon and/or rectum by abnormal tissue folds in the inner lining called “polyps”. These abnormal growths are considered benign in nature without further mutations. In early stages of CRC the growth has not spread to surrounding tissue (benign, early adenoma, *etc.*) but over time, and with accumulated mutations, the benign polyps can undergo a shift to metastatic forms of CRC. In later stages such as the late adenoma and metastatic stages, the cancer cells begin to invade the walls of the colon and rectum. The late adenoma stage then leads to metastasis wherein there is uncontrolled cell growth and spreading to neighboring and distant tissue and lymph nodes.



**Figure 1.** Three stepwise pathways of CRC pathogenesis (A) In the CIN pathway, a mutation of the APC (adenomatous polyposis coli, a known tumor suppressor) gene in the Wnt/ $\beta$ -catenin signaling pathway is needed for polyp formation and progression to early adenoma. For transition to a late adenoma stage mutations in KRAS and BRAF, and loss of heterogeneity (LOH) of the long arm of chromosome 18q which contains SMAD2 and SMAD4 increase chromosomal instability and promotes proliferation. Inactivation of TP53 gene in the p53 signaling pathway is necessary for development of metastatic CRC. (B) APC also drives the MSI to a late adenoma stage and is characterized by mutations in transforming growth factor  $\beta$  receptor II (TGF $\beta$ RII) and apoptosis regulation gene Bcl-2 associated X (BAX), which promotes rapid development of metastatic cancer. (C) The Serrated Pathway involves hypermethylation of non-APC genes. Activation of mutant KRAS or BRAF through MAPK signaling pathways promotes MAPK activation and the formation of a Serrated adenoma phenotype. Inactivation of TP53 is also necessary for development to a metastatic cancer phenotype. Adapted from Martin et al 2018.

CRC has several different risk factors and genetic causes. CRC known risk factors include age, diet, smoking, low physical activity and obesity all of which can cause somatic mutations that promote the development of cancer (Slattery, 2000). Additionally, disease that cause intestinal inflammation such as ulcerative colitis can increase risk of developing CRC (Lakatos, 2008).

The genetic causes of CRC have been studied in great detail in comparison to more environmental risk factors. CRC can be broadly classified as having either a sporadic, familial, or hereditary components. Of these cases, 15-30% are familial and hereditary CRCs which arose from specific genetic predispositions towards CRC. Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) have been studied in some detail (Taylor, 2010). HNPCC is characterized by mutations in DNA mismatch repair genes, which are needed for DNA repair, such as MutS protein homolog 2 (MSH2) and MutL homolog 1 (MLH1), in about 30% of cases (Jaspersen, 2010). FAP instead exhibits germline mutations in the adenomatous polyposis coli (APC) gene, causing increased polyp formation in the colon and rectum (Jaspersen, 2010).

Sporadic CRCs represent the majority of CRC cases (70-80% of cases) and are the result of sequential accumulation of multiple gene mutations (Fearon, 2011 and Mundade, 2014). Despite the fact there are no specific genetic risk factors associated with sporadic CRC, there are distinct major pathways of genomic instability involved in sporadic CRC development namely the chromosomal instability (CIN), microsatellite instability (MSI), and Serrated pathways (**Figure 1**).

Many sporadic CRCs are characterized by stepwise mutations that develop in the CIN pathway through activation and inactivation of oncogenes and tumor suppressor genes respectively as shown in **Figure 1A** (Mundade, 2014). Inactivation of APC can promote early adenoma development (Powell, 1992) while transition from early to late adenomas usually requires activation of proto-oncogenes V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog (KRAS)/murine sarcoma viral oncogene homolog B (BRAF) (Schubbert, 2007 and Tan, 2012). Besides these mutations there is a frequent loss of heterozygosity (LOH) for the long arm of chromosome 18 (18q) in sporadic CRC which relates to tumor suppressor genes Mothers against decapentaplegic homolog 2, (SMAD2) and Mothers against decapentaplegic homolog 4 (SMAD4). Eventual development of carcinoma can be promoted by inactivation mutations of tumor suppressor p53 (TP53) gene (Vogelstein, 1993).

The MSI pathway of CRC development is characterized by genetic instability resulting from an impaired DNA mismatch repair (MMR) system (Geiersbach, 2011). Impaired MMR machinery can cause accumulation of so-called insertion or deletion mutations (indels) in microsatellite regions, resulting in nonspecific frameshift mutations and genetic instability that contributes to CRC development (Armaghany, 2012 and Sinicrope, 2009).

As shown in **Figure 1**, loss of function of APC is also important for MSI-related CRC development. Since there is variability in the degree of MSI in cancer, tumors can be clinically classified as MSI-high (MSI-H), MSI-low (MSI-L), or microsatellite stable (MSS) (Fang, 1999, Boland, 2010, Gatalica, 2016). Mutations in the transition between late adenoma to metastatic CRC tend to be in genes related to proliferation and apoptotic

regulation that are critically regulated by MMR, such as transforming growth factor  $\beta$  receptor II (TGF $\beta$ RII) and Bcl-2 associated X gene (BAX) which lead to rapid development of CRC (Saridaki, 2014).

The third pathway of development of CRC is the lesser studied and more recently defined Serrated pathway, named for the morphological Serrated (saw-tooth) appearance of precursor lesions (Leggett, 2010 and Muto, 2014). Infoldings of the crypt epithelium cause this phenotype. The serrated pathway accounts for about 10%-20% of CRCs and exhibits its own distinct genetic and epigenetic profiles (Kedrin, 2015). As illustrated in **Figure 1C**, there are critical differences between this pathway and the CIN and MSI pathways. Mass hypermethylation of non-APC genes can promote development of Serrated-related CRC as well as activating mutations in KRAS/BRAF proteins. Additionally, there can be activation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) which acts as a proto-oncogene. An important difference in this pathway is that the Serrated pathway does not require inactivation of the APC gene (**Figure 1**) (Mundade, 2014). As shown in **Figure 1C**, the serrated phenotype requires mass epigenetic hypermethylation of CpG (5'—Cytosine—phosphate—Guanine—3') islands, which result in inactivation of tumor suppressor or other tumor-related genes (Lao, 2011 and Hughes, 2012). Similarly, to MSI and CIN pathways, the serrated pathway also exhibits inactivation of TP53, (Michaloglou, 2005).

As I described above, CRC progression is frequently caused by specific gene mutations. Some of these mutations are known to have critical roles in CRC development and have been studied in detail. Two of these critical mutations act as tumor suppressors in their normal functions. Specifically, mutations in the APC/Wnt/ $\beta$ -catenin

pathway plays a major role in CRC carcinogenesis. As shown in **Figure 1**, loss of function of APC tumor suppressor activity is a key step for the formation of adenoma for sporadic colorectal cancer (Fearon, 2011). Many APC mutations result in the frameshift or nonsense mutations leading to a truncated and inactive protein occurring in around 30%–70% of sporadic adenomas and sporadic CRCs (Schell, 2016). Wnt signaling functions is a critical signaling pathway in colonic crypts which are areas of high intestinal cell stemness. Wnt acts to regulate proliferation, differentiation, and tissue homeostasis in these crypts (Song, 2009). Normally, APC acts to block transition from G1 to S phase of the cell cycle, thereby limiting excessive proliferation of colonic epithelial cells by inducing degradation of  $\beta$ -catenin (Song, 2009). If APC is mutated,  $\beta$ -catenin accumulates and results in prolonged activation of the Wnt pathway causing dysregulation of intestinal cells proliferation (Song, 2009). Mutated or abnormal APC function is therefore common in CRC. The TP53 gene is mutated in approximately 40%-50% of sporadic CRC cases (Li, 2015 and Takayama, 2006). TP53 acts as a critical transcription factor that induces cell cycle arrest and apoptosis under stressful cellular conditions (Takayama, 2006). Therefore, it is not surprising that inactivating TP53 mutations promote uncontrolled cell growth, usually causing the development of late adenocarcinoma (**Figure 1**) (Li, 2015). TP53 mutations have been shown to be associated with lymphatic invasion in proximal CRC. These specific mutations in tumor suppressors suggest the importance of tumor suppressors and tumor suppressing factors in CRC development and progression which will be discussed in upcoming sections.

Cell lines exhibiting these specific stepwise mutations are frequently used to answer questions about cancer development and progression. I will therefore delineate

the characteristics of three CRC cell lines below which are all in later stages of CRC but exhibit different mutational landscapes. DLD-1, a colorectal adenocarcinoma line, is MSI-H, has a G13D activating mutation in KRAS, an E545K/D549N activating mutation in PIK3CA and an activating S241F mutation in TP53 (Ahmed, 2013). DLD1 also has a truncated mutated APC. HCT116, a colorectal carcinoma line, is MSI+, has an activating G13D mutation in KRAS and an activating H1047R mutation in PIK3CA but wild type TP53. HCT116 has wild type APC (Chandra, 2012). HT29, a colorectal adenocarcinoma line, is MSS has a V600E activating mutation in BRAF, a P449T activating mutation in PI3KCA and an activating R273H mutation in TP53 (Ahmed, 2013). HT29 has a different truncation of APC causing its mutant function (Chandra, 2012). As seen in **Figure 1** these mutations in each respective cell line can be mapped to the stepwise progression of sporadic CRC. PI3KCA while not listed on the figure is involved in the transition to carcinoma. Initial mutations can be attributed to MSI or mutated APC *etc.* While these cell lines have similar staging of CRC their genetic makeup it is very different in the specific mutations that lead to progression of the cells to a later stage of CRC. These cell lines can therefore be compared in different components of a cancer cell such as its proliferative ability, migratory ability and tumor formation ability. These cell lines are also useful as a panel of cell lines to study the progression and development of CRC. Also, these cell lines are useful for identifying if a specific novel phenomenon discovered in a singular cell line is a cell-line specific effect or is seen in multiple CRC cell lines. Together these factors make these three cell lines a good panel for the experiments described later in this work.

### 1.1.2 Treatment options and limitations of treating CRC

CRC costs are a burden on public health, typically involve invasive screening procedures, and are projected to cost upwards of \$17 billion by 2020 (Mariotto, 2011 and Ray, 2013). This means CRC is very expensive for clinical treatment and provides a major burden on public health costs (Mariotto, 2011 and Ray, 2013). Of even further concern is while the costs of treatments for CRC are increasing, they are not necessarily effective especially for mCRC. This promotes prolonged treatment for several years further reducing the quality of life of patients, resulting in detrimental effects on their lives. Therefore, more effective therapeutic treatments are needed to address these significant quality of life concerns (Polat, 2014).

Therapeutic strategies for treatment of CRC depend upon the particular stage of cancer treated. As shown on **Table 1**, staging of CRC is determined by invasiveness and severity. For example, Stage I CRC can be defined as a CRC that has grown into the cell wall but has not spread outside the colon itself. Typically, single cancerous polyps can be removed during routine colonoscopies and is the most common therapeutic treatment. Stage I CRC can commonly be treated by a partial colectomy to remove their section of cancerous colon (Kornmann, 2008). In contrast, Stage II CRC has grown through the colon cell wall and possibly into other tissues, but has not spread to the lymph nodes. Surgery is also common as a treatment for Stage II CRC as well as chemotherapy (Kornmann, 2008). Common chemotherapeutics to treat Stage II CRC include single use agents of fluorouracil, leucovorin, oxaliplatin, and capecitabine. These therapies target DNA damage pathways and are not particularly specific to targeting the specific mutational landscape of CRC. Stage III CRC has spread to the lymph nodes

**Table 1** Stages of CRC with 5-year survival rates following current therapeutic strategies

Stage of CRC	Description of spread of colorectal cancer by stage	Common therapeutic strategy	5 Year survival rate
Stage I	cancer has grown through the muscularis mucosa into the submucosa	Surgery, colonoscopy	92%
Stage IIA	The cancer has grown into the outermost layers of the colon or rectum but has not gone through them	Fluorouracil, leucovorin, oxaliplatin, capecitabine, surgery	87%
Stage IIB	The cancer has grown through the wall of the colon or rectum but has not grown into other nearby tissues or organs	Fluorouracil, leucovorin, oxaliplatin, capecitabine, surgery	63%
Stage IIIA	cancer has grown through the muscularis mucosa into the submucosa and cancer has spread to nearby lymph nodes	Surgery, FOLFOX, CapeOX	89%
Stage IIIB	The cancer has grown into the outermost layers of the colon or rectum but has not gone through them and has spread to lymph nodes	Surgery, FOLFOX, CapeOX	69%
Stage IIIC	The cancer has grown through the wall of the colon or rectum but has not reached nearby organs and has spread to lymph nodes	Surgery, FOLFOX, CapeOX	53%
Stage IV	The cancer might or might not have grown through the wall of the colon or rectum. It might or might not have spread to lymph nodes. It has spread to more than 1 distant organ or distant set of lymph nodes	Bastuzimab, cetuximab, CapeOX, FOLFOX, radiation therapy	11%

but has not spread to distant organs in the body. While surgery is less effective to treat this stage, it is still used to remove affected lymph nodes alongside treatment with chemotherapies. Combinations of FOLFOX [leucovorin (also called folinic acid), fluorouracil, and oxaliplatin] or CapeOx (capecitabine and oxaliplatin) are used for adjunct chemotherapy (Mundade, 2014). Stage IV CRC has spread to distant organs and tissues. Chemotherapy is the main therapeutic option for Stage IV CRC patients as the effectiveness of surgery at this stage tends to be negligible. Stage IV therapies usually consists of drug cocktails such as FOLFOX or CapeOX, as well as treatment with cetuximab or irinotecan (Mundade, 2014). In addition to chemotherapy, radiation therapy is occasionally used to prevent symptoms associated with CRC. However, radiation therapy has not been shown to have great efficacy in treating patients with late stage CRC (Abbot, 2015).

To date, chemotherapy remains the second most commonly used therapeutic strategy following surgery of CRC. Despite known chemotherapeutic efficacy there are several concerns for using these treatments. For instance, a major concern of current therapeutics for CRC is their non-specific nature. Commonly, the DNA damage pathways are targeted, usually through increasing DNA damage in systems in the cancer where there is constant DNA damage repair (Sussman, 2007). Furthermore, chemotherapeutic resistance is an increasing real problem for CRC chemotherapy. Chemotherapy can arise from several areas including reduced drug uptake, increased drug efflux or metabolism and altered DNA repair, all of which contribute to chemoresistance in CRC (Sussman, 2007). These are combinations of innate resistance to therapies, in the case of active metabolism or heterogeneity of tumors, and developed

resistance, such as activation of alternative proto-oncogenes to drive tumor development or through changes in the tumor microenvironment. Together, developed and innate chemoresistance mechanisms can result in reduced efficacy of treatments. To further complicate matters, chemotherapeutic treatments tend to have detrimental side effects to normal tissues and organs. For instance, the combination therapies very commonly used for treatment of CRC, FOLFOX and FOLFIRI (leucovorin, fluorouracil, and irinotecan), can induce major peripheral neuropathy, nausea, weight loss, and severe diarrhea, which lead to eventual reduced dosing of chemotherapies (McQuade, 2014). This then reduces efficacy of treatment leading to a decreased quality of life for patients and ineffective treatments (Stringer, 2007).

There is a great need for effective therapeutics for CRC patients with disease progression (**Table 1**), especially in later stages of CRC. Fortunately, there are now emerging targeted therapies that can be used in later stages of CRC to specifically target CRC cells over noncancerous cells. In order to treat metastatic CRC monoclonal antibodies specific for the epidermal growth factor receptor (EGFR) cetuximab, and, specifically targeting the vascular endothelial growth factor A (VEGFA) bevacizumab, are used. Both EGFR and VEGFA are highly expressed in CRC cells but not in normal cells (Finger, 2007 and Misale, 2012). VEGF is heavily involved in angiogenesis and its disruption can prevent CRC development (Misale, 2012). EGFR on the other hand is critical for cell proliferation and migration (Finger, 2007). There are several problems with these proteins as therapeutic targets. Namely there can be off-target effects in patients. Cetuximab has been shown to produce eczema, acneiform eruption, paronychia, and xerosis cutis, which can cause serious discomfort and may be dangerous for patients

(Hu, 2007). Furthermore, there are increasing reports of cetuximab resistance. KRAS-mutation status is a predictive biomarker to anti-EGFR monoclonal antibodies in CRC patients and necessitate bevacizumab treatment can only be given to a subset of all CRC patients (Di Fiore, 2007 and Lièvre, 2008). Many wild type-RAS CRC tumors do not respond to the VEGFA and EGFR antibodies. In short, these adverse reactions to targeted therapies in CRC patients indicate more targeted and novel therapies of CRC are necessary to improve patient outcome.

Fortunately, advances in genomic sequencing may help us better understand patients' responses to current and future therapeutics. Several studies have sequenced the genomes of CRC patients which helped provide better indication of therapeutic efficacy prior to treatments (Stoehlmacher, 2004, Bass, 2011 and Leary, 2010). Other novel therapeutics are also being developed to treat CRC or other therapeutics are being repurposed for use in CRC. Atezolizumab, a PD-L1 antibody used to treat bladder cancer and nivolumab, an IgG4 PD-1 antibody used to treat melanoma, are novel antibody therapies for CRC (Bendell, 2016). PD-1 acts as a checkpoint on the immune response and when inhibited can promote immune function. This may be relevant to MSI high tumors which have shown a greater response to PD-1 inhibitors (Bendell, 2016).

Unfortunately, PD-1 inhibitors have shown less efficacy in MSS tumors and PD-L1 expression is not consistent in CRC patients (Yaghoubi, 2019). Cobimetinib, a MEK inhibitor was previously used to treat melanoma and is now used for CRC (Hammers, 2014). Inhibition of MEK also effects the immune cell response by inhibiting T cell activity. Treatments with cobimetinib in conjunction with atezolizumab have shown variable effectiveness depending on the MSI status of tumors (Bendell, 2018). Besides

repurposed therapeutics, novel therapeutic targets for CRC are also being pursued. One target is MGN1703, a toll-like receptor 9 (TLR9) agonist (Schmoll, 2014). Encorafenib is a novel therapy which acts as a BRAF inhibitor and Binimetinib, which is a novel MEK inhibitor (Sullivan, 2015 and Huijberts, 2017). These therapeutics have been used in clinical trials but so far have not been studied in detail. More studies remain to show the efficacy of these therapeutics. While these new therapeutic directions are promising for CRC treatment more must be understood about CRC cell signaling in order to design other novel therapeutic inhibitors of CRC functions and discover novel therapeutic targets.

## **1.2 NF- $\kappa$ B signaling and its role in CRC**

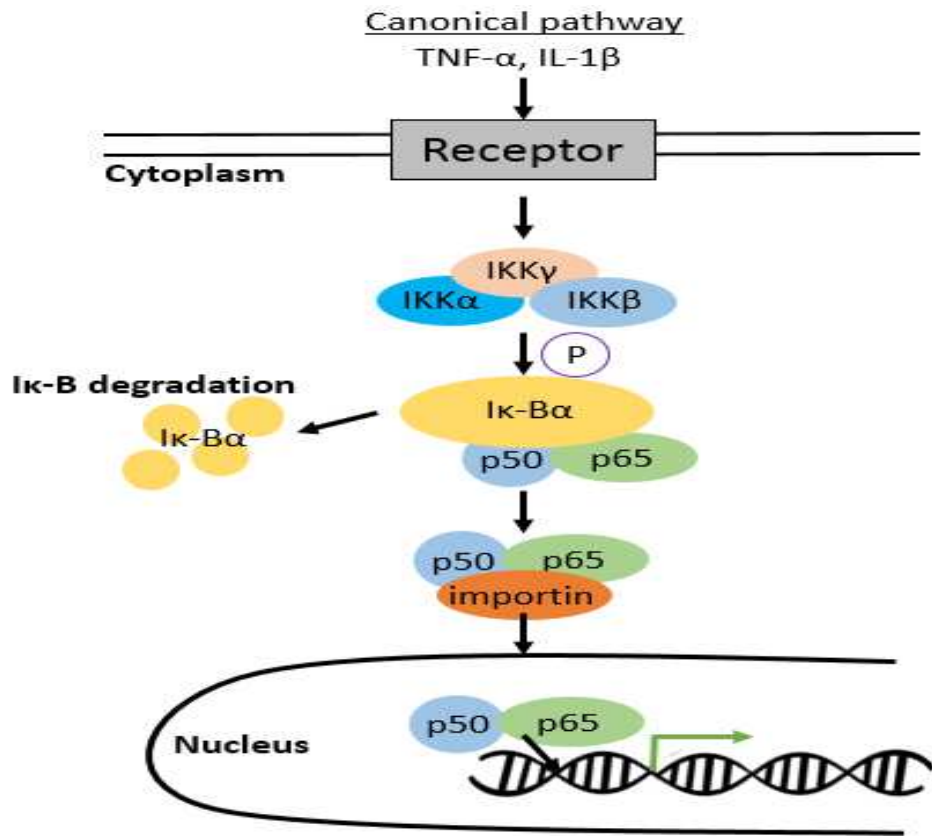
In mammals, the NF- $\kappa$ B family consists of five members: RelA (p65), RelB, Rel (cRel), NF- $\kappa$ B1 (p50 and its precursor p105) and NF- $\kappa$ B2 (p52 and its precursor p100) (Ghosh et al., 1998). All the members of this family contain a Rel homology domain in the N-terminus, which is required for dimerization and binding to cognate DNA sequences. This family of proteins was initially identified several years ago as a regulator of  $\kappa$ B light chain expression in mature B and plasma cells (Sen, 1986). From that point, NF- $\kappa$ B has been studied in great detail and it has been demonstrated NF- $\kappa$ B is expressed in many cell types and tissues, and NF- $\kappa$ B has specific binding sites for a large number of genes. A plethora of work since NF- $\kappa$ B's discovery have also identified different stimuli can cause NF- $\kappa$ B activation resulting in cell specific responses. These specific responses have been identified as different to normal responses in cancerous states. The NF- $\kappa$ B response to stimuli is achieved through the NF- $\kappa$ B signaling pathway described below.

### 1.2.1 NF- $\kappa$ B signaling pathway

NF- $\kappa$ B signaling can be classified into either the canonical or the non-canonical pathway. The canonical pathway plays a role in regulating the transcription of genes involved in inflammation, innate immunity and cell survival. The non-canonical pathway regulates in the transcription of genes regulating B-cell maturation, humoral immunity and lymphoid organ development. The canonical pathway has been documented to primarily play a role in CRC, in which an inhibitor complex named I $\kappa$ B $\alpha$  (inhibitor of  $\kappa$ B alpha) sequesters the p65-p50 heterodimer in an inactive state in the cytoplasm (**Figure 2**). Extracellular signals like stress, cytokine binding, *etc.*, activate the I $\kappa$ B kinase which phosphorylates I $\kappa$ B $\alpha$ , leading to its release and proteasomal degradation, thus freeing the p65-p50 complex, and enabling it to migrate to the nucleus and bind its  $\kappa$ B cognate DNA elements resulting in the activation of specific target genes. Abnormal upregulation of NF- $\kappa$ B target genes contribute to the pathology of CRC and can promote the six hallmarks of cancer; inflammation, cell survival, angiogenesis, cell death and anti-proliferative effects, tumor promotion and metastasis and proliferation. Therefore, constitutive activity of the canonical NF- $\kappa$ B pathway can drive cancer progression.

### 1.2.2 NF- $\kappa$ B signaling in CRC

Instances of a hyperactive NF- $\kappa$ B pathway have been well documented in CRC (Prabhu, 2017). In CRC, NF- $\kappa$ B is constitutively activated in the majority of cancers which promotes cell growth, metastasis and chemotherapeutic resistance (Hassanzadeh, 2011). Hyperactivated NF- $\kappa$ B has been linked to deregulation of the inflammatory immune response through overexpression of NF- $\kappa$ B activating cytokines Il-1 $\beta$  and TNF- $\alpha$ .



**Figure 2.** Canonical pathway of NF- $\kappa$ B activation: NF- $\kappa$ B activity is regulated by interactions between I $\kappa$ Bs (inhibitor of  $\kappa$ B) proteins and NF- $\kappa$ B subunits p50 and p65. I $\kappa$ B proteins hold NF- $\kappa$ B proteins in inactive conformations by binding in the cytoplasm and preventing nuclear localization. Extracellular signals, including; cytokines, stress, free radicals, or radiation cause I $\kappa$ B kinase (IKK) to phosphorylate I $\kappa$ B leading to ubiquitination and proteasomal degradation of I $\kappa$ B. After p65 and p50 are released from I $\kappa$ B, the two-unit NF- $\kappa$ B complex binds to the protein importin and translocates to the nucleus where it binds to DNA and signals for increased expression of NF- $\kappa$ B target genes.

causing dysregulation of NF- $\kappa$ B activity in the innate inflammatory immune response (Ben-Neriah, 2011, Neurath, 1996). Deregulation of the inflammatory immune response can cause Inflammatory Bowel Disease (IBD) and possibly CRC (Itzkowitz, 2006). Furthermore, in the colon, colonic tumor polyps have been shown to have increased NF- $\kappa$ B activity compared to normal tissue (Hardwick, 2001). Hyperactivation of NF- $\kappa$ B has been identified in several mouse models of inflammation. Knock down of the subunit of NF- $\kappa$ B, p65, in IL-10 deficient mice with hyperactivated NF- $\kappa$ B was shown to reduce IBD symptoms and CRC risk (Kühn, 1993). NF- $\kappa$ B hyperactivation has been observed both in mouse models with colitis-associated colon cancer (CAC) induced by a combination of Azoxymethane (AOM)/ and Dextran Sodium Sulfate (DSS), and mouse models of conditional knockouts of IKK $\beta$  leading to hyperactivation of NF- $\kappa$ B, increased inflammation and the development of CRC (Okayasu, 1996). Besides its role in the inflammatory response to cancer, the NF- $\kappa$ B pathway plays roles in angiogenesis and tumor growth as well as apoptosis and cellular proliferation (Neurath, 1996). These reports of NF- $\kappa$ B's role in inflammation and CRC progression suggest the critical importance of NF- $\kappa$ B in CRC and suggest the pathway of NF- $\kappa$ B as an attractive therapeutic target for CRC treatment.

### 1.2.3 Tumor microenvironment in CRC and NF- $\kappa$ B

Since NF- $\kappa$ B signaling has critical roles in inflammation and the immune response it appears to be a good area to target therapeutically in CRC. However, one must also consider the interplay between NF- $\kappa$ B, the tumor microenvironment and the immune response in CRC.

A common concept of the microenvironment, or stroma, is known as the “seed and soil” concept where the cancer cell “seeds” can grow in the microenvironment “soil.” Furthermore, metastasis can be equating to growing new “seeds” in areas of new “soil” (Mathot, 2012). However, the microenvironment can be very complex and includes secreted factors, extracellular matrix (ECM) and several types of cells.

The ECM is needed for formation of a tumor, through attachments of cancer cells to the ECM which allows communication with other cells including such as cancer-associated fibroblasts (CAFs), and myeloid-derived suppressor cells. Following attachment cancer cells can form into a tumor and metastasize (Nguyen, 2009). Cells respond and adapt to the local microenvironment ECM to progress to a malignant state. In order to become malignant, there is decreased regulation of proliferation of tumor cells and promotion of factors in the microenvironment that promote cell survival, angiogenesis, and spread of the tumor. These include  $TNF\alpha$  which when overexpressed can promote tumor formation in mice (Tracey, 1990). Destabilization and fragmentation of the ECM can also promote tumor formation and development. Matrix metalloproteinases (MMPs) have been discovered to play a role in degradation of basement membranes and ECM, which promotes tumor cell invasion and metastasis (Chambers, 1997).

Epithelial-mesenchymal transition (EMT) can also promote metastasis and invasion of cancer cells. EMT can regulate invasiveness and production of ECM (Kalluri, 2003). Furthermore, induction of EMT can be caused by upregulation of EGF and  $TGF\beta$  which promote transcription of EMT-inducing transcription factors (Kalluri, 2003).  $TGF\beta$  in particular can repress e-cadherin and other cell adhesion molecules, leading to

activation of SMAD and MAPK signaling pathways, both of which promote EMT induction (Oft, 1998).

Metastasis is made up of several steps. Intravasation, invasion of cancer through the basal membrane into a blood or lymphatic vessel, extravasation, movement through blood circulation, formation of metastases, and colonization (Yang, 2008). Other cells in the microenvironment can promote an environment for tumor growth (Yang, 2008). For example, macrophages in a tumor microenvironment can produce EGF which promotes tumor growth.

Constant cell proliferation in the stroma of tumors has been associated with secreted factors which promote tumor growth (Personnet, 1991). Furthermore, these secreted factors can promote inflammatory responses. Inflammation is induced in tumors angiogenesis and lymphangiogenesis. These processes promote organization of blood vessels and lymphatics with mesenchymal, hematopoietic, lymphoid cells, and the ECM. Additionally, this leads to production of cytokines and chemokines that are chemoattractants for granulocytes, mast cells, monocytes/macrophages, fibroblasts and endothelial cells, which promotes tumor formation (Yang, 2008). MMPs can also be secreted through activated fibroblasts and infiltrating inflammatory cells promoting a tumor microenvironment state. Together these factors can remodel the ECM and promote activation of the tumor microenvironment through tumor growth, angiogenesis and metastasis. Besides factors upregulated by cancer, non-cancerous cells in the tumor environment can signal and be signaled by released chemokines and cytokines from cancerous and non-cancerous cells (Oft, 1998). These cytokines include NF- $\kappa$ B target

genes such as TNF $\alpha$  and TGF $\beta$ . This allows for promotion of areas of inflammation in the tumor microenvironment (Oft, 1998).

Chronic overexpression of TNF $\alpha$  and TGF $\beta$  can also promote metastasis and tumor growth in several types of cancer (Moustakas, 2002). Hypoxic conditions can promote hypoxia-sensitive gene expression in tumor cells, resulting in the recruitment of macrophages and granulocytes to the tumor microenvironment (Aller, 2004). This then promotes production of reactive oxygen species which activate the NF- $\kappa$ B pathway in tumor cells causing secretion of TNF $\alpha$  to promote cell proliferation (Aller, 2004). Our lab's work has shown NF- $\kappa$ B can also be phosphorylated at multiple sites (S316, S529 and S536) in response to IL-1 $\beta$  treatment. These posttranslational modifications allow regulation of secretion of distinct NF- $\kappa$ B-dependent cytokines and growth factors which promote a NF- $\kappa$ B-inducible autocrine loop in the tumor microenvironment of CRC cells (Prabhu, 2015).

The tumor microenvironment can affect the host immune response and the host immune response can in turn effect the tumor microenvironment. NF- $\kappa$ B signaling can be key in these interactions. Inflammatory cells act through immune surveillance to infiltrate tumors slow tumor progression (Aller, 2004). Immune cell infiltrates are made of cells from both the adaptive and innate immune responses. Adaptive immune response cells include tumor-infiltrating lymphocytes (TILs), dendritic cells, and B cells, while innate immune response cells include macrophages, polymorphonuclear leukocytes and natural killer cells (Whiteside, 2008). TILs make up much of the immune cell infiltrate. Antigens are released in the tumor microenvironment by tumor cells and cells in the microenvironment through activation of NF- $\kappa$ B which result in a net immune suppressive

effect from TILs (Uzzo, 1999). This then allows the tumor to escape destruction from the immune system.

These data also suggest possible complications in targeting CRC with attractive novel therapies such as PD-L1 inhibitors as NF- $\kappa$ B has such a critical role in regulation of inflammatory responses and in the tumor microenvironment. Since PD-1 inhibitors inhibit the immune response this may have a negative impact on NF- $\kappa$ B function. Further exploration of this interplay will be necessary when considering immune-based therapeutic strategies for CRC. Together all of these data suggest an extremely interconnected relationship between NF- $\kappa$ B signaling, the immune response and inflammation. Furthermore, there is a complicated relationship between NF- $\kappa$ B signaling and cancer in the tumor microenvironment. These factors should be considered when pursuing the NF- $\kappa$ B pathway using targeted therapies and potential off target effects of NF- $\kappa$ B inhibitors.

#### 1.2.4 Status of NF- $\kappa$ B inhibitors and their limitations

The NF- $\kappa$ B pathway is critical in CRC and contributes to disease initiation and progression. Unfortunately, directly targeting NF- $\kappa$ B signaling is not practical due to crosstalk between NF- $\kappa$ B signaling and other signaling pathways including the Wnt pathway (Karimaian, 2017).

Many inhibitors of the NF- $\kappa$ B pathway have been developed, including small molecules, peptides, small DNA/RNA, viral proteins, and natural compounds (Prabhu, 2017). Despite these advancements there are currently no direct NF- $\kappa$ B inhibitors used in treatment of humans and few indirect inhibitors. Furthermore, inhibitors of NF- $\kappa$ B have

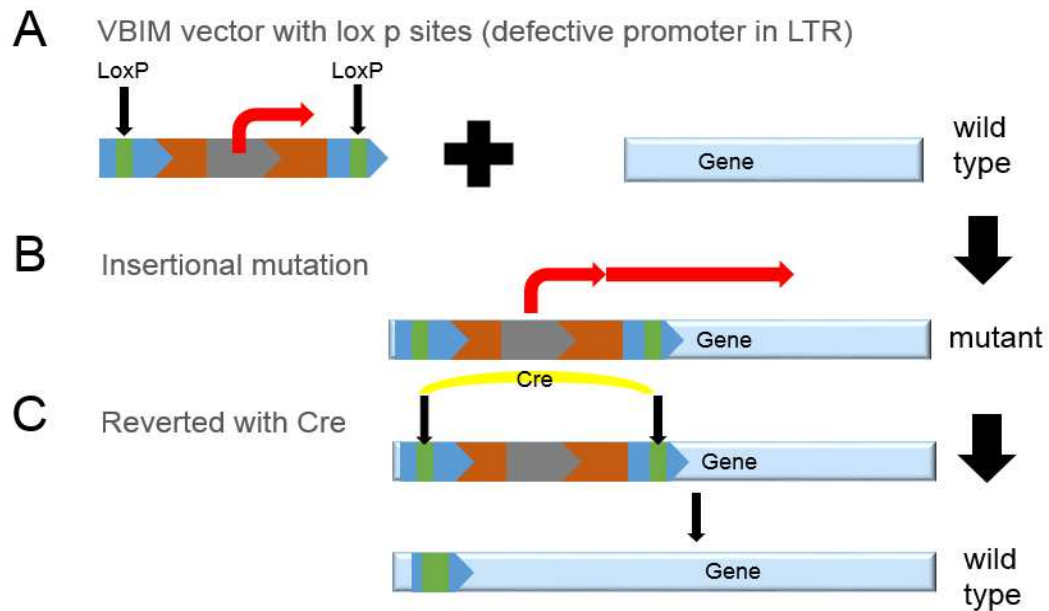
been shown to have suboptimal bioavailability and are decreased in concentration by natural metabolic processes. Additionally, these are no highly selective inhibitors of NF- $\kappa$ B, and there are concerns that selective inhibitors of NF- $\kappa$ B could also result in off-target effects (Karimain, 2017). Therefore, targeting to important regulatory proteins in NF- $\kappa$ B signaling specifically active in cancerous conditions could offer an interesting avenue of therapeutic treatment. This would offer an avenue of treatment that could affect NF- $\kappa$ B activity in cancer while sparing noncancerous cells. There are few clinical trials currently ongoing in regard to NF- $\kappa$ B targeting in CRC. One trial recently closed last year looked at p53 activation and NF- $\kappa$ B activation in response to radiation therapy (Biomarkers in patients..., 2018). This trial was using an indirect way to target NF- $\kappa$ B through radiation but may not have been specifically targeted to CRC. Another current trial is using Aspirin (ASA) and Metformin (MET) to observe the effects of these inhibitors of mammalian target of rapamycin (mTOR) and NF- $\kappa$ B activation (A Randomized 2x2, 2019). This trial is aimed to identify indirect activation of NF- $\kappa$ B through crosstalk with mTOR signaling. This trial also exhibits that few clinical trials are ongoing regarding specific inhibition of NF- $\kappa$ B activity and are only looking at an indirect effect on NF- $\kappa$ B activity. There is therefore a great need for identification and characterization of novel therapeutic targets of NF- $\kappa$ B signaling in CRC. In this thesis, a novel area of NF- $\kappa$ B regulation has been discovered. In this body of work, I demonstrate that armadillo repeat containing protein 4 (ARMC4), functions as a novel negative regulator of NF- $\kappa$ B and offers a novel node of regulation of NF- $\kappa$ B signaling and potentially CRC signaling.

### 1.3 ARMC4 a novel negative regulator of NF- $\kappa$ B

Using validation based insertional mutagenesis (VBIM) our lab identified ARMC4 as a novel negative regulator of NF- $\kappa$ B. This process will be described in detail in the next section. ARMC4 is also identified as primary ciliary dyskinesia 23 (CILD23), and is in the same family of proteins, known as ARM containing proteins, as APC, a well characterized tumor suppressor, and  $\beta$ -catenin. Little is known about the regulation and function of ARMC4. The ARMC4 gene is located on human chromosome 8. It encodes 1,044 amino acids. The ARMC4 gene has 10 tandem armadillo repeat motifs (ARMs) and one HEAT repeat. The ARM repeats are known to be important for transduction of Wnt signaling in embryonic development and are a common motif among proteins in the same family including  $\beta$ -catenin and APC. The tandem ARMs are proposed to form a superhelix that mediates protein-protein interaction with its ligands. The VBIM system is described in **Figures 3 and 4**. VBIM virus contains a GFP tag. To use the VBIM virus for **Figure 4** VBIM virus titer was determined by treating HEK293 cells with virus and determining GFP expression then adjusting the viral titer for infection of 50% of cells by dilution of the virus with media. Control of the viral infection for 50% of the cells had been determined previously as controlling for a single viral insertion per gene and for allowing for the cells to remain healthy for therapeutic selection (Lu, 2009). The exact number of viral particles were not determined.

#### 1.3.1 Discovery of ARMC4 in cancer

Z3 cells, HEK293 cells with constitutive NF- $\kappa$ B activity, described in **Figure 4**, were used to generate a selected mutant clone with low NF- $\kappa$ B activity. This selection

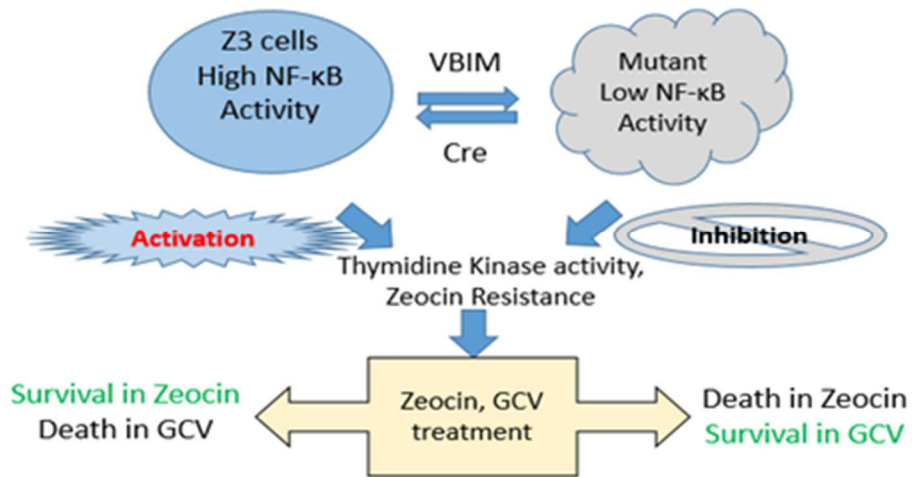


**Figure 3.** Schematic of VBIM Libraries of cells were generated by integration of VBIM lentiviral vectors that integrated a CMV promoter randomly into the genomes of mammalian cells. VBIM vectors contained LoxP sites on both ends of the vector, allowing excision through Cre-recombinase. The promoter stimulated high expression of downstream genes which generated dominant mutants. The mutant phenotype was attributed to the overexpressed protein. Results were validated with Cre recombinase that reverted cells from a mutant to a wild type phenotype. This data provided conclusive genetic evidence that the insertion of the CMV promoter caused the mutant phenotype.

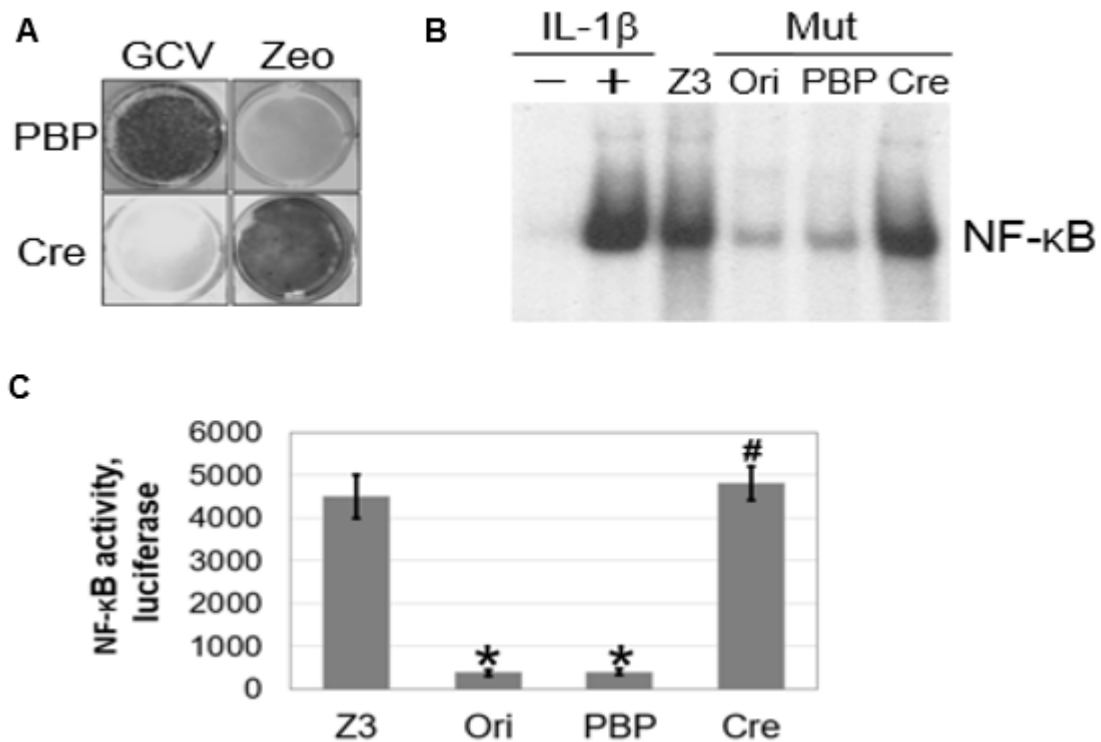
process is shown in **Figure 5A**. Activation of NF- $\kappa$ B was confirmed by the electrophoretic mobility shift assay (EMSA) wherein the amount of p65 binding to DNA correlated with the intensity of the observed band. Increased binding to the DNA correlates with increased expression of NF- $\kappa$ B target genes. Interleukin 1  $\beta$  (IL-1 $\beta$ ) is a cytokine and has an obligatory role in the activation of NF- $\kappa$ B. Therefore IL-1 $\beta$  was used to induce NF- $\kappa$ B activity.

As shown in **Figure 5B**, 293 cells without IL-1 $\beta$  stimulation showed low NF- $\kappa$ B binding. After cytokine stimulation, NF- $\kappa$ B was significantly activated in 293 cells and p65 bound more strongly to DNA. The mutant clone (Ori) derived from Z3 cells showed low NF- $\kappa$ B activity and weak DNA binding. A cre-Lox recombination system was used to determine if the mutant clone was real and reversible and had low NF- $\kappa$ B activity. Cre recombinase targeted the loxP sites flanking the VBIM vector and excised the vector to revert the mutant to a WT phenotype. After Cre infection, the CMV promoter was removed and the DNA binding ability of NF- $\kappa$ B was restored suggesting that Ori contains the VBIM vector and removing the mutation restored constitutive NF- $\kappa$ B activity. There was no change in DNA binding ability of mutant O clones after infection with pBabe vector control (PBP) confirming there was no change due to transfection.

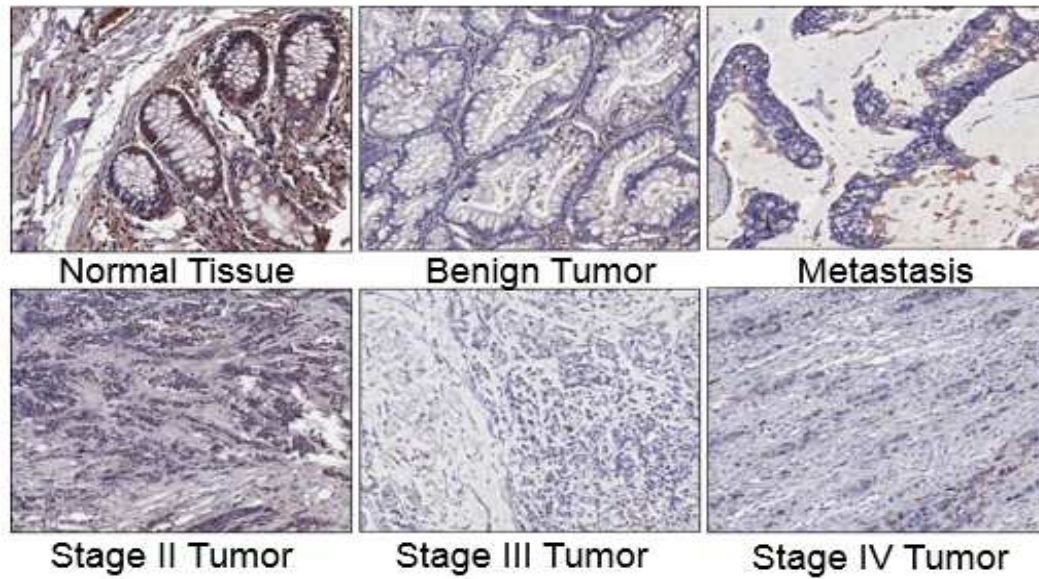
**Figure 5C**, shows a luciferase-based reporter gene assay showing mutant Ori cells exhibited decreased NF- $\kappa$ B activity compared to the Z3 cells which had a high basal level of NF- $\kappa$ B activity as expected. PBP treated cells exhibited similar NF- $\kappa$ B activity to Ori cells which was reverted to a low NF- $\kappa$ B activity phenotype by Cre-recombinase. To determine the gene of interest in the mutant Ori cells, we amplified the DNA containing



**Figure 4.** Schematic of Thymidine Kinase/Ganciclovir and Zeocin system mutant selection strategy Z3 cells carrying a Zeocin resistance gene and Thymidine Kinase (TK) gene survive in Zeocin but die in Ganciclovir (GCV) due to TK conversion of GCV into a toxic product. This is mediated by the high NF-κB activity of the Z3 cells. Mutations were caused by random insertion of VBIM vector into the genome which resulted in loss of TK and Zeocin resistant gene and a low NF-κB activity phenotype. Cells containing low NF-κB activity survived in GCV but died in Zeocin. The mutant cells of interest were then expanded from the population of mutant clones.



**Figure 5.** Validation of clone with low NF- $\kappa$ B binding activity is due to VBIM overexpression. A) Drug sensitivity of an original mutant (Ori) clone under pbabe vector control (PBP) or Cre-recombinase treatment. An original mutant (Ori) treated with a vector control treatment (PBP) for Cre-recombinase (Cre) was identified as a clone with a low NF- $\kappa$ B ability that died in Zeocin and survived in GCV. (B) EMSA assay showing Ori cells have decreased DNA binding ability of the p65/p50 heterodimer of NF- $\kappa$ B as compared to the parental Z3 cells in unstimulated conditions. HEK293 cells had increased DNA binding ability upon stimulation with IL-1 $\beta$  and Z3 cells had basal high DNA binding ability. PBP conditions had comparable DNA binding ability to Ori cells but DNA binding ability could be restored with use of Cre-recombinase treatment. (C) NF- $\kappa$ B transactivation luciferase assay showing mutant Ori cells exhibited decreased NF- $\kappa$ B activity compared to the Z3 cells. PBP treatment exhibited similar NF- $\kappa$ B activity to Ori cells which was reverted by use of Cre-recombinase to a low NF- $\kappa$ B activity phenotype. \* $p < 0.01$  Ori and PBP vs. Z3 group; # $p < 0.01$  Cre vs. PBP group.



**Figure 6.** ARMC4 expression decreases with tumor advancement in human colon tissues. Images of ARMC4 staining in normal tissue and progressively worse staging of CRC showing overall loss of ARMC4 expression. Highest loss of expression is seen in later stages. ARMC4 expression is shown by brown nuclei. Images shown are at 20X.

the VBIM insert using VBIM-specific primers with PCR based methods. This band was excised from a DNA gel and cloned into a TA cloning vector. The TA construct with the target band was sequenced and aligned with the human genome database to determine the overexpressed gene. GAPDH was used as the internal control. The gene thus identified was ARMC4. After determining ARMC4 as a gene involved in control of the NF- $\kappa$ B pathway, I theorized the expression of ARMC4 could be different in cancerous tissue compared to non-cancerous tissue. Previously our lab had shown that positive regulators of NF- $\kappa$ B can be increased in expression in later stages of CRC and negative regulators can be decreased in expression in later stages of CRC (Prabhu, 2018, Lu, 2010). I wondered if there was a correlation between the expression of ARMC4 as a negative regulator of NF- $\kappa$ B and the severity of the cancer by staging benign through metastasis. We used a tissue microarray (TMA) shown in **Figure 6**, and immunohistochemistry (IHC) results showed that the ARMC4 protein was more heavily stained in normal colon tissue (shown in a brown stain), which decreased in later stages of CRC. Nuclei not stained for ARMC4 are shown in a purple stain in **Figure 6**. The expression of the ARMC4 protein was observed and scored by IHC on the TMA by Dr. George Sandusky of the IUSM Immunohistochemistry Core which included 40 colon adenocarcinoma and 8 normal colon tissues. I observed the advanced stages of CRC showed ARMC4 negative tumor nuclei. These data suggests that later stages of CRC exhibit a lower ARMC4 expression in the colon.

Together all of these data suggests a potential novel role for ARMC4 as a negative regulator of NF- $\kappa$ B and a potential role of ARMC4 in CRC. I therefore wanted

to explore other known roles of ARMC4 and to determine if it had ever been studied in the context of cancer.

### 1.3.2 Role of ARMC4 in other diseases

Despite there being a plethora of information on ARMC4's superfamily members, to date, little is known about ARMC4's regulation and function. ARMC4 is known to play a critical role in the rare disorder primary ciliary dyskinesia (PCD) (Onoufriadis, 2013 and Hjeij, 2013) and mouse spermatogenesis (Cheng, 2013). In PCD patients, multiple different specific point mutations of ARMC4 cause dysfunction of binding with cilia leading to a PCD phenotype and dysfunction of the lungs and heart (Onoufriadis 2013, Hjeij 2013). In terms of ARMC4's functions in cancer, previous studies have identified ARMC4 as a theoretical therapeutic target to study through genome wide association studies (GWAS) in gastric, ovarian, and breast cancers respectively (Pongor, 2015, Xu, 2019 and Zhang, 2015). While little is known about ARMC4's functions in cancer it is interesting that the known tumor suppressor APC and ARMC4 share some major structural similarities. The human APC gene is located on chromosome 5 and encodes a protein of 2,843 amino acids. APC contains 8 ARM domains and 4 coiled coil motifs. Since both APC and ARMC4 have tandem ARM domains, they can both form an ARM superhelical structure. APC's function as a tumor suppressor is related to its ability to bind proteins in the Wnt/ $\beta$ -catenin pathway (Novellademunt, 2017). While ARMC4's functions in cancer were generally unknown prior to this work, it is purported that ARMC4's functions in other diseases is reliant on ARMC4's ability to interact with other proteins. This suggests to me an interesting potential role of ARMC4 in the context of

regulating NF- $\kappa$ B signaling through potential interactions with proteins in the NF- $\kappa$ B signaling pathways.

#### **1.4 Summary and Hypothesis**

Previous studies have clearly shown the importance of regulation of NF- $\kappa$ B signaling and its importance and relevance in the progression and development of CRC. Current therapeutics for targeting to CRC specifically are not particularly prevalent or have known complications with treatment and therefore identification of novel therapeutic targets in CRC is critical for future therapeutic development. NF- $\kappa$ B signaling is an attractive therapeutic target in CRC as there is constitutive NF- $\kappa$ B activity that can regulate the cancer. In my preliminary studies, I have successfully identified and confirmed the role of ARMC4 as a novel negative regulator of NF- $\kappa$ B. My preliminary data supports ARMC4's role as an endogenous regulator of NF- $\kappa$ B that regulates activity and DNA binding ability. Expression of ARMC4 decreases in later stages of CRC as shown in a TMA. Prior to our lab's pioneering work, the ARMC4 protein had only been tangentially identified as a potential target to study through genome wide association studies (GWAS). The role ARMC4 plays in cancer progression and the mechanism of regulation of the NF- $\kappa$ B pathway by ARMC4 still remain to be discovered. My work here aims to better explain those questions. Thus, the novel hypothesis of this proposal is that ARMC4 functions as a tumor suppressor that blocks CRC progression through regulation of the NF- $\kappa$ B pathway.

## CHAPTER 2. METHODS

### 2.1 *In Vitro* Experiments

#### 2.1.1 Cell lines and materials

The normal colon cell line (FHS74) and CRC (HT29 adenocarcinoma, HCT116 carcinoma, DLD1 adenocarcinoma) cell lines were purchased from the American Type Culture Collection (ATCC). CRC cells were maintained in Roswell Park Memorial Institute Medium (RPMI 1640) (GE Healthcare), containing 1% penicillin/streptomycin and 5% fetal bovine serum (FBS), while FHS74 cells were cultured in RPMI 1640 under 10% FBS, 1% penicillin/streptomycin, plus 30 ng/ml EGF. All cell lines were cultured at 37°C under 5% CO<sub>2</sub> and used between passages 2 to 6. Cells were passaged when 90-95% confluent on cell culture dishes. Cell lines were authenticated using 9 Marker STR Profile by IDEXX BioResearch.

#### 2.1.2 Generation of stable ARMC4 overexpressing and knockdown cell lines

Flag-tagged WT-ARMC4 or shARMC4 cDNA was generated from total mRNA derived from 293 cells. After confirming the insertion by DNA sequencing, the cDNA constructs were cloned into pLVX-IRES-puro vector (Lu, 2010). An empty vector construct of the WT-ARMC4 virus was used as a control for the WT-ARMC4 generation. A pool of 5 shRNAs against ARMC4 (Sigma-Aldrich) were used to ensure efficient knockdown of this target. A separate pool of 5 shscramble shRNAs not specific to ARMC4 (Sigma-Aldrich) were used as a control for the shRNA knockdown. The respective lentiviral plasmids containing empty vector, WT-ARMC4, shscramble or shRNAs against ARMC4 were transected into a 293T packaging cell line that presents a

T antigen allowing high efficiency of virus production in order to produce viral preps that were used to infect HT29, DLD1, and HCT116 cells. 293 cells were infected as well. Following 48h of infection, cells were exposed to 1 µg/mL of puromycin and separately 0.5 µg/mL of puromycin to allow for further selection of virus-infected cells, as the lentiviral vector contains a puromycin resistance gene. Cells were then collected for Western Blotting and otherwise were maintained with 1 µg/mL puromycin in their media when cultured. Western Blotting using an ARMC4 specific Antibody (Abcam) 1:2000 primary, goat anti-rabbit 1:3000 secondary was used to confirm the overexpression and knockdown in these cells in comparison to the shscramble and vector controls. All lines were used between passages 2-6.

### 2.1.3 Western blotting

Cultured cells were pelleted by scraping them in 1ml of 1X phosphate buffered saline (PBS), then centrifuged at 5,500 rpm for 5 min in a 4°C centrifuge. The supernatant was aspirated, and pellets were frozen for a minimum of overnight at -80°C. The next day or later, 500 µl RIPA buffer [10mM Tris-Cl pH 8.0, 1mM EDTA, 1% Triton X, 0.1% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate; electrophoresis grade), 14mM NaCl, 1mM PMSF] was added to each pellet, vortexed several times for 5-10 seconds every 5 minutes and incubated on ice for 20 minutes total to promote lysis. The cells were pelleted at 5,500 rpm for 5 min at 4°C. The supernatant for each sample was then tested for its protein concentration using the Protein Assay Reagent (Biorad) and absorbance values measured using a Genesys 10S Vis spectrophotometer (Thermo Fisher Scientific), by comparison to a standard curve generated with Protein

Assay Reagent to ensure equal loading of the samples on the gel. A set amount of protein depending on the amount needed for equal loading of samples was determined when determining protein concentration with the spectrophotometer. Equal protein concentrations were then mixed with 2X SDS sample loading buffer [100mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200mM  $\beta$ -mercaptoethanol] and 1X SDS sample loading buffer [100mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200mM  $\beta$ -mercaptoethanol] to equalize volumes of loading to the same volume. Each sample mixture was then heated at 100°C for 5 min and gently spun down in a microcentrifuge at maximum speed at room temperature to gather all the sample at the bottom of the tube. These protein samples were then run on a 7% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel at 110V for 2 hours and then transferred overnight 14-16 hours on a polyvinylidene fluoride (PVDF) membrane (Fisher Scientific) in 1x Western Transfer Buffer [25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol/vol] at 4°C. Membranes were blocked from nonspecific binding with 5% non-fat milk powder in tris-buffered saline with Tween20 (TBST). Membranes were washed in between primary and secondary antibody additions 6x5min each. Primary and secondary antibodies were also made with 5% non-fat milk powder in tris-buffered saline with Tween20 (TBST). Primary antibodies for anti-ARMC4 1:2000 (Abcam), anti-Flag 1:3000 (Sigma-Aldrich), anti-p65 1:3000 (Abcam), anti-I $\kappa$ B $\alpha$  1:3000 (Sigma-Aldrich) and their corresponding secondary antibodies, goat anti mouse 1:5000, goat anti rabbit 1:3000 were used. All primary antibodies were incubated 1 hour at room temperature and 1% NaN<sub>3</sub> was added to prevent bacterial growth following use. Primary antibodies were used a maximum of 3

times before discarding. To visualize signal the enhanced chemiluminescence (ECL) detection method (PerkinElmer) was conducted to detect the protein signal by detecting hydrogen peroxide conjugated to the secondary antibody. Autoradiography films were used to detect light emission signal with a film developer in a dark room. Membranes were then washed with TBST 4x5 minutes and incubated at 50C for 30 minutes in fresh stripping buffer [1M Tris-HCl pH 6.8, 20% SDS and  $\beta$ -mercaptoethanol]. The membrane was then washed again 3x5 minutes and reblocked for probing with anti-  $\beta$ -actin anti-  $\beta$ -actin 1:5000 (Sigma-Aldrich) as a loading control.

#### 2.1.4 Immunohistochemistry assay

The colon cancer TMA CO951 (30 cases/95 cores) was purchased from US Biomax, Inc. (Rockville, MD, USA). Samples were used in duplicate; 30 cancer samples and 8 of these samples has matched normal adjacent tissue and 10 cases of matched metastasis. All histochemical stains were carried out at the IUSM Immunohistochemistry (IHC) Core, by Dr. Constance Temm and included standard deparaffinization in xylene, quenching in 1% hydrogen peroxide/methanol for 10 min, and rehydration through sequentially graded ethanols. Antigen retrieval was performed by Ethylenediamine-tetraacetic acid (EDTA). Using DAKO automated immunostainers (DAKO, Carpinteria, CA, USA), the slides were blocked for 30 min in horse serum and incubated with ARMC4 antibody (Abcam), followed by incubation with secondary antibody (goat anti rabbit). 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 1 hour before use.

#### 2.1.5 Cell proliferation assay

Stable HT29, DLD1 and HCT116 cells with WT-ARMC4, shARMC4 and empty vector control expressing cells respectively were seeded in triplicates in 6-well plates. To plate cells they were washed with PBS and Trypsin-EDTA (Fisher Scientific) was added to cover the plate before aspiration and incubation at 37°C. The cells were resuspended in media, counted using a hemocytometer, and then seeded at  $2 \times 10^4$  cells/well. Cells were trypsinized using Trypsin-EDTA at days 3, 5, 7, 9 and counted using a hemocytometer chamber. The number of cells were then averaged, and standard error was determined from each day in each group. Groups were then compared to each other using student's t-test and a graph was generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for each replicate for a n=3 of each condition.

#### 2.1.6 Anchorage-independent growth assay

Stable HT29, DLD1 and HCT116 cells with WT-ARMC4, shARMC4 and empty vector control were used for this experiment. 6-well plates were plated with 2.5% agar to prepare a bottom layer of agar and 1.25% agar was used to prepare the top layer of soft agar. Cells were prepared with PBS, trypsinized and counted as described in method 2.1.5.  $2 \times 10^5$  cells for each cell line in triplicate were then mixed with the 1.25% top agar solution and added to the top of the bottom layer. The plates were incubated for 14-21

days at 37°C and 5% CO<sub>2</sub>. Images were captured using a Canon EOS Rebel T3i Digital SLR camera and microscope and quantification of colony size and number was performed using ImageJ. Colonies smaller than 100mm<sup>3</sup> were removed from the dataset as too small and total number and size were counted for each triplicate and standard error was determined. Groups were compared by student t-test and graphs were generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for a n=3 of each condition.

#### 2.1.7 Migration assay

S table HT29, DLD1 and HCT116 cells with WT-ARMC4, shARMC4 and empty vector control were used for this experiment. Migration assay was conducted using the Boyden chamber assay. Cells were prepared with PBS, trypsinized and counted as described in method 2.1.5. 8µm pore size cell culture inserts (Corning) were placed in a 24-well plate. 2X10<sup>5</sup> cells per condition were suspended in triplicate in serum-starved media (no FBS added) in the upper chamber of the well. Media containing 5% FBS was placed in the lower chamber. Cells were allowed to migrate for 48h at 37°C and 5% CO<sub>2</sub>. Migrated cells that had passed through the pore were fixed using 4% formaldehyde (sigma-aldrich) followed by crystal violet staining (sigma-aldrich) and counted using a microscope at 20X magnification under light. The images were captured using a Canon EOS Rebel T3i Digital SLR camera. Groups were compared by student t-test and graphs were generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for a n=3 of each condition.

### 2.1.8 NF- $\kappa$ B luciferase assay

Stable HT29, DLD1 and HCT116 cells with WT-ARMC4, shARMC4, shscramble and empty vector control were used for this experiment. The NF- $\kappa$ B luciferase construct p5XIP10 (containing five tandem copies of the NF- $\kappa$ B site from the IP10 gene) was transfected transiently in the cell lines using Lipofectamine™ LTX Reagent and PLUS Reagents (Thermo Fisher Scientific). Luciferase activity was assayed 48h later by using the Luciferase Assay System with Reporter Lysis Buffer kit (Promega) as per the manufacturer's instructions. Cells were rinsed in PBS and lysis buffer was added to lyse cells. Cells were centrifuged and pelleted. The cell lysates were then added to Luciferase Assay reagent and detected by plate reader. Each sample was normalized to the amount of protein per sample determined by Spectrophotometer. The activity was measured using a Synergy H1 Multi-Mode Reader (BioTek Instruments Inc). Groups were compared by student t-test and graphs were generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for a n=3 of each condition.

### 2.1.9 Quantitative PCR

Cells were cultured to 90% confluence and treated or untreated with IL-1 $\beta$  for 1 hour and total RNA was isolated using Trizol as described previously (Wei, 2014). cDNA was made by reverse PCR from total RNA of 293IL1R cells by using the SuperScript III First-Strand Synthesis System (Invitrogen). FastStart Universal SYBR Green Master ROX (Roche) was used for the qPCR reactions. Primers were designed by Primer Express 3.0 software. Plates were read using an Applied Biosystems 7500 Fast

Real-Time PCR machine and each condition was plated in triplicate. Primers were designed by Primer Express 3.0 software and are listed in **Appendix A**. Groups were compared by student t-test and graphs were generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for a n=3 of each condition.

#### 2.1.10 Co-immunoprecipitation assay

293 and HT29 cells, either vector control or ARMC4 overexpressing, were cultured in 15-cm plates to 95% confluency were lysed in coimmunoprecipitation buffer [1% Triton X-100 (vol/vol), 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 20  $\mu$ M aprotinin, and 1 mM phenylmethanesulfonyl fluoride and pepstatin A]. Prewashed immobilized protein A/G (Pierce) was premixed with anti-p65, or anti-PRMT5 for 1 h, then mixed with cell lysates with equivalent amount of proteins at 4°C overnight. Gel beads were washed four times with 20 volumes of coimmunoprecipitation 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 SDS sample loading buffer [6% (vol/vol) glycerol, 1% (vol/vol)  $\beta$ -mercaptoethanol, 2% (wt/vol) SDS, 50 mM Tris·HCl, pH 6.7, 0.004% (wt/vol) bromophenol blue]. For anti-Flag-M2 antibody, EZView beads were used (Sigma).

#### 2.1.11 Conditioned media assay

HT29, DLD1, and HCT116 cells were seeded into 12-well plates, cultivated to 90% confluency, and transfected with different plasmids: empty vector, WT-ARMC4,

shscramble, and shARMC4. 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 described above. Groups were compared by student t-test and graphs were generated using Microsoft Office 2010 software. This experiment was performed a minimum of 3 separate times for a n=3 of each condition.

#### 2.1.12 Cell fractionation assay

Based on a Promega kit assay, stable HT29 cells and 293 cells with empty vector, and WT-ARMC4 plus or minus 1L-1β 1 hour treatment were washed with (R.T.) PBS solution, scraped down with a cell scraper and centrifuged for 5 minutes at 200 x g in a centrifuge pre-cooled at 4°C. The cell pellet was then resuspended in 500 μl 1X Hypotonic Buffer and incubated on ice for 15 minutes. 25 μl Detergent was added and vortexed. Then sample was centrifuged for 30 seconds at 14,000 x g in a microcentrifuge at 4°C. The supernatant (cytoplasmic fraction) was then collected. Then the nuclear pellet was resuspended in 50 μl Complete Lysis Buffer by pipetting up and down and vortexed. Samples were then incubated for 30 minutes on ice on a rocking platform set at 150 rpm. Then samples were vortexed 30 seconds at the highest setting and centrifuge for 10 minutes at 14,000 x g at 4°C. The nuclear fraction was then collected. Western Blotting was performed as described above. Tubulin (abcam) 1:2000 primary and Lamin (abcam)

1:2000 primary were used as loading controls for cytoplasmic and nuclear fractions respectively.

## 2.2 *In vivo* Experiments

### 2.2.1 Mice

Male NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from the In Vivo Therapeutics Core (IVT) at Indiana University Melvin and Bren Simon Cancer Center. These mice are an extremely immunodeficient strain and they lack mature T cells, B cells, and natural killer (NK) cells. Male mice were used for experiments described here. In the future I will use a combination of male and female mice. A minimum of n=3 mice were used per each condition. Animals were housed in a pathogen-free environment and maintained on Teklad Lab Animal Diet (Harlan Laboratories). Access to sterile water and food was provided ad libitum under a 12 h light-dark cycle. The room temperature was maintained at 22-24°C. All studies described here were performed in accordance with the guidelines and standards of the Institutional Animal Care and Use Committee (IACUC) and under the approved animal protocol #11073 by Indiana University.

### 2.2.2 Subcutaneous xenograft model of CRC

Male NSG mice were obtained from the In Vivo Therapeutics Core at IUSM. After acclimation for 7 days, NSG mice (6-8 weeks old) were xenografted with Mycoplasma-free HCT116 or HT29 cells with either empty vector, WT-ARMC4 or shARMC4 stable lines subcutaneously (s.c.) ( $3 \times 10^6$  HT29 or  $3 \times 10^6$  HCT116 cells used per mouse) in 0.2 ml of a 1:1 mix of PBS and Matrigel (BD Biosciences). Tumor

volumes and body weights were measured twice a week using calipers and weighing scale respectively.  $V = (W^2 \times L)/2$  for caliper measurements where V is tumor volume, W is tumor width and L is tumor length. A minimum of n=3 mice were used for each condition of each experiment. At the endpoint of 21 days or tumor size of 2000 mm<sup>3</sup> the mice were sacrificed and the tumors were collected in liquid nitrogen and stored at –80°C.

### 2.3 Statistical analysis

Statistical analysis for experiments mentioned in the entire Methods section was performed using Prism 6 software (GraphPad). Results have been presented as mean ± SD or mean ± SEM, as specified. A two-tailed Student's t test was used while comparing two means to test for significant differences for all other experiments. All statistics were calculated on triplicate experiments and p value < 0.05 was considered statistically significant.

## CHAPTER 3. ASSESSING THE TUMOR SUPPRESSIVE ROLE OF ARMC4 ON NF- $\kappa$ B SIGNALING IN CRC

### 3.1 Rationale and Background

CRC is the second highest cause of cancer-related death in the United States. Therapeutic treatments particularly in metastatic CRC remain limited. Therefore, identification of new therapeutic targets in CRC will promote development of novel treatment strategies.

NF- $\kappa$ B is a critical transcription factor whose family consists of five members: RelA (p65), RelB, cRel, NF- $\kappa$ B1 (p50 and precursor p105), and NF- $\kappa$ B2 (p52 and precursor p100) (Ghosh, 1988). The canonical pathway has been well established as a key contributor to CRC (Martin, 2018). Upon receiving a stimulus such as a pro-inflammatory cytokine such as IL-1 $\beta$ , I $\kappa$ B kinase can phosphorylate the inhibitor of  $\kappa$ B (I $\kappa$ B $\alpha$ ), leading to its degradation. This process results in the release of the p65:p50 complex, and the activation of NF- $\kappa$ B target genes, many of which have been implicated in cancer. Furthermore hyperactive NF- $\kappa$ B can promote chemotherapeutic resistance in CRC (Martin. 2018). Ergo, controlling NF- $\kappa$ B activity allows control of CRC signaling.

Recently, I have discovered a novel role for ARMC4 in regulating NF- $\kappa$ B signaling as a novel negative regulator. ARMC4's role as a negative regulator may also be important for control of NF- $\kappa$ B signaling in CRC. Our lab's preliminary data suggests ARMC4 may play a role in regulating NF- $\kappa$ B activity in HEK293 cells and is decreased in expression in CRC cancer tissues. Because of our promising preliminary data supporting this work, I aim to provide evidence that ARMC4 expression is correlated with the regulation of several hallmarks of cancer including cell growth, anchorage-

independent growth, cell migration and tumor growth which will support my proposed hypothesis of ARMC4 acting as a tumor suppressing factor in CRC. The results discussed in this chapter indeed indicate a regulatory role of ARMC4 in CRC. Overall the results presented in this chapter provide the evidence that overexpression of ARMC4 correlated with decreases in several hallmarks of cancer including cell growth, anchorage-independent growth, and cell migration, at least partly via decreased activation of NF- $\kappa$ B. Furthermore, ARMC4 expression also correlated with tumor growth in a xenograft mouse model. Discovery of potentially pathway-specific novel regulators of NF- $\kappa$ B like ARMC4 may prove significant for classification of tumor types and grade and direct future therapeutic targeting of CRC.

## **3.2 Results**

### **3.2.1 ARMC4 has reduced expression in CRC Cells**

Before examining the potential role of ARMC4 in CRC, endogenous ARMC4 levels were checked in cancerous and noncancerous colon cell lines. This was done to further validate previous results seen in the initial TMA wherein there was reduced ARMC4 expression in cancerous tissue. This set of cell lines (HT29, DLD1, and HCT116) are all well characterized in the literature and commonly used as a panel of cells for CRC *in vitro* experiments (Ahmed, 2013). As shown in **Figure 7**, ARMC4 expression was decreased in cancerous HT29, HCT116, and DLD1 cell lines compared to normal non-cancerous FHS74 cells. These data clearly demonstrated that ARMC4 is reduced in expression in this panel of CRC cell lines. For further experiments, the three

CRC cell lines and HEK293 cells were used to generate stable cell lines as described previously (Wei, 2014).

### 3.2.2 Generation of stable ARMC4 overexpression and shRNA knockdown cell lines

Stable cells with either ARMC4 overexpression or shRNA knockdown were established in HEK293, HT29, DLD1, and HCT116 cells by cloning Flag-tagged WT-ARMC4 or shARMC4 cDNA into pLVX-IRES-puro vector (Lu, 2010). Here, I knocked down ARMC4 further in cancer cells which already have lower ARMC4 expression to show the significance of modulating a single factor such as ARMC4 in the context of regulation of NF- $\kappa$ B. Empty vector pLVX or shscramble pools were used as controls for WT-ARMC4 and shARMC4 respectively.

After transfecting the respective lentiviral plasmids containing empty vector, WT-ARMC4 or shRNAs into a 293T packaging cell line, viral preps were generated to infect my CRC cells and HEK293 cells. 1  $\mu$ g/mL and 0.5  $\mu$ g/mL of puromycin was used for further selection, as the pLVX vector contains a puromycin resistance gene. Cells that went under 1  $\mu$ g/mL selection were determined to have better overexpression or knockdown expression respectively and were used for the other experiments described here (data not shown). All the experiments done with stable cell lines were done using a minimum three biological replicates and pools of early passage infected stable cells that were frozen down at -80°C from passages 2-6. Finally, Western blotting was conducted to confirm the expected ARMC4 overexpression or knockdown. As shown in **Figure 8**, significant overexpression was observed in WT-ARMC4-HT29, DLD1, and HCT116 cells as compared to the empty vector control. Conversely, shARMC4-HT29, DLD1, and

HCT116 cells showed knockdown of ARMC4 in these cells, compared to the shscramble control.  $\beta$ -actin was used as a loading control. Upon confirmation of ARMC4 expression, I used these cell lines to pursue further assays that examined the different hallmarks of cancer regulated through NF- $\kappa$ B signaling.

### 3.2.3 Overexpression of ARMC4 decreases NF- $\kappa$ B activity in CRC cells

Previously, I have shown in our preliminary data that ARMC4 overexpression can decrease NF- $\kappa$ B activity in HEK293 cells but I wondered if overexpression of ARMC4 could affect NF- $\kappa$ B activity in CRC cells as well. NF- $\kappa$ B is known to be critical for both normal cell and cancer cell function. This makes direct targeting of NF- $\kappa$ B difficult and therefore negative regulators of NF- $\kappa$ B offer an alternative targeting approach to regulate NF- $\kappa$ B signaling through indirect targeting of NF- $\kappa$ B.

I therefore wanted to determine ARMC4's impact on NF- $\kappa$ B activation. NF- $\kappa$ B activity was measured using transient transfection of the construct p5XIP10 (containing five tandem copies of the NF- $\kappa$ B site from the IP10 gene upstream of the luciferase reporter gene) and addition of luciferase substrate 48 h after transfection. If ARMC4 decreased NF- $\kappa$ B activation, then I would expect to see decreased binding of the active p65 subunit of NF- $\kappa$ B to its consensus site of the IP10 gene, leading to decreased transcription and translation of the downstream luciferase reporter gene. Upon addition of luciferase substrate, a fluorescent product is formed by the luciferase enzyme which is directly proportional to the level of NF- $\kappa$ B activity in the cells, thus allowing determination of NF- $\kappa$ B-dependent activity. As illustrated in **Figure 9**, overexpression of ARMC4 significantly decreased NF- $\kappa$ B activity, while shARMC4 knockdown exhibited

a marked increase in activity compared to the control cells and shscramble cells respectively, indicating that ARMC4 indeed significantly reduced NF- $\kappa$ B activity in CRC cells.

#### 3.2.4 Overexpression of ARMC4 decreases CRC cell growth

Naturally the next step after determining if NF- $\kappa$ B target gene expression was regulated by NF- $\kappa$ B was to determine if the hallmarks of cancer regulated through NF- $\kappa$ B signaling are regulated by expression of ARMC4. Proliferation is one of the hallmarks of cancer that can be regulated by NF- $\kappa$ B signaling. In order to test if elevated levels of ARMC4 correlated with decreasing the cell proliferating ability of CRC cells, cells were seeded in 6-well plates and checked for growth over a period of 7-9 days. These cells were then counted using a hemocytometer chamber.

I showed that overexpression of ARMC4 decreased cell growth, while shRNA knockdown increased growth in CRC cells around day 5-7 (**Figure 10**) as compared to the vector control cells. This data suggests that ARMC4 played an important role in regulating cell proliferation in CRC and ARMC4 overexpression decreased cellular proliferation.

#### 3.2.5 Overexpression of ARMC4 decreases migratory ability of CRC cells

Migration is another hallmark of cancer critical for its importance in tumor growth and invasion (Agarwal 2005). There are several well-characterized methods for migration including scratch-wound assays and Boyden Chamber assays. The Boyden chamber assay is described in detail in my methods **chapter 2**. I performed these Boyden

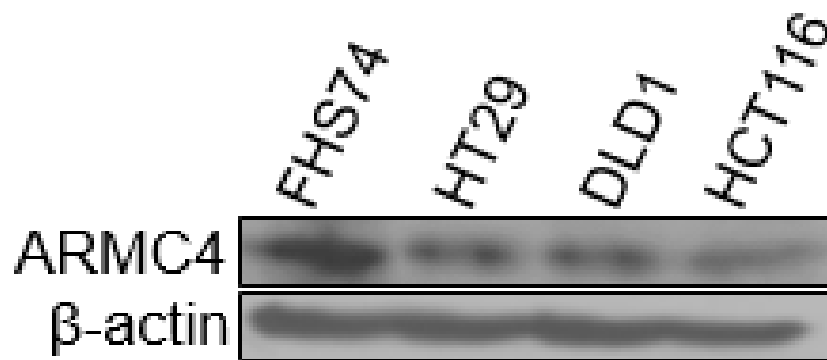
Chamber assays as shown in **Figure 11**. The Boyden Chamber assay uses a plastic chamber with a porous membrane at its bottom to separate cells from an area of high and area of low nutrition. This allows for cells to migrate across the porous membrane. The migrated cells are finally stained with crystal violet and counted using the hemocytometer chamber. As observed in **Figure 11**, overexpression of ARMC4 decreased the number of migrated cells (seen in purple), whereas shARMC4 knockdown increased migration dramatically. Representative pictures for each condition were taken in 20X magnification. Overall, these results indicate that ARMC4 overexpression decreased the migratory ability of CRC cells.

### 3.2.6 Overexpression of ARMC4 decreases anchorage-independent growth of CRC cells

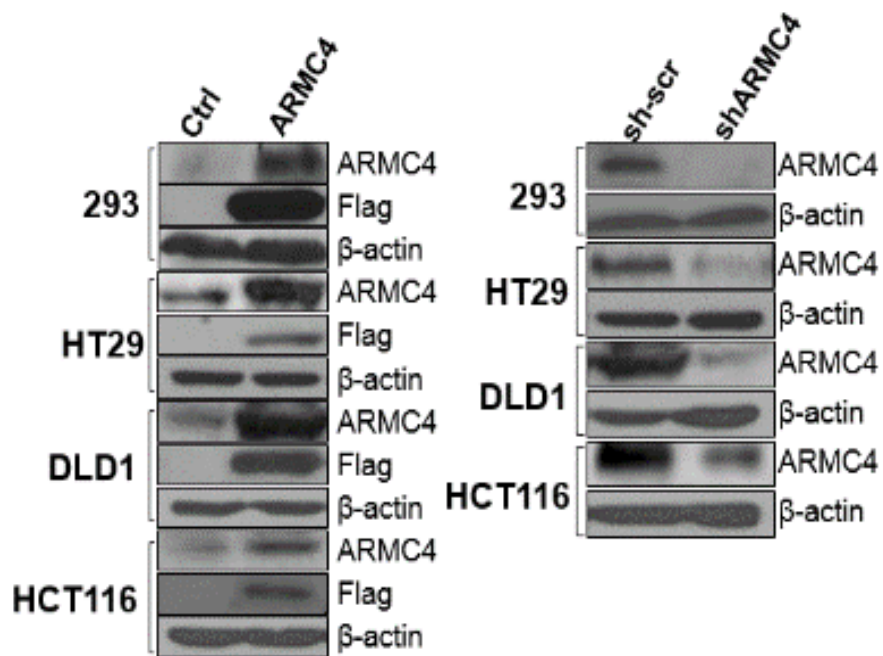
The ability to grow without anchorage is a so-called hallmark of cancer as it describes the invasiveness and metastasis of cancer cells. In order to test if ARMC4 expression can affect anchorage-independent growth I performed an anchorage-independent growth assay as described in **chapter 2**. As shown in **Figure 12**, my data suggested that ARMC4 overexpression led to a decrease in both the colony size and number in CRC cell lines while shARMC4 knockdown significantly increased colony size and number, suggesting a critical role of ARMC4 in regulating anchorage-independent growth in CRC.

### 3.2.7 Tumor Growth for HCT116 using Subcutaneous Injection

While ARMC4 seems to have several important effects *in vivo* I wished to determine if there is any translational aspect of my work with ARMC4 by moving

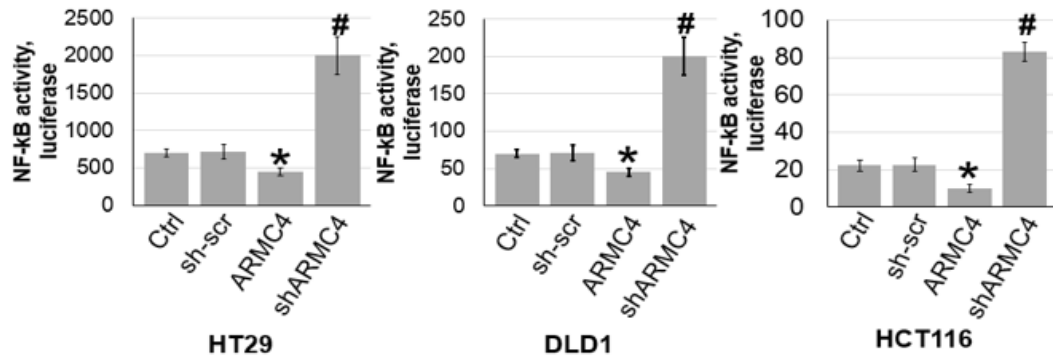


**Figure 7.** ARMC4 has reduced expression in CRC Cells ARMC4 expression is lower in CRC cells (HT29, HCT116 and DLD1) as compared to control colon cells FHS74.  $\beta$ -actin was used as a loading control.

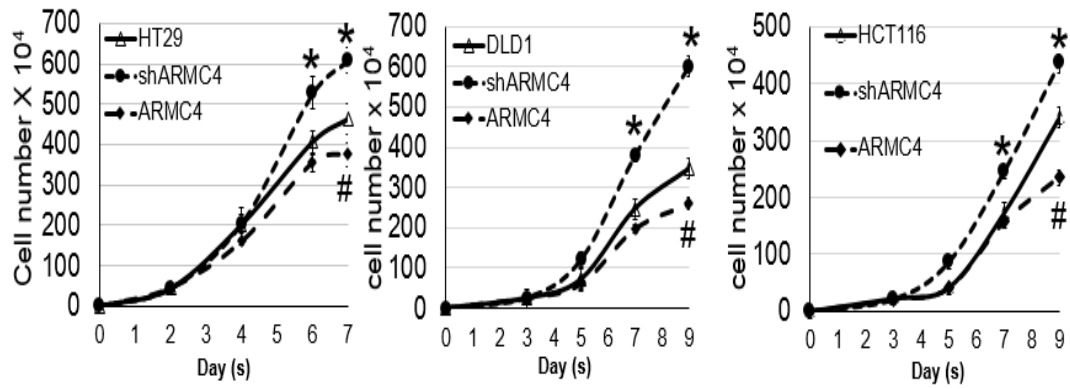


**Figure 8.** Generation of stable ARMC4 overexpression and shRNA knockdown cell lines

Western blot, confirming stable ARMC4 overexpression and shARMC4 knockdown in 293 and CRC cell lines.  $\beta$ -actin was used as a loading control.



**Figure 9.** Overexpression of ARMC4 decreases NF- $\kappa$ B activity in CRC Cells NF- $\kappa$ B luciferase assay, showing that overexpression of WT-ARMC4 led to decreased NF- $\kappa$ B activation compared to the vector control, while shARMC4 increased activation compared to the shscramble. \* $P < 0.05$  ARMC4 vs. Ctrl. # $<0.05$  shARMC4 vs. shscramble using student's t-test. N=3



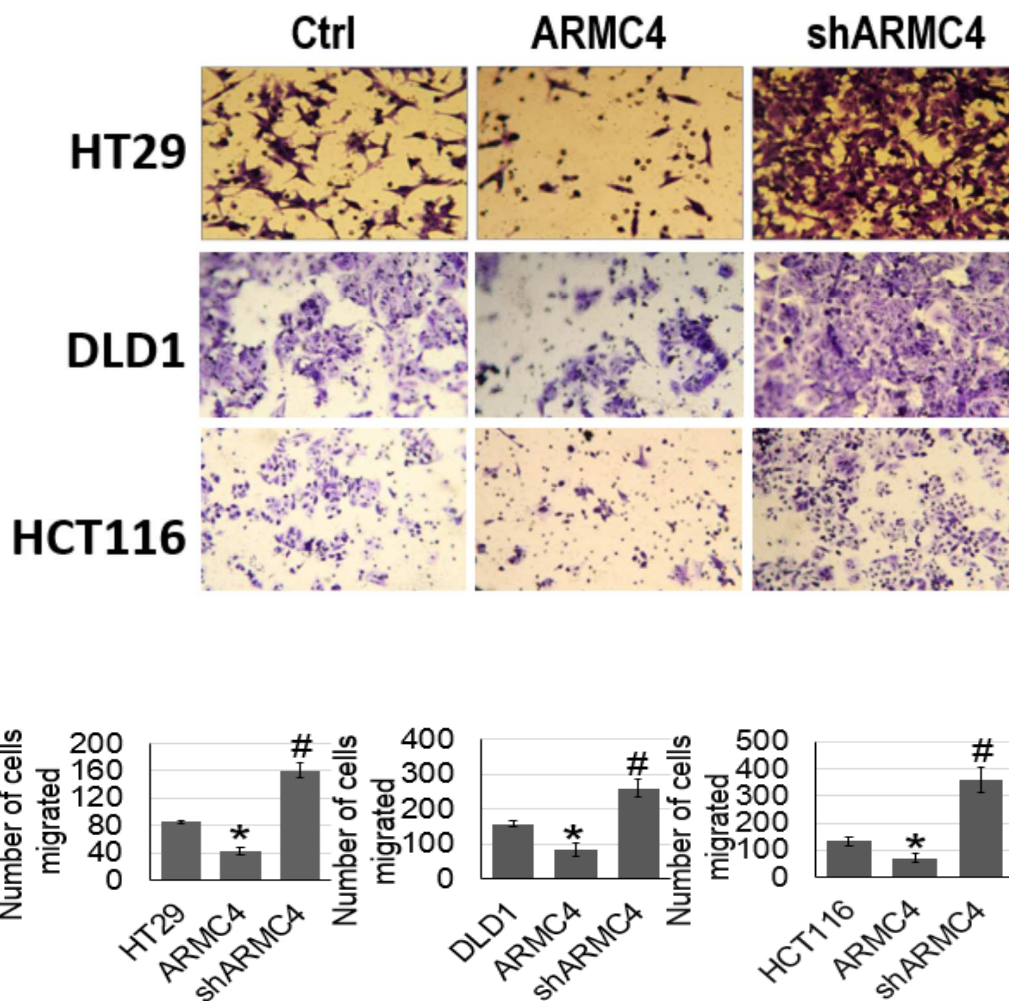
**Figure 10.** Overexpression of ARMC4 decreases CRC Cell Growth

Cell proliferation assay, showing that cell proliferation was significantly higher in the ARMC4 overexpression cell lines, while shARMC4 cells exhibited increased proliferation compared to empty vector control. \*P < 0.05 shARMC4 vs. Ctrl. #P < 0.05 ARMC4 vs. Ctrl. Using student's t-test. N=3

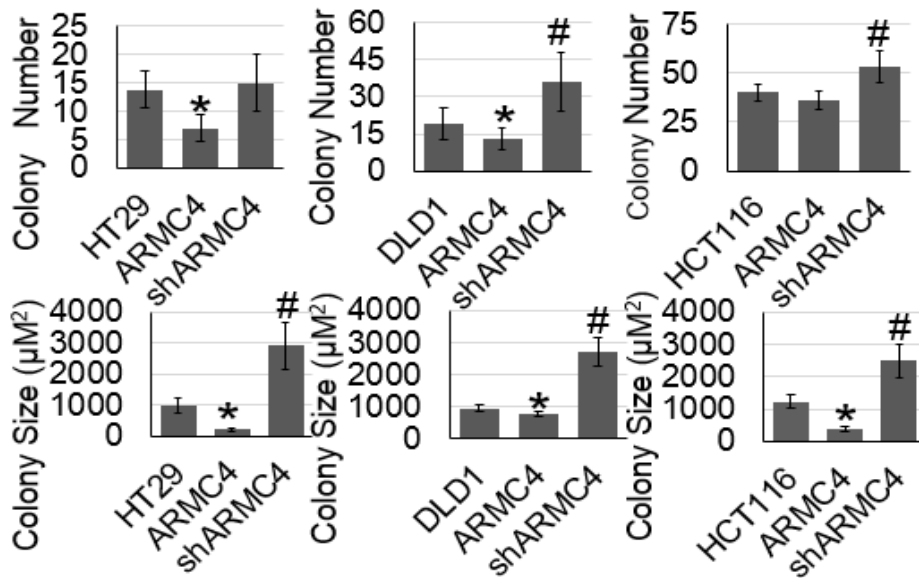
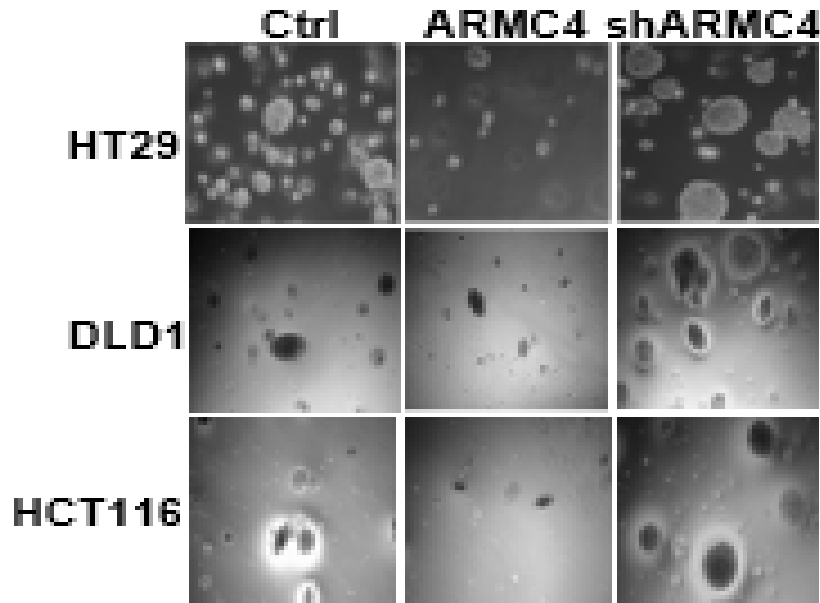
into an *in vitro* model. I wondered whether ARMC4 can regulate tumor growth in addition to proliferation, migration and anchorage-independent growth. In order to study this, it was necessary to move into a xenograft mouse model and determine if ARMC4 expression could alter tumor growth. However, before beginning tumor growth studies, it was important to determine the proper implant conditions for my CRC cells to be used. Previously, I had determined a good number of cells to use for implantation of HT29 cells was  $3 \times 10^6$  which would allow a tumor to grow for several weeks with vector control HT29 cells. However, I had not determined the number of HCT116 cells to implant. In order to achieve this goal, I injected three different cell numbers of HCT116 cells in NSG mice:  $3 \times 10^6$ ,  $5 \times 10^6$  and  $10 \times 10^6$  cells per mouse. I then checked for growth kinetics over a period of time by measuring tumor volume at regular intervals (**Figure 13**).  $2000 \text{ mm}^3$  is the cutoff for tumor size for sacrifice as per the ethical regulations under the approved protocol. As shown in **Figure 13**,  $3 \times 10^6$  cells managed to reach a lower tumor volume following three weeks of growth and was below the maximal volume of  $2000 \text{ mm}^3$ . Hence, I decided to use a cell number of  $3 \times 10^6$  to allow for tumor growth with and without knockdown and overexpression of ARMC4.

### 3.2.8 ARMC4 overexpression decreases tumor growth in a CRC xenograft model

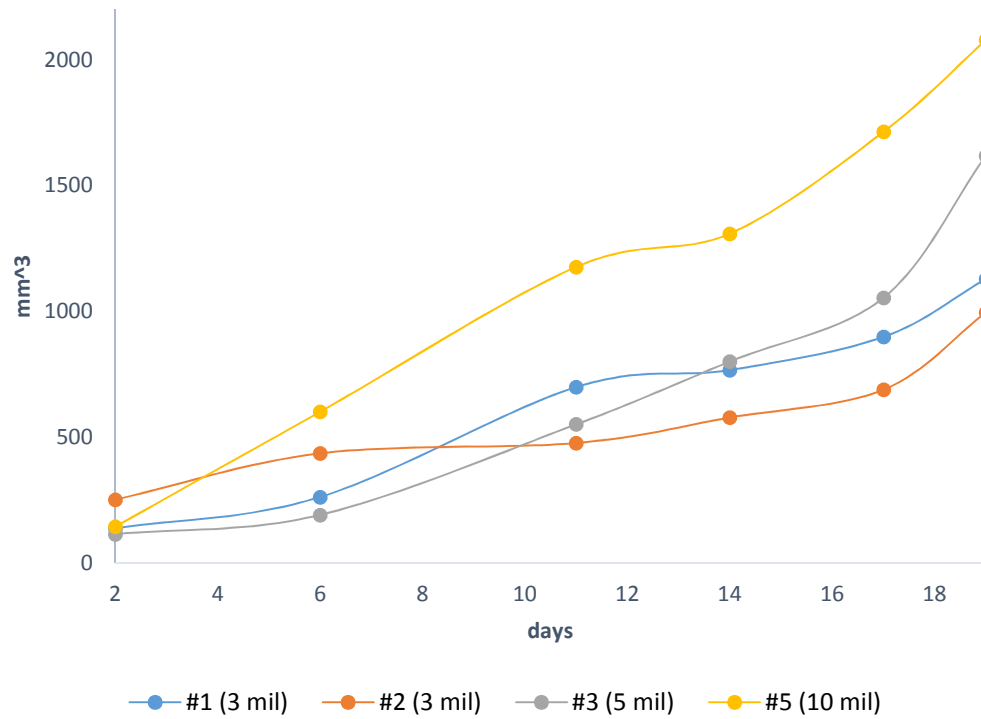
Once I had optimized my implant conditions, the next logical step was to determine if ARMC4 expression could affect tumor growth *in vivo*. Either HT29 or HCT116 cells were subcutaneously xenografted into NSG mice and allowed to grow until a maximal  $2000 \text{ mm}^3$  tumor developed. Both body weight and tumor size were monitored



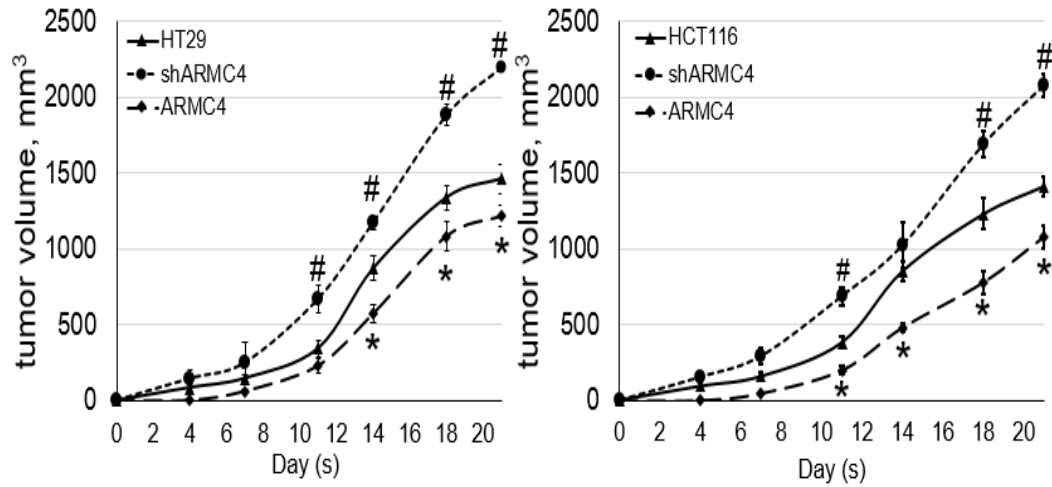
**Figure 11.** Overexpression of ARMC4 decreases migration of CRC cells  
 Cell migration assay, showing that cell migration was significantly higher in the shARMC4 overexpression cells, while significantly reduced in the ARMC4 overexpressing cells. Upper panels, representative pictures in 20X magnification. Lower panel, quantification for the change in migration. \*P < 0.05 ARMC4 vs. Ctrl. #P < 0.05 shARMC4 vs. Ctrl. Using student t-test. N=3



**Figure 12.** Overexpression of ARMC4 decreases anchorage-independent growth of CRC cells Anchorage-independent growth (Soft agar assay), showing ARMC4 overexpression cells exhibited decreased colony size and number compared with vector control cells, in contrast, shARMC4 CRC cells showed increased colony size and number compared with Ctrl cells. The lower panel is a quantification of the upper colony number counts and size analyzed by ImageJ software. \* $p < 0.05$  Ctrl vs. ARMC4 group; # $p < 0.05$  Ctrl vs. shARMC4 group. Using student t-test. N=3



**Figure 13.** Tumor growth pilot study for HCT116 Using subcutaneous injection Graph depicting tumor growth over time for HCT116 cells when they were subcutaneously implanted respectively in NSG mice. N=1 for each condition.



**Figure 14.** ARMC4 overexpression decreases tumor growth in a CRC xenograft model. Tumor growth of cells in a NSG mouse sub-cutaneous xenograft model showed ARMC4 overexpression led to decreased tumor growth compared to the vector control. shARMC4 led to increased tumor growth compared to the vector control. \* $p < 0.05$  Ctrl vs. ARMC4 group; # $p < 0.05$  Ctrl vs. shARMC4 group. Using student t-test. N=3 separate experiments for all experiments described here.

during this process. As shown in **Figure 14** overexpression of ARMC4 decreased tumor growth in comparison to the vector control. Tumor growth of cells in a NSG mouse subcutaneous xenograft model showed ARMC4 overexpression led to decreased tumor growth compared to the vector control in both HCT116 and HT29 xenografts. shARMC4 knockdown led to increased tumor growth compared to the vector control. These data suggest ARMC4 expression regulates tumor growth of CRC cells.

### **3.3 Concluding Remarks**

Here, I provide the first link for an ARMC4-mediated NF- $\kappa$ B signaling pathway in CRC. I have shown here ARMC4 overexpression decreased NF- $\kappa$ B activity. My findings show that ARMC4 overexpression downregulates cell proliferation, migration and anchorage-independent growth all of which are associated with cancer.

Knockdown of ARMC4 leads to an inverse of these effects. Furthermore, ARMC4 overexpression can also decrease tumor growth. These data together suggest a novel signaling mechanism responsible for NF- $\kappa$ B regulation in CRC through ARMC4. Furthermore, these findings indicate a possible clinical relevance of ARMC4 in the context of CRC. Regulating ARMC4 expression by regulating the negative regulator of NF- $\kappa$ B could serve as a means to indirectly control constitutive NF- $\kappa$ B activation in CRC. However, while I have seen potential functional significance of ARMC4 in the context of CRC, I have not explored the mechanisms by which ARMC4 is acting upon NF- $\kappa$ B signaling. In the next chapter I will aim to investigate this aspect.

## CHAPTER 4. DETERMINING THE MECHANISMS BEHIND ARMC4'S REGULATION OF NF- $\kappa$ B SIGNALING IN CRC

### 4.1 Rationale and Background

So far in **chapter 3** I explored ARMC4 as a potential tumor suppressor in the context of CRC. My overall conclusions indeed suggested the importance of ARMC4 in negatively regulating NF- $\kappa$ B but in order to consider the future ramifications and importance of ARMC4 in the context of cancer I decided I need to better understand the mechanism or mechanisms leading to regulation of NF- $\kappa$ B signaling by ARMC4. My preliminary data showed NF- $\kappa$ B activity was decreased when ARMC4 was overexpressed. Since NF- $\kappa$ B activity is directly linked to NF- $\kappa$ B target gene expression, I wish to consider whether ARMC4 expression can affect NF- $\kappa$ B target expression. To study this I will perform an Illumina microarray with cells overexpressing ARMC4 to determine the effects of ARMC4 expression on NF- $\kappa$ B target expression. I also wish to better understand ARMC4's roles in disease and regulation of ARMC4's interaction with NF- $\kappa$ B signaling. I can study this through IPA (ingenuity pathway analysis) of my microarray data. Additionally, I propose ARMC4 may affect NF- $\kappa$ B activity in an autocrine fashion as NF- $\kappa$ B signaling is known to self-regulate in autocrine loops (Agarwal, 2005).

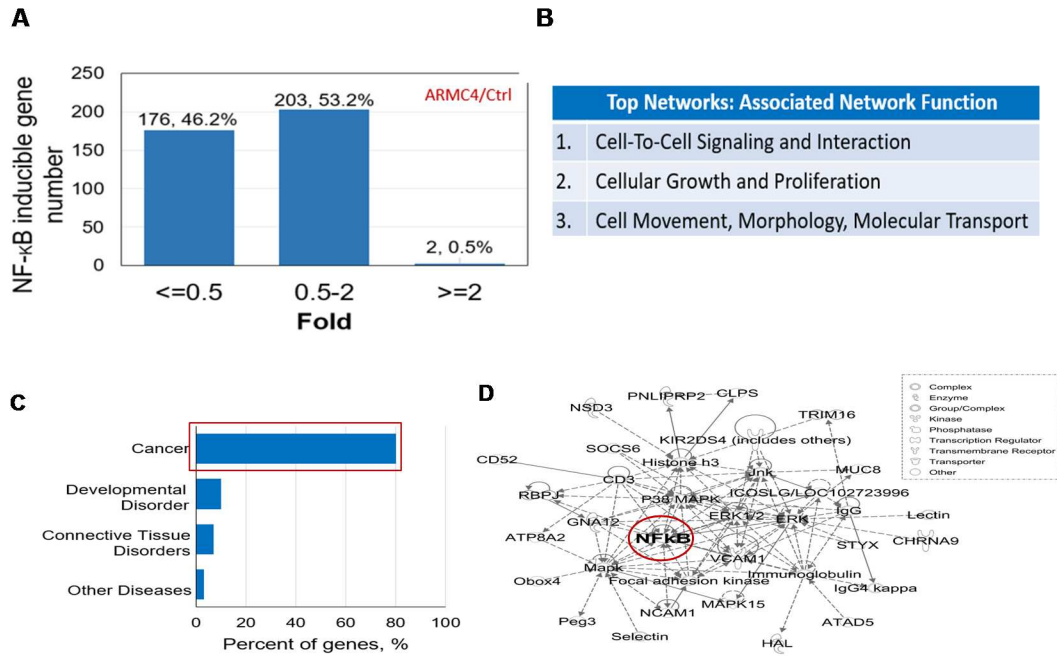
In terms of known functions of ARMC4 most known associated functions are in the context of binding to other proteins in both PCD and in ARMC4's functions in mouse spermatogenesis. To study this I will determine if ARMC4 interacts with NF- $\kappa$ B prior to or after the critical I $\kappa$ B $\alpha$  degradation which allows release of the p65/p50 heterodimer into the cytoplasm. These known roles lead me to propose that ARMC4 may play an

important role in binding proteins in the NF- $\kappa$ B pathway in order to regulate its function. Furthermore, other questions of where interactions are occurring in the cell and when regulation of NF- $\kappa$ B by ARMC4 is occurring also still need to be answered. To study these questions, I performed co-immunoprecipitation assays to pulldown my Flag-tagged ARMC4 and probed for a subunit of NF- $\kappa$ B signaling known to be involved in regulation of CRC progression, p65. I performed cell fractionation experiments to determine the localization of ARMC4 and the p65 subunit of NF- $\kappa$ B. Together, the results in this chapter suggest ARMC4 can regulate NF- $\kappa$ B target gene expression and potentially complex with the p65 subunit of NF- $\kappa$ B after the degradation of I $\kappa$ B $\alpha$ . The results depicted in this chapter provide the evidence that overexpression of ARMC4 correlated with similar degradation of I $\kappa$ B $\alpha$  as control and shARMC4 knockdown as well as a potential complexing of p65 with ARMC4 in the presence and absence of IL-1 $\beta$ . Preliminary localization data suggests ARMC4 may interact with normal p65 translocation to the nucleus. These data together suggest novel and exciting avenues by which ARMC4 can regulate NF- $\kappa$ B signaling. This chapter's work is the beginning for understanding the mechanism of ARMC4 mediated NF- $\kappa$ B activity.

## 4.2 Results

### 4.2.1 ARMC4 expression affects NF- $\kappa$ B cell signaling and gene expression

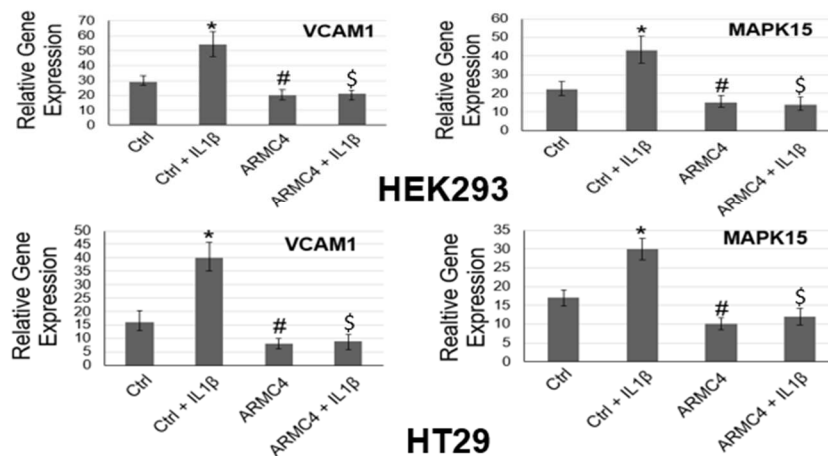
Since I have shown ARMC4 expression can affect NF- $\kappa$ B activity in **chapter 3** I wondered whether negative regulation of NF- $\kappa$ B activity through ARMC4 may directly downregulate downstream NF- $\kappa$ B target gene expression. In order to test this,



**Figure 15.** ARMC4 expression affects NF- $\kappa$ B cell signaling and gene expression  
 A) An Illumina Microarray with HEK293 cells and HEK293 cells overexpressing ARMC4 showed a 2-fold decrease of about 46% of NF- $\kappa$ B target genes in overexpressed ARMC4 293 cells compared to normal 293 cells. (B) Ingenuity Pathway Analysis identified ARMC4 has several cellular functions including cell-cell signaling, proliferation, cell morphology. (C) Ingenuity Pathway Analysis identified many genes regulated by ARMC4 have important roles in cancer. (D) Ingenuity Pathway Analysis identified ARMC4 as in signaling networks with NF- $\kappa$ B.

**A**

Gene Accession	Symbol	Definition	ARMC4/Ctrl
NM_080615.1	GNA12	Guanine nucleotide binding protein a12	0.4
NM_139021.1	MAPK15	Mitogen-activated protein kinase 15	0.3
NM_001076682.2	NCAM1	Neural cell adhesion molecule 1	0.4
NM_001078.2	VCAM1	Vascular cell adhesion molecule 1	0.5

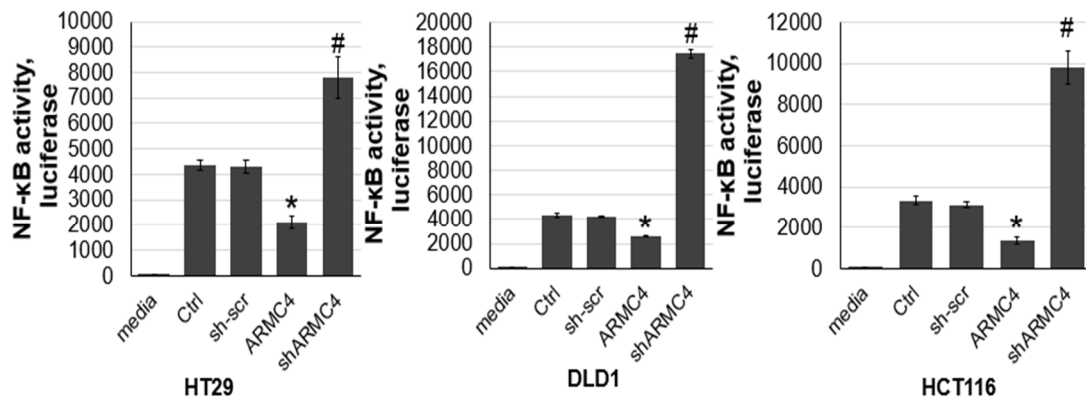
**B**

**Figure 16.** ARMC4 expression affects NF- $\kappa$ B target gene expression A) A short list of NF- $\kappa$ B target genes downregulated by ARMC4 overexpression in the microarray are shown here including VCAM1 and MAPK15 which were downregulated by several fold. B) qPCRs were done to confirm VCAM1 and MAPK15 as NF- $\kappa$ B inducible genes in HEK293 and HT29 control and ARMC4 overexpression cells with and without IL-1 $\beta$ . Upon stimulation of IL-1 $\beta$  ctrl cells in both HEK293 and HT29 cells were able to induce VCAM1 and MAPK15 gene expression but ARMC4 overexpression decreased induction of VCAM1 and MAPK15 gene expression in HEK293 and HT29 cell lines. \* $p < 0.05$  Ctrl vs. Ctrl +IL-1 $\beta$  group; # $p < 0.05$  Ctrl vs. ARMC4 group; \$ $p < 0.05$  Ctr + IL-1 $\beta$  vs ARMC4 + IL-1 $\beta$ ; Using student t-test. N=3.

I used HEK293 cells and HEK293 cells overexpressing ARMC4 in an Illumina microarray with and without NF- $\kappa$ B induction by IL-1 $\beta$  for 1 hour. This method used a specific panel of common NF- $\kappa$ B inducible genes to determine if ARMC4 expression could affect expression of some of the most common NF- $\kappa$ B-inducible genes. This is a commonly used methodology for studying genes involved in NF- $\kappa$ B signaling and can screen for many genes in one screen allowing for a bigger picture approach to identifying genes that are upregulated and downregulated. As shown in **Figure 15**, HEK293 cells overexpressing ARMC4 showed a 2-fold decrease of about 46% of NF- $\kappa$ B target genes in overexpressed ARMC4 293 cells compared to normal 293 cells. These data were then used to determine ARMC4's known and unknown signaling associations through IPA. IPA showed ARMC4 has several cellular functions including cell-cell signaling, proliferation, and cell morphology. Some of these functions have been well characterized but others such as a role in proliferation have not been studied. Furthermore, IPA identified many genes regulated by ARMC4 have important roles in cancer. IPA also identified ARMC4's involvement in signaling networks with NF- $\kappa$ B. These data suggest ARMC4 had several previously unstudied functions through regulation of many NF- $\kappa$ B target genes.

#### 4.2.2 ARMC4 expression affects NF- $\kappa$ B target gene expression.

While the Illumina Microarray data was valuable for a general sense of genes regulated by ARMC4 I wanted to confirm particular target gene expression that was changed when ARMC4 was overexpressed. Therefore, to confirm the results I saw in



**Figure 17.** Conditioned media from ARMC4 overexpressing cells reduces NF- $\kappa$ B activity. Conditioned media from HT29, DLD1 and HCT116 cells overexpressing ARMC4 had much lower NF- $\kappa$ B-inducing activity than vector control cells. Media is a control of untreated media. Conditioned media from shARMC4 knockdown cells in HT29, DLD1, and HCT116 cell lines had higher NF- $\kappa$ B-inducing activity than shscramble cells. Stable 293-NF- $\kappa$ B 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  $\pm$  SD from three independent experiments. \* $p < 0.05$  ARMC4 vs. Ctrl group; # $p < 0.05$  shARMC4 vs. shscramble group. Using student t-test. N=3.

**Figure 15** I performed qPCR analysis of several well-known NF- $\kappa$ B target genes as shown in **Figure 16**. Among these genes downregulated by ARMC4 overexpression in the microarray are VCAM1 and MAPK15 both of which have been associated with cellular adhesion. Upon stimulation with IL-1 $\beta$  in both HEK293 and HT29 cells there was induction of VCAM1 and MAPK15 gene expression but ARMC4 overexpression decreased induction of VCAM1 and MAPK15 gene expression in HEK293 and HT29 cell lines. These data suggest ARMC4 expression is able to control induction of NF- $\kappa$ B target gene expression.

#### 4.2.3 Conditioned media from ARMC4 overexpressing cells reduces NF- $\kappa$ B activity

Constitutive NF- $\kappa$ B activation in cancer can be enhanced by autocrine cytokine secretion in NF- $\kappa$ B activation feedback loops (Agarwal 2005). To test if ARMC4 expression could affect the secretion of activators in culture medium, I assayed conditioned media from HT29, DLD1 and HCT116 cells with a well-established 293- $\kappa$ B reporter cell line (Lu, 2004). Different media secreted from the overexpression and knockdown of ARMC4 cells were used to treat this 293- $\kappa$ B reporter cell line. As shown in **Figure 17**, the data suggested that media from the HT29 cells with ARMC4 overexpression had decreased NF- $\kappa$ B inducing ability as compared to that of the vector control cells. On the other hand, media from shARMC4 condition showed greatly increased NF- $\kappa$ B inducing ability compared to the shscramble suggesting that ARMC4 overexpression can negatively regulate NF- $\kappa$ B activity through autocrine feedback. However, the caveat to this approach is that the signal seen may correspond to the concentration of factors released by the cancerous cells which can correspond to cell

density and health. Media was taken from plates at very similar cell density and apparent health but in the future this experiment may be done with a cell death marker to identify health of the cells as well.

#### 4.2.4 ARMC4 expression does not significantly affect I $\kappa$ B $\alpha$ degradation

While my data so far suggested ARMC4 expression regulated target gene expression I wanted to understand if ARMC4's interaction is upstream or downstream of I $\kappa$ B $\alpha$  in my normal, overexpressing and knockdown ARMC4 lines. If ARMC4 interacted upstream of I $\kappa$ B $\alpha$  degradation, there could potentially be a lag or increase in speed of degradation of I $\kappa$ B $\alpha$  which could then promote the differences I observed in NF- $\kappa$ B activity and effects on target gene expression. I $\kappa$ B $\alpha$  is a target gene of NF- $\kappa$ B and can become resynthesized with longer induction via IL-1 $\beta$ . I therefore performed a western analysis as shown in **Figure 18**, comparing my CRC stable cell lines with either vector control or shscramble control respectively. Upon treatment with IL-1 $\beta$ , the expression of ARMC4 did not affect either the I $\kappa$ B $\alpha$  degradation or its resynthesis significantly, indicating that ARMC4 probably functions downstream of I $\kappa$ B $\alpha$  degradation.

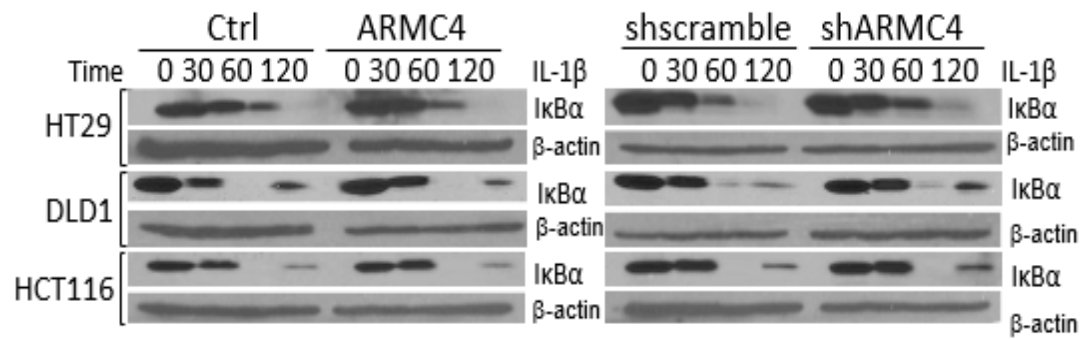
#### 4.2.5 ARMC4 potentially complexes with p65 in CRC

Following my studies of determining where ARMC4 is interacting with NF- $\kappa$ B I wanted to determine if NF- $\kappa$ B could complex or interact with ARMC4 as ARMC4's known functions almost exclusively involve binding to and regulating transport or interactions of other proteins. The p65 subunit is known to be involved in regulation of NF- $\kappa$ B activity through interactions with positive regulators of NF- $\kappa$ B signaling in CRC

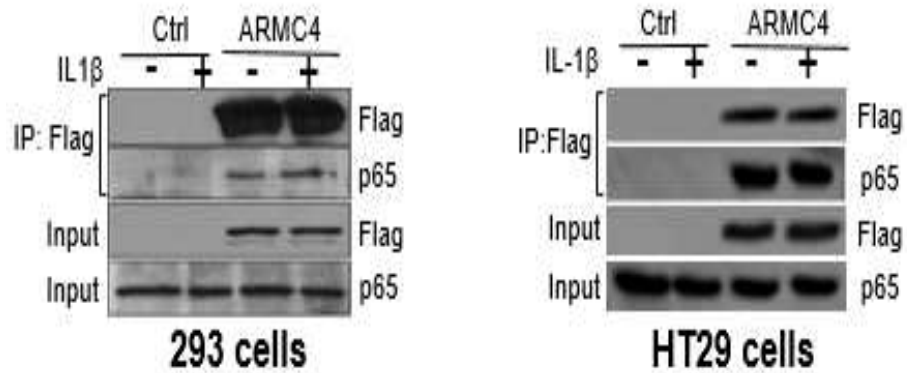
(Prabhu, 2017). I therefore decided to explore if p65 could interact with ARMC4. To determine if p65 could interact with Flag-tagged ARMC4 in the context of CRC, I performed co-immunoprecipitation experiments in HEK293 and HT29 cells with or without IL-1 $\beta$  treatment and determined Flag-tagged ARMC4 in HT29 cells could pull down p65 when probed with p65 antibody. This is shown in **Figure 19**. This suggests a potential formation of a complex between ARMC4 and p65 and interaction with and without stimulation of NF- $\kappa$ B signaling. This suggests there is some interaction between ARMC4 and p65 downstream of I $\kappa$ B $\alpha$  degradation.

#### 4.2.6 ARMC4 exhibits a mostly cytoplasmic localization in HEK293 cells and potentially interacts with p65 in the cytoplasm

While I think there is some interaction between the p65 subunit of NF- $\kappa$ B and ARMC4 I wondered whether ARMC4 expression could regulate the transport of p65 to the nucleus. As described previously, ARMC4 is known to bind to proteins and regulate their function in other disorders of PCD and mouse spermatogenesis. I therefore decided to perform a pilot study with 293 cells overexpressing Flag-tagged ARMC4 to determine the cytoplasmic and nuclear localization of p65 and ARMC4. As seen in **Figure 20**, I observed a mostly cytoplasmic localization of ARMC4 as indicated by both Flag and ARMC4 expression. IL-1 $\beta$  treatment stimulated translocation of p65 to the nucleus in 293 and 293 ARMC4 overexpression cells as anticipated. However, in 293 ARMC4 cells p65 expression trended towards a slight decrease in the translocation to the nucleus when stimulated by IL-1 $\beta$  in comparison to the 293 parental cell condition. These

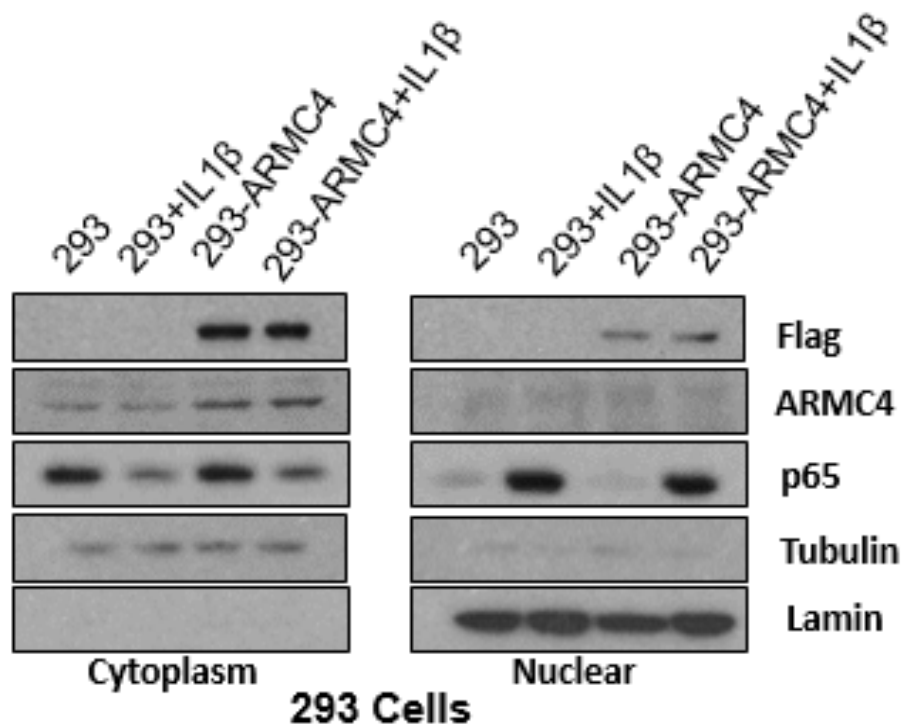


**Figure 18.** ARMC4 expression does not significantly affect IκBα degradation. Western Blot showed similar pattern in the levels of total IκBα in colon cancer HT29, DLD1 and HCT116 cells across vector-control, shscramble, ARMC4 overexpression and shARMC4 knockdown. B-actin was used as a loading control. N=3.



**Figure 19.** ARMC4 potentially complexes with p65 in CRC

Co-immunoprecipitation experiments in HEK293 and HT29 representative cell line pulled down by Flag antibody and probed with p65 antibody. Cells were treated or untreated with IL-1 $\beta$  for 1h to stimulate the NF- $\kappa$ B pathway. Flag-ARMC4 was then pulled down with Flag antibody. Samples were then subjected to Western analysis and probed with p65 antibody. ARMC4 was pulled down with and without NF- $\kappa$ B activation by IL-1 $\beta$ . Anti-Flag antibody was used to determine the input of Flag-tagged ARMC4. N=3.



**Figure 20** ARMC4 exhibits a mostly cytoplasmic localization in HEK293 cells and potentially holds p65 in the cytoplasm  
 Cell fractionation experiment, showing localization of HEK293 nuclei cells parental and overexpressing Flag-tagged ARMC4 to both the cytoplasm and nucleus. IL-1 $\beta$  induced translocation of p65 from the cytoplasm to the nucleus. ARMC4 localized mostly to the cytoplasm and ARMC4 overexpression reduced translocation of p65 to the nucleus. Tubulin and lamin were used as loading controls for cytoplasmic and nuclear fractions, respectively. N=1.

data suggest there may be some interaction, sequestration or other phenomena reducing the p65 translocation in 293 cells overexpressing ARMC4. This is a potential exciting way in which ARMC4 may regulate NF- $\kappa$ B signaling and therefore regulate NF- $\kappa$ B target gene expression.

### **4.3 Concluding Remarks**

Collectively, potential mechanisms by which ARMC4 regulates NF- $\kappa$ B activity remain largely undiscovered. My data here shows the beginning of understanding of these mechanisms. I have shown here that NF- $\kappa$ B target gene expression can be regulated by ARMC4 as well as ARMC4 may act to regulate NF- $\kappa$ B activity through an autocrine fashion. I have also determined there is a potential interaction between the p65 subunit of NF- $\kappa$ B and ARMC4 downstream of I $\kappa$ B $\alpha$  and there may be regulation of NF- $\kappa$ B activity through sequestration or lagging of p65 transport to the nucleus. In order to confirm this several other experiments may be done in the future including looking at the promoter level of NF- $\kappa$ B target genes in cells overexpressing and knockdown of ARMC4 as well as confirm this phenomenon of potential co-localization of ARMC4 and p65 in cancer cell lines. While many more questions remain regarding the potential mechanisms of ARMC4-mediated NF- $\kappa$ B activity regulation there is potential for future studies into the regulation of ARMC4. These experiments give me a good foundation to start building the story of the mechanisms of action of ARMC4-mediated regulation of NF- $\kappa$ B signaling. With the work from the previous chapter and this chapter I have a solid basis for further study and characterization of ARMC4's functions in CRC.

## CHAPTER 5. DISCUSSION

### 5.1 Summary of Findings and Discussion

As summarized in **Figure 21** based on our preliminary work and data from **chapters 3 and 4** I hypothesize ARMC4 acts as a tumor suppressing factor in CRC.

#### 5.1.1 Current therapeutic limitations in CRC treatment

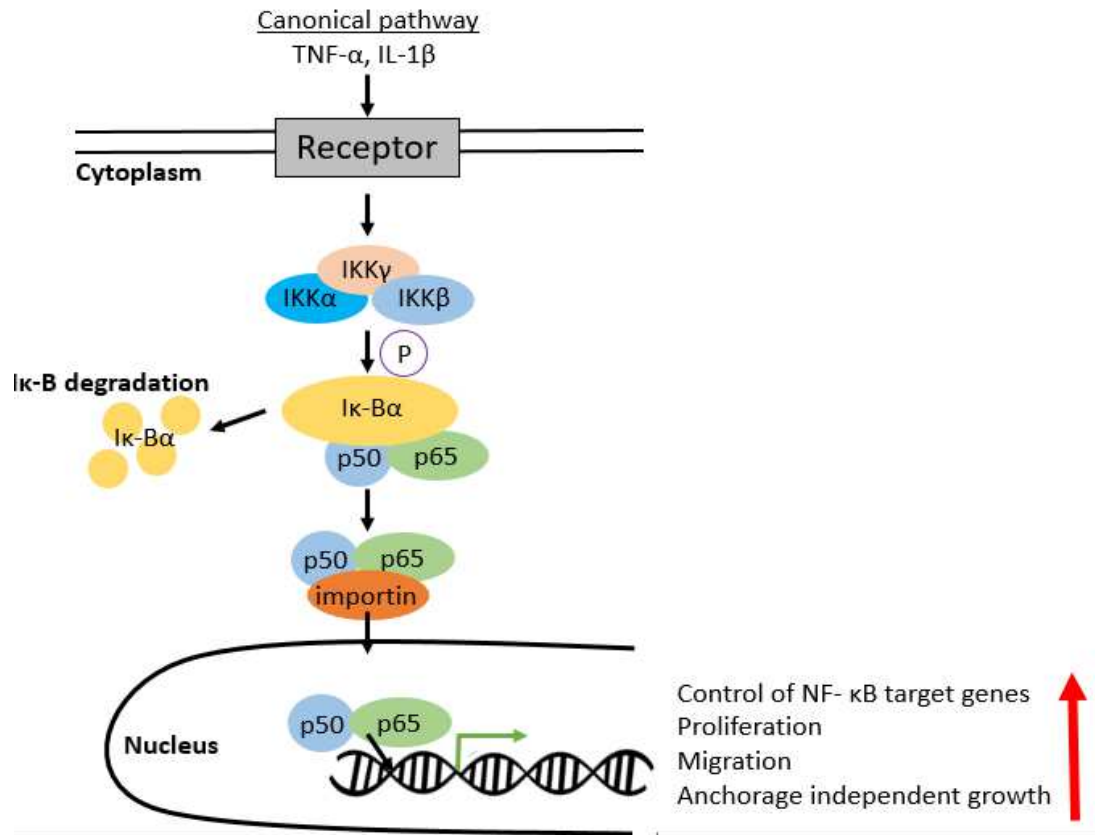
There are about 155,000 new cases of CRC expected in 2019 in the United States and one third of those patients will die from CRC or complications with treatment of CRC (Alteri 2019). It is a grim reality that the number of cases of CRC has been rising and will continue to rise in the foreseeable future. The largest problem associated with treatment of CRC arises from metastatic CRC. Later stages of CRC exhibit low survival rates due to high amounts of chemoresistance and ineffectiveness of radiation and surgery (Dallas 2009). Additionally, disease recurrence can be particularly prevalent in cancers originating from sporadic CRC due to the nature of sporadic CRC arising from a series of somatic mutations. These hard facts can mean there is not a lot of hope for patients who are diagnosed with metastatic CRC.

Besides these problems there are also significant issues with currently used therapeutic treatments for metastatic CRC (Dy, 2009, Hurwitz 2004). Standard care for patients prescribed therapeutics in later stages of CRC are usually FOLFOX which was described in **chapter 1**. These therapeutics can interfere with DNA synthesis pathways and also, due to their low specificity, they can have particularly devastating side effects such as fatigue, bleeding, nausea, vomiting all of which can push a patient to reduce their dosing of chemotherapy rendering the therapy much less effective. The question becomes

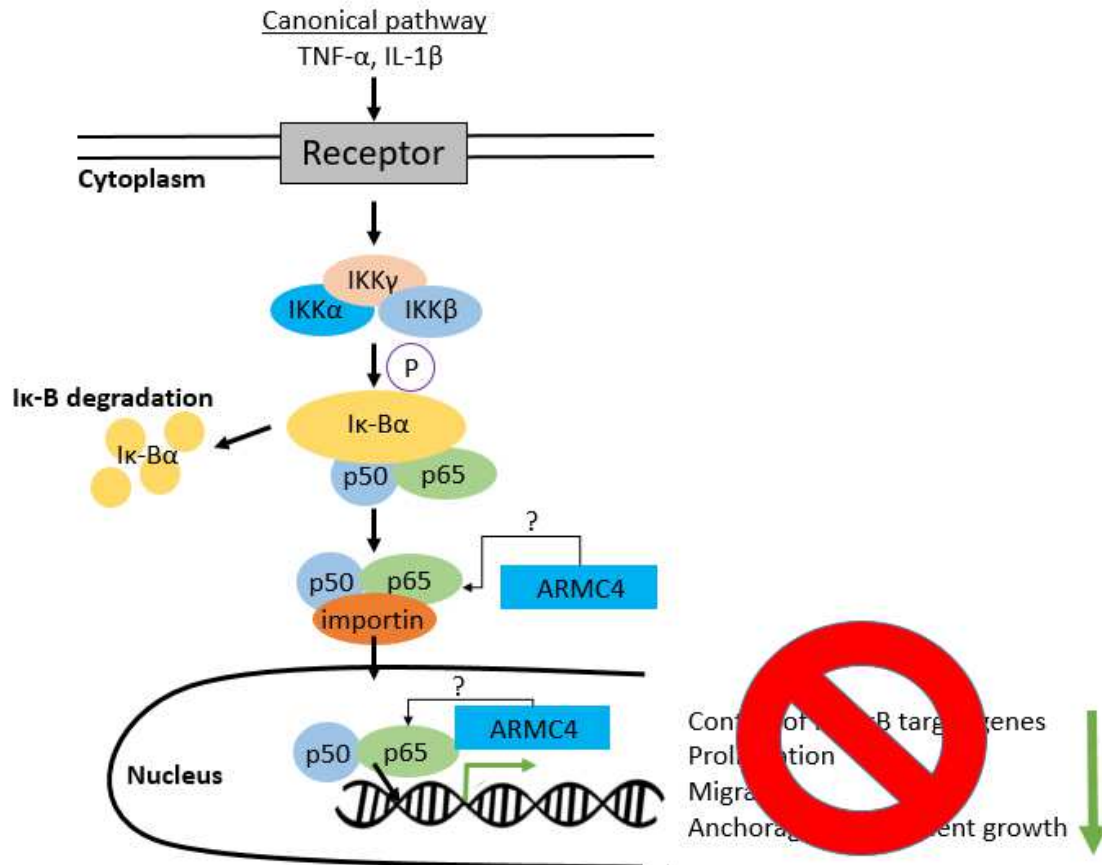
whether or not a treatment is worth the pain and suffering for a patient if the treatments themselves have their own issues and may not be very effective in treating metastatic CRC. Besides this, I must consider the overarching negative effects on the patients who must go through grueling regimens of chemotherapy. The quality of life of these patients who are treated with these drug cocktails is much lower. This is primarily due to the lack of specificity of therapies. Since current therapies are ineffective in metastatic CRC with 5-year survival rates as low as 11% in stage IV CRC, as seen in **Table 1**, it makes sense to look for other avenues of targeting CRC more specifically. Fortunately, some specific therapies for CRC have been developed. EGFR and VEGF antibodies are promising therapeutic inhibitors for treating later stages of CRC but still exhibit difficulties with targeting portions of the metastatic CRC population due to expression differences in EGFR and VEGF as well as differences in response to antibody therapy depending on the subtype of colorectal cancer. There are also known adverse side effects such as eczema with cetuximab, an EGFR inhibitor (Di Fiore, 2007 Lièvre, 2008, and Kerr 2017). Clearly more targeted therapies are needed for treatment of CRC and particularly for late stages of CRC.

When considering targeting to CRC the natural consideration is to consider whatever proteins, lipids *etc.* are in a differential state in cancer *vs.* in a normal cell. Currently, when I consider my work done here with ARMC4 the state of regulation of ARMC4 in cancer and in normal cells is still somewhat unknown. What I have shown are novel regulatory functions for ARMC4 in the context of NF- $\kappa$ B signaling and a few potential mechanisms by which this regulation of signaling occurs. However, this is in colorectal cancer cells and I accept there are several limitations in the scope of my

# A



# B



**Figure 21.** Hypothetical model of ARMC4's regulation of NF- $\kappa$ B signaling in CRC (A). Under normal CRC conditions, NF- $\kappa$ B activity is hyperactivated promoting I $\kappa$ B $\alpha$  degradation and release of NF- $\kappa$ B subunits p50 and p65 into the cytoplasm. I $\kappa$ B proteins hold NF- $\kappa$ B proteins in inactive conformations by binding in the cytoplasm and translocation to the nucleus where p65 and p50 bind to DNA and signals for increased expression of NF- $\kappa$ B target genes, resulting in upregulation of cancerous phenotypes such as proliferation, migration and anchorage-independent growth. (B). In the presence of ARMC4 an interaction with p65 downstream of I $\kappa$ B $\alpha$  degradation leads to decreased NF- $\kappa$ B target gene expression resulting in a significant decrease in cellular proliferation, migration, and anchorage-independent growth and a reduction of the cancerous phenotype.

system. Firstly, I am primarily focused on *in vitro* work and while I have some introductory *in vivo* work with NSG mouse experiments I still have a number of interesting questions to answer regarding ARMC4's functions in tissues and organisms.

One of the biggest pitfalls for therapeutic development of CRC is the heterogeneity of the disease colorectal cancer. As shown in **Figure 1**, colorectal cancer can arise from many different mutations in just the subtype of sporadic CRC. While there are familial and hereditary forms of CRC sporadic CRC is the form with the most variability in classifications. First, I can consider some of the specific hallmark mutations of CRC such as APC and TP53. While these are critical tumor suppressors, the specific mutation of only APC or only TP53 will not drive CRC to metastasis. This is why CRC is considered to have a stepwise form of cancer development. Furthermore, CRC can develop from microsite instability or chromosomal instability. All of these mutations can drive development of CRC and are not necessarily mutually exclusive. Because there is such heterogeneity in sporadic CRC development it makes sense to use cell lines for my experiments that have different genetic landscapes of mutation that resulted in the development of CRC. This is seen in the cell lines I used for my work with DLD1, HCT116 and HT29 which while they are all later stages of CRC they have specific differences in mutations. For example, DLD1 and HT29 both have a mutant form of APC which is very relevant to my interest in ARMC4 as a therapeutic target in CRC. HCT116 however has wild type APC.

In my experiments described in this work I saw similar trends among DLD1, HCT116, and HT29 in relation to the effects on hallmarks of cancer when I overexpressed or knocked down ARMC4. This data is heartening as these effects are seen

regardless of the mutational background of my three CRC cell lines. This also suggests regulation of NF- $\kappa$ B signaling by ARMC4 may be APC independent. However, to confirm this I may in the future need to determine APC expression in my different cell lines where I have overexpressed and knocked down ARMC4. The structural similarities of ARMC4 with APC could suggest its role in regulation of cell signaling and while there has been crosstalk of NF- $\kappa$ B and Wnt shown (Baghi, 2017) there may be other potential causes for such similar results in my three cell lines when looking at things such as proliferation, migration, and tumor growth. All of these data together suggest molecules regulating ARMC4 as potential attractive therapeutic targets in CRC. However, there are several caveats and potential pitfalls in targeting ARMC4 which I will describe in the next sections.

#### 5.1.2 ARMC4 knockdown leads to CRC cancerous phenotypes via NF- $\kappa$ B activation

Negative regulators such as APC and p53 have been previously shown to have critical roles in progression and modulation of the cancerous phenotype in CRC (Powell, 1992 and Michaloglou, 2005). Additionally, constitutive NF- $\kappa$ B activation has been well characterized to aggressively promote CRC cancerous phenotypes and chemotherapeutic resistance (Sussman, 2007). It is therefore unsurprising that my identification of ARMC4 as a novel negative regulator of NF- $\kappa$ B is a potentially interesting and novel therapeutic target for CRC as well as a prognostic biomarker. Identification of novel factors in NF- $\kappa$ B signaling is critical for my overall understanding of CRC cell signaling as well as for better understanding of the complex signaling network of NF- $\kappa$ B. Based on my preliminary data regarding ARMC4's effect on DNA binding ability and activity of Z3

cells (described in **chapter 1**), which have constitutive NF- $\kappa$ B activity, I hypothesized ARMC4 overexpression could mediate NF- $\kappa$ B signaling and reduce the cancerous phenotype by acting as a tumor suppressor.

In this thesis, I showed that ARMC4 expression was significantly downregulated in CRC cell lines as well as tumor tissue from patients. To determine ARMC4's effect on NF- $\kappa$ B activity, I conducted luciferase assays with a consensus binding sequence for NF- $\kappa$ B located upstream of the luciferase reporter gene and showed that ARMC4 indeed promoted NF- $\kappa$ B activation in CRC. Decreased expression of ARMC4 in cancer cells also led to diminished cancer-promoting characteristics, including cell proliferation, anchorage-independent growth and cell migration, clearly highlighting the role of ARMC4 as a tumor suppressor in CRC. This work is described in greater detail in **chapter 3**. Overall, these studies emphasize the significance of ARMC4-mediated NF- $\kappa$ B activation resulting in reduction of the cancerous phenotype in CRC.

Some of the experiments described in **chapter 3** do have a few caveats involved. For instance, in my cell proliferation experiments I saw differences in proliferation, but the differences were observed after 5 to 7 days of growth. There could be several potential reasons for this. For example, ARMC4 regulation of NF- $\kappa$ B activity could be lagging proliferation and not preventing it. Also, ARMC4 is most likely not the sole regulator of proliferative ability of CRC cells. ARMC4 regulates several different NF- $\kappa$ B target genes but the exact functions of all of those target genes have not been studied in great detail. So far, representative NF- $\kappa$ B target genes regulated by ARMC4 expression seem to have more relation to migratory ability. To confirm proliferation differences were not due to differences in health of the cells I will also consider in the future using

cell death specific dyes such as methylene blue. My migration assay in **chapter 3** may also have the same caveats as I did not initially determine if there were differences in cell death of the cell lines when plated in the Boyden chamber assay. I will therefore consider doing a similar dye staining of methylene blue of the cells at 48 hours which is the same time in which the cells have migrated and determine the number of living and dead cells on each side of the membrane which are with and without the nutrition of FBS.

While ARMC4 appears to have an important role in NF- $\kappa$ B signaling, it is possible ARMC4 mediated signaling can be much more complicated than activating one signaling pathway. While ARMC4 is not known to be involved in other signaling pathways, NF- $\kappa$ B signaling can activate other pathways. NF- $\kappa$ B is known to crosstalk with the Wnt/ $\beta$ -catenin pathway in particular (Karimaian, 2017). I therefore acknowledge the possibility of other potential signaling through NF- $\kappa$ B when regulated by ARMC4 or by ARMC4 itself. The similarities between ARMC4 and APC also suggest potential binding partners for ARMC4 in the Wnt signaling pathway. This will need to be considered in future experiments whether ARMC4 is solely regulating NF- $\kappa$ B activity or is also modulating other signaling pathways.

Even though much regarding cell signaling of ARMC4 and NF- $\kappa$ B is not fully clear I feel the insights provided by this study are highly novel in understanding a previously unknown function of ARMC4 and a previously unknown regulatory mechanism for NF- $\kappa$ B.

### 5.1.3 ARMC4 as a potential therapeutic target and a prognostic biomarker for CRC

Almost all research regarding ARMC4 has been done *in vitro* with the exception of some work in flies and mice in regard to ARMC4's role in mouse spermatogenesis (Chenge, 2013). These are also studies done in disease states in one form or the other (Onoufriadis, 2013). Interestingly, there has been some evidence demonstrating high ARMC4 expression levels in liver, testes, and brain of male mice with a much lower expression in female mice (Wang, 2014). This may also relate to ARMC4's normal functions in mouse spermatogenesis. However, the impact of higher expression levels of ARMC4 in other sections of the mouse body is not yet understood.

The relatively understudied protein ARMC4 brings several questions to mind. What is ARMC4's function in normal cells? How is it regulated? Is it regulated differently in disease states such as cancer? Of interest to me is when I consider whether ARMC4 is upregulated, downregulated or alternatively regulated through cancer-specific mechanisms. In theory, I can modulate ARMC4's functions in NF- $\kappa$ B signaling. Here is where more studies of ARMC4's functions remain. I do not know the functions of ARMC4 in normal cell. Therefore, it also is sensible to consider upstream or downstream regulators of ARMC4 signaling to indirectly target the CRC NF- $\kappa$ B signaling. My studies suggest in **Chapter 4** that interactions between ARMC4 and NF- $\kappa$ B are occurring downstream of IKK degradation. While I indeed have seen differences in NF- $\kappa$ B target gene expression in **Chapter 4** when I overexpress ARMC4 I are still unable to answer what is modulating ARMC4's functions.

In terms of ARMC4 as a feasible therapeutic target in CRC it may make more sense to further investigate the mechanisms by which ARMC4 expression is regulated.

Theoretically, if I identify a regulator of ARMC4, I can indirectly regulate NF- $\kappa$ B activity and indirectly reduce the cancerous phenotype. Another question is how ARMC4 would be regulated. It is possible there are differences in degradation or synthesis of ARMC4 in the normal versus the CRC cancerous states. I am quite interested in regulation of ARMC4 expression as I identified decreased ARMC4 expression in CRC cell lines and in tumors compared to normal cells and tissues (**chapter 1**). What exactly causes ARMC4 expression to decrease is a question I am considering for future research. If solely decreased ARMC4 expression can promote a cancerous phenotype as suggested by my shARMC4 data then it seems logical to pursue the regulator of ARMC4 as a potential biomarker for CRC severity. This is a potentially interesting problem as it indicates mutant or normal ARMC4 regulation could be an important factor in the known disorder PCD and other disorders. This then leads into a different issue for targeting ARMC4 in CRC. Specificity. If I inhibit NF- $\kappa$ B activity what are the potential off-target effects of inhibiting NF- $\kappa$ B signaling? As described in **chapter 1**, NF- $\kappa$ B has critical roles in inflammation and the tumor microenvironment. I also showed in **chapter 4** that when HEK293 cells were treated with conditioned media from my cells overexpressing ARMC4 there was a reduction in NF- $\kappa$ B activity as shown through a luciferase assay. This is suggesting ARMC4 may regulate secretion of cytokines into the microenvironment. This can be studied in further detail by using the tumors and tissues I collected from the NSG mouse experiments in **chapter 3**. Currently I have only shown *in vitro* that there are inhibitory effects on the microenvironment when ARMC4 is upregulated but IHC analysis of the mouse tumors may give me a better idea of ARMC4's effects on the microenvironment and immune response in CRC. I also will

consider looking at immune cell infiltration of tumors with ARMC4 and inflammatory cytokine expression. Together these data will better help me understand ARMC4's potential regulation of the tumor microenvironment.

ARMC4 expression is not just low in colon cancers as I showed in my TMA in **chapter 1**. Breast cancer and lung cancer exhibit lower expression levels of ARMC4 in cancers when compared to normal tissues (Chen, 2013 and Xu, 2019). Due to the commonality of low expression of ARMC4 levels in some cancers my current work could have broader impacts on the understanding of cancer progression as a whole and the role of ARMC4 in cancer.

There are some considerations that are important if I wish to potentially pursue ARMC4 or a regulator of ARMC4 as a novel therapeutic target in CRC. ARMC4 as a potential therapeutic target has several potential difficulties for treatment as well as unknown interactions. Previous work on ARMC4 has identified specific point mutations as regulators of its function in the heart and in mouse sperm (Hjiej 2013, Onoufriadis 2013). However, specific mutations in sporadic CRC are extremely infrequent and usually somatic mutations (**Table 2**). **Table 2** is showing all mutations that have been identified at least once in patients with sporadic CRC. Furthermore, the exact functional significance of the mutations is unknown as the structure of ARMC4 is not known. Since the crystal structure of ARMC4 has not been determined the secondary structure is a theoretical mass of  $\beta$ -helices purported to form into a superhelical structure allowing its interaction with other proteins. Therefore, knowing specific mutations will only give a theoretical idea of how a specific mutation could affect the secondary structure of ARMC4. This then begs the question if there are potential mutations of ARMC4 in

**Table 2** Currently known ARMC4 mutations in CRC

<b>N914H</b>	<b>Missense</b>
<b>G395E</b>	<b>Missense</b>
<b>R340C</b>	<b>Missense</b>
<b>E387*</b>	<b>Nonsense</b>
<b>V498M</b>	<b>Missense</b>
<b>E913K</b>	<b>Missense</b>
<b>D911Y</b>	<b>Missense</b>
<b>A150T</b>	<b>Missense</b>

cancer that can affect the functions of ARMC4. Clearly in the case of other disorders of PCD and spermatogenesis this is the cause wherein mutant ARMC4 is unable to bind tubulin preventing its function (Onoufriadis, 2013). However, it is not clear what the potential is for mutant ARMC4 to cause dysfunction in cancerous ARMC4-mediated NF- $\kappa$ B activity. Furthermore, specific mutations of ARMC4 have unknown effects on ARMC4's normal function. Together these data suggest there may be potential problems or considerations if I aim to therapeutically regulate ARMC4-mediated NF- $\kappa$ B activity.

#### 5.1.4 ARMC4 interacts with NF- $\kappa$ B through several potential mechanisms

In my studies in **chapter 4** I have shown a few potential mechanisms by which ARMC4 can interact with and potentially regulate NF- $\kappa$ B signaling. An Illumina microarray showed ARMC4 can promote specific NF- $\kappa$ B target gene expression. There is a small caveat to this approach in that the microarray used is a plate with well-characterized or well-studied NF- $\kappa$ B target genes. This may then bias me towards looking at only well-known target genes and is a less random approach to other methodology such as a traditional RNA-seq which would be a more discover-based approach. qPCR analysis showed that ARMC4-mediated NF- $\kappa$ B activation decreased the expression of NF- $\kappa$ B's well-known downstream target genes, indicative of the critical role of ARMC4 in NF- $\kappa$ B activity in CRC. IKK degradation indicated ARMC4 may be regulating NF- $\kappa$ B downstream of I $\kappa$ B $\alpha$  degradation. Additionally, I have shown ARMC4 can interact with the p65 subunit of NF- $\kappa$ B and potentially regulate translocation of p65 to the nucleus. I acknowledge that so far little has been studied regarding ARMC4's regulation. Because of this, many potential avenues are available for study but based on my current work I think it is most feasible to continue to study the cellular localization of

ARMC4 and p65 as well as the promoter occupancy of p65 at known NF- $\kappa$ B target genes. Additionally, since so little is known in regards to the structure-function relationship of ARMC4 it is important to consider attempting to disrupt the secondary structure of ARMC4 in future studies and consider the impact this will have on NF- $\kappa$ B signaling as well as on the potential DNA binding ability of p65 in the presence of mutant ARMC4. So far, my studies of the mechanism of ARMC4 and NF- $\kappa$ B's interaction have not been fully realized but in the future, it may be important to pin down what regulates ARMC4 expression and therefore can regulate NF- $\kappa$ B signaling.

## **5.2 Key Points for Consideration**

In this work, I highlighted a novel mechanism by which ARMC4 overexpression suppressed NF- $\kappa$ B activation and target gene expression which affected cell proliferation, migration, and anchorage independent growth of cancer cells. This is a novel therapeutic target and in fact a novel role for ARMC4 in cancer. Overall, I did not encounter any major hurdles while performing the work described in this thesis. However, some of these results have inherent pitfalls or concerns that have not been described yet in detail. In some of my early work I had not yet generated a scramble control for each of my CRC cell lines. Because of this, my comparisons to my common vector control in my cell proliferation data, migration, anchorage-independent growth and tumor growth data in **chapter 2** are only in comparison to the vector control. Later studies showed no difference in comparison between shscramble and vector control cells in terms of proliferation. My ARMC4 gene was cloned into a lentiviral vector which is a similar lentiviral vector as the shRNAs constructs I used. I would then expect similar phenotypes

in my shscramble and vector control conditions. Despite this in terms of using more proper controls, in the future, the shscramble control will be included alongside any experiments that include the shARMC4 cell line as an appropriate control though both control vectors are very similar and went under the same puromycin selection process. Besides these concerns, I used NSG mice, which are a commonly used immunodeficient strain that lacks mature T cells, B cells, and NK cells. This is a valuable tool for quick tumor growth in a xenograft model as it is simple to grow a xenograft in an immunodeficient model. Unfortunately, the lack of a functioning immune system means there is not a comparable microenvironment as compared to other potential models which means the tumor that grows is not pressured by the immune system. This may be particularly critical for my future studies as in CRC there are well known roles for NF- $\kappa$ B regulation of inflammation and the immune response. Some of the immune response is lacking from the NSG immunodeficient mice. In the future if the tumor growth of ARMC4 overexpressed or deficient tumors will be further pursued, I will plan to identify immunocompetent models of CRC that can be chemically induced, or genetically engineered as more accurate representations of the immune microenvironment when compared to NSG mice.

### **5.3 Future Directions**

The extremely interesting and novel work presented in this study highlights the critical role of ARMC4 in regulation of NF- $\kappa$ B activation and the hallmarks of cancer in CRC (**chapter 3**) as well as novel mechanisms by which ARMC4 regulates NF- $\kappa$ B

signaling (**chapter 4**). Most importantly this work opens many potential avenues of research in this field.

In terms of my immediate goals I wish to focus on potential mechanistic regulation of NF- $\kappa$ B by ARMC4 in CRC by further studies of cell fractionation with CRC cell lines overexpressing and knockdown of ARMC4. I also have not explored how ARMC4 knockdown can affect potential sequestration of p65. I want to determine if ARMC4 expression can change the promoter occupancy of known NF- $\kappa$ B target genes. Other potential mechanisms of interaction between ARMC4 and NF- $\kappa$ B may lie in protein-protein interactions. To study this, I aim to perform truncation experiments of ARMC4 at purported turns of its supposed superhelical structure. I aim to determine if the full structure of ARMC4 is necessary for its function by generating truncated versions of ARMC4 and determining if truncating ARMC4 will affect its ability to inhibit NF- $\kappa$ B activity and potentially DNA binding ability.

Furthermore, I want to determine the mutational status of ARMC4 in my cell lines and patient samples. This will give me a better grasp of the frequency of ARMC4 mutations in the context of cancer. I will also be able to determine expression levels of ARMC4 in tissue samples in addition to cell lines and determine if the mutational status of ARMC4 can affect the expression level of ARMC4. I have investigated a few different databases regarding ARMC4 expression in normal tissues and I have identified a higher protein expression of ARMC4 in normal tissue of the colon, lung, endocrine, and male and female tissues as well as in muscle. I also have identified expression of ARMC4 in breast, prostate, lung and liver cancers. I have not yet identified if the expression of ARMC4 is decreased in those cancers and have only identified the presence of ARMC4

protein expression. These data suggest potential interesting functions for ARMC4 in multiple tissues and cancer types. Also, some of this data is in line with the currently known functions of ARMC4 from previous studies in PCD and spermatogenesis in respect to known lung and testes functions for ARMC4. However, these data also suggest potential for off-target effects of a potential pan-ARM4 regulator protein inhibitor. As ARMC4 has known critical roles in other systems than the colon it is very important to consider targeting to ARMC4 in relation to CRC specifically before considering targeting to other cancers. I will also consider current therapeutic strategies used in treatment of known tumor suppressors in CRC such as p53 and APC to better target ARMC4 therapeutically.

One critical consideration is the potential for ARMC4 to be involved in crosstalk with other signaling pathways than NF- $\kappa$ B, in particular in the Wnt/ $\beta$ -catenin signaling pathway. It would be interesting to see if the structural similarities of ARMC4 and APC allow ARMC4 to inhibit Wnt signaling in addition to NF- $\kappa$ B signaling.

Lastly, the therapeutic role of ARMC4 may be extended beyond CRC. More research into ARMC4 functions in cancer will also give me more understanding of ARMC4's functions in normal cells. ARMC4's functions in normal cells are also relatively unknown so understanding of those functions may be applicable in other ARMC4-related disease states. Furthermore, NF- $\kappa$ B is constitutively activated in a many cancers and ARMC4 expression is decreased in several of those cancers as well. While I cannot directly correlate ARMC4 expression to cancer progression it will be interesting to consider ARMC4 as a potential biomarker for CRC and other cancers' progression. While I have discussed the importance of stepwise development of CRC in great detail, I

have not considered if ARMC4 expression is protective of CRC development as has been shown to be the case in several models of APC knockout of CRC. I may then consider knocking out ARMC4 expression in a mouse model and determine if ARMC4 expression is protective against tumor development. In the end, my overarching goal and the importance of these current and future studies of ARMC4 are to better understand cancer cell signaling and tumor suppressor functions so that I can reduce the cancer burden of CRC and provide aid to those who are suffering from CRC.

**Appendix A. List of qPCR Primers**

<b>Primer number</b>	<b>Primer identification</b>	<b>sequence</b>
<b>P1</b>	<b>GAPDH-F 326(Max)</b>	<b>CCATCACCATCTTCCAGGAGCG</b>
<b>P2</b>	<b>GAPDHR 468(Max)</b>	<b>AGAGATGATGACCCTTTTGGC</b>
<b>P114</b>	<b>VCAM1-F1 1550</b>	<b>TGGTCAGCCCTTCCTCCAT</b>
<b>P115</b>	<b>VCAM1-R1 1648</b>	<b>GCTGCCTGCTCCACAGGAT</b>
<b>P122</b>	<b>MAPK15-F1 459</b>	<b>GGACCAGAAGCCGTCCAAT</b>
<b>P123</b>	<b>MAPK15-R1 523</b>	<b>GGCCAAAGTCACACAGCTTCA</b>

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# CURRICULUM VITAE

Matthew Peter Martin

## EDUCATION

2014-2020 Doctor of Philosophy in Pharmacology

Indiana University

Indianapolis, IN

Mentor: Tao Lu, Ph.D.

2010-2014 MS, BS. Biology

Purdue University

West Lafayette, IN

## PROFESSIONAL EXPERIENCE

5/2013-8/2012, 5/2013-7/2013 **Student Intern**

Stark Neuroscience Research Institute

Mentor Andy Hudmon, Ph.D.

2014-2020 **Graduate Research Assistant**

Indiana University School of Medicine, Indianapolis, IN

Department of Pharmacology and Toxicology

## PEER-REVIEWED PUBLICATIONS

1. **Martin, M.**, Hua, L., Wang, B., Wei, H., Prabhu, L., Hartley, A. V., ... & Lu, T. (2017). Novel Serine 176 phosphorylation of YBX1 activates NF- $\kappa$ B in colon cancer. *Journal of Biological Chemistry*, 292(8), 3433-3444.

2. Prabhu, L., Mundade, R., Wang, B., Wei, H., Hartley, A. V., **Martin, M.**, ... & Lu, T. (2015). Critical role of phosphorylation of serine 165 of YBX1 on the activation of NF- $\kappa$ B in colon cancer. *Oncotarget*, 6(30), 29396.

3. Ashpole, N. M., Chawla, A. R., **Martin, M. P.**, Brustovetsky, T., Brustovetsky, N., & Hudmon, A. (2013). Loss of calcium/calmodulin-dependent protein kinase II activity in cortical astrocytes decreases glutamate uptake and induces neurotoxic release of ATP. *Journal of Biological Chemistry*, 288(20), 14599-14611.

### **REVIEW ARTICLES**

1. Jiamin, J., **Martin, M.**, Lu, T. (2019). PRMTs and miRNAs, functional cooperation in cancer and other biological conditions. *Cell Cycle*.

2. **Martin, M.**, Wei, H., & Lu, T. (2016). Targeting microenvironment in cancer therapeutics. *Oncotarget*, 7(32), 52575.

3. Prabhu, L., Hartley, A. V., **Martin, M.**, Warsame, F., Sun, E., & Lu, T. (2015). Role of post-translational modification of the Y box binding protein 1 in human cancers. *Genes & Diseases*, 2(3), 240-246.

### **BOOK CHAPTERS**

1. Hartley, A.V., **Martin, M.**, Lu, T. (2019) Epigenetic biomarkers and their therapeutic applications in colorectal cancer. In: *Advances in the Molecular Understanding of Colorectal Cancer*. InTech.

2. Hartley, A. V., **Martin, M.**, & Lu, T. (2017). Aging: Cancer—an unlikely couple. *Aging (Albany NY)*, 9(9), 1949.

3. **Martin, M.**, Hartley, A.V., Lu, T.. (2018) Colorectal Cancer Therapeutics: Present and the Future. In: *Cancer Therapeutics*. Hyderabad, India: Avid Science.; 2-32. ISBN:978-93-86337-78-8.

4. Wei, H., Prabhu, L., Hartley, A., **Martin, M.**, Sun, E., Jiang, G., Liu, Y., & Lu, T. (2017). Methylation of NF- $\kappa$ B and its role in gene regulation. Gene expression and regulation in mammalian cells, InTechOpen Publishing.

5. Hartley, A., Wei, H., Prabhu, L., **Martin, M.**, & Lu, T. (2017). NF- $\kappa$ B: Its Role in Colorectal Cancer. In *Role of Transcription Factors in Gastrointestinal Malignancies* (pp. 247-260). Springer, Singapore.

### **PRESENTATIONS**

1. **Martin, M.**, Hua, L., Wang, B., Wei, H., Prabhu, L., Hartley, A. V., ... & Lu, T. (2017). Novel Serine 176 phosphorylation of YBX1 activates NF- $\kappa$ B in colon cancer. *Biochemistry Research Day, Indianapolis, IN 2017*.

2. **Martin, M.**, Hua, L., Wang, B., Wei, H., Prabhu, L., Hartley, A. V., ... & Lu, T. (2017). Novel Serine 176 phosphorylation of YBX1 activates NF- $\kappa$ B in colon cancer. Poster Presentation. Indiana University School of Medicine Poster Showcase, Indianapolis, IN, 2017.

3. **Martin, M.**, Mundade, R, Lu, T., (2018). Adenomatous Polyposis Coli like protein (APCLP) functions as a novel negative regulator of NF- $\kappa$ B signaling in colon cancer cells. Poster Presentation. American Association of Cancer Research, Chicago, IL, 2018.

4, **Martin, M.**, Mundade, R, Lu, T., (2018). Adenomatous Polyposis Coli like protein (APCLP) functions as a novel negative regulator of NF- $\kappa$ B signaling in colon cancer cells. Poster Presentation. IU Simon Cancer Center Annual Research Day, Indianapolis, IN 2018.

5. **Martin, M.**, Mundade, R, Lu, T., (2019). Adenomatous Polyposis Coli like protein (APCLP) functions as a novel negative regulator of NF- $\kappa$ B signaling in colon cancer cells. Poster Presentation. ASBMB Annual Meeting, Orlando, Fl 2019.

### **HONORS/AWARDS**

Paradise Travel Award Spring 2019

IUPUI travel award Spring 2019

Paradise Travel Award Spring 2018

### **SERVICE**

Vice President of Membership- Communicators at IUPUI, Toastmasters International  
*September 2015-2017*

Secretary- Communicators at IUPUI, Toastmasters International  
*September 2018-present*

Boy Scouts of America – *Eagle Scout*  
*September 2009*