Tumor Suppressor APC and Musashi1: Double-Negative Feedback, Wnt Signaling and Colon Cancer

By

Erick Spears

Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

Chairperson Kristi L. Neufeld

_____

Yoshiaki Azuma

_____

Robert S. Cohen

_____

T. Christopher Gamblin

_____

Linheng Li

_____

Robert E. Ward

Date Defended: July 14, 2011

The Dissertation Committee for Erick Spears

certifies that this is the approved version of the following dissertation:

Tumor Suppressor APC and Musashi1: Double-Negative Feedback, Wnt Signaling and Colon Cancer

_____

Chairperson Kristi L. Neufeld

Date approved: July 14, 2011

Abstract

Colorectal cancer is the second leading cause of cancer related deaths in the United States. Approximately 80% of all colon cancers are associated with a mutation in the Adenomatous polyposis coli (*APC*) tumor suppressor gene. We study the intracellular functions of normal APC and how loss of APC function leads to the formation of precancerous polyps in the intestines of mice and humans. The studies presented here are particularly concerned with the role of APC in maintenance of homeostasis in the intestinal epithelium. I have identified a double-negative feedback loop between APC and a sequence specific RNA binding protein, Mushashi-1 (MSI1). I hypothesize that this feedback loop serves to maintain a critical balance and that disruption of this balance leads to loss of homeostasis and ultimately tumorigenesis in the intestinal epithelium. Studies described here and by others have shown that MSI1 is a target of β-catenin transcriptional activation through the canonical Wnt signaling pathway. My work has demonstrated that *APC* mRNA is a target of MSI1 binding and translational repression. Further study has indicated that MSI1 binding also results in stabilization of the *APC* mRNA, presumably as a rapid response mechanism to repress Wnt signal transduction once the signal is no longer present. Finally, my studies indicate that *MSI1* expression is stimulated by the transcription factor c-MYC, the most commonly deregulated human oncoprotein. Though overexpression of c-*MYC* stimulates *MSI1* expression, c-*MYC* expression is not stimulated by loss of APC in our cell culture system. The implication is that c-MYC stimulation of *MSI1* expression may only occur in tumors with deregulated c-*MYC* expression. The studies presented here have uncovered novel molecular mechanisms involved in regulation of the canonical Wnt signaling pathway in the intestinal epithelium, thereby expanding the potential methods by which deregulation of this pathway contributes to colorectal carcinogenesis.

Acknowledgements

I would like to thank my graduate advisor Dr. Kristi Neufeld for dedicating the necessary time and resources needed for the successful completion of the research presented in this dissertation. Thanks also go to the fellow graduate students in the Neufeld lab, Maged Zein El Din and Amanda Ernlund, as well as former graduate students, Yang Wang and Jamie Cunningham, for their critical input on experiments. Former lab members Dr. Kimberly Honeycutt, Lauren Baehner and Shane Stecklein participated directly in early experiments on APC and MSI1. Technician William McGuinness also provided support for this project. My graduate advisory committee, consisting of Dr. Yoshiaki Azuma, Dr. Robert Cohen, Dr. Christopher Gamblin, Dr. Linheng Li and Dr. Robert Ward, offered a great deal of support for my dissertation research as well as other aspects of my graduate studies.

Reagents for these studies were graciously provided by many researchers around the world. Dr. Hideyuki Okano provided the FlagMsi1 expression constructs, the Numb 3'UTR luciferase reporter construct, and most recently, the Msi1 knock-out mice. Dr. Bert Vogelstein provided the HCT116βw cells that were used for the bulk of these experiments. The TOPFlash reporter construct was provided by Dr. Randall Moon and the LEF-1 expression construct was provided by Dr. Rudolf Grosschedl. Local production of APC and MSI1 antibodies was overseen by Dr. Yoshiaki Azuma.

Funding for this project was provided by the Kansas Masonic Cancer Research Institute, the Kansas IDeA Network of Biomedical Research Excellence, and the National Institutes and Health (NIH R01 CA10922-01). Personal stipends were funded by University of Kansas Graduate Teaching Assistantships, Graduate Research Assistantships, the National Science Foundation

Graduate STEM Fellows in K-12 Education (GK-12) Program, and the William King Candlin Memorial Fellowship from the Department of Molecular Biosciences at the University of Kansas.

Finally, I must offer thanks to the faculty, staff and students of the University of Kansas, Department of Molecular Biosciences for their support and camaraderie during my graduate studies.

This work is dedicated to my family. My beautiful wife Sheri and my children, Jacob, Ashley and Tyler, have been extremely supportive during my graduate studies.

Table of Contents

Chapter 1

Introduction

Colorectal Cancer

Colorectal cancer is the second leading cause of cancer related death in the United States. There has been significant progress made in the past thirty years regarding the successful treatment of colorectal malignancies. In the mid-1970s the overall 5-year survival rate was approximately 50%, but by 2008 this rate had risen to greater than 65% [1]. Though the post-diagnostic survival has increased, there is still significant morbidity associated with treatment. The standard treatment for colorectal cancer is surgical resection of the tumor followed by adjuvant chemotherapy [2]. Surgical outcomes range from almost complete recovery of normal bowel function to complete colectomy depending upon site and severity of the disease. Typical chemotherapeutics are toxic to both normal cells and cancer cells. Even with recent developments in molecular screening and more personalized chemotherapy regimens, a significant number of patients will have recurrent disease [3].

Along with increasing success in the treatment of colorectal cancer, scientists have made progress in understanding the development of colorectal tumors [4, 5], with recent insight regarding the cellular and molecular mechanisms of intestinal epithelial morphogenesis. Of particular importance is the identification of stem/progenitor cells of the intestinal epithelium [6-8]. There is still much to be learned about the development of normal intestinal epithelium from stem/progenitor cells to mature absorptive and secretory cells. Understanding the molecular mechanisms involved in this development will help us to grasp the abnormalities in these processes that ultimately lead to colorectal carcinogenesis.

Anatomy of the Intestinal Epithelium

The luminal surface of the intestine is covered by a single layer of cuboidal epithelial cells [4]. Epithelia, which cover the outside surfaces of the body, are in a constant state of mitotic turnover. Stem/progenitor cells divide asymmetrically on a regular, but relatively infrequent, basis and this

asymmetric stem cell division produces phenotypically different daughter cells [9, 10]. One daughter is a regeneration of the slow cycling stem cell, which remains in the stem cell compartment, called the stem cell niche. The stem cell niche has a specific microenvironment with specialized signals, such as opposing Wnt/bone morphogenetic protein (BMP) gradients, which maintain the multipotency of these cells [10]. The other product of an asymmetric stem cell division enters the rapidly proliferating transit amplifying (TA) cell compartment. TA cells reproduce rapidly, but remain in a relatively undifferentiated state [5]. This rapid proliferation is typically accompanied by migration away from the stem cells niche. Once TA cells reach a critical distance from the stem cell niche, where their microenvironment is dramatically different, proliferation slows and maturation occurs [5]. Fully differentiated epithelial cells have a finite life span during which they perform the functions necessary to maintain the integrity of the epithelium. The life of a differentiated epithelial cells ends with apoptosis and removal from the epithelium [10].

The intestinal epithelium is organized into sac-like structures, known as crypts, which extend away from the luminal surface (Fig. 1.1A & B). Recently, two populations of intestinal cells residing at or near the base of the crypt have been determined to be stem/progenitor cells ([6, 7]; Fig. 1.1A). The first intestinal stem cells identified were the crypt-base columnar cells (CBCs) residing between the Paneth cells at the base of the small intestinal crypt [6]. These cells were marked by the specific expression of the *Lgr5* gene and were shown to regenerate all of the differentiated cell types in the intestinal epithelium under normal growth conditions. Because these cells cycle on a regular basis in order to maintain the intestinal epithelium, these cells were classified as the “active” stem cells [10]. Shortly after the discovery of the CBCs as the active stem cells in the adult intestinal epithelium, another stem cell population with the ability to regenerate the entire intestinal epithelium was discovered. These cells reside at the +4 position of the crypt, just above the Paneth cell compartment, and are identified by the specific expression of *Bmi1* or the mouse homolog of *DCAMKL-1* [7, 8]. In contrast to the CBCs,

these cells do not cycle under normal intestinal conditions. Instead, cell cycle is activated by intestinal injury. Thus, this stem cell population has been classified as the "quiescent" stem cell population [10].

In the case of either of these two intestinal stem cell populations, asymmetric division produces the rapidly proliferating TA cells which divide and migrate up the crypt, away from the stem cell niche. There are three main intracellular signal transduction pathways involved in the division and differentiation of the intestinal epithelium: Wnt, BMP and Notch [reviewed in [4, 5]]. Briefly, opposing Wnt and BMP gradients define the epithelial stem cell niche ([10]; Figure 1.1C). In the case of the intestinal epithelium, strong Wnt signaling is required for stem cell proliferation, thus the Wnt signal is strongest at the base of the crypt [4, 6, 10]. The opposing BMP signal is most highly expressed from the stroma of the villi and thus, intracellular BMP signaling activity is highest in the more differentiated cells of the crypt that are closer to the luminal surface [5, 11]. This opposing interaction between Wnt and BMP gradients is thought to be the signal for differentiation in the intestinal epithelium [10]. The Notch signaling pathway also plays a role in development of mature intestinal epithelium [5]. Notch differs from Wnt and BMP in that the ligands for the Notch receptor are not soluble, but are cell membrane-bound [12]. Thus, Notch signaling requires cell-cell contact. In general, Notch signaling has been shown to maintain cells in an undifferentiated state [13, 14]. Specifically in the intestine, Notch signaling has been shown to influence the decision between the two major differentiated cell types in the intestinal epithelium [15, 16].

Approximately two-thirds of the way up the crypt differentiated intestinal epithelium can be detected [17]. There are two major cell lineages in the intestinal epithelium, absorptive and secretory. In the small intestine, the secretory cells include goblet cells, enteroendocrine cells and Paneth cells. The colon does not have Paneth cells. Each differentiated cell type is readily identifiable by molecular markers [4].

Mutations of genes involved in pathways leading to proliferation and differentiation result in tumor formation from the crypt compartment [4]. Colorectal cancer is an excellent model for genetic studies of cancer development. Morphological staging of colorectal cancers is highly correlated with genetic alterations that have occurred [18-20]. There are five signaling pathways that have been shown to be important for colorectal cancer development: Wnt, Ras, PI3 Kinase, TGFβ and p53 [21]. While mutations that drive the progression of tumors occur in variable order and frequency, it was recognized, even before the specific gene was identified, that mutations in the chromosomal region that contains the tumor suppressor Adenomatous Polyposis coli (*APC)* were important for initiating colorectal tumorigenesis [18, 22]. It is now known that the tumor suppressor *APC* is mutated in over 80% of all colorectal tumors and these initiating mutations result in the deregulation of the canonical Wnt signaling pathway [23, 24]. Thus, while the alterations to components of the other pathways are required for tumor progression, deregulation of Wnt signaling is the initiating event in most colorectal tumorigenesis. This underscores the importance of understanding the functional interactions of APC in the intestinal epithelium.

History of the Adenomatous Polyposis Coli gene

In the late 1980s the discovery of the first tumor suppressor gene, retinoblastoma (*Rb)*, sparked a search for recessive mutations within inherited cancers [25-27]. During this time, allelotype studies of chromosomes from human colorectal tumors were revealing that a strict pattern of allelic deletion was associated with severity of these tumors [18, 22]. Colorectal tumorigenesis involves a step-wise progression from hyperplastic epithelium to premalignant adenomatous polyps to adenocarcinoma ([18]; Fig. 1.2). Even early on in the study of colorectal carcinogenesis it was recognized that these different morphological stages were associated with definable genetic aberrations [18, 22]. In the late 1980s the loss of chromosome 5q21 was identified as an initiating event in colorectal tumorigenesis [18,

22]. In the early 1990s mutations associated with an inherited form of colorectal cancer known as Familial Adenomatous Polyposis coli (FAP) were identified [28-30]. These mutations were mapped to a gene on chromosome 5q21 and the affected gene was named after the resultant disease, Adenomatous Polyposis Coli (*APC*). Over 20 years and 6000 publications later, mutation of the *APC* locus is still defined as the initiating mutation in over 80% of all colorectal cancers, inherited and sporadic alike [31, 32].

APC Gene Function and Tumorigenesis

Although identified as a causative genetic change in what was recognized at the time as the most common form of hereditary colorectal cancer, the function of the *APC* gene product remained elusive for several years. It was not until the late 1990s when the canonical Wnt/Wingless signaling pathway was further delineated that the APC protein was recognized as an antagonist of this pathway [23, 24]. Shortly after its identification, APC was shown to bind the E-cadherin- associated protein, β-catenin [33]. The complexing of APC and β-catenin is regulated, in part, by the serine-threonine kinase GSK3β [34] and results in targeting β-catenin for ubiquitin-mediated proteasomal degradation [35-37]. β-catenin was shown to be involved in cell-cell adhesion and was also shown to be a transcriptional activator through its homology to the Drosophila segment polarity gene, *Armadillo* [38] and through interaction with the Tcf/LEF family of DNA binding proteins [39]. APC can repress β-catenin transcriptional activity by sequestering nuclear β-catenin from transcription co-factor TCF/LEF-1 [40, 41] and also by associating with transcriptional repressors β-TrCP, C-terminal binding protein, and histone deacetylase [42, 43]. Truncation mutations of APC found in colorectal cancers result in deregulation of β-catenin transcriptional activation [23, 24].

The role of β-catenin as a transcriptional co-factor led to the discovery of genes activated by β-catenin [44-49]. The first gene identified as a target of β-catenin transcriptional activation, *c-Myc*, is the oncogene that is most commonly deregulated in human cancers and is well known to stimulate cell

proliferation [44]. *Cyclin D1* was also shown to be upregulated by transcriptionally active β-catenin [45, 46], though not without controversy [50]. Further study of the canonical Wnt signaling pathway has clarified this tumor suppressor function of APC and the carcinogenic outcomes associated with its loss from colonic epithelial cells. Loss of functional APC results in the deregulation of β-catenin transcriptional activation and uncontrolled proliferation through overexpression of target genes that drive proliferation of the intestinal epithelium.

The Canonical Wnt Signaling Pathway

The previous section was a historical account of the discovery of the role of APC in the Wnt/wingless signaling pathway. We now know that the transduction of a Wnt signal is much more complex than what I described. The canonical Wnt signaling pathway is conserved in all metazoans [51] and one of the most widely studied intracellular signaling pathways. Results from studies in standard genetic model organisms such as *Drosophila* and *C. elegans* have been readily translated into mammalian systems and applied to human disease [52]. Described here is a basic overview of the current understanding of Wnt signal transduction as it relates to the intestinal epithelia [Figure 1.3, reviewed in [53] and [54]]. The signaling molecules, Wnts, are soluble growth factors that are secreted by many different cell types including stromal cells underlying the base of intestinal crypts [4]. Wnt is recognized on the cell surface by the Frizzled (FZD) family of seven-transmembrane receptors and this binding initiates a cascade of events that ultimately results in expression of genes that stimulate cell proliferation and inhibit differentiation, the Wnt target genes [17, 44-49]. When FZD is in a Wnt unbound state, a cytoplasmic complex involving APC, GSK3β, the scaffold protein Axin and casein kinase 1α (CK1α) binds and phosphorylates β-catenin, targeting it for ubiquitin-mediated proteolytic degradation. This so-called β-catenin destruction complex maintains low levels of cytoplasmic β-catenin, thus inhibiting the expression of Wnt target genes (Fig. 1.3A). Once Wnt binds to FZD, the

receptor rapidly complexes with an LRP5/6 co-receptor and recruits Dishevelled (DVL) and Axin to the plasma membrane [55]. LRP binding to Axin inhibits the kinase activity of the β-catenin destruction complex [56]. This Wnt signal results in hypophosphorylated β-catenin which is not ubiquitinated and accumulates in the cytoplasm. Cytoplasmic accumulation of β-catenin results in its translocation into the nucleus. Nuclear β-catenin functions as a co-transcription factor with the TCF family of DNA binding proteins [39]. Generally, β-catenin functions to stimulate target gene expression, which in turn functions to stimulate cell cycle progression (Fig. 1.3B). This is a skeletal description of the canonical Wnt signaling pathway that does not account for interactions with other intracellular signaling pathways that can also affect Wnt pathway activity.

It has been hypothesized that the events following the receipt of Wnt signal on the cell surface ultimately result in disrupted assembly of the β-catenin destruction complex [54]. Experimentally, either disruption of complex assembly or inhibition of its various components have been shown to stimulate aberrant Wnt target gene expression ([53, 54], Chapter 2) and this is the primary known mechanism by which *APC* mutations result in colorectal cancers. Biallelic mutation of *APC* disrupts the assembly of the β-catenin destruction complex, leading to aberrant Wnt signaling and ultimately to uncontrolled cell proliferation and loss of differentiation in the intestinal epithelium [17]. The question of why mutation of *APC* is so strongly associated with colorectal cancers will be reviewed in the following section.

Eighty percent of all colorectal cancers, including both hereditary and sporadic cancers, involve mutations in *APC* that result in the loss of its tumor suppressor function [31, 53, 54]. Carcinogenic mutations in *APC* are typically frame-shift or nonsense mutations that result in a premature stop codon [57]. In the majority of colorectal cancers, the stop codons produced by these mutations are clustered in the region between codons 1250 and 1550 of the *APC* gene which is referred to as the mutation cluster region [58]. The implication is that proteins produced from most mutant *APC* genes are

truncated by half when compared with the wild-type proteins. Colorectal cancer-associated APC truncations lack all of the Axin binding sites and most of the β-catenin interaction domains, the 20 amino acid repeats (Figure 1.4). As illustrated in the previous discussion of the Wnt signaling pathway, the loss of this ability of APC to interact with Axin and/or β-catenin is catastrophic to the regulation of the Wnt signaling cascade. Indeed, the loss of APC in colonic epithelium has been shown to result in the deregulation of Wnt signaling [23, 24] and lost control of proliferation and differentiation [17].

Why are loss-of-function mutations in *APC* so highly correlated with colorectal cancers? While this question is difficult to answer, it does emphasize the importance of the Wnt signaling pathway in regulation of homeostasis in the colonic epithelium. APC is thought to function as a scaffold to bring GSK3β and β-catenin into close enough proximity for the regulatory phosphorylations to occur [54]. While it is likely that APC function in the β-catenin destruction complex extends beyond that of a scaffold protein, in tumors in which *APC* mutations occur, it has been shown that the loss of both alleles of *APC* is required to initiate tumorigenesis. Of the roughly 20% of colorectal cancers with wild-type *APC*, around 50% have activating mutations in the gene encoding β-catenin, *CTNNB1* [59]. This implies that deregulation of Wnt signaling through the loss of APC or activation of β-catenin contributes to the initiation or progression of 90% of all colorectal tumors, again underscoring the importance of the Wnt signaling pathway in the intestinal epithelium.

As described above, the most well defined tumor suppressor function of APC is as an antagonist of the canonical Wnt signaling pathway. APC is a relatively large protein of 2843 amino acids and approximately 312 KDa. Reviewed extensively by van Es, *et al.* [2001; [32]], APC has multiple well-defined intracellular roles, with some intracellular functions yet to be revealed. The finding of APC in the nucleus underscores the need to consider APC as a multifunctional intracellular component. APC has been shown to shuttle between the nucleus and cytoplasm [60-62]. APC is too large to passively transit the nuclear envelope. Nuclear export signals and nuclear localization signals have been identified

near the N-terminus and in the central region, respectively [60, 62]. As mentioned, one proposed function of nuclear APC is to bind DNA-associated β-catenin and inhibit β-catenin transcriptional activity by sequestration and removal from the nucleus [40, 42]. Recently, APC has been shown to interact with the DNA decatenation enzyme Topoisomerase IIα (TopoIIα) in the nucleus [63]. Previously observed functions of APC in the G2/M cell cycle transition have been attributed to the APC-TopoIIα interaction [64]. The nuclear function of APC illustrates its importance outside the canonical Wnt signaling pathway and should be considered when assessing intracellular functions of APC.

One might expect that the discovery of the causative gene for colorectal cancer would have led quickly to a cure for the disease. Unfortunately, we are 20 years from the identification of APC and its role as a suppressor of colorectal cancer initiation, and the disease still has a significant impact on the population in the US. The difficulty with treatments aimed at APC stems from its nature as a tumor suppressor. Since functional APC is lost in colorectal cancers, traditional targeted therapies which seek to inhibit target activity are not effective in this system. Replacement of a genetic loss-of-function has yet to be shown as a viable option for treating human cancers. Thus, we collectively turn our focus to genes and gene products that interact with APC, in the hopes of finding the critical gain-of-function events that result from APC loss-of-function. In this way the standard therapeutic strategy of target inhibition could be employed as a means to attenuate the effects of loss of APC tumor suppressor activity.

Musashi1

The *musashi* (*msi*) gene locus was first identified and cloned from a P element transposon mobilization screen in *Drosophila* [65]. The gene is so named because of the phenotype observed in adult flies homozygous for mutant *msi* alleles. The dorsal portion of the thorax (notum) of an adult fly contains mechanosensory organs consisting of one shaft cell (bristle) per socket. In *msi* mutant animals,

it was found that many sockets contained two bristles. The gene name is attributed to the great samurai warrior Musashi Miyamoto who instituted a fighting style that involved the use of two swords [65]. A protein domain structure was deduced from the cDNA sequence and was predicted to contain two RNA-binding domains because of homology with known RNA-binding proteins [66].

Musashi (MSI) protein is required, along with Seven in absentia (SINA), for proper eye development in *Drosophila* [67]. These studies indicated that the products of the *tramtrack* (*ttk*) gene were regulated by MSI and that MSI and SINA are required for proper differentiation of photoreceptor cells. Further study of the bristles indicated the mechanism by which MSI regulates the expression of *ttk* ([68], Figure 1.5). Asymmetric division of the sensory organ precursor cell (SOP) leads to the production of the IIa shaft-socket precursor and IIb neuron/glial precursor daughter cells [65, 68]. These cells can be distinguished by the presence of the *ttk* gene product, TTK69, in IIa only [68]. Studies indicated that the production of TTK69 was inhibited by the sequence-specific binding of MSI to the 3' untranslated region (3'UTR) of the *ttk*69 mRNA and subsequent inhibition of its translation. These data indicated that MSI played a key role in the determination of cell fate during *Drosophila* nervous system development [68].

Concurrent with the *Drosophila* studies, mammalian homologs of MSI were identified and tested for RNA binding activity [69]. Mouse studies demonstrated that Msi1 binds to *Numb* mRNA, which encodes a Notch signaling antagonist, and inhibits its translation. The result of Msi1 depression of Numb is an activation of intracellular Notch signal [69]. Subsequent studies indicated that Msi family proteins maintain the undifferentiated nature of neural stem cells and related this functional activity of Msi1 to its inhibition of *Numb* expression [70]. Thus, in mammalian cells, high levels of Msi1 result in increased Notch signaling, through the translational inhibition of *Numb*. In general, cells with high Notch signaling activity are less differentiated [13, 14].

Musashi1 as a Stem Cell Marker

As described above, MSI function was first elucidated in the sensory organ precursors of adult *Drosophila* [65]. Shortly thereafter it was observed that the mammalian homolog, Msi1, was highly expressed in neural precursor cells in the mouse brain, but expression was lost in the differentiated neurons and glial cells [71]. These data, along with the link between Msi1 and maintenance of cells in an undifferentiated state through its interaction with the Notch signaling pathway, led to the description of Msi1 as a stem cell marker in the mammalian nervous system [66, 70]. In the case of the nervous system specific precursor cells, their multipotency had been established, they were readily identifiable, and the expression of Msi1 was easily observed. The definition of Msi1 as a stem cell marker was generalized and applied to epithelial tissues [66]. While this generalization likely helped to drive discovery of the stem cells in the intestinal epithelium [6-8], in retrospect it is not likely that Msi1 is a good stem cell marker in the intestinal epithelium, as its expression is not limited to one of the previously described stem cell compartments [72].

Studies on the expression of *Msi1* in the intestines of mice and humans have shown mixed results with respect to Msi1 as a stem cell marker in the intestinal epithelium. Early studies showed high levels of Msi1 mRNA and protein in developing crypts of embryonic mice [72]. The data indicated a sensitive and specific detection of Msi1 protein and mRNA, but the pattern of expression was not restricted to the putative stem cell compartment. Other studies demonstrated the presence of Msi1 in the crypt-base columnar cells (CBCs) that were later shown to function as intestinal epithelial progenitors [73]. In this case, *Msi1* co-expression with another putative stem cell marker, *Hes1*, was correlated with stem cell specificity. Unfortunately, the co-expression did not occur in the CBCs, but instead in the cells just above the Paneth cells, the location of the so called quiescent, *Bmi1*/*DCAMKL-1*-expressing stem cells [7, 8]. Evaluating these data retrospectively with the knowledge of the epithelial progenitor cells that have

recently been identified [6-8] it is clear that *Msi1* expression is not restricted to the stem cell compartment. Rather, it appears that Msi1 is present in both the quiescent and cycling stem cells as well as in the transit-amplifying cells. Many subsequent studies have successfully identified Msi1-positive cells driving epithelial regeneration in the intestine both under normal conditions [74], after injury [75] and in genetic models where aberrant crypt formation occurs [76]. More recent studies of primary human colon cells from surgical resections showed MSI1 expression in sorted stem cell populations and indicated the presence of MSI1 is necessary for stem cell properties [77]. These mouse and human studies implicate a significant role for Msi1 in regeneration of crypt structures.

Disease-based studies have indicated high *Msi1* expression in intestinal tumors from mice and humans [72, 78] and have shown reduction of MSI1 levels in human colon cancer cells lines decreases tumorigenicity in a mouse xenograft model [78]. In a PTEN knock-out mouse model, cells expressing Msi1 were sufficient to drive intestinal polyp formation [76]. These data further solidify the importance of proper regulation of *Msi1* to the maintenance of homeostasis in the intestinal epithelium.

The conclusions from this evaluation of the literature on Msi1 are two-fold. First, looking back on data acquired prior to the identification of stem cells in the intestinal epithelium it is now clear that Msi1 is not restricted to the stem cell compartment. Thus, while it is important for crypt regeneration and maintenance, Msi1 is not a classic stem cell marker in the intestinal epithelium. Other stem cell markers such as Lgr5, Bmi1, and DCAMKL-1 are more strict indicators of the two stem cell subpopulations [6-8]. Secondly, it is apparent from the reviewed studies that Msi1 plays a significant role in the regeneration and maintenance of the intestinal epithelium [77]. Overexpression of *Msi1* has been implicated in aberrant crypt formation and intestinal tumorigenesis in both mice and humans [72, 76, 78]. Thus, Msi1 association with the stem cell compartment is critical for maintaining homeostasis in the intestinal epithelium and deregulation of Msi1 expression may be sufficient for initiation of intestinal

tumorigenesis. The rest of this introduction will focus on the molecular mechanisms involved in the regulation of *Msi1* expression specifically in the intestinal epithelium.

***In vivo* Interaction between APC and MSI1**

The finding that mutations resulting in the loss of APC tumor suppressor function are so highly correlated with the initiation of colorectal tumors has made the *APC* gene and its protein product the topic of significant study. Mouse models completely lacking APC have been difficult to obtain because germline loss-of-function mutations in *APC* result in embryonic lethality [79-81]. While mice heterozygous for mutations in *Apc* have served as models for colorectal tumorigenesis for over 20 years [79], the study of complete loss of Apc function in the intestines of these mice is impossible.

Only recently, a technological advance allowed analysis of complete loss of Apc in the intestines of mice [17]. An inducible, Apc loss-of-function model which utilizes Cre-lox technology was employed to assess the overall impact of the loss of Apc on the intestines of adult mice. Adult animals homozygous for lox-flanked *Apc* ($Apc^{fl/fl}$) alleles could be treated in order to induce Cre recombinase expression specifically in the intestine. The subsequent Cre-induced recombination led to complete loss of Apc in the intestines of adult mice. The induced $Apc^{fl/fl}$ mice were critically ill within five days post-treatment. Assessment of the intestine of these mice showed that crypts were longer and contained more cells indicating the expected hyperproliferation from deregulation of the canonical Wnt signaling pathway. The more unexpected finding was that Apc had a significant impact on differentiation of the intestinal epithelium. There was a complete loss of differentiation within the intestinal crypt as defined by the generalized villus marker alkaline phosphatase. Further analysis of the $Apc^{fl/fl}$ intestines indicated a loss of proper differentiation into absoptive, goblet and enteroendocrine cells. A microarray analysis was also performed on intestinal tissue from induced $Apc^{fl/fl}$ animals to assess the overall impact of Apc on gene expression in the intestine. Of particular interest to us was an almost 13-fold increase in the

expression of *Msi1*. As discussed previously, Msi1, while not restricted to the stem cell compartment, is associated with maintenance of stem cells.

The main focus of the Neufeld lab is to evaluate alternative functions of APC in the intestinal epithelium as a means to further elucidate its tumor suppressor function. Previous work has made significant inroads into the function of APC in the nucleus [64, 82, 83] and identification of novel APC interactions [63, 84] as a means of understanding the role of APC in normal intestinal homeostasis and carcinogenesis. As was previously mentioned, there is difficulty in therapeutic replacement to compensate for a loss-of-function, however, understanding potential gains-of-function that result could allow for standard therapeutic inhibitors to be employed. My specific interests have stemmed from the observation of a potential interaction between Apc and Msi1 in mouse intestine [17] and I sought to evaluate the molecular mechanisms involved using cultured human colonocytes.

A Novel Double-negative Feedback Circuit Involving APC and MSI1

My first challenge was to select a cell line in which to perform our assessment of the APC-MSI1 interaction. The first obstacle came about because of a desire to study this interaction under normal cellular conditions. There are no normal human intestinal cell lines. The only intestinal cell lines available are cancer cell lines. As mentioned above, 80% of all colorectal cancers have biallelic mutations in *APC*. This translates into the intestinal cell lines as well. Secondly, of the few cell lines that contain wild-type APC, there are typically mutations in other components of the canonical Wnt signaling pathway, particularly the gene for β-catenin [85]. We expected the canonical Wnt signaling pathway to play some role in the interaction between APC and MSI1. In order to evaluate this interaction in a relatively normal background, we used a cell line, termed HCT116βw, engineered in the Vogelstein lab [86]. The parental HCT116 colon cancer cell line expresses wild-type *APC*, but contains a mutant allele of *CTNNB1* (β-catenin). In order to normalize the canonical Wnt signaling pathway, a CTNNB1 deletion

series was produced and a cell line hemizygous for wild-type *CTNNB1* (HCT116βw) was isolated. These cells are derived from human colon epithelium and express wild-type canonical Wnt signaling pathway components. I tested the these cells using the TOPFlash reporter assay and demonstrated that they have normal β-catenin transcriptional response to canonical Wnt signal (Chapter 2).

Once I characterized the Wnt signaling response in the HCT116βw cell line, I assessed the interaction between APC and MSI1 (Chapter 2) by recapitulating the results observed in mice using human cell culture experiments. Short-hairpin RNA (shRNA) knockdown of APC led to an increase in MSI1 protein and mRNA. Further supporting the idea that MSI1 is a target of β-catenin transcriptional activation, I demonstrated that over-expression of stabilized β-catenin led to a dose-dependent increase in MSI1 protein. Concurrent with my work, studies in mouse intestine demonstrated that a 7Kb region corresponding to the Msi1 promoter could drive luciferase expression in response to β-catenin over-expression [87]. These data combined, demonstrate that *MSI1* is a previously unidentified target of the canonical Wnt signaling pathway. Thus, deregulation of the canonical Wnt signaling pathway by the loss of functional APC leads to deregulation of *MSI1* expression (Chapter 2).

A further probe into the mechanism of the APC-MSI1 interaction led us to identify a double-negative feedback loop between APC and MSI1. Again, employing shRNA to specifically knock down MSI1 levels, I observed a coincident increase in APC protein, but no change in *APC* mRNA levels (Chapter 2). Transient over-expression of *Msi1* cDNA decreased APC protein and actually slightly increased *APC* mRNA. These data are consistent with the previously described function of Msi1 as a translational inhibitor [68, 69, 88]. The mechanism of MSI1 translational inhibition has been delineated [89]. Briefly, MSI1 binding to the 3'UTR of its target mRNA binds to poly(A) binding protein (PABP) and inhibits its interaction with eukaryotic initiation factor 4G (eIF4G). The interaction of PABP with eIF4G is critical for initiation of translation. Luciferase reporter assessment using the 3'UTR of APC (as described in Imai 2001 [69]) demonstrated that *APC* mRNA is a target of MSI1 binding and translational silencing. This

type of reciprocal regulation, termed “double-negative feedback”, results in a bistable circuit that is often observed in biological systems balancing on the edge of considerable change.

Implications of APC-MSI1 Double-negative Feedback in the Intestinal Epithelium

The occurrence of bistable biological systems has been extensively reviewed by James Ferrell (Current opinion in cell biology, 2002 [90]). To summarized his definition, a bistable circuit is one in which feedback produces a balance between two states, *i.e.*, *on* or *off*, *stop* or *go*. External signals result in an imbalance in the feedback that leads to a stable, long-term, signal-independent response. The simplest, non-biological, analogy is that of a light switch [91, 92]. Your finger, the signal, pushing the light switch up stimulates a cascade of events that ultimately result in a response, the light turns on. This response then becomes independent of the initial signal such that the light remains on when you remove your finger. The light remains in the “on” state until this state is actively reversed, *i.e.*, your finger pushes the light switch down. Bistable circuits require either positive feedback, *i.e.*, an effector activates its own stimulation, or double-negative feedback, *i.e.*, an effector represses its own inhibition. Other hallmarks of bistable circuits are a toggle-like response to the signal, high sensitivity to the signal and ultimate acquisition of independence from the original signal. It is intuitive that, in isolation, both positive feedback and double-negative feedback circuits have all of the characteristics of bistability. However, these circuits are not necessarily bistable when placed into the context of a biological system. In fact, understanding the regulation of bistability may be just as important as understanding its role in controlling complex biological processes.

While understanding the functional aspects of bistability is important, another, potentially more intriguing question presents itself: Why would biological systems adapt a signal transduction circuit that becomes signal independent and has the potential for irreversibility? The answer may come from the significant complexity of cellular processes such as proliferation and differentiation. Both of these processes require significant changes to the intracellular environment. Recent studies have

demonstrated that multiple bistable events are required for cells to progress through cell cycle checkpoints to the ultimate end of cell division [93]. Not only are these bistable events necessary, but they are required for progression through the cell cycle. It is likely that bistable molecular switches are responsible for many of the processes that result in global cellular change. In the instance of differentiation, the need for a signal independent response is implicit. Particularly in the intestinal crypts, differentiated cells are likely in different environments than their undifferentiated ancestors, yet the program for differentiation must be maintained independent of the original stimulating signal and persist for the life of the cell.

Summary

MSI1 is a sequence-specific RNA binding protein that functions to inhibit translation of target mRNAs. This function is conserved from flies to humans ([68, 69], Chapter 2). Early on, the effect of *Msi1* expression on the cellular environment was best defined in the developing nervous system [67, 68, 70] where Msi1 was convincingly defined as a stem cell marker. These findings have resulted in the application of Msi1 as a generalized stem cell marker, especially in the intestinal epithelium [72, 74, 94]. While *Msi1* is expressed in the stem cell compartment in cycling tissues, such as the intestinal epithelium, its expression is not restricted to the stem cell compartment [72]. Thus, while the interaction between Msi1 and the Notch signaling pathway does help maintain cells in a relatively undifferentiated state [70], this activity does not appear to be restricted to stem cells in the intestinal epithelium.

Early studies of Msi1 in the intestinal epithelium have demonstrated that *Msi1* expression is up-regulated in mouse and human intestinal tumors [72, 78]. Furthermore, siRNA knockdown of *MSI1* in human colorectal cancer cells decreased their tumorigenicity upon Xenograft [78]. More recent mechanistic studies of Msi1 in the intestinal epithelium have further expanded our understanding. *Msi1*

has been identified as a target gene of the canonical Wnt signaling pathway in mouse intestine [87] and in cultured human colonocytes (Chapter 2). Studies of the interaction of MSI1 with the Wnt signaling pathway have revealed a double-negative feedback circuit with the tumor suppressor APC (Chapter 2). In this circuit, the function of APC as an antagonist of the canonical Wnt signaling pathway leads to inhibition of *MSI1* transcription; MSI1 inhibits translation of *APC* mRNA. While further study is warranted, the implications of these results on the prevention and treatment of colorectal cancers are potentially far reaching. The loss of APC, an initiating event in 80% of all colorectal cancers, results in the deregulation of MSI1. The chain of events leading to highly proliferative, undifferentiated colorectal tumors may hinge on this deregulation of MSI1 activity. Flipping the "bistable switch" of the APC-MSI1 double-negative feedback circuit to the "off" position, by inhibiting MSI1 activity, may be the key to successful treatment of colorectal cancers.

Figures

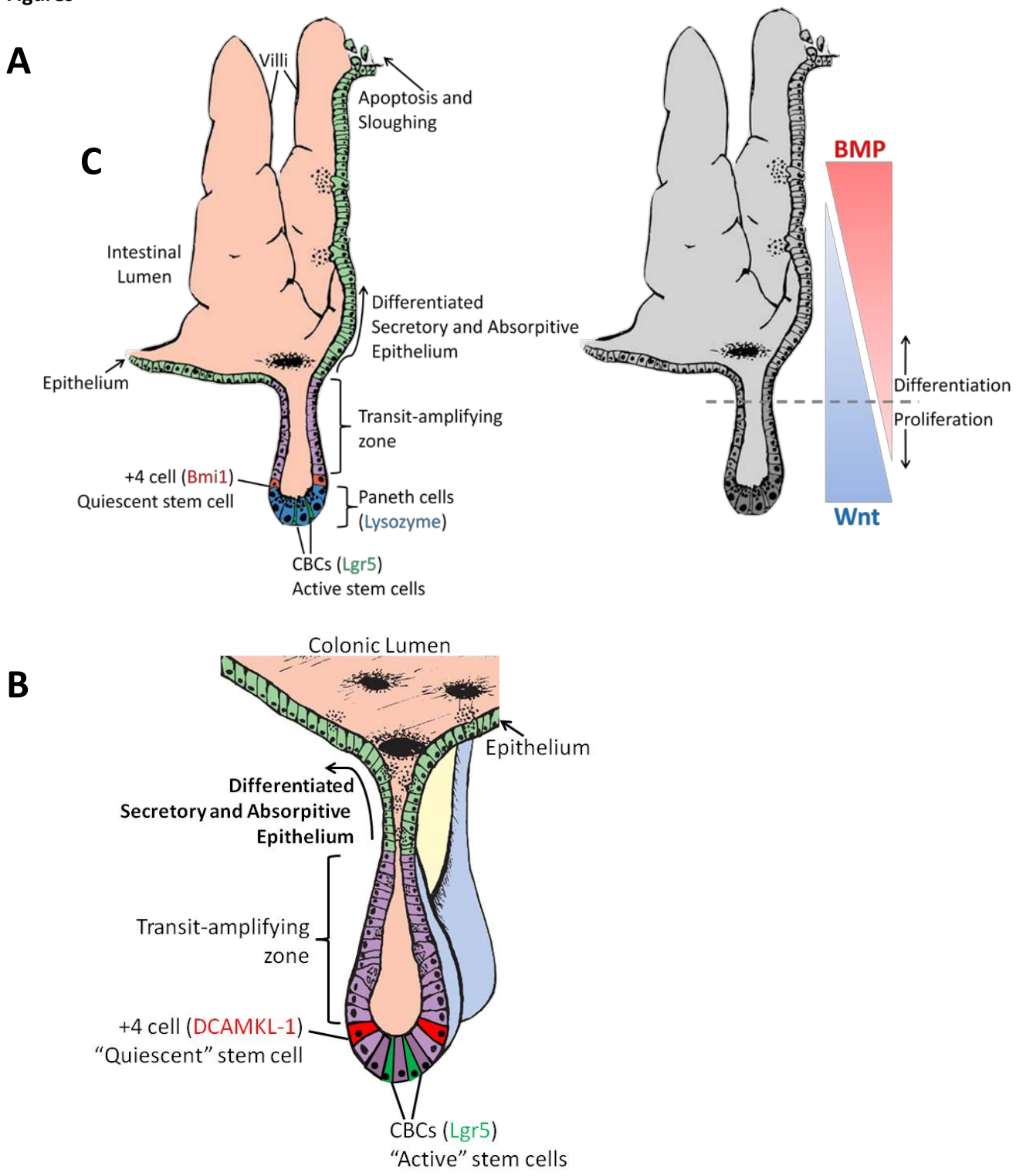


Figure 1.1. *The intestinal epithelium is organized into crypt structures.* A: The anatomy of the small intestinal epithelium is shown. Specifically, the two stem cell populations are identified with respect to

the Paneth cell compartment and the transit-amplifying zone. The differentiated intestinal epithelial cells cover the villi which extend into the lumen of the intestine. Cells are programmed for a finite life span and somewhere in the villus structure, they die by apoptosis and are sloughed into the lumen of the intestine. B: The structure of the colonic epithelium is shown identifying the same structures as in A. There are two major differences between the small intestinal and colonic epithelia. First, though the $Lgr5^+$ active stem cells reside in the same position, there are no Paneth cells in the colon. Secondly, the surface of the colon is smooth, there are no villi. Fully differentiated colonic epithelium die and are sloughed as in the small intestine. C: The structural depiction of the small intestinal epithelium is shown next to indications of the soluble signal gradients that regulate proliferation and differentiation in the intestinal epithelium. As indicated in the text, Wnt concentration is highest at the base of the crypt and decreases toward the villus. Alternatively, BMP signal is strongest in the villus and decreases toward the base of the crypt. The synchronization of these two soluble signal gradients results in a line of differentiation (dashed line). Cells above this line arrest, become quiescent with regard to cell cycle, and terminally differentiate into the absorptive or secretory lineages. Not depicted is the contribution to Notch signaling to the determination of cell fate in the intestinal epithelium. (Adapted from Sancho, E., Batlle, E., and Clevers, H., *Ann. Rev. Cell. Dev. Bio.*, 2004 [4])

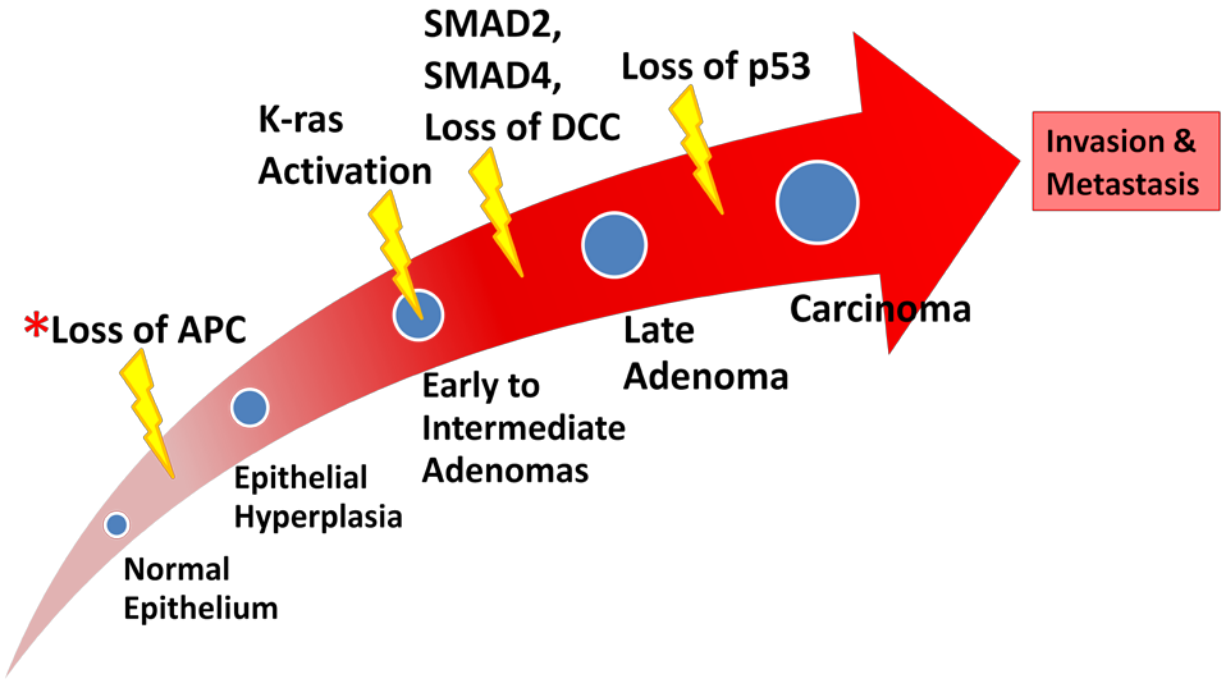


Figure 1.2. *Progressive genetic alterations define colorectal tumorigenesis.* The morphological staging of colorectal cancer is shown as a progression along the arrow. At each stage of the initiation and progression of tumor growth, genetic alterations (lightning bolts) occur that allow the tumors to be staged genetically as well as morphologically. The red asterisk indicates that, while other genetic alterations may occur in varying order during tumor progression, the vast majority of colorectal tumors initiate from loss of function mutations in *APC*. (Adapted from Fearon, E.R. and Vogelstein, B., *Cell*, 1990 [18])

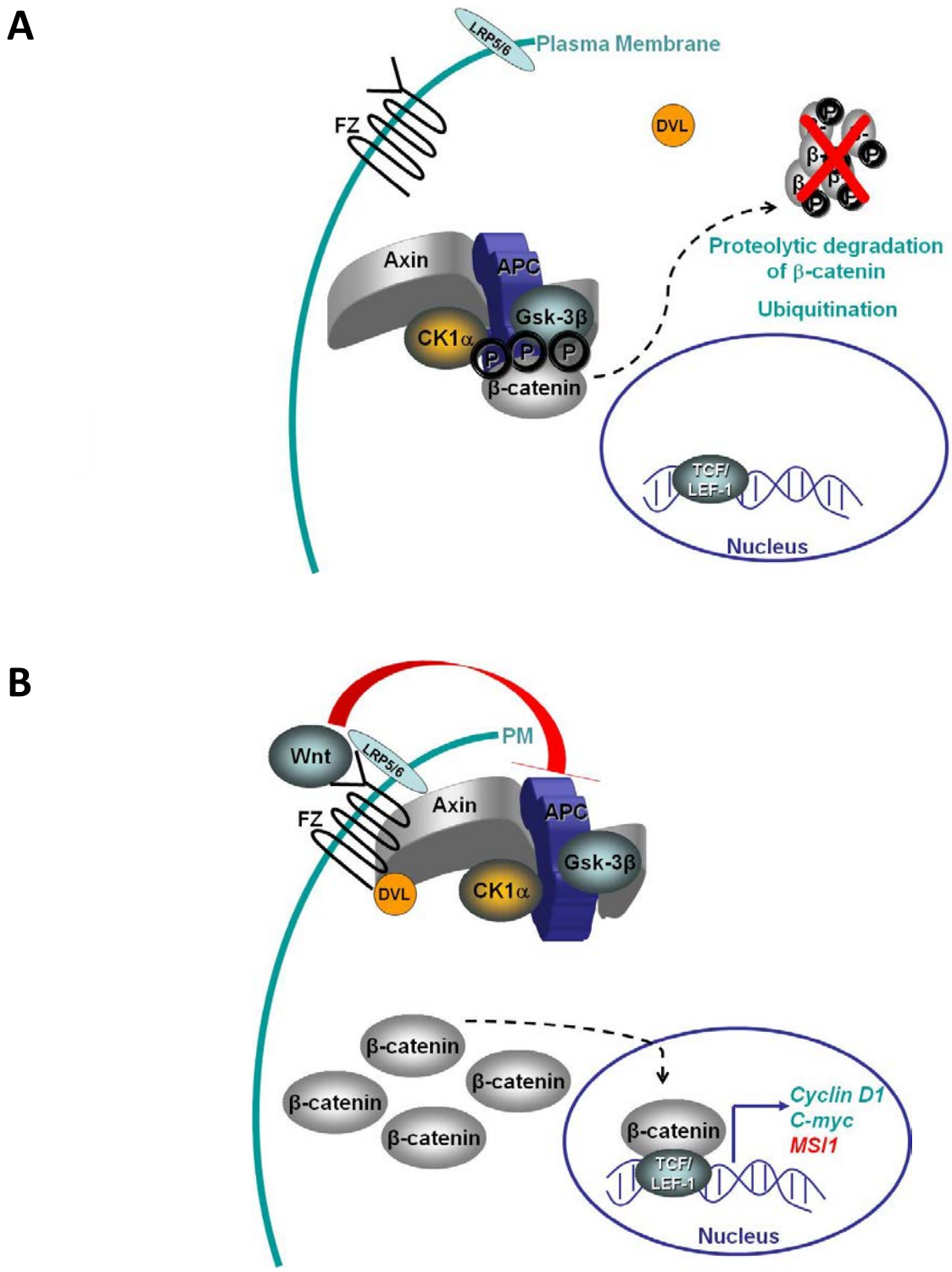


Figure 1.3. *Wnt signal is transduced through β-catenin and regulated by the APC-containing complex.* The canonical Wnt signaling pathway is depicted. A: When the Frizzled (FZ) receptor is unbound, the APC-containing β-catenin destruction complex is intact and functional. The complex, which includes

APC, Axin, GSK3β and CK1α, binds to and phosphorylates β-catenin. This phosphorylation targets β-catenin for ubiquitination and degradation in the proteosome (dashed line). Wnt target gene expression is low when β-catenin is actively degraded. B: In the presence of soluble Wnt signal, Wnt-FZ binding recruits LRP5/6 and Dishevelled (DVL) to the receptor complex. The receptor complex recruits the APC-complex to the plasma membrane and the binding of LRPs and DVL to Axin inactivates the kinase activity of the β-catenin destruction complex. Hypophosphorylated β-catenin builds up in the cytoplasm and is subsequently translocated into the nucleus. In the nucleus, β-catenin functions as a transcription co-factor with the TCF/LEF-1 family of DNA binding proteins and stimulates target gene expression. Typical Wnt target genes, such as Cyclin D1, c-MYC and MSI1 (identified recently), function to stimulate cell proliferation.

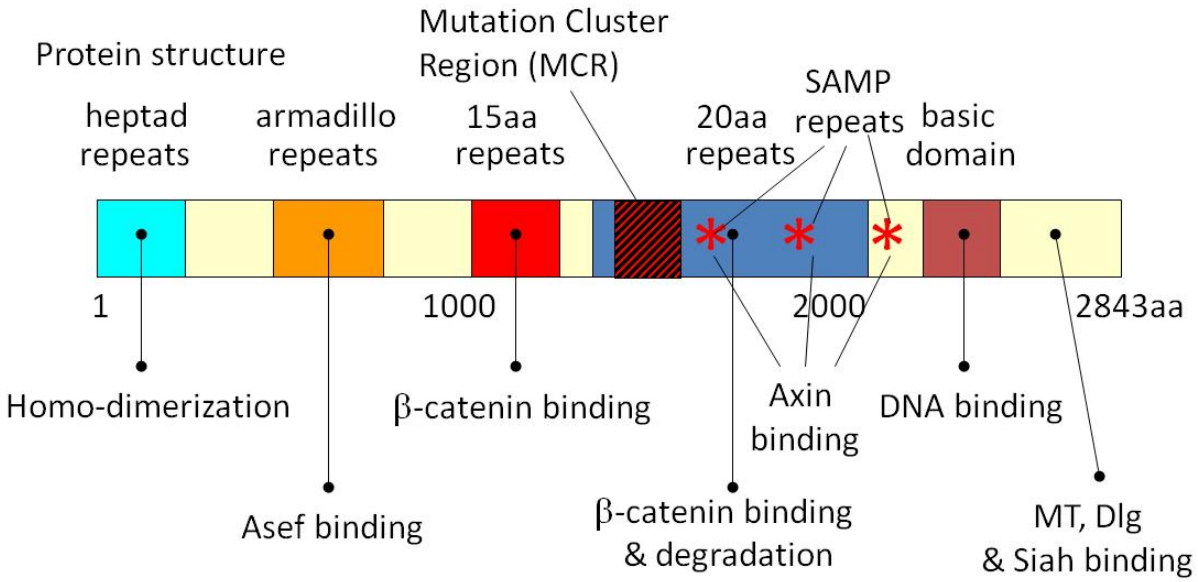
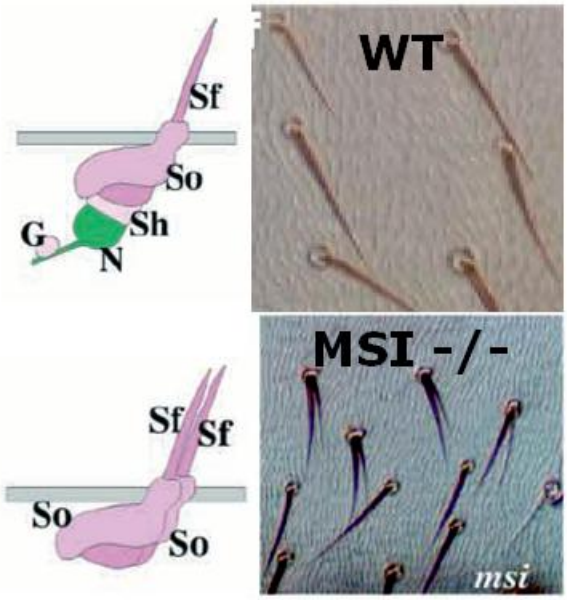


Figure 1.4. *APC protein domain structure.* APC is a large, multi-domain protein. The domain map of the APC protein is depicted with the codon/amino acid designation below. The black and red hatched box indicates the mutation cluster region in which premature stop codons occur as the result of the majority of carcinogenic *APC* mutations. These mutations result in truncation of the C-terminal half of the protein, removing the Axin binding (SAMP repeat) domain and the bulk of the β-catenin binding (20 amino acid repeats) domain. Truncation of APC inhibits its ability to participate in the β-catenin destruction complex and inactivates this complex (Fig. 1.3). This leads to deregulation of the Wnt signaling pathway and loss of proliferative control. (Adapted from Fernhead, N., *et al.*, *Human Molecular Genetics*, 2001 [57])

A



B

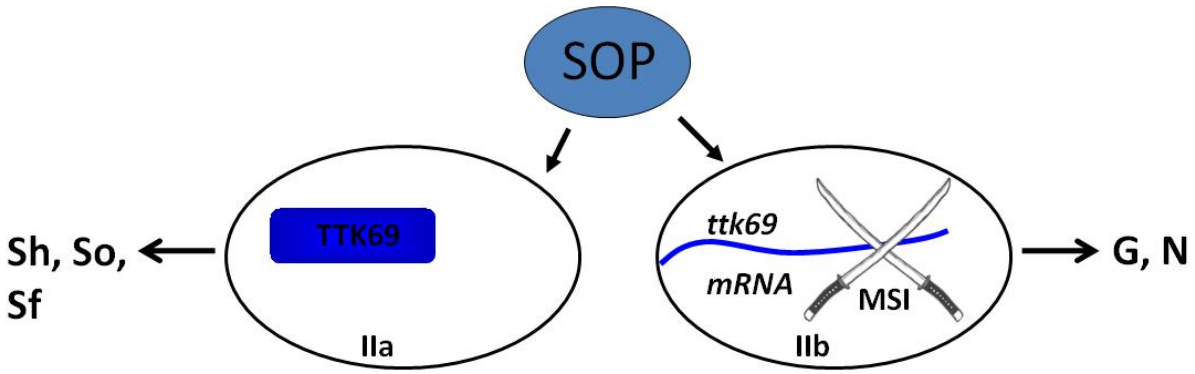


Figure 1.5. *msi is a translational regulator of cell fate.* A: The *msi* gene was identified based on a double-bristle phenotype obtained from a P element excision screen in *Drosophila*. The loss of Glia (G) and Neurons from the neuronal lineage in the bristle structure led to the duplication of the non-neuronal Sheath (Sh), Socket (So) and Shaft (Sf) cells and thus two bristles. B: The two cell lineages that compose the bristle come from a single sensory organ precursor cell (SOP). The SOP gives rise to two daughter cells, IIa and IIb, which ultimately give rise to the non-neuronal and neuronal lineages, respectively. The molecular determinant of SOP daughter cell fate is the presence of TTK69 protein. Both IIa and IIb have ttk69 mRNA, but protein is only produced in the IIa cell, the precursor to the non-neuronal cells. In the IIb cell, the translation of *ttk69* mRNA is repressed by MSI. MSI repression of ttk69 expression determines the neuronal cell fate in the IIb progeny. Homozygous loss of function of *msi* allows the production of TTK69 in both SOP progeny and duplication of the non-neuronal cell lineage. (Adapted from Okabe, M. *et al.*, *Nature*, 2001 [68])

Chapter 2

Novel Double-negative Feedback Loop between Adenomatous Polyposis Coli and Musashi1 in Colon Epithelia

The data and opinions in this chapter were published previously [95] and reformatted for this dissertation:

Spears, E. and K. L. Neufeld (2011). "Novel double-negative feedback loop between adenomatous polyposis coli and Musashi1 in colon epithelia." *The Journal of biological chemistry* **286**(7): 4946-4950.

Abstract

Loss of tumor suppressor adenomatous polyposis coli (APC) is thought to initiate the majority of all colorectal cancers. The predominant theory of colorectal carcinogenesis implicates stem cells as the initiating cells. However, relatively little is known about the function of APC in governing the homeostasis of normal intestinal stem cells. Here, we identify a novel double-negative feedback loop between APC and a translation inhibitor protein, Musashi1 (MSI1), in cultured human colonocytes. We show APC as a key factor in MSI1 regulation through Wnt signaling and identify APC mRNA as a novel target of translational inhibition by MSI1. We propose that APC/MSI1 interactions maintain homeostatic balance in the intestinal epithelium.

Introduction

Two subpopulations of stem cells have recently been identified in the intestinal epithelium [6-8]. It has been shown that loss of Apc in one of these stem cell subpopulations results in intestinal adenomas [96]. Loss of Apc throughout the small intestine leads to expansion of undifferentiated, stem cell-like epithelium into the normally well differentiated villus compartment and a 12-fold increase in Msi1 mRNA [17].

Musashi proteins function to inhibit translation initiation by binding to the 3-untranslated region (3-UTR) of target mRNAs and competing with poly(A)-binding protein for eukaryotic initiation factor 4G

binding [89]. The two best characterized targets of mammalian MSI1 are the Notch antagonist Numb and the cyclin- dependent kinase inhibitor p21 [69, 88]. Msi12 maintains cells in a more undifferentiated state, in part, through activation of Notch signaling and cell cycle progression [66]. MSI1 is expressed in the putative intestinal stem cells [75, 76].

The most well defined function for APC is as an antagonist in the canonical Wnt signaling pathway [5]. A complex containing APC, Axin, and glycogen synthase kinase 3 binds to and phosphorylates β-catenin, leading to ubiquitination and degradation of β-catenin by the proteasome. Wnt binding to its cognate receptor or APC loss each lead to β-catenin accumulation in the cytoplasm, translocation into the nucleus, and interaction with co-activator TCF/LEF-1, resulting in transcription of Wnt target genes. In addition, there is evidence that nuclear APC also brings the transcriptional repressor C-terminal binding protein to the TCF-β-catenin complex [42], sequesters β-catenin from TCF/LEF-1 [40] and facilitates nuclear export of β-catenin [60, 61, 97]. Approximately 80% of all colorectal cancers are associated with mutation of both APC alleles, resulting in APC truncation and loss of this tumor suppressor function [19].

In the current study, we used cultured human colonocytes to examine the functional relationship between APC and MSI1, first observed in mouse intestines [17]. Msi1 was recently shown to be a Wnt target gene in mouse intestinal epithelium [87], and our studies in human colon cells support this finding. Our study further reveals that MSI1 regulates APC levels, providing the first evidence for a double-negative feedback loop between APC and MSI1. We have confirmed that MSI1 regulates APC translation. We hypothesize that this double-negative feedback system is central to the maintenance of homeostasis, the critical balance of proliferation and differentiation, in the intestinal epithelium.

Experimental Procedures

Plasmids and Cloning.

SureSilencing short-hairpin RNA (shRNA) plasmids (SuperArray, Frederick, MD) were used to reduce levels of APC and MSI1. For Msi1 overexpression, pCDNA3-Flag-Msi1 (Flag-Msi1) and pCDNA3-Flag-Msi1mutR1 (Flag-mutR1) were generously provided by Hideyuki Okano (Tokyo, Japan). The mutR1 Msi1 mutant contains three phenylalanine-to-leucine substitutions in the first RNA binding domain [69]. These phenylalanines are required for RNA binding and subsequent translational inhibition by Msi1, and these substitutions have been shown to ablate binding of the mutant to *Numb* mRNA [69, 98]. Mouse *Msi1* cDNA was employed for these studies. Mouse and human cDNAs are 93% identical, and the proteins differ by two amino acids (Q127H and T251S) that do not affect the first RNA recognition motifs, required for RNA binding [98]. The *Numb* 3'-UTR-luciferase reporter, pGVP2-Numb, was provided by H. Okano. The *APC* 3'-UTR luciferase reporter was made by replacing the Numb 3'-UTR in pGVP2-Numb3'-UTR with the APC 3-UTR amplified from HCT116βw genomic DNA using the following primers: APC 3'-UTR (forward), 5'-AAGAGAGGAAGAATGAAACTAAG-3' and APC 3'-UTR (reverse), 5'-GCATGTATCTCCATTGTTTATG-3'. The pGVP2-APC 3'-UTR 5' deletion mutant was made by cutting the APC 3'UTR reporter DNA with the XbaI restriction enzyme, gel-purifying the fragments, and religating the truncated APC 3'-UTR into the pGVP2 backbone.

In order to look at Wnt target gene expression in HCT116βw cells we overexpressed a mutant form of exogenous β-catenin that is not phosphorylated by the APC containing complex and is thus not targeted for ubiquitin-mediated degradation described previously [99]. As with the other overexpression constructs, this β-catenin is Flag-tagged and contains a S33F and T45A substitutions which alter the GSK3β phosphorylation sites and stabilize the protein in a transcriptionally active state (Flag-β-catenin Ub-). This stabilized, exogenous β-catenin can stimulate target gene activity regardless of the presence or activity of the APC-containing destruction complex.

Cell Culture and Transfection.

HCT116βw cells, a generous gift from Bert Vogelstein, were cultured as described [86]. NIH3T3 cells (ATCC) were grown in DMEM:10% Cosmic Calf® serum. GeneExpresso (Lab Supply Mall, Gaithersburg, MD) was used to transfect cells at 30–40% confluency, grown in 6-well plates. For RNA interference, 2.5 μg of shRNA plasmid was used; for overexpression, 0.5 μg and 1 μg of Flag-Msi1 or Flag-mutR1 were used with DNA content equalized by the addition of pCDNA3.1.

Protein Analysis.

Cells were washed twice with cold PBS and then collected in reporter lysis buffer (Promega, Madison, WI) with protease inhibitors 48 h after transfection. Lysates were sonicated (10 s, Output 1) using a Heat Systems-Ultrasonics, Inc. cell disruptor (Plainview, NY). An aliquot was immediately removed, added to 4X protein sample buffer, and heated to 95°C for SDS-PAGE. Another aliquot was added to 1 ml of TRIzol reagent (Invitrogen), incubated at room temperature for 5 min, and stored at -80 °C for RNA isolation. Proteins were separated using SDS-PAGE and 7% polyacrylamide gels and blotted onto nitrocellulose. Antibodies used for protein detection were as follows: APC [M2-APC [63]], rabbit anti-MSI1 (locally produced), and α-tubulin (12G10, Developmental Studies Hybridoma Bank (DSHB), University of Iowa. Images acquired with a Kodak image station 4000R (Molecular Imaging Systems, Rochester, NY) were analyzed using the ImageJ 1.4.1o software (rsb.info.nih.gov/ij).

RNA analysis.

Quantitative real-time reverse transcriptase polymerase chain reaction was used to assess mRNA content. RNA was isolated from TRIzol solution following the manufacturer's instructions. First strand cDNA was prepared from 0.5 μg of RNA using 200 units of M-MuLV reverse transcriptase and Random 6 primer (New England Biolabs, Ipswich, MA). Quantitative PCR for APC, MSI1, and hypoxanthine-guanine phosphoribosyl-transferase (HGPRT; internal control) was performed using the

DyNAmo HS SYBR Green quantitative PCR kit (New England Biolabs) and analyzed on a DNA Engine Opticon 2 thermal cycler for continuous fluorescence detection (Bio-Rad). Target mRNA estimates were made by comparing fold change to levels in control transfections using the $\Delta\Delta C(T)$ calculation method.

Luciferase Reporter Assay.

Numb and APC 3'-UTR-Luciferase reporters were co-transfected with Flag-Msi1 or FlagmutR1 and Renilla luciferase (transfection control) expression constructs with total DNA adjusted to 2 μg using pCDNA3.1 vector. 48 h after transfection, cell lysates were prepared as described for protein analysis, and luciferase activity was assessed using a Dual-Luciferase reporter assay system (Promega) and a LMAXII384 microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase data were normalized to Renilla luciferase and expressed as a percentage of the negative control (vector only) luminescence. MSI1 shRNA experiments were performed similarly with 3 μg of total DNA.

The TOPFlash luciferase reporter system has been adopted as a standard for detecting β-catenin driven transcriptional activation [23]. The reporter used for the current experiments is a 16X TOPFlash reporter meaning that it contains 16 TCF/LEF-1 binding sites upstream of a minimal promoter driving expression of Firefly luciferase and was a generous gift from Randall T. Moon (Seattle, WA). A FOPFlash reporter, which contains scrambled sequence in the enhancer region, is used as a negative control to show that the change in luciferase activity is specifically due to β-catenin transcriptional activity. The TOPFlash reporter was co- transfected with plasmids expressing stabilized, Flag-tagged β-catenin (Flag-β-catenin Ub-), LEF-1, and APC shRNA and firefly luciferase activities were assessed as described in Materials and Methods. The TOPFlash reporter was also used to assess the upregulation of β-catenin transcriptional activation in Wnt3a treated cells. Cell treatment involved mixing Wnt3a or control medium with fresh culture on dividing cultures 24 h after transfection with TOPFlash reporter and incubating for another 24 h.

Wnt3a Conditioned Medium.

Wnt3a conditioned medium was derived from mouse L cells stably expressing a transgene for the secreted signal [100]. As a control, L cells lacking the Wnt3a transgene were grown in parallel. Wnt3a expressing and control L cells were a generous gift from Shinji Takada (Kyoto, Japan). Briefly, Wnt3a secreting cells, and counterpart control cells, were cultured in McCoy's 5A with 10% FBS and expanded through multiple passages until the desired cell volume was reached. Cells were allowed to grow for 24 h after the last passage and medium was collected from cells that were 50-75% confluent. Wnt3a and control media were stored at -80°C in aliquots until use.

Results

Loss of APC Leads to Increased MSI1 Protein and mRNA in HCT116βw Cells.

In mice, inducible, intestine-specific loss of Apc led to a greater than 12-fold increase in Msi1 mRNA in intestinal tissue. To further evaluate the interaction between APC and MSI1 in human colonocytes, we used HCT116βw cells, which have a stable karyotype and express wild-type APC. HCT116w cells were also manipulated to only express wild-type β-catenin [86]. The intact Wnt signaling pathway in HCT116βw cells is critical for our studies because both APC and MSI1 can impact Wnt signaling [4, 87]. APC mRNA and protein levels were both reduced in HCT116βw cells transiently transfected with plasmids expressing shRNA corresponding to the APC mRNA sequence (Fig. 2.1). This knockdown of APC resulted in a reproducible increase in MSI1 protein (Fig. 2.1B) and a 2–4-fold increase in MSI1 mRNA (Fig. 2.1C).

Canonical Wnt signaling is functional and stimulates MSI1 expression in HCT116βw cells.

In order to demonstrate that the canonical Wnt signaling pathway is functional and stimulates target gene expression in HCT116βw cells we employed the TOPFlash luciferase reporter system. The TOPFlash system was developed as a read-out for β-catenin transcription activation, the major end-point of the canonical Wnt signaling pathway, and is used extensively in the field [23]. Co-transfection of the TOPFlash plasmid with a mutant *CTNNB1* cDNA results in the expression of a Flag-epitope tagged, stabilized, transcriptionally active form of β-catenin [Flag-β-catenin Ub^-; [99]]. As expected, this stabilized β-catenin increases the luciferase activity and supplementation with LEF-1 further increased the transcriptional read-out (Fig. 2.2A). Co-transfection of the TOPFlash reporter with APC shRNAs leads to a 14-fold increase in luciferase activity in these cells (Fig. 2.2B). Finally, exposure of transfected cells to Wnt3a conditioned medium also increased TOPFlash activity (Fig. 2.2C). Because the source of Wnt3a was conditioned media harvested from Wnt3a-expressing cells, it is expected that there was depletion of growth factors and nutrients from this "used" culture medium. Thus, in the case where 75% Wnt-conditioned media was mixed with 25% fresh media, the added Wnt3a did not appear to compensate for the depletion of other growth factors that might stimulate proliferation and the TOPFlash activity was not as high as with a 50:50 mix. These data indicate that β-catenin transcriptional activation is properly functioning in the HCT116βw cells and is readily stimulated by loss of APC tumor suppressor function.

Not only is the canonical Wnt signaling pathway functional in HCT116βw cells, but it stimulates *MSI1* expression. Figure 2.2 D and E show results from overexpression of Flag-β-catenin Ub^- on MSI1 in these cells. Western blot analysis and subsequent quantification of protein band intensities indicate that MSI increases with increasing amounts of Flag-β-catenin Ub^-. Recently *Msi1* was shown to be a direct target of β-catenin transcriptional activation in mice [87] and our results indicate the same in human colonocytes.

Loss of MSI1 Leads to Increased APC Protein in HCT116βw Cells.

The canonical Wnt signaling pathway is functional and stimulates target gene expression in HCT116w cells (Fig. 2.2, A–C). Moreover, MSI1 expression is up-regulated in HCT116βw cells with overexpressed β-catenin (Fig. 2.2, D and E). MSI1 has recently been shown to activate the Wnt signaling pathway [87]. To determine whether this regulation involves APC, we reduced MSI1 protein and mRNA in HCT116βw cells by transient transfection with plasmids expressing shRNA corresponding to the MSI1 mRNA sequence (Fig. 2.3). This knockdown of MSI1 resulted in a reproducible increase in APC protein (Fig. 2B) but no significant change in *APC* mRNA level (Fig. 2.3C), consistent with MSI1 functioning as a translational inhibitor of APC.

Msi1 Overexpression Decreases APC Protein in HCT116βw Cells.

Consistent with the loss-of-function data, overexpression of wild-type but not mutant Msi1 in HCT116βw cells led to a decrease in APC protein (Fig. 2.4, A and B). Unexpectedly, *APC* mRNA did not decrease but rather showed an increase at the highest level of exogenous Msi1 (Fig. 2.4C). These data are consistent with the known function of MSI1 as a translational inhibitor. Even in the presence of increased *APC* mRNA, *Msi1* overexpression led to decreased APC protein levels.

MSI1 Inhibits Translation of mRNA Containing the APC 3'-UTR.

MSI1 binds the 3'-UTR of target mRNAs such as *Numb* and *Cdkn1a* and inhibits their translation [69, 88]. The sequence (G/AU$_{1–5}$AGU) has been established as a MSI1 consensus binding sequence [MCS; [69]. The APC 3'-UTR contains four putative MCSs, two near the 5' end and two closer to the 3' end (Fig. 2.5A). Of the four potential MCS, only the first is conserved in sequence and position in human and mouse (Fig. 2.5B). This first MCS is predicted to locate in a hairpin structure, which is optimal for MSI1 binding (Fig. 2.5C).

To test whether APC is a novel target of MSI1 translational inhibition, we generated a luciferase reporter that incorporated the entire APC 3'-UTR or a deletion mutant lacking the first two potential MCSs. NIH3T3 cells were used for this study because they lack endogenous MSI1 [69]. Cells transfected with the APC 3'-UTR luciferase reporter showed a marked reduction in luciferase activity when co-transfected with the Flag-Msi1 expression construct (Fig. 2.5D). This reduction was similar to that seen using the Numb 3'-UTR reporter and did not occur in NIH3T3 cells expressing a mutant form of Msi1 (Fig. 2.5D). The APC 3'-UTR reporter lacking MCS1 and -2 did not show a decline in activity significantly different from the complete APC 3'-UTR reporter in the presence of mutant Msi1 (Fig. 2.5D). HCT116βw cells transfected with the APC 3'-UTR luciferase reporter showed no significant decline in luciferase activity when cells co-expressed either Msi1 or mutant Msi1, nor was a decline apparent for the Numb 3'-UTR luciferase reporter, which served as a positive control (Fig. 2.6). To examine whether endogenous MSI1 in HCT116βw cells was suppressing translation of the APC 3'-UTR reporter, endogenous MSI1 levels were reduced using MSI1 shRNA (Fig. 2.5E). Reduced MSI1 levels were accompanied by an increase in APC 3'-UTR reporter activity. We conclude that the dampened response of the reporters to MSI1 overexpression seen in HCT116βw cells likely resulted from endogenous MSI1. Combined, these data indicate that MSI1 regulates APC levels in HCT116βw cells through its activity as an RNA sequence-specific inhibitor of translation.

Discussion

Here, we report a novel double-negative feedback loop between APC and MSI1 that has potential relevance to the homeostasis of intestinal epithelia (Fig. 2.7). Our data indicate that APC, acting through β-catenin in the canonical Wnt signaling pathway, is responsible for regulation of MSI1 in cultured human colonocytes. These results confirm the recent report that murine *Msi1* is a direct target of β-catenin transcriptional activation [87] and extend these findings to human colonocytes.

Furthermore, we show that the APC 3'-UTR is a novel target of translational inhibition by MSI1. This reciprocal regulation of APC by MSI1 is likely responsible for the dramatic effect on differentiation observed upon *Apc* mutation in the adult mouse intestine [17]. Double-negative feedback is thought to be important in cells undergoing significant change, such as cells that are differentiating or undergoing asymmetric cell division, because the effect of double-negative feedback is similar to that of positive feedback [90]. APC is involved in regulating cell division in the intestinal epithelium through inhibition of Wnt signaling [101]. Loss of APC is an initiating event in a majority of colorectal cancers, further underscoring the importance of APC in the maintenance of the intestinal epithelium. MSI1 inhibition of p21 translation has been shown to increase cell proliferation [69, 88]. By activating Notch signaling, MSI1 has also been implicated in the maintenance of a dedifferentiated state in mammalian cells [66]. In the intestinal epithelium, MSI1 is present in the stem cell compartment and is predicted to have a role in symmetric stem cell division [72]. It is noteworthy that colorectal cancers show increased MSI1 levels, and decreasing MSI1 in colorectal cancer cell lines decreases tumorigenicity in mouse xenografts [78]. It is likely that loss of differentiation observed *in vivo* following induced *Apc* mutation results from the double-negative feedback between APC and MSI1 that we have now identified [17]. Imbalance in this double-negative feedback loop leads to loss of homeostasis in the intestinal epithelium and may ultimately result in cancer. Colorectal cancer is unique because APC loss is associated with ~80% of all disease [19]. Replacement of APC in colorectal cancers through gene therapy remains a complicated therapeutic strategy. Our data indicate that APC loss results in the up-regulation of MSI1 and the disruption of a delicate balance between these two effectors. Thus, we propose that MSI1 represents a potential target for future colorectal cancer therapies.

Figures

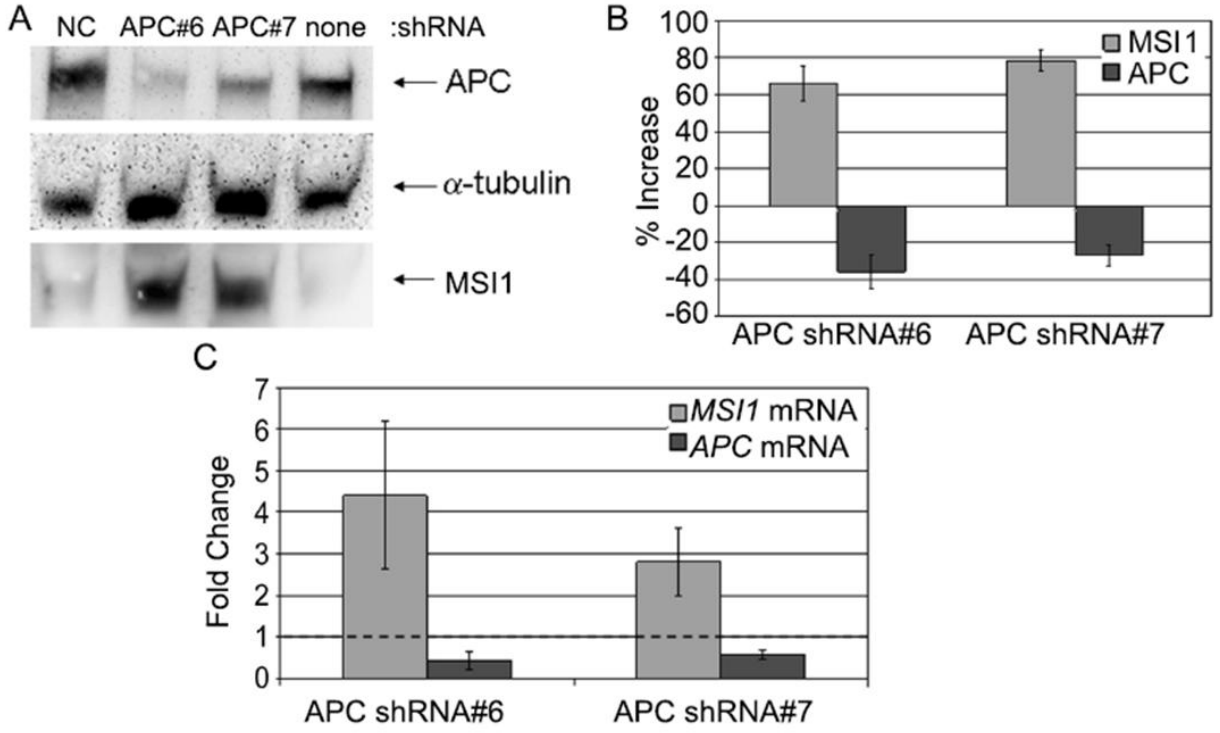


Figure 2.1. *Loss of APC leads to an increase in MSI1 protein and mRNA in cultured human colonocytes.* A, a representative Western blot reveals decreased APC protein and increased MSI1 protein in cells expressing either of two *APC* shRNAs as compared with cells with scrambled shRNA (NC). B, MSI1 and APC band intensities from 3 independent experiments were normalized to α-tubulin. Results are expressed as the percent increase in APC or MSI1 in cells transfected with *APC* shRNA as compared with cells transfected with scrambled shRNA ± S.E. C, quantitative real-time RT-PCR analysis was performed on cells transiently expressing APC or scrambled shRNA. Results from 3–5 independent experiments are expressed as average -fold change of *APC*- or *MSI1*-specific mRNA in *APC* shRNA transfected cells as compared with scrambled shRNA-transfected cells (dashed line)± S.E.

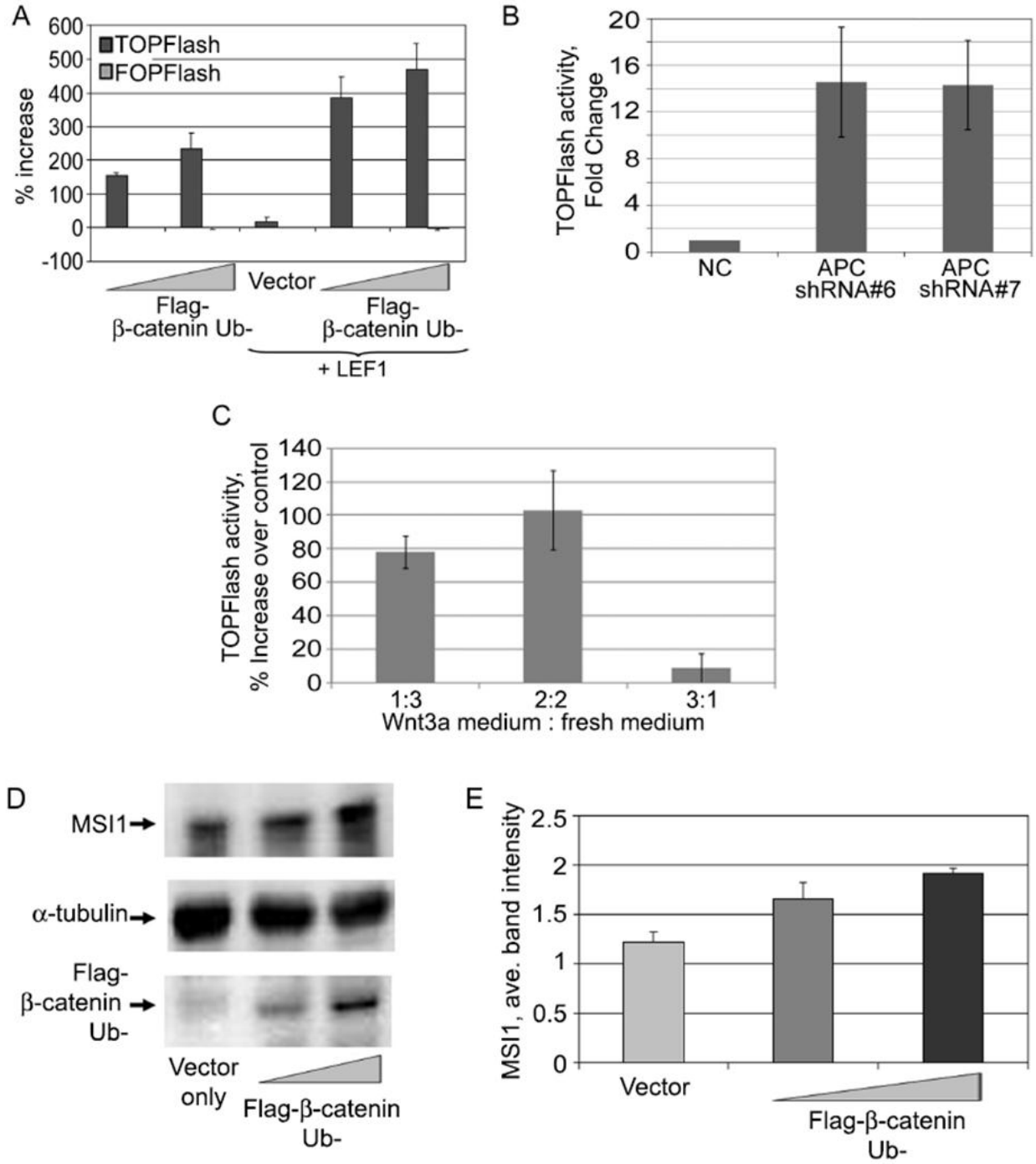


Figure 2.2. *MSI1 is a Wnt target in HCT116βw cells.* A: TOPFlash, a luciferase reporter for LEF1/β-catenin activity, is up-regulated in response to β-catenin overexpression and this effect is augmented by exogenous expression of co-transcription factor, LEF-1. Data are expressed as the average % increase from three independent experiments comparing cells transiently transfected with the indicated expression plasmids to cells co-transfected with empty vector ± SEM. B: Loss of APC by shRNA treatment results in increased β-catenin transcriptional activation as indicated by increased luciferase activity from the TOPFlash reporter system. APC shRNA data are shown as Fold Change compared to

cells transfected with NC shRNA. C: Addition of Wnt3a conditioned medium to HCT116βw cells increases TOPflash reporter expression. FOPFlash control luciferase activity did not vary significantly from the vector only control in any of the experiments. D: Representative western blot showing increased MSI1 protein cells expressing exogenous Flag-tagged, stabilized β-catenin (Flag-β-catenin Ub^-). E: Western blot Band intensity data from three independent experiments showing a reproducible and statistically significant ($p << 0.05$) increase in MSI1 protein level in cells expressing exogenous Flag-β-catenin Ub^-.

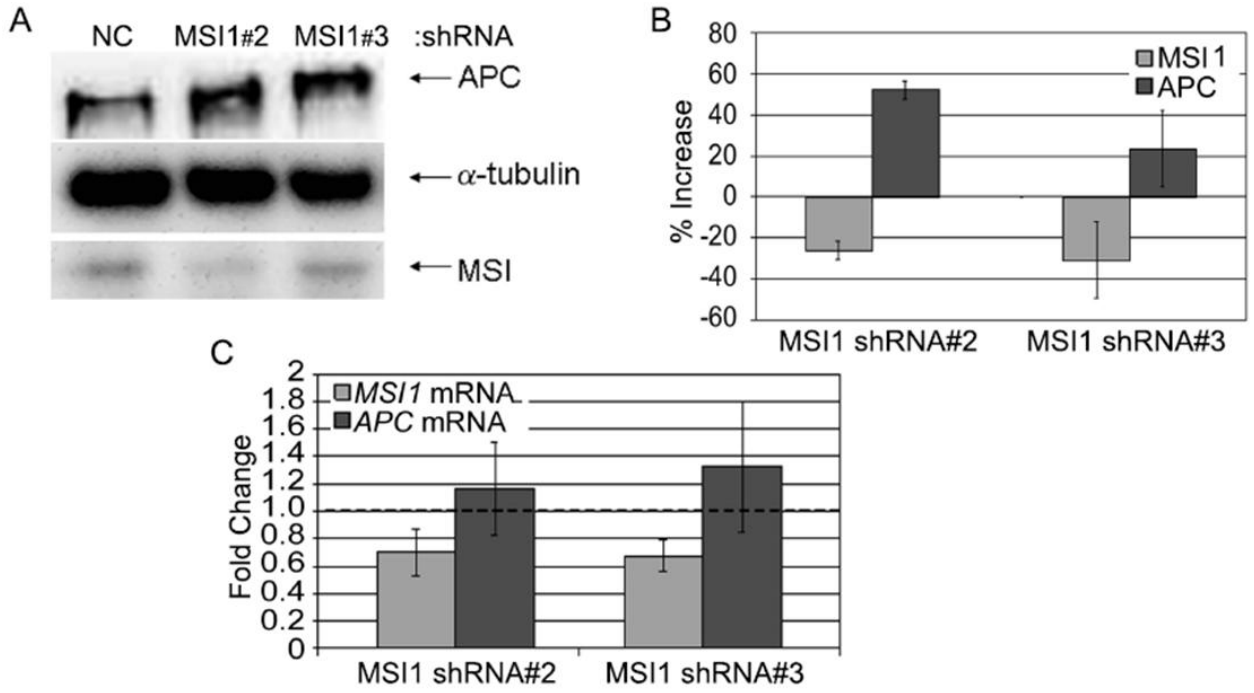


Figure 2.3. *Loss of MSI1 leads to an increase in APC protein, but not mRNA. A*, a representative Western blot reveals decreased MSI1 protein and increased APC protein in cells expressing either of two *MSI* shRNAs as compared with cells with scrambled shRNA (*NC*). *B*, MSI1 and APC band intensities from 3 independent experiments were normalized to α-tubulin. Results are expressed as percentage of increase in APC or MSI1 in cells transfected with *MSI1* shRNA as compared with cells transfected with scrambled shRNA ± S.E. *C*, quantitative real-time RT-PCR analysis was performed on cells transiently expressing *MSI1* or scrambled shRNA. Results from 4–5 independent experiments are expressed as average -fold change of *APC*- or *MSI1*-specific mRNA in *MSI* shRNA-transfected cells as compared with scrambled shRNA-transfected cells (*dashed line*)±S.E.

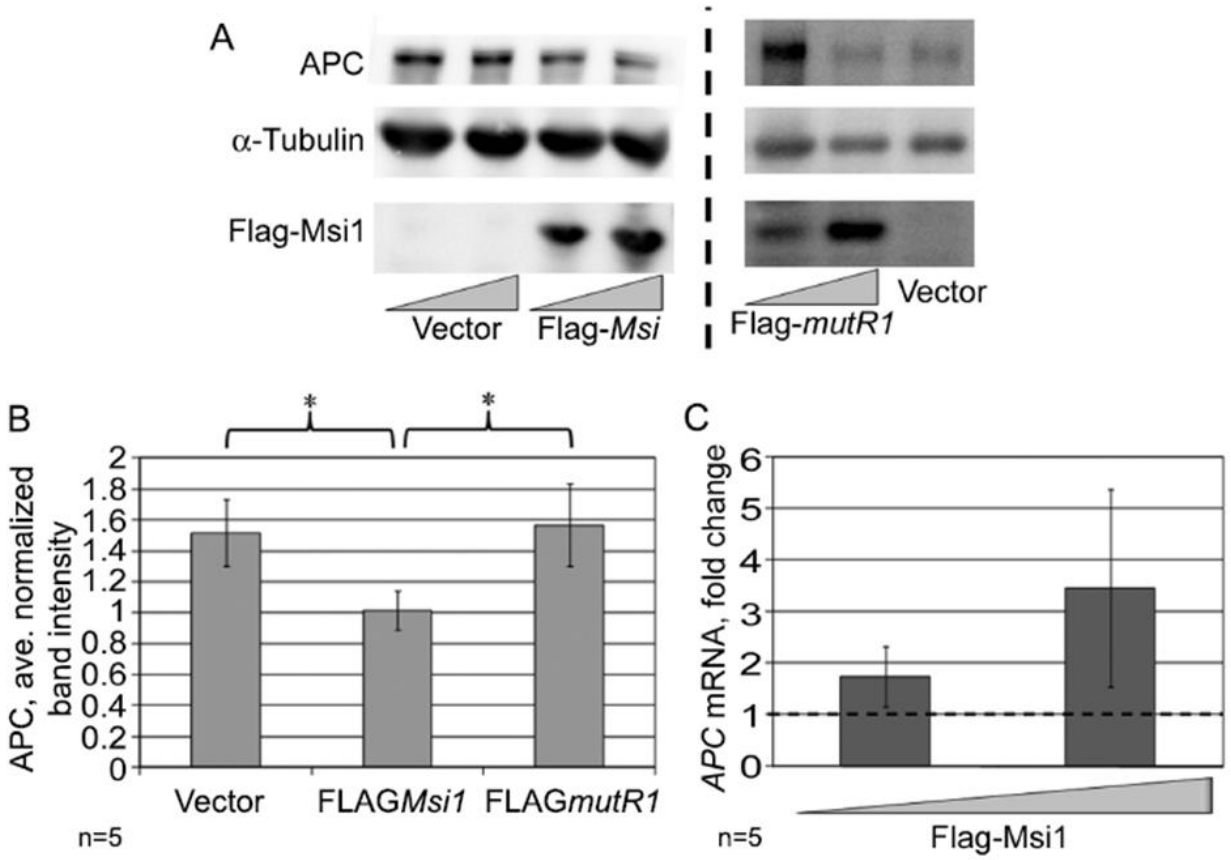


Figure 2.4. *Overexpression of Msi1 results in decreased APC protein. A*, representative Western blots of HCT116βw cells transiently transfected with *Flag-Msi1* or mutant *Msi* (*Flag-mutR1*) expression constructs. *B*, APC band intensities for the lowest *Msi1* cDNA concentrations, from 5 independent experiments, were normalized to α-tubulin and expressed as average (*ave.*) band intensity ± S.E. *Asterisks* indicate a statistically significant decrease in APC band intensity from vector alone ($p < 0.05$) and *Flag-mutR1* controls. *Flag-mutR1* overexpression did not alter APC band intensity when compared with vector alone control ($p > 0.4$). *C*, quantitative real-time RTPCR analysis was used to measure *APC* mRNA in HCT116_w cells transfected with increasing concentration of *Flag-Msi1* expression construct or empty vector. Results from 5 independent experiments are expressed as average -fold change of *APC* mRNA in *Flag-Msi1*-transfected cells as compared with empty vector-transfected cells (*dashed line*)±S.E.

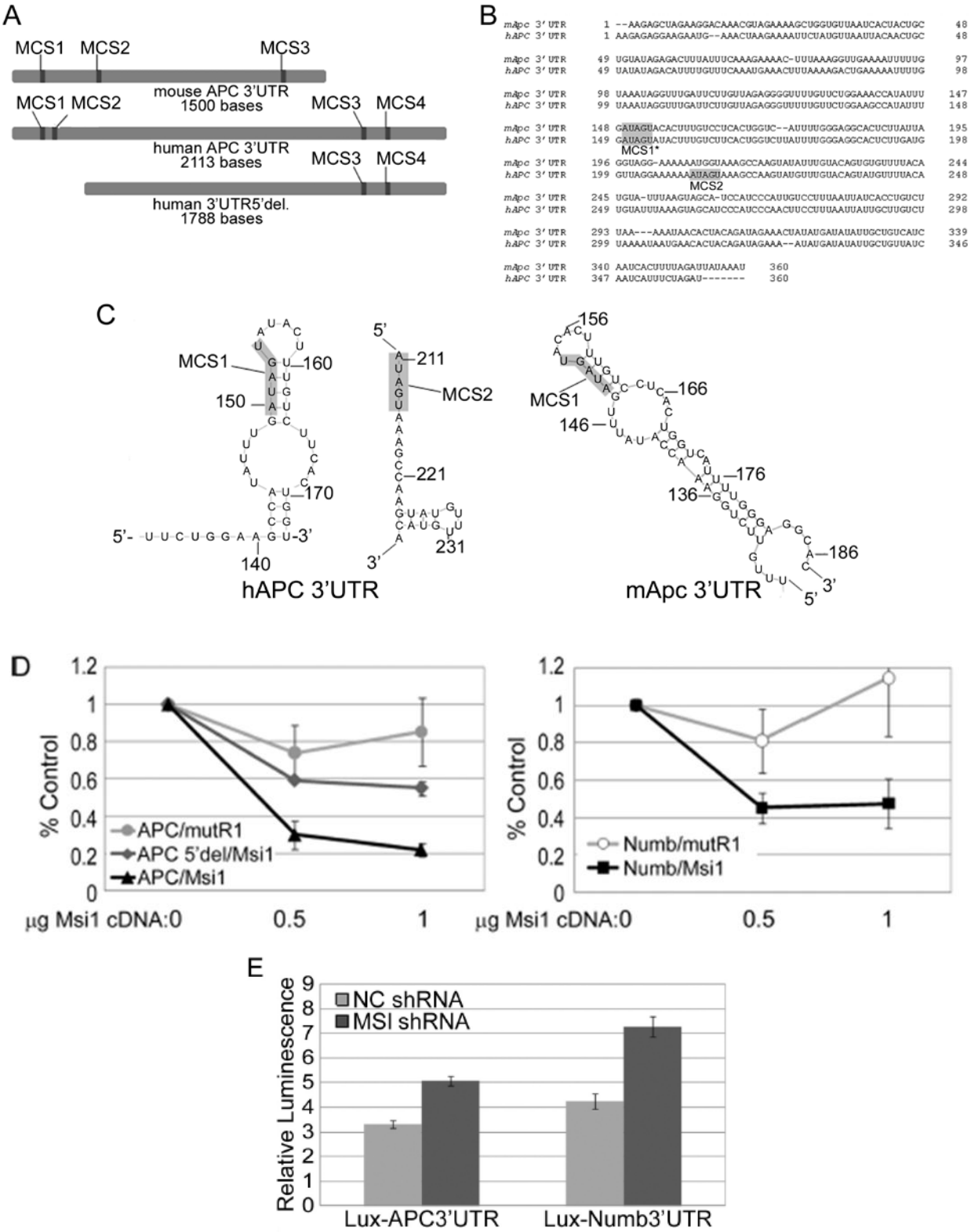


Figure 2.5. *MSI1 binds to APC 3_-UTR and inhibits translation. A*, schematic representation of mouse and human *APC* 3_-UTRs showing position of MCSs. A mutant with the first 360 bp deleted (*human 3'-UTR5'del.*) was incorporated into a luciferase reporter. *B*, alignment of the first 360 bp of mouse and human APC 3'-UTR (*mApc 3'UTR* and *hAPC 3'UTR*, respectively). The *asterisk* indicates MCS conservation

in both position and sequence. *C*, secondary RNA structure surrounding MCS1 and -2 (*shaded*) as predicted by the RNAfold WebServer. Structural images were produced by the mfold Web Server. *D*, *left panel*, luciferase activity in NIH3T3 cells transfected with a luciferase reporter containing *APC* 3'-UTR (*APC*), *APC* 3'-UTR 5' deletion (*APC 5'del.*), and *Flag-Msi1* cDNA (*Msi*) or a *Flag* mutant *Msi1* cDNA (*mutR1*). *Right panel*, luciferase activity in NIH3T3 cells transfected with luciferase reporter containing the positive control *Numb 3'-UTR* (Numb) and Msi or mutR1. Data are expressed as the average percentage of luciferase reporter activity in Msi or mutR1 co-transfected cells *versus* Vector alone (*% Control* ± S.E.). Co-transfection of the wild-type *APC* 3'-UTR construct with *Flag-Msi1* significantly decreased the luciferase activity when compared with mutR1 co-transfectants ($p < 0.05$). Co-transfection of *APC* 3'-UTR 5' deletion with *Msi1* did not significantly decrease luciferase activity from mutR1 ($p < 0.05$) and resulted in a significant loss of Msi1 repression ($p < 0.05$). *E*, luciferase activity in HCT116βw cells co-transfected with either scrambled (*NC*) or MSI shRNA and luciferase reporters containing either *APC* or *Numb* 3'-UTR. Data are expressed as average relative luminescence ± S.E. after normalizing for transfection efficiency with *Renilla* luciferase. In HCT116βw cells, reporters containing either the *APC* or the *Numb* 3'-UTR each produced more luciferase when co-transfected with *MSI1* shRNA than when co-transfected with scrambled shRNA ($p < 0.05$).

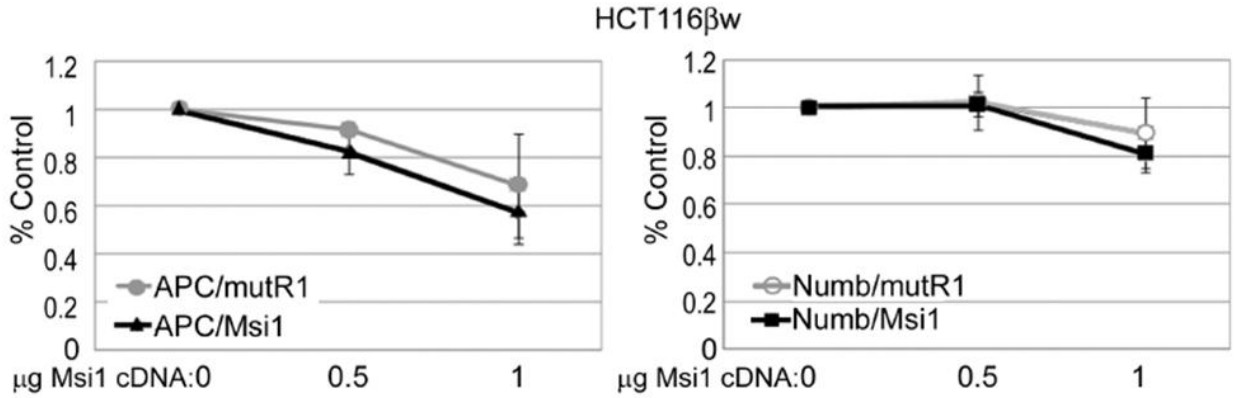


Figure 2.6. *APC 3'UTR reporter assay in HCT116βw cells.* Luciferase activity in HCT116βw cells co-transfected as in Fig. 4D with luciferase reporter containing APC 3'UTR (APC) or Numb 3'UTR (left and right panels, respectively) and Msi or mutR1.

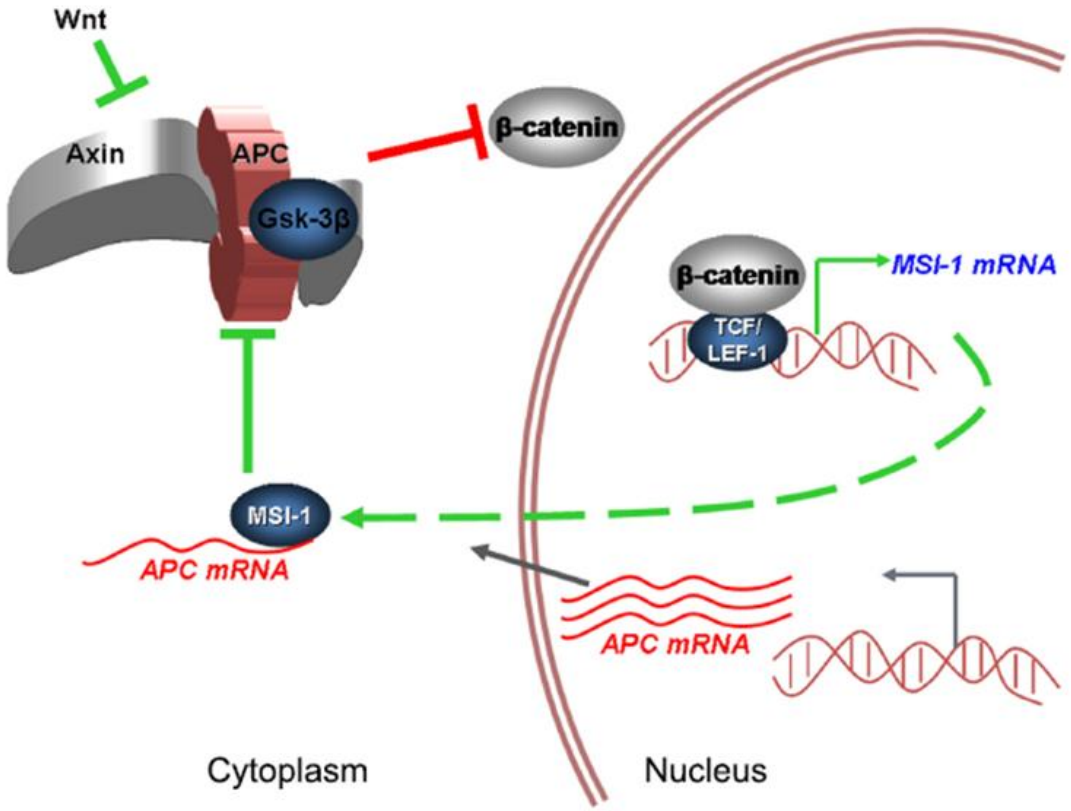


Figure 2.7. *APC-MSI1 double-negative feedback loop in human intestinal epithelium.* Under normal cellular conditions, APC represses Wnt target gene expression by targeting _-catenin for destruction (*red line*) and sequestering nuclear β-catenin from transcription co-factor TCF/LEF-1 (not shown). In the presence of Wnt or following loss of APC function, β-catenin drives expression of Wnt target genes including *MSI1* (*green lines* and *arrows*). Current studies expand this model by demonstrating that MSI1 protein inhibits translation of *APC* mRNA, completing a double-negative feedback loop between APC and MSI1.

Chapter 3

MSI1 Stabilizes *APC* mRNA

Abstract

MSI1 is an RNA-binding protein that binds to the 3' untranslated region of target mRNAs in a sequence specific manner and inhibits their translation. In Chapter 2 we described a unique interaction between MSI1 and the tumor suppressor APC in which each represses the expression of the other in a double-negative feedback circuit. MSI1 bound to a specific sequence in the 3' untranslated region of the *APC* mRNA and inhibited protein production. Overexpression of exogenous *Msi1* decreased APC protein levels, as expected, but *APC* mRNA levels increased. The data presented here demonstrate that *APC* mRNA is stabilized by MSI1 binding. We hypothesize that stabilization of *APC* mRNA by MSI1 allows a rapid restoration of APC protein upon Wnt removal. The inherent bistability of a double-negative feedback circuit, such as the one between APC and MSI1, requires a signal, in this case Wnt removal, to return the circuit back to the APC dominant state. Such a signal would involve the release of the *APC* mRNA by MSI1. We tested whether MSI1 was inherently unstable or conditionally unstable in response to removal of Wnt signal. We did not observe destabilization of MSI1, indicating that another mechanism is involved stimulating the release of the *APC* mRNA. We expect that the stabilization and subsequent rapid translation of *APC* mRNA is the first step in response to a Wnt signal. Further study is needed to understand the mechanisms involved in this process.

Introduction

The Musashi (MSI) family of sequence specific RNA binding proteins has been shown to significantly impact cell fate determination in developing central nervous systems of *Drosophila* and mice [70, 102]. The importance of Msi1 in driving epithelial regeneration in the intestines of mice has been established [74-76]. Msi1 has been shown to stimulate proliferation and inhibit differentiation of

cultured cells in which it is up-regulated [69, 88]. Mechanistic studies have revealed that these cellular phenotypes are the direct result of MSI1 mRNA binding and subsequent translational repression of target mRNAs ([69, 88], Chapter 2). This translational repression leads to decreases in critical regulatory elements of the Wnt, Notch and Cyclin-dependant kinase signal transduction pathways.

The tumor suppressor APC is mutated in approximately 80% of all colorectal cancers [31, 32]. APC is a critical component of the Wnt signaling pathway which regulates the expression of genes required for cell proliferation such as Cyclin D1 and c-Myc [44-46]. Extracellular Wnt ligand results in an intracellular signal that inactivates a complex containing APC. The inactivation of the APC-containing complex results in β-catenin-mediated transcriptional activation of target genes [103]. Recently, *Msi1* was identified as a target of the Wnt signaling pathway in mouse and human intestinal epithelium ([87], Chapter 2). Moreover, *APC* mRNA is a target of MSI1 binding and translational inhibition resulting in a double-negative feedback loop indicating the potential of bistable regulation of the Wnt signaling pathway in the intestinal epithelium (Chapter 2).

Our previous work showed that overexpression of exogenous *Msi1* in human colon cancer cells leads to a decrease in APC protein, in accordance with the role of MSI1 as a translational inhibitor (Chapter 2). Unexpectedly, in this same experiment, *APC* mRNA levels increased, indicating that MSI1 binding to the APC 3'UTR may serve to both stabilize and inhibit translation of the *APC* message. In the current studies we demonstrate that MSI1 binding results in stabilization of *APC* mRNA. We expect that the stabilization of *APC* mRNA serves as a rapid response mechanism to turn off the canonical Wnt signaling pathway once the Wnt signal has been removed. In this case, MSI1 would need to be released from the *APC* 3'UTR once Wnt signal is removed from these cells. To this end, we also tested whether MSI1 is an inherently unstable protein or if MSI1 protein stability is decreased after removal of a Wnt signal in these cells. This study is the first demonstration of a role for MSI1 in mRNA stabilization.

Materials and Methods

Cell culture.

HCT116βw were acquired and cultured as described previously (Chapter 2). Briefly, cells were cultured in McCoy's 5A medium with 5% fetal bovine serum (FBS) at 37°C with 5% carbon dioxide. Experiments involving transfection and/or treatment with drug were performed in 6-well plates as described below.

Plasmids and Transfection.

FlagMsi expression and RNAi knockdown experiments employed plasmids described previously (Chapter 2). FlagMsi expression constructs were a generous gift from Hideyuki Okano (Tokyo, Japan). Cells were transfected with 1μg of FlagMsi1 expression construct or 2.5μg of shRNA construct with GeneExpresso lipofection reagent (Lab Supply Mall, Gaithersburg, MD) at a reagent(μL):DNA(μg) ratio of 3.5μL:1μg. Transfection medium was prepared by adding GeneExpresso and DNA to fresh McCoy's 5A medium with 7.5% FBS. Cells were grown in 2mL of transfection medium for 24-48 hours depending on experimental design.

Drug treatments.

The RNA polymerase II inhibitor, Actinomycin D, was used to assess the stability of APC mRNA in response to exogenous FlagMsi1 or shRNA knockdown of endogenous MSI1. Cells were incubated for 40-48 hours in transfection medium prior to drug treatment. Transfection medium was replaced with 2mL of fresh medium containing 5μg/mL Actinomycin D. Cells from a single well were collected immediately after addition of Actinomycin D (zero time point) and remaining cells were cultured under normal culture conditions until harvest at designated time points (Fig. 3.1). Upon harvest, cells were

washed twice with ice cold PBS, collected in Reporter Lysis Buffer (Promega, Madison, WI) with protease inhibitors and sonicated briefly (Output 1 for 10 sec.). 100μL of cell lysate was added to 1mL of Trizol reagent (Invitrogen) and stored at -80°C until use.

Protein stability experiments employed Cycloheximide (CHX, Sigma), an inhibitor of translation. CHX stock was prepared in Dulbecco's Modified Eagle Medium (DMEM) and diluted in culture medium to a final concentration of 100μg/mL for treatment. The experiment sought to assess the stability of MSI1 protein before and after removal of a Wnt signal. In this case, cells were treated with Wnt3a conditioned medium, as described below, for 24 hours. Wnt medium was removed, cells were washed with PBS and fresh medium containing Cycloheximide was added. Cells were harvested at different time points as described above (Fig. 3.2B). Cells from a single well were harvested immediately after addition of drug (zero time point). For protein analysis, 75μL of cell lysate was added to 25μL of 4x protein sample buffer immediately after sonication. Samples were heated to 95°C for 5 minutes, centrifuged at maximum speed for 1 minute and stored at -80°C until use.

RNA analysis.

Total RNA was extracted from Trizol reagent and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to assess levels of target mRNAs as described previously (Chapter 2). C(T) values for target mRNAs, *APC* and *c-MYC*, were normalized to *GAPDH* and expressed as a Fold Change from the zero time point for each target. For MSI1 shRNA studies, *MSI1* mRNA was analyzed in zero time point samples to verify that *MSI1* expression was reduced due to RNA interference. *MSI1* mRNA decreased by an average of 71.6% ± 6.8% (four independent experiments) in *MSI1* shRNA treated samples as when compared to negative control shRNA treated cells. Sequences of target specific primers were as follows: APC primers – forward 5'-AACTGCGGTCAAAAATGTCC-3', reverse 5'-TGTCAAGATCAGC-AAGAAGCA-3'; c-MYC primers – forward 5'- GAGGCTATTCTGCCCATTTG-3', reverse

5'- ACCGAGTCGTAG-TCGAGGTC-3'; MSI1 primers – forward 5'-GACTCGCCGCACGACCCC-3', reverse 5'-GGCCACCTTAGGGTC-AATTG-3'; GAPDH primers – forward 5'-TG-CTGTTGAAGTCGCAGGAG-3', reverse 5'-CCAATGTGTCCGTCGTGGA-3'.

Protein analysis.

Protein samples were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Blots were probed for MSI1 with a rabbit polyclonal antiserum produced locally (1:2000) and for Histone H2B (1:1000, abcam, cat. no. ab1790). MSI1 band intensities were calculated using Image J software (Java 1.6.0_26, NIH, http://rsb.info.nih.gov/ij) normalized to Histone H2B and expressed as fold change from time zero to assess protein stability.

TOPFlash Luciferase Reporter Assay.

The TOPFlash reporter system was used as described previously (Chapter 2) to assess Wnt pathway signal transduction by assessing β-catenin transcriptional activation. Cells treated with Wnt3a or control conditioned medium were harvested in lysis buffer and analyzed for luciferase activity to demonstrate stimulation of the canonical Wnt signaling pathway.

Wnt3a Conditioned Medium.

Wnt3a and control conditioned media were produced from mouse L cell lines as described previously (Chapter2). Previous data indicated that a 1:1 volume:volume ratio of Wnt3a medium to fresh culture medium stimulates an optimal Wnt signaling response in HCT116βw cells. This ratio was used for all subsequent experiments.

Results

Msi1 overexpression in HCT116βw cells results in the stabilization of APC mRNA.

Previous studies showed that the overexpression of exogenous Msi1 in human colonocytes led to decreases in APC protein, consistent with the role of MSI1 as a translational inhibitor, but a concomitant increase in *APC* mRNA also occurred (Chapter 2). This indicated that MSI1 binding to the 3'UTR of the *APC* mRNA may function not only to repress *APC* expression, but also to stabilize the *APC* mRNA. To test this hypothesis, we used the RNA polymerase II inhibitor, Actinomycin D in order to halt mRNA production in FlagMsi1-transfected cells and monitored mRNA levels over time (Fig. 3.1A). Cells transfected with FlagMsi1 had significantly more APC mRNA at two and four hour time points post-Actinomycin D treatment when compared to cells transfected with empty vector or the RNA binding-incompetent Msi1 mutant, FlagMsimutR1. The expected rapid degradation of c-*MYC* mRNA indicated that mRNA production had ceased following addition of Actinomycin D (Figure 3.1B). c-*MYC* is an immediate early gene that codes for a transcription factor involved in cell cycle progression. The activity of the c-MYC protein is regulated by the relatively unstable nature of the mRNA and protein [104]. These data suggest that Msi1 binding to *APC* mRNA results in its stabilization.

In order to demonstrate that the RNA stabilization effect was due to the sequence-specific binding of MSI1 to its target mRNA we analyzed the effect of exogenous expression of *Msi1* on c-*MYC* mRNA. If the effect of FlagMsi1 overexpression was a generalized mRNA stabilization, then we would expect to see stabilization of c-*MYC* mRNA as well. We observed no stabilization of c-*MYC* mRNA associated with the expression of FlagMsi1 when compared to the RNA binding incompetent mutant, FlagMsimutR1 (Fig. 3.1B). This indicates a target specific stabilization of *APC* mRNA by Msi1.

Finally, we wanted to demonstrate that the stabilization of *APC* mRNA by MSI1 takes place under physiological conditions and not just a result of exogenous protein overexpression. Since HCT116βw cells express detectable amounts of endogenous MSI1, we predicted that depletion of this

MSI1 would destabilize the *APC* mRNA. Consistent with this prediction, after 4 hours of Actinomycin D treatment cells transfected with *MSI1*-specific shRNA had approximately 60% less *APC* mRNA than cells transfected with a negative control (NC) shRNA, that does not have a specific cellular target (Fig. 3.1C). Taken together these data indicate that the sequence-specific binding of MSI1 to the 3'UTR of APC mRNA not only results in translational repression (Chapter 2), but also results in the stabilization and accumulation of the *APC* mRNA.

Msi1 protein is stable and this stability does not decrease in response to removal of a Wnt signal from HCT116βw cells.

We have shown previously, and have repeated the experiment here, that HCT116βw cells respond to treatment with Wnt conditioned medium by upregulation of β-catenin transcriptional activity as indicated by the TOPFlash luciferase reporter assay system (Chapter 2, Fig. 3.2A). Given the potential bistability of the APC-MSI1 double-negative feedback system, it is possible that the stabilization of the *APC* mRNA by MSI1 functions as a response to Wnt signal upregulation of *MSI1* expression and subsequent downregulation of APC. In order for the accumulated *APC* mRNA to be translated, MSI1 must release it. We have proposed three potential scenarios by which MSI1 releases the APC mRNA allowing translation to initiate (Fig. 3.3). The first and second scenarios involve MSI1 protein stability. In the first scenario, MSI1 protein is inherently unstable and decreased expression through the loss of Wnt signal is sufficient to remove it from the *APC* mRNA. The second scenario indicates that the loss of Wnt signal is required to destabilize MSI1 protein. In this case decreased expression coupled with decreased protein stability would combine to remove MSI1 from the *APC* mRNA. In the final scenario, MSI1 protein is modified or bound in some way that would cause a conformational change allowing for the release of the *APC* mRNA. We did not venture to test this final scenario in these studies, but have plans for future experiments to this end. In the second scenario we

expect an observable response by MSI1 to the removal of Wnt signal. The most obvious response would be a decrease in MSI1 protein stability subsequent to the removal of Wnt conditioned medium as indicated by the second scenario. The destabilization of MSI1 protein after removal of the Wnt signal would allow production of APC protein from the accumulated *APC* mRNA. This upregulation of *APC* expression would allow for regulation of the Wnt signaling cascade to be reestablished.

To explore the first and second scenarios relating to MSI1 protein stability, we treated cells with cycloheximide arrest protein synthesis. Cells were treated with Wnt-conditioned or control medium for 24 hours. After this Wnt stimulation, cells were washed, treated with cycloheximide-containing medium and collected at different time points. MSI1 protein stability was assessed by western blot analysis and normalized to Histone H2B (Figure 3.2B). MSI1 protein was stable over the eight hour time course, showing no drop in normalized protein levels in control medium-treated cells. Likewise, there was no change in MSI1 protein stability in response to removal of the Wnt conditioned medium. Therefore, having no evidence to support the first two scenarios, we are left to test the third scenario in which other modifications of MSI1 are required to remove it from the *APC* 3'UTR and allow translation of the target. Further experimentation is required to establish the response of APC and MSI1 to the stimulation with Wnt-conditioned medium and removal of this stimulus.

Discussion

The importance of post-transcriptional events in regulation of cellular processes is well established. RNA binding proteins play a crucial role in these events, particularly with respect to mRNA trafficking, stability and translational regulation [105-107]. The MSI family of sequence-specific RNA binding proteins has been studied in depth in neural precursor cells from flies to mammals [108]. The importance of MSI1 in the homeostasis of the intestinal epithelium in mammals has recently come to light ([76, 78, 87], Chapter 2). There is quite convincing evidence that Msi1 levels are elevated in

intestinal tumors from mice [72] and humans [78]. Concurrent studies demonstrated that human colon cancer cells require elevated levels of MSI1 for tumorigenicity [78]. Subsequently, our lab and others demonstrated that MSI1 is a target of the canonical Wnt signaling pathway, which is central to the initiation of tumorigenesis in the intestine ([87], Chapter 2). Our studies further demonstrated that MSI1 is involved in feedback regulation of the Wnt signaling pathway through translational repression of the tumor suppressor *APC* (Chapter 2). Taken together, these data emphasize the importance of MSI1 for proper homeostatic balance of the intestinal epithelium and suggest that deregulation of MSI1 expression contributes to tumor development in the intestine.

Previously we demonstrated the presence of a double-negative feedback circuit between MSI1 and APC in the intestinal epithelium (Chapter 2). The potential bistablilty of this circuit has implications for the regulation of proliferation and differentiation in the intestinal epithelium [90]. The bistable nature of double-negative feedback systems indicates that these pathways exist in a toggle-like state. Though not yet defined experimentally, the toggle-like nature of Wnt pathway regulation through the APC-MSI1 double-negative feedback loop can easily be envisioned. When an extracellular Wnt signal is received, the inactivation of the APC-containing complex leads to β-catenin-driven expression of MSI1 ([87], Chapter 2). MSI1 binds to and inhibits the translation of target mRNAs, one of which is *APC*. The loss of functional APC either decreasing expression or mutation expression leads to perpetuation of the Wnt signaling pathway independent of the extracellular Wnt signal ([17, 23, 24], Chapter 2). It seems reasonable that, under normal conditions, there is some mechanism to reverse this signal independent Wnt pathway stimulation by MSI1, mostly because we know that inappropriate Wnt pathway stimulation, usually through biallelic mutation of *APC*, is an initiating event in colorectal cancer development [18, 23, 24, 32, 103]. It is this secondary signal that “flips the bistable switch” back toward APC which is the topic of these studies.

During our previously studies we noticed that MSI1 upregulation in cultured human colonocytes led not only to decreased APC protein levels, through translational inhibition, but also to increased APC mRNA levels (Chapter 2). This led us to hypothesize that MSI1 both inhibits *APC* translation and stabilizes *APC* mRNA, resulting in its accumulation. We provide experimental evidence that MSI1 does stabilize the APC mRNA, that this stabilization is specific to the RNA binding ability of MSI1, and that it occurs under normal cellular conditions, not merely as an artifact of MSI1 overexpression.

Considering the nature of bistable systems, such as the APC-MSI1 double-negative feedback circuit, we wondered if *APC* mRNA stabilization might be part of the regulatory mechanism to return the system back to one of APC dominance once the Wnt signal has dissipated. If this were the case, we expected that the removal of the Wnt signal would create a signal that causes MSI1 to release the *APC* mRNA. We first hypothesized that MSI1 protein is inherently unstable and that the loss of Wnt signal would rapidly decrease the intracellular pool of MSI1. The data do not indicate this as cells treated with control medium showed no decrease in MSI1 protein levels after eight hours. In a second scenario, we explored whether the loss of Wnt signal would decrease MSI1 protein stability, thus releasing *APC* mRNA for translation. Again, our data do not support this model.

We acknowledge that these studies have created more questions than they have answered. Is there a rapid response of APC protein production after removal of a Wnt signal? Is this response MSI1 specific? What is the signal that causes MSI1 to release the *APC* mRNA? Post-translational modification? Protein-protein interaction? The observation that MSI1 stabilizes APC mRNA is an important cornerstone for the generation of these and other questions. Further study of this system will help to elucidate the mechanisms involved in this complex double-negative feedback circuit which will be important to understanding the regulation of homeostasis in the intestinal epithelium. Ultimately, we expect that this knowledge will broaden our understanding of colorectal carcinogenesis.

Figures

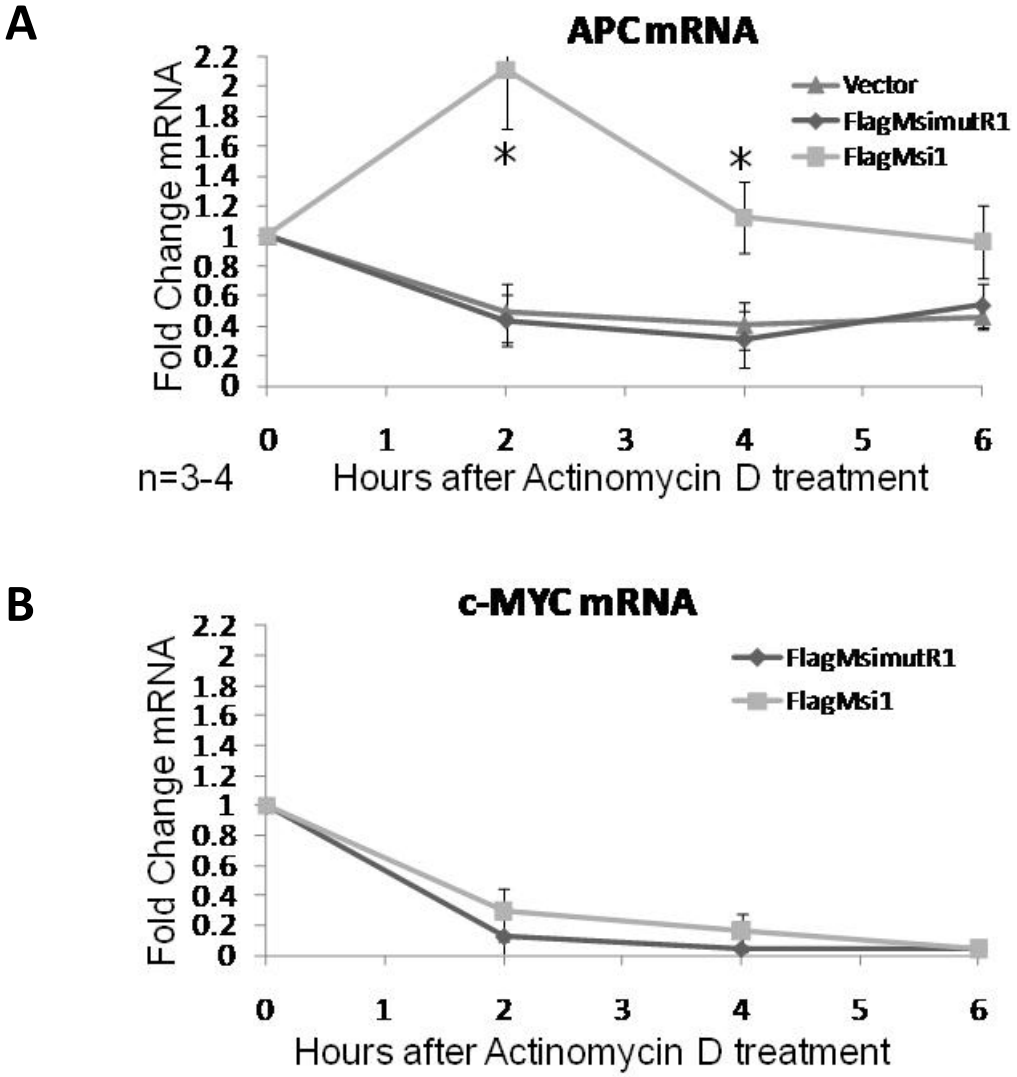


Figure 3.1. *MSI1 stabilizes APC mRNA in HCT116βw cells.* Cells transfected with *FlagMsi1*, MSI1 shRNA or controls for 40-48 hours were treated with Actinomycin D and collected for qRT-PCR analysis of RNA as described in Materials and Methods. A: Fold change data from qRT-PCR analysis of *APC* mRNA from cells transfected with FlagMsi1, FlagMsimutR1 (an RNA binding incompetent Msi1 mutant cDNA) or pCDNA3 vector showing a stabilization of *APC* mRNA at 2 and 4 hour time points. B: Fold change data from qRT-PCR analysis of *c-MYC* mRNA from the same cells showing no stabilization of *c-MYC* mRNA. * Data from FlagMsi1- and FlagMsimutR1-transfected cultures show statistically significant differences ($p < 0.05$, Student's T-test). Error bars represent SEM from 4 independent experiments.

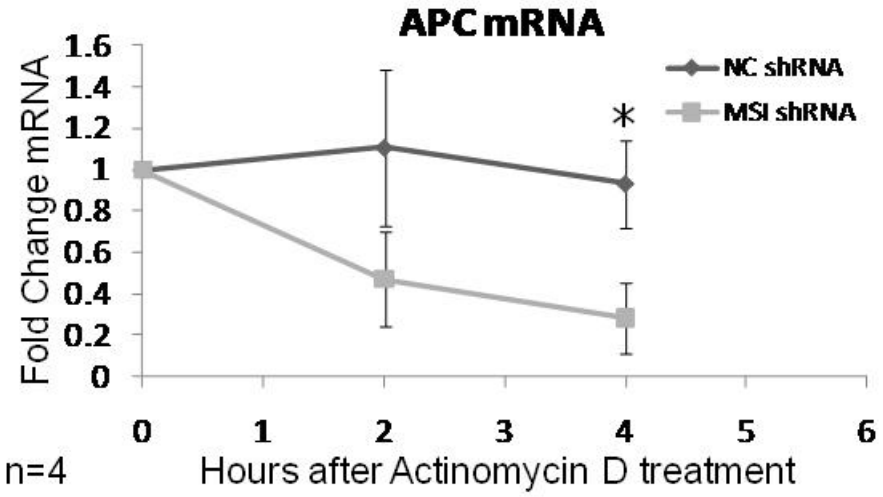


Figure 3.2. *Loss of MSI1 from HCT116βw cells destabilizes APC mRNA.* Fold change data from qRT-PCR analysis of *APC* mRNA from cells transfected with *MSI1* shRNA or negative control (NC) shRNA showing a destabilizing effect on *APC* mRNA when endogenous MSI1 is decreased. * Data from MSI1 and NC shRNA transfected cultures show statistically significant differences ($p < 0.05$, Student's T-test). Error bars represent SEM from 4 independent experiments.

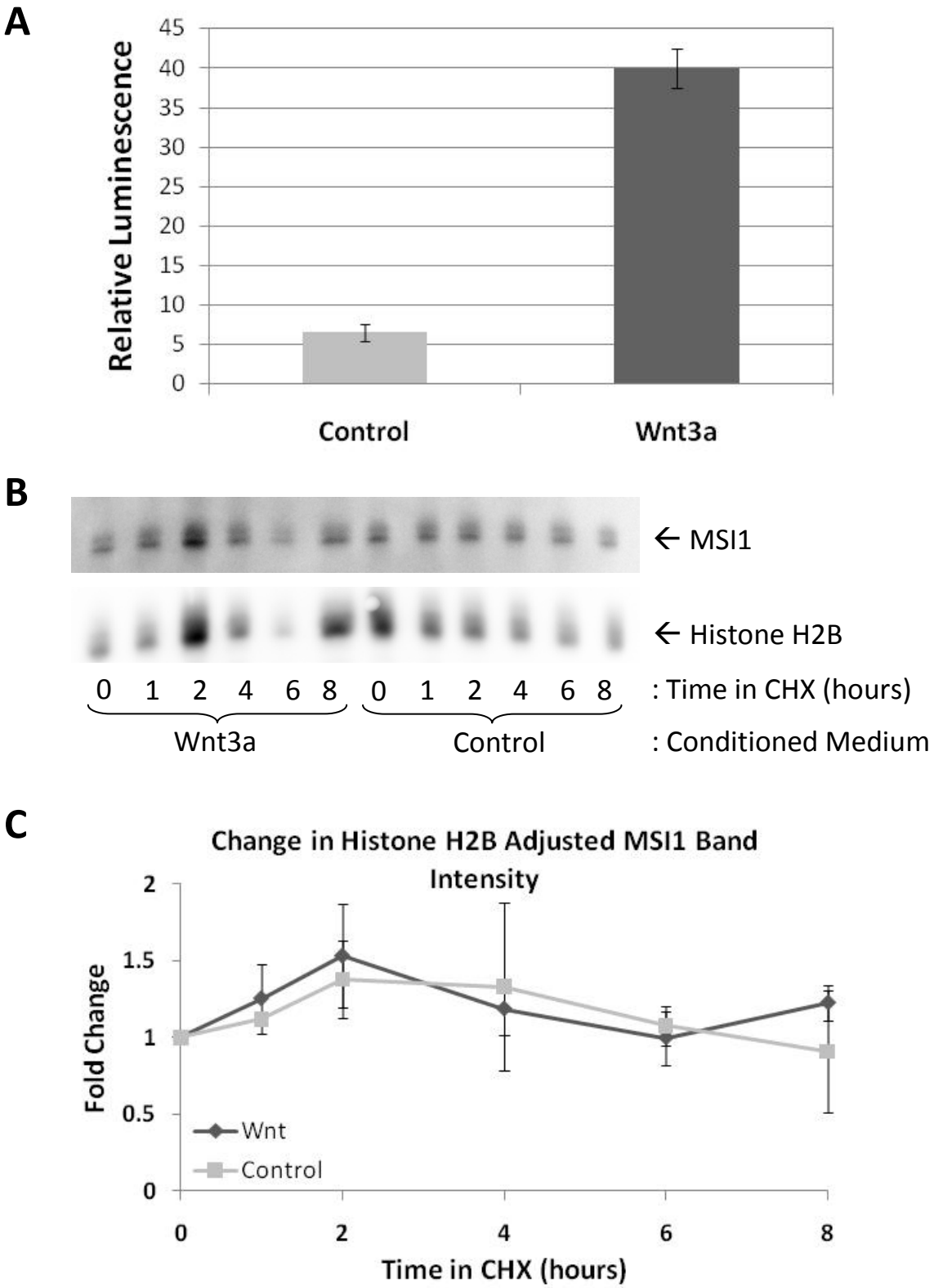


Figure 3.3. *MSI1 protein stability does not change after removal of Wnt signal from HCT116βw cells.* Untransfected cells were cultured in Wnt3a or control conditioned medium for 24 hours, washed, treated with Cycloheximide (CHX), and collected at different time points for MSI1 protein analysis. A:

Cells transfected with TOPFlash reporter 24 hours prior to Wnt3a treatment were collected at zero time point and analyzed for luciferase activity. The TOPFlash assay indicates that cells treated with Wnt3a conditioned medium have a >7 fold increase in β-catenin transcriptional activity indicating stimulation of the canonical Wnt signaling pathway. B: Representative western blot of MSI1 and Histone H2B in CHX-treated cells after wash-out of Wnt3a signal. C: Quantification of Histone H2B-normalized MSI1 band intensities showing no difference in MSI1 stability between Wnt3a and control treated cells. Error bars represent SEM from 2-3 independent experiments.

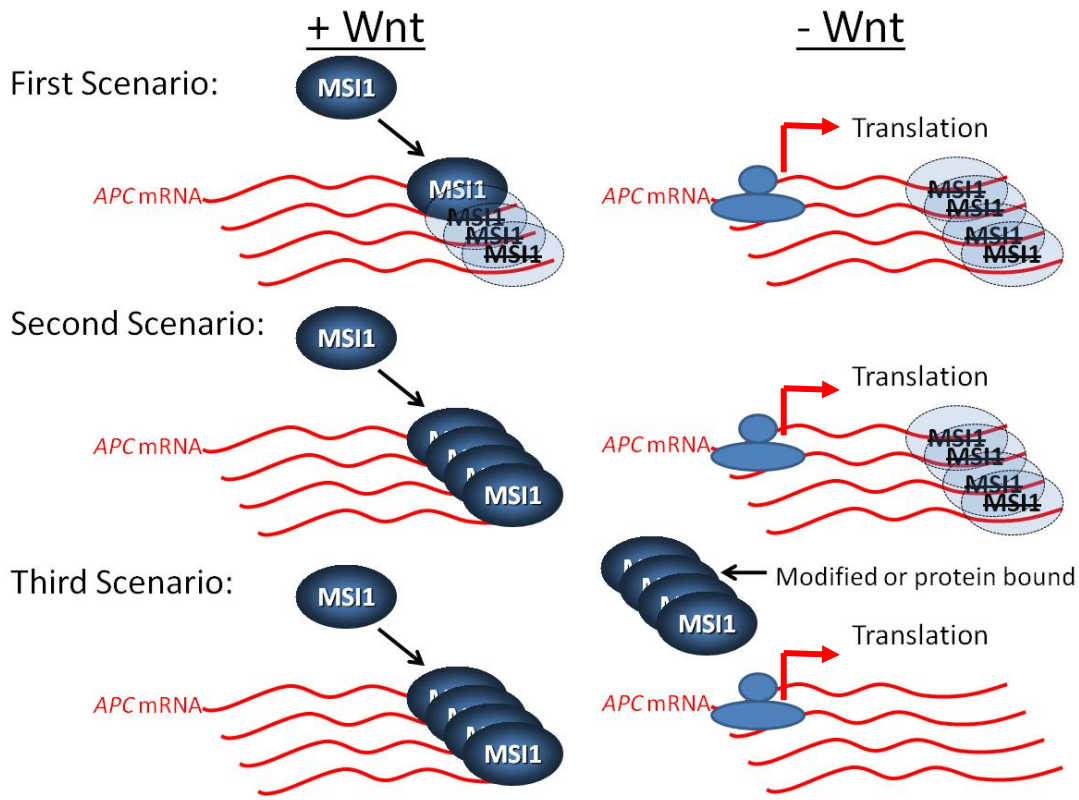


Figure 3.4. *Multiple scenarios for the release of APC mRNA by MSI1 and subsequent stimulation of APC expression.* Three hypothetical scenarios for the release of APC by MSI1 after Wnt signal depletion are depicted. For all scenarios the presence of Wnt signal is indicated on the left side (Wnt+) and the absence of Wnt signal is indicated on the right (Wnt-). The first scenario shows inherently unstable MSI1 protein. In the presence of Wnt signal *MSI1* expression is stimulated (Chapter 2) and MSI1 binding to *APC* mRNA is constantly replenished. When Wnt signal is lost, MSI1 is no longer replenished and MSI1 bound to *APC* mRNA is degraded, freeing the *APC* mRNA for translation (the ribosome indicated by large and small ovals and translation is indicated by the arrow). In the second scenario, MSI1 protein is conditionally unstable, with stability decreasing after Wnt signal is lost thus freeing *APC* mRNA for translation. The third scenario does not require a change in MSI1 stability, but instead indicates a post translational modification of MSI1 or a third protein binding to MSI1 that causes it to release the APC mRNA.

Chapter 4

c-MYC Stimulates *MSI1* Expression

Abstract

The MYC family of transcription factors is the most commonly deregulated group of oncoproteins in human cancers. c-*MYC* is expressed in actively proliferating tissues, such as the intestinal epithelium. Studies in mice hinted of a link between c-MYC and the sequence specific RNA binding protein MSI1 that hinges on the tumor suppressor APC [109]. Inducible, intestine specific loss of Apc in mice leads to decreased differentiation, increased proliferation and a significant increase in *Msi1* expression. These intestinal phenotypes are rescued by inducing the losses of c-Myc and Apc together. The data presented here indicate that c-MYC can stimulate MSI1 expression in cultured human colonocytes. Because c-*MYC* is a stimulated Wnt target gene, decreasing APC in these cells would be expected to stimulate c-*MYC* expression. In fact, the opposite was observed. Reduction in APC levels, following introduction of specific shRNA, resulted in a decrease in c-*MYC* expression indicating that the stimulation of MSI1 by c-MYC is not a result of Wnt pathway stimulation. This implies that c-MYC has the ability to stimulate *MSI1* expression, but may not be involved in regulation of *MSI1* expression under normal cellular conditions. We propose that MSI1 regulation by c-MYC is specifically associated with cancers that have deregulated c-*MYC*.

Introduction

Deregulation of the c-*MYC* gene is the most common genetic alteration associated with the global array of human cancers [110]. In contrast to other common oncogenes such as ras, deregulation of c-MYC is not typically associated with a point mutation which creates constituitive activity. Instead, c-MYC deregulation is commonly associated with overproduction of a normal protein as a result of gene amplification, translocation, or aberrant induction of oncogenic pathways, particularly the Wnt signaling

pathway, for which c-MYC is a stimulated target [44, 111]. Early studies of c-*MYC* revealed it as an immediate early response gene for growth factor signaling [112]. By definition, this indicates that c-*MYC* mRNA and protein are elevated in actively proliferating cells and low in cells that are not actively cycling, or quiescent [104]. Consequently, the major regulatory mechanism for c-*MYC* activity is the rapid degradation of the mRNA and protein products in normal cells [113]. Though study of c-*MYC* regulation at multiple levels is ongoing, one picture has become clear, deregulated c-MYC activity, either through overexpression or stabilization of the gene products, is important to the development of human cancers [110]. The pleiotropic nature of c-MYC indicates that its deregulation may play different roles in the development and progression of different cancers [110]. When studying any one of the vast array of human cancers, the role of c-*MYC* must be considered because of its prevalence as an oncogene.

Initial studies in an inducible, intestine-specific *Apc* knockout system indicated that loss of Apc stimulates *Msi1* expression in the mouse intestine [17]. This Msi1 upregulation was accompanied by decreased differentiation and increased proliferation in the intestinal epithelium. Mechanistic studies of the regulation of *MSI1* expression have linked the observed phenotypes to *MSI1* by demonstrating that MSI1 is a target of β-catenin transcriptional activation through the canonical Wnt signaling pathway ([87], Chapter 2). Furthermore, we demonstrated that MSI1 feeds back to stimulate the Wnt signaling pathway through translational inhibition of *APC* (Chapter 2). There is some indication however, that c-Myc may play a role in the regulation of the Msi1 associated phenotypes *in vivo*. Combining inducible loss of both *Apc* and *c-Myc* in the mouse intestine rescues the intestinal phenotypes observed in the *Apc* knockout alone [109]. These data indicate a role for c-MYC in the regulation of MSI1 expression in the intestinal epithelium.

The studies in this chapter were performed to test whether c-MYC effects *MSI1* expression in intestinal epithelial cells. We first performed bioinformatic analyses on the putative *MSI1* promoter region to ascertain the presence of c-MYC binding elements. Experimentally, we tested the potential

impact of exogenously expressed c-*MYC* on MSI1 levels both by western blot analysis of whole cultures and through the use of immunofluorescence microscopy, comparing transfected and untransfected cells. As demonstrated previously, the subject HCT116βw cell line responds appropriately to stimulation of the canonical Wnt signaling pathway (Chapter 2). Since c-*MYC* has been shown to be a stimulated target of the Wnt signaling pathway [44], we tested the effect of loss of APC on expression of c-*MYC* in these cells as well, with a surprising outcome. Contrary to expectations, we observed a decrease in c-*MYC* expression in response to decreased APC levels. Together, the data presented here indicate that only the overproduction of c-MYC has that ability to stimulate MSI1 expression. These data have implications in the treatment of c-*MYC* overexpressing cancers.

Materials and Methods

Bioinformatics.

The mouse and human *MSI1* genomic sequences were obtained from the UCSC Genome Bioinformatics website (genome.ucsc.edu). Sequence searches and alignments were performed with GENtle DNA analysis software (Magnus Manske, University of Cologne).

Cell culture.

HCT116βw were acquired and cultured as described previously (Chapter 2). As mentioned, transfections were performed in 10cm^2 per well 6-well plates (Chapter 3). For immunofluorescence staining, a 2.2cm^2 glass cover slip was ethanol/flame sterilized and placed in the bottom of the well prior to addition of cells.

Plasmids and Transfection.

The c-*MYC* overexpression construct from which expression of c-*MYC* cDNA is driven by the Cytomegalovirus immediate-early promoter was a generous gift from Don Ayer (University of Utah). The dominant negative MYC cDNA was subcloned from pGEM-T based construct into a pCDNA3 backbone and in-frame with an amino-terminal Flag-epitope tag (Flag-dnMYC). Transfections were performed as described previously (Chapter 3) and cells were grown in transfection reagent for 40-48 hours.

Protein analysis.

Cells were harvested and lysates prepared for western blot as described previously (Chapters 2 and 3). MSI1 was detected on western blots using a rabbit polyclonal antiserum produced locally (1:2500). β-actin was detected with a mouse monoclonal antibody from Sigma (1:1000, cat no. 128K4813).

Samples for immunofluorescent staining were washed twice with ice cold PBS and fixed on ice for 30 minutes in 4% paraformaldehyde solution in PBS with 0.1% Triton X-100. Cells were permeabilized at room temperature in 0.2% Triton/TBS for 5 minutes with agitation. Antibody solutions were prepared in 0.1% Triton/TBS with 1% Bovine serum albumin and 3% normal goat serum. MSI1 was detected with rabbit anti-Musashi-1 polyclonal antibody (1:300, Millipore, cat. no. AB5977), MYC was detected with the mouse monoclonal c-Myc antibody (1:1000, 9E10, Santa Cruz Biotechnology, cat. no. sc-40), and Flag-dnMYC was detected with anti-FLAG M2 monoclonal antibody (1:1000, Stratagene, cat. no. 200472-21). Cells fixed on coverslips were incubated with primary antibody solutions for 90 minutes at room temperature in a humidified chamber. Cells were washed three times with TBS prior to addition of secondary antibody. Secondary antibodies against mouse and rabbit were conjugated to AlexaFluor 488 and 568, respectively (1:1000 each, Invitrogen, cat. nos. A11029 and A11036). Secondary antibodies were applied for 30 minutes at room temperature. During the last 5 minutes of

the secondary antibody incubation, DAPI was added to visualize the nuclei. Stained cells were washed as before, coverslips were mounted onto slides using Pro-Long Anti-Fade reagent (Molecular Probes, cat. no. P7481) and slides were stored in the dark at 4°C until use. Fluorescence microscopy was performed on a Nikon Eclipse 80i with an Optronics MagnaFire SP digital camera attachment. Images were acquired with MagnaFire software and colors merged with Adobe Photoshop.

RNA analysis.

Quantitative real-time reverse transcriptase polymerase chain reaction analysis of mRNAs was performed as described previously (Chapters 2 and 3). C(T) values for *c-MYC* and *APC* mRNAs were normalized to *HGPRT* and expressed as fold change from NC shRNA-transfected cells as calculated using the $\Delta\Delta$C(T) method. HGPRT primer sequences were as follows: forward 5'-TGACACTGGCAAAAC-AATGCA-3', reverse 5'-GGTCCTTTTCACCAGCAAGCT-3'. Primers for the detection *APC* and c-*MYC* mRNA were the same as those used previously (Chapter 3).

Results

The MSI1 promoter contains non-canonical Ebox elements.

Sequence analysis of the 7 kilobase region upstream of the human *MSI1* coding region, the putative *MSI1* promoter, identified four candidate c-MYC-binding, E-box, sequences (Fig. 4.1). While no canonical E-box (CACGTG) sequences were identified, all four candidate sequences were CATGTG. The MYC DNA binding complex has been shown to bind this non-canonical E-box sequence, but with a lower affinity than the canonical CACGTG (Young 2011). In comparison, the mouse *Msi1* promoter region contains three of the same non-canonical CATGTG E-box sequences. Since *MSI1* has been identified as a

target of the canonical Wnt signaling pathway ([87], Chapter 2), we postulate that c-MYC plays a supplementary role in *MSI1* expression.

Exogenous c-MYC expression increases MSI1 protein levels in HCT116βw cells.

In order to test whether *MSI1* expression was responsive to changes in c-MYC levels, we expressed exogenous c-*MYC* in HCT116βw cells. Western blots from cultures transfected with c-MYC cDNA indicate a dose-dependent increase in MSI1 levels (Fig. 4.2A). Immunofluorescence microscopy indicates that MSI1 levels are higher in cells with exogenous *c-MYC* than in cells in the same culture that were not transfected (Fig. 4.2B). MYC was easily detected using an anti-MYC antibody (Materials and Methods) as there was no detectable exogenous c-MYC in these cells. These data indicate that c-MYC can impact *MSI1* expression and that it has a stimulatory effect.

Dominant-negative MYC overexpression decreases MSI1 protein levels in HCT116βw cells.

A dominant-negative c-*MYC* cDNA, which produces a protein lacking the transactivation domain, was cloned into an expression vector in-frame with an amino-terminal Flag-epitope tag (Flag-dnMYC). To further assess the impact of c-MYC on MSI1 expression in our cell line, the dominant-negative MYC was employed. Exogenous dnMYC was specifically detected using an anti-FLAG antibody (Materials and Methods). Overexpression of Flag-dnMYC in HCT116βw cells led to a decrease in MSI1 levels as compared to untransfected cells from the same culture (Fig. 4.3). This depression of *MSI1* expression can be explained by the exogenous dominant negative MYC competing with any endogenous wild-type c-MYC for DNA binding in these cells and thus inhibiting c-MYC induced target gene expression. These data further indicate a role for c-MYC in the regulation of *MSI1* expression and further emphasize that wild-type c-MYC has a stimulatory effect on *MSI1* expression.

c-MYC mRNA decreases in response to the loss of APC in HCT116βw cells.

c-*MYC* was the first gene whose expression was shown to be stimulated by the canonical Wnt signaling pathway [44]. Previous studies have shown that loss of APC in the intestinal epithelium results in upregulation of *MSI1* expression ([17], Chapter 2). We now know that this effect is associated with stimulation of β-catenin transcriptional activation through the canonical Wnt signaling pathway ([87], Chapter 2). The current studies have indicated that c-MYC has a stimulatory effect on *MSI1* expression. Based the data described above, we expected that loss of APC would result in increased *c-MYC* expression. In fact, our data revealed the opposite effect. An shRNA-mediated decrease in APC in these cells led to decreased c-*MYC* mRNA (Fig. 4.4). Coupled with the observation that there is no detectable endogenous c-MYC in the HCT116βw cell line, these data indicate that the observed upregulation of MSI1 in response to the loss of APC is solely driven by the canonical Wnt signaling pathway. Though our data indicate that c-MYC has the ability to upregulate MSI1 expression, this mechanism is likely not functional in these cells under normal circumstances.

Discussion

The product of the *c-MYC* proto-oncogene is a transcription factor with a number of cellular targets [114]. The results of transcriptional activations by MYC family proteins are global cellular changes such as proliferation, growth, and inhibition of terminal differentiation. Deregulation of MYC activity, most commonly through amplification, occurs in a wide range of human cancers making it the most prevalent human oncogene [110, 115].

Studies in mice have indicated the importance of c-*Myc* in phenotypes observed in inducible *Apc*$^{-/-}$ mouse intestine. The loss of c-Myc concurrently with the loss Apc rescued phenotypes and restored normal gene expression as compared to the deletion of Apc alone [109]. These data along with

our observation of double-negative feedback between APC and MSI1 (Chapter2) led us to investigate this apparent convergence of MSI1 and c-MYC at APC.

Our data indicate that c-MYC, when overexpressed, has the ability to stimulate *MSI1* expression in human colonocytes. We have also demonstrated that the transactivating function of c-MYC is required for this stimulation through the overexpression of a dominant negative form of MYC. These data are confounded by the observation that c-*MYC* expression is downregulated in response to loss of APC. The simplest interpretation of these data is that these observations were an artifact of overexpression of a potent oncogene, such as *c-MYC*. However, we submit that the observations from the dominant negative *MYC* overexpression, a suppression of *MSI1* expression, indicate that these data are not merely an artifact. It is instead likely that c-MYC has the ability to stimulate *MSI1* expression, but that this activity is not required for the regulation of *MSI1* expression under normal circumstances in this colon epithelial cell line with low or nonexistent c-MYC.

Based on our data, it is likely that the normal regulation of *MSI1* expression in these intestinal epithelial cells is independent of c-MYC transcriptional activity. An interesting question that arises from these studies is: What happens to *MSI1* expression in cells in which c-MYC is deregulated as is the case in a majority of cancers? Given that MSI1 is involved in a double-negative feedback loop with APC and overexpression of c-MYC leads to increased MSI1, it is likely that deregulation of c-MYC would lead to a change in the balance between APC and MSI1 that would result in a cancer phenotype ([78], Chapter 2). There is still much work to do to test this hypothesis. Direct study of the MSI1 promoter region, particularly through the deletion of the candidate E-box sequences, would indicate the importance of c-MYC in *MSI1* expression during normal and tumorigenic cell states. In any case, the interaction of c-MYC with the APC-MSI1 double-negative feedback loop is an intriguing topic of study that may have significant impact on cancer therapeutics.

Figures

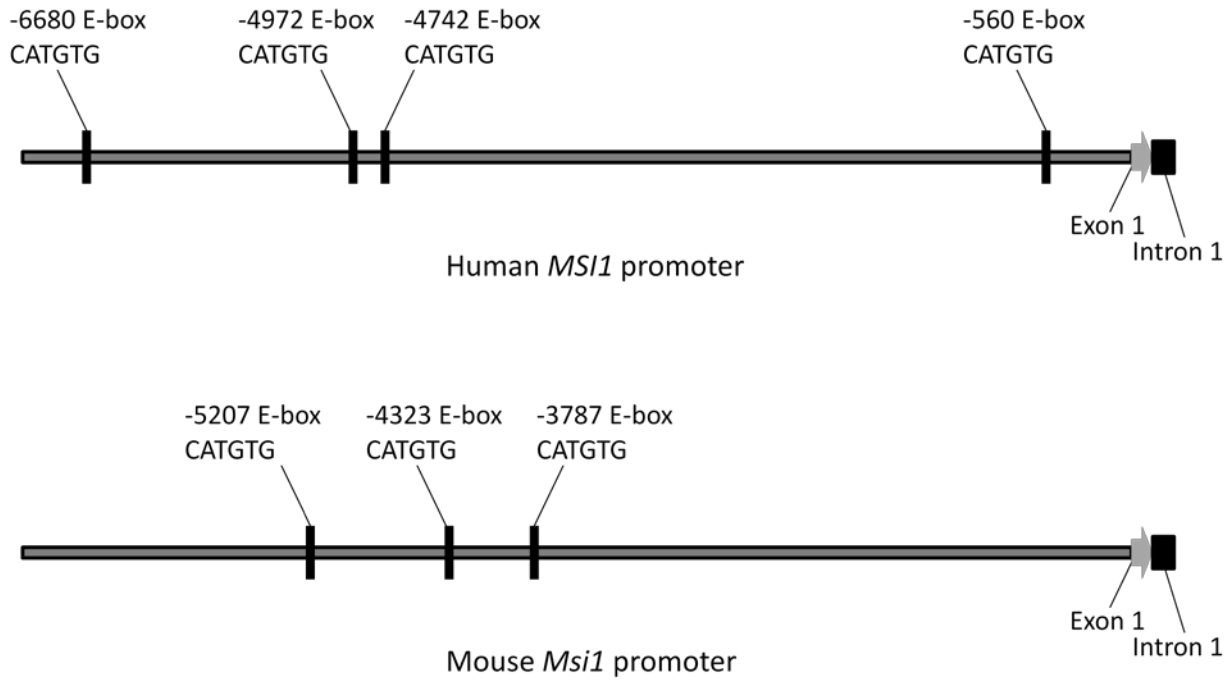


Figure 4.1. *The MSI1 promoter region contains non-canonical E-box elements.* Representative diagrams of human and mouse *Msi1* promoter regions designating the positions of the non-canonical (CATGTG) E-box elements. The mouse promoter region contains three and the human promoter contains four putative c-Myc-binding elements.

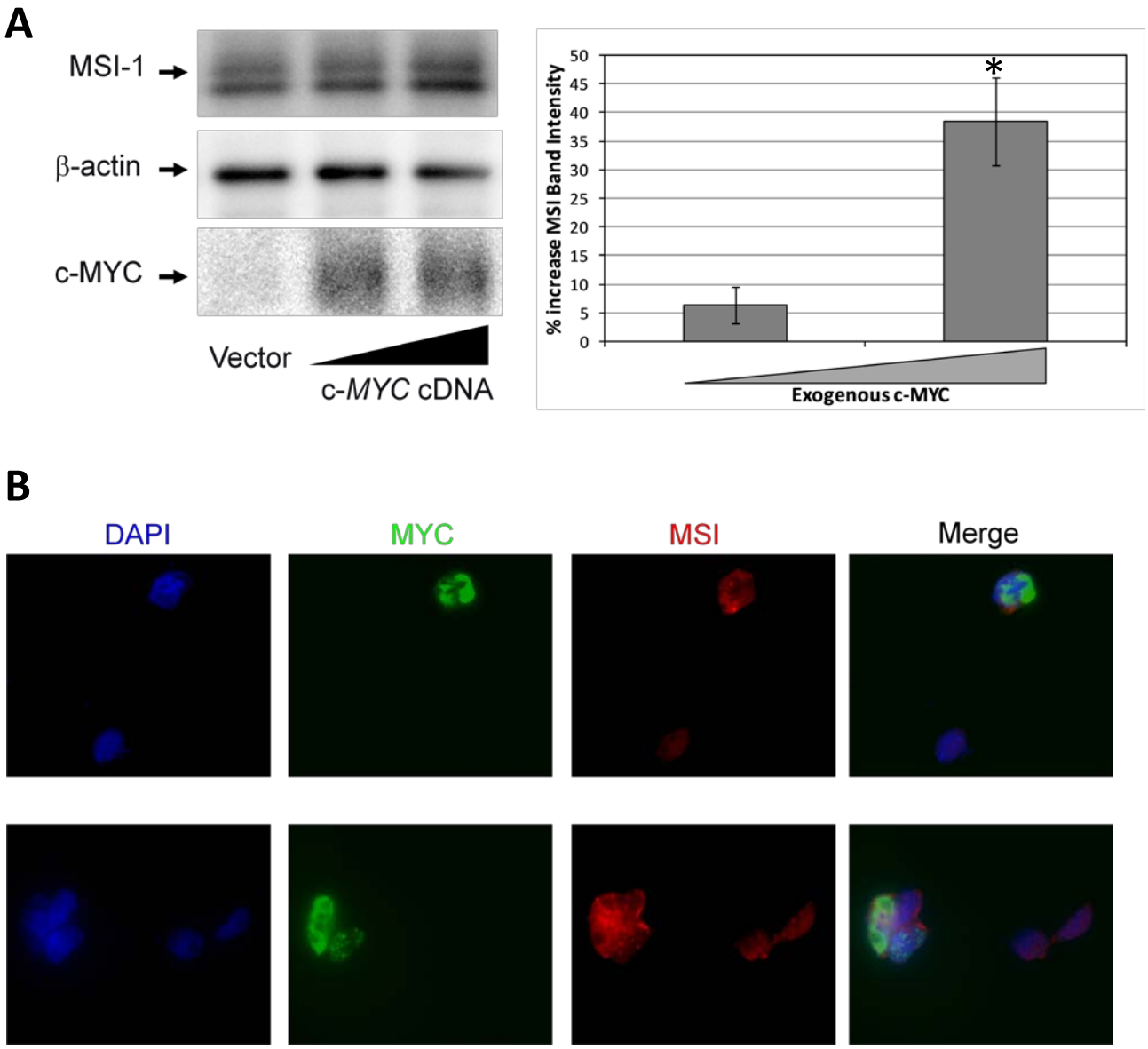


Figure 4.2. *c-MYC overexpression increases MSI1 levels in HCT116βw cells.* A: Cells transfected with c-MYC cDNA were collected and analyzed for the presence of MSI1 protein by western blot analysis of whole cell lysates. (Left) A representative western blot showing an increase in MSI1 protein in response to increasing amounts of exogenous c-MYC with β-actin as a loading control. (Right) MSI1 band intensities were quantified, normalized to β-actin, and expressed as % increase over vector alone transfected cultures. Error bars represent SEM from three independent experiments. * c-MYC transfected cells show a statistically significant increase over cells transfected with vector alone ($p<0.05$, Student's T-test). B: Immunofluorescence analysis of MSI1 (red) in c-*MYC*–transfected (green) cells compared to untransfected cells in the same field. DAPI (blue) marks nuclei of all cells in the field.

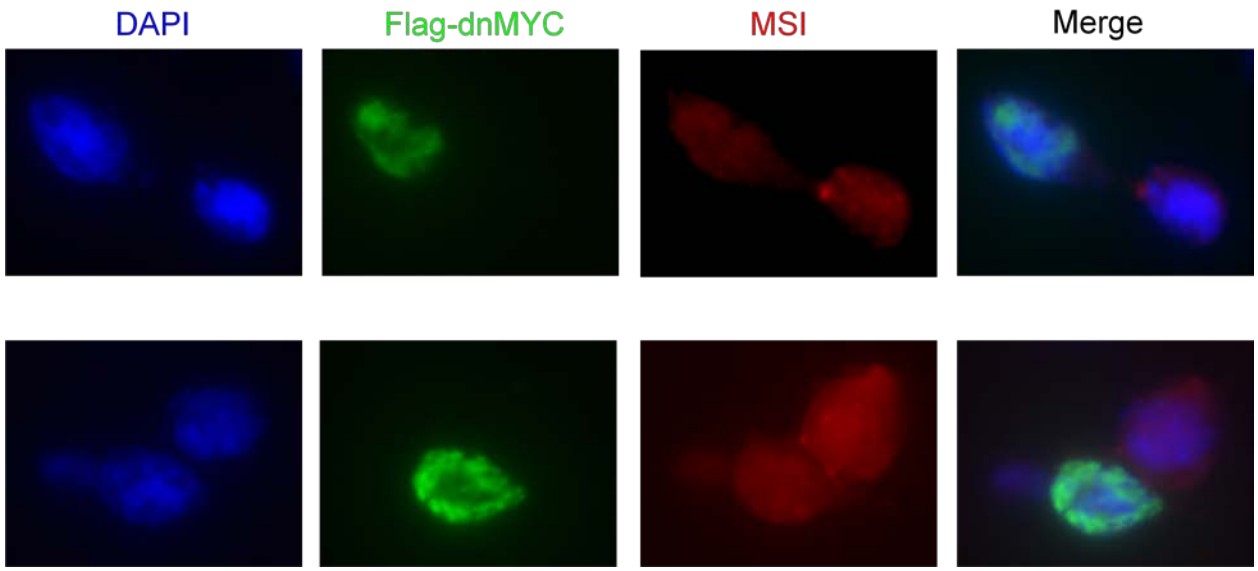


Figure 4.3. *Exogenous dominant negative MYC decreases MSI1 levels in HCT116βw cells.* Immunofluorescence analysis of MSI1 in Flag-dnMYC-transfected cells compared to untransfected cells in the same field. Exogenous dnMYC (green) was detected using an antibody specific to the amino-terminal FLAG epitope tag. Endogenous MSI1 (red) was detected as in Fig. 4.2 and DAPI (blue) stained the nuclei.

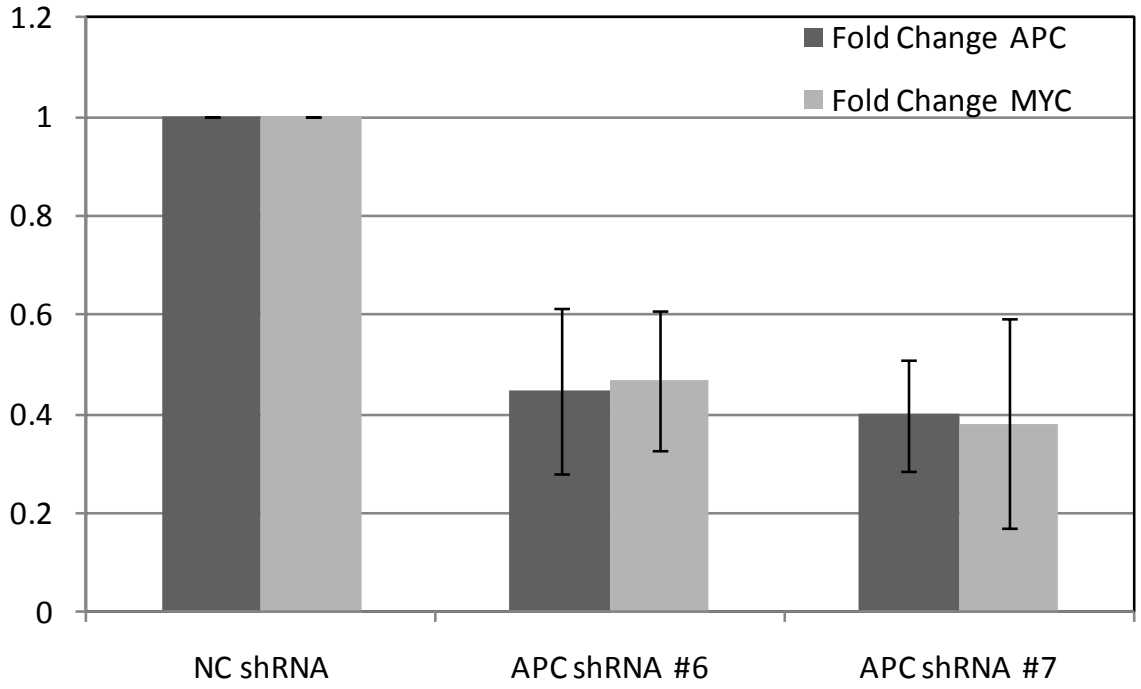


Figure 4.4. *shRNA knockdown of APC in HCT116βw cells leads to decreased c-MYC expression.* Quantitative real-time reverse transcriptase PCR analysis of *APC* and c-*MYC* mRNAs in cells transfected with *APC* shRNAs. C(T) values were calculated for APC and c-MYC mRNAs, normalized to C(T) values for *HGPRT* mRNA and expressed as fold change from negative control (NC) shRNA-transfected cells using the ΔΔC(T) method. Error bars indicate SEM for three independent experiments. *HGPRT* adjusted C(T) values for **c-MYC** mRNA were significantly lower in APC shRNA-transfected cells than in negative control-transfected cells (p-value < 0.05, Student's T-test).

Chapter 5

Discussion

Summary of Results

The data presented in this dissertation have expanded our understanding of the molecular mechanisms involved in the maintenance of homeostasis in the intestinal epithelium and how defects in the process can lead to colorectal cancer. We have demonstrated the presence of a double-negative feedback circuit between the tumor suppressor protein APC and the RNA binding protein MSI1 (Chapter 2). Furthermore, we have shown that *MSI1* expression is regulated by the canonical Wnt signaling pathway. The most well defined, tumor suppressor function of APC is as an antagonist of the Wnt signaling pathway. In this capacity, APC functions in the degradation of β-catenin, the central transcription co-factor in the Wnt pathway [23, 24]. On the other side of the feedback system, we have demonstrated that the RNA-binding properties of MSI1 allow it to regulate *APC* expression. MSI1 binds a specific sequence in the 3'untranslated region of the *APC* mRNA and inhibits translation (Chapter 2). This double-negative feedback system has important implications for the regulation of Wnt signal transduction. By nature, a double-negative feedback circuit is bistable. This means that it functions as a "toggle-like" switch in which a graded signal, upon reaching threshold intensity, causes a change in the state of the system that remains independently of persistence of the original signal [see "light switch analogy" in Chapter 1, [90, 92]]. The idea that the Wnt signaling pathway, which is critical to maintaining homeostasis in the intestinal epithelium [101, 116], is regulated by a bistable switch has important implications for the understanding of normal maintenance of the intestine as well as the understanding of colorectal carcinogenesis.

One striking aspect of the APC-MSI1 double-negative feedback circuit and its role in regulation of the canonical Wnt signaling pathway is the potential "switch-like" nature of this system. The implication is that a Wnt signal, received at the cells surface, inhibits the function of the APC-containing complex charged with signaling the degradation of β-catenin. β-catenin in turn activates expression of

target genes, one of which is MSI1 which inhibits *APC* expression and consequently stimulates its own further expression in a self-perpetuating loop. The question that arises is how does this system "switch" back to the APC-dominated state? Under normal cellular conditions, a second signal must be received that allows the expression of *APC* and the inhibition of Wnt signal transduction through the degradation of β-catenin. I expect this to be true for two reasons. First, it is well established that inappropriate stabilization of β-catenin through the loss of functional APC, thus mimicking a constant Wnt signal, is an initiating event in colorectal carcinogenesis [reviewed in Morin 1999 [103]]. Second, study of the APC-MSI1 interaction showed an unexpected increase in APC mRNA when *Msi1* was overexpressed in our system (Chapter 2). This "priming" the cell with an abundance of APC mRNA might then facilitate a rapid response upon Wnt signal removal.

I decided to test whether this increase in *APC* mRNA in response to Msi1 upregulation represented a stabilization of the *APC* mRNA. Using the RNA polymerase II inhibitor, Actinomycin D, I demonstrated that *APC* mRNA is stabilized in response to exogenous expression of *Msi1*. Furthermore, I showed that this stabilization was specific to the mRNA binding capacity of Msi1, as a binding incompetent Msi1 mutant did not stabilize *APC* mRNA (Chapter 3). I further demonstrated that shRNA-mediated decrease in levels of endogenous MSI1 in our subject cell line resulted in destabilization of *APC* mRNA. I hypothesize that the MSI1-specific stabilization of *APC* mRNA is the first step toward flipping the bistable switch regulating the Wnt signaling pathway back to the APC-dominated state. Since MSI1 binding to the *APC* mRNA inhibits translation, this binding must be relieved in order for the accumulated *APC* mRNA to be translated. I tested the stability of MSI1 protein and whether it was destabilized after removal of Wnt signal from stimulated cells. MSI1 levels did not change for eight hours and stimulation with Wnt did not alter this stability (*APC* mRNA was stabilized for four to six hours after Actinomycin D treatment). Experiments are under way to answer the questions of how MSI1 releases the *APC* mRNA and whether *APC* mRNA is stabilized as a rapid response to Wnt signal.

Finally, to more fully understand the regulatory mechanisms involved in the APC-MSI1 double-negative feedback circuit, I tested whether c-MYC had an effect on *MSI1* expression. The MYC genes comprise a family of the most common human oncogenes, being deregulated in an estimated 70% of all cancers [110]. c-*MYC* is the family member that is most often associated with tissues in which cells actively proliferate [117]. There is precedent in the literature for an interaction between c-*MYC* and *MSI1* as mice with inducible, intestine specific knock-out of both *Apc* and c-*Myc* show less severe intestinal phenotypes, including the increased expression of *Msi1*, compared to mice with *Apc* knock-out only [17, 109]. I tested whether c-MYC could affect expression of *MSI1* in human colonocytes and found expression of exogenous c-*MYC* in the system did lead to increased MSI1 levels and expression of a dominant negative MYC mutant decreased MSI1 levels (Chapter 4). Surprisingly, shRNA-induced reduction of APC levels in these cells resulted in decreased endogenous c-*MYC* expression. These data indicated to me that c-MYC has the ability to stimulate *MSI1* expression, but that c-MYC is not likely functioning to regulate *MSI1* under normal cellular conditions. The implication is that c-MYC upregulation of *MSI1* may only occur in cancer cells with deregulated c-*MYC* expression.

Implications of APC-MSI1 Double-negative feedback on Wnt Signal Regulation

With respect to the APC-MSI1 double-negative feedback circuit, there are still unanswered questions. One question is whether or not this double-negative feedback truly acts as a bistable circuit. While I have demonstrated the mechanisms involved in the APC-MSI1 interaction, I have not extensively evaluated the different cellular states resulting from changes in the balance between APC and MSI1. For example, it is clear that stabilization of β-catenin, either through a Wnt signal or the loss of APC, results in an increase in MSI1 which feeds back to inhibit the production of APC. As previously mentioned, in isolation, a double-negative feedback system is bistable, *i.e.*, tipping the balance toward MSI1 would

result in self-perpetuation and signal independence. Studies have demonstrated that upregulation of MSI1 leads to decreased differentiation, increased proliferation and tumorigenicity, thus I expect the MSI1 dominated, "lights on" state to involve these cellular conditions [69, 78, 88]. In this instance the "lights off" state, where the balance is tipped toward APC, would involve cell cycle quiescence and differentiation. Once resultant cellular states are directly linked to the APC-MSI1 double-negative feedback circuit, the bistability of the circuit can be evaluated. Whether or not the opposing cellular states are persistent and signal independent is the true test of bistability.

A second, and likely more interesting, question about the APC-MSI1 double-negative feedback system will follow its establishment as a bistable circuit. What is the impact of this bistable circuit on the homeostasis of the intestinal epithelium? It is well established that the canonical Wnt signaling pathway plays a critical role in homeostasis of the intestinal epithelium [4, 5, 101] and that APC is a key component of that pathway [17]. As mentioned in Chapter 1, MSI1 expression is seen in, though not restricted to, the stem cell compartment in the intestinal crypt. Studies of MSI1 in the intestinal epithelium have demonstrated the presence of MSI1 in cells at the site of epithelial regeneration and indicate the importance of MSI1 in the stem/progenitor cells of the intestinal epithelium [75, 76, 118]. Isolation of MSI1-expressing cells from disaggregated human colonic crypts led to an enrichment of cells with stem cell properties [77]. All of these studies indicate an important role for MSI1 in intestinal stem cells. These cells are critical for the maintenance of homeostasis in the intestinal epithelium under normal growth conditions. The potential bistability associated with the regulation of *MSI1* expression might be important for the asymmetric stem division and differentiation of the intestinal epithelium.

The implication that the intracellular regulation of Wnt signaling hinges on a bistable, "toggle-like", circuit would have significant impact on our approach to prevention and treatment of colorectal cancer. Elevated levels of Msi1 have been observed in tumors of mice and humans [72, 78]. Further study demonstrated the dependence of colorectal cancer cells on MSI1 for their tumorigenic properties

[78]. It has been demonstrated that β-catenin stabilization, either through loss of activity of APC or other pathway components, leads to transcriptional upregulation of MSI1 ([87], Chapter 2). We have also shown that MSI1 downregulates, in a double-negative feedback fashion, translation of APC, further perpetuating the effect of β-catenin transcriptional activation (Chapter 2). It is well accepted that colorectal tumorigenesis is a complex, multistep process. However, if the initiation of tumorigenesis is a bistable, "light switch-like" system, then prevention and/or treatment of colorectal cancer may be as simple as finding a signal to "flip the switch".

Flipping the Switch

Chapters 3 and 4 were devoted to understanding one way in which the bistable switch implied by the APC-MSI1 double-negative feedback circuit is returned to the APC-dominant, or Wnt-off, state. The stabilization of the *APC* mRNA by MSI1 is an intriguing phenomenon. I hypothesize that this is a component of the signal required to turn the Wnt signal "off", effectively preloading the mRNA in preparation for the production of APC protein required to turn off the signal once Wnt has been removed. My data are the first to demonstrate the stabilization of an mRNA target by MSI1. It will be intriguing to study other MSI1 target mRNAs, such as *NUMB* and *CDKN1a* (p21), to see if mRNA stabilization is a general effect of MSI1 binding or if this is an APC-specific phenomenon. In terms of whether or not this is a part of the response to activation of the Wnt signaling cascade, three important steps must follow. First, it will be important to demonstrate that there is a response of APC production after stimulation of the Wnt pathway. Secondly, it will be important to identify the mechanism by which MSI1 releases the *APC* mRNA after Wnt signal activation. Finally, it will be important to test whether the translational inhibition and mRNA stabilization activities of MSI1 are coupled.

The MYC effect

Of further interest to the regulation of MSI1 expression is the observation that the transcription factor, and prevalent oncoprotein, c-MYC has the ability to stimulate *MSI1* expression. Admittedly, there is still work to do to demonstrate whether this is a direct effect, *i.e.*, c-MYC binding to the *MSI1* promoter, or a secondary effect, but the implications of our discovery that c-MYC stimulates *MSI1* expression are profound. Probably most intriguing is the observation that c-*MYC* expression is not stimulated, and in fact is depressed, when APC levels are decreased in our human cell culture system. This indicates that under normal cellular conditions, in intestinal epithelium, c-MYC does not participate in regulation of *MSI1* expression. Only when c-*MYC* is overexpressed, or deregulated as it is in approximately 70% of all cancers, does it stimulate *MSI1* expression. Based on the data that we have collected, the impact of MSI1 upregulation could be dramatic. We have shown that MSI1 upregulation depresses *APC* expression tipping the homeostatic balance of the cells (Chapter 2). Based on our data, the deregulation of c-MYC would mimick this effect. Although further experimentation is required to establish this effect, we postulate that c-MYC stimulation of *MSI1* expression might be a cancer-specific phenomenon. In this case, MSI1 would be upregulated as a part of colorectal tumor initiation (through the loss of APC) and also during progression (through the deregulation of c-MYC). This would mean that MSI1 plays a central role throughout colorectal cancer development making it a good candidate as a target for therapies.

Future Studies

My studies have illuminated a double-negative feedback circuit between APC and MSI1 and described the mechanisms of this reciprocal regulation. While this is valuable information, significant questions still remain regarding the functional implications of this double-negative feedback. Generally, it is necessary to perform functional studies *in vivo* and the definitive studies of this feedback circuit will likely involve a mouse model. However, with the discovery of the stem cells in the intestinal epithelium [6-8] a new technique for culturing the intestinal epithelium was established [119]. Stem cells or whole crypts are isolated from mouse intestine and placed in 3-dimensional matrix (Matrigel™, BD Biosciences). Under the proper culturing conditions, the isolated epithelium will form a hollow crypt organoid structure that develops and turns-over like normal intestinal epithelium [119]. This 3-D crypt culturing technique will be extremely useful in evaluating the functional effects of the APC-MSI1 double-negative feedback circuit on intestinal epithelial homeostasis.

Using the crypt culturing system I propose to evaluate the effects of exogenous expression of *Msi1* on the stem cell compartment. Introducing exogenous Msi1 specifically into the stem cells will allow us to assess the effect of deregulation of Msi1 on proliferation and differentiation in the intestinal epithelium. This will also allow evaluation of bistablilty of the APC-MSI1 interaction in the context of a whole epithelial structure. Transient expression of Msi1 will test whether or not the response is signal independent, *i.e.*, does the signal persist in progeny of the cells in which Msi1 was expressed? All of these experiments will help gain a better understanding of the functional effects of the APC-MSI1 double-negative feedback on homeostasis in the intestinal epithelium.

Finally, as mentioned earlier, *in vivo* models will be important for the evaluation of the APC-MSI1 double-negative feedback circuit, especially as it pertains to tumorigenesis. We have acquired Msi1$^{-/-}$ mice and have plans to breed them with standard intestinal cancer models carrying Apc mutations. Comparing tumorigenesis in Apc mouse models with and without Msi1 will allow direct

genetic examination of the role of the APC-MSI1 double-negative feedback circuit in intestinal tumorigenesis.

In summary, I have identified a novel double-negative feedback circuit between the tumor suppressor APC and the sequence-specific RNA binding protein MSI1 in the intestinal epithelium. This double-negative feedback is involved in regulation of the canonical Wnt signaling pathway and may confer bistability onto this already complex signal transduction pathway. Given the bistable nature of double-negative feedback, strong signals are required to reverse the effect of stimulation of the Wnt signaling pathway. The first part of this signal may be the stabilization and accumulation of *APC* mRNA in Wnt stimulated cells followed by the release and subsequent translation of APC when Wnt signal is removed. Finally, a prevalent oncoprotein, c-MYC, has the ability to stimulate MSI1 expression. This stimulation does not seem to occur under normal cellular conditions and we speculate that it is a cancer-specific response. Taken together, these data have expanded our understanding of the molecular mechanisms involved in regulation of the canonical Wnt signaling pathway and the maintenance of homeostasis in the intestinal epithelium. With this knowledge, we have gained insight into the molecular basis of colorectal cancer. It is our hope that one day these basic, molecular principles will be applied to the clinical setting for the development of improved prevention and treatment plans for colorectal cancer.

Appendix I

References

References

1. Altekruse SF K.C., Krapcho M, et al (eds). *SEER Cancer Statistics Review, 1975-2008*. 2011 (Updated April 15) Available from: http://seer.cancer.gov/csr/1975_2008/.
2. Institute N.C. *Physician Data Query (PDQ). Colon Cancer Treatment.* [cited 2011 June 2, 2011]; Available from: www.cancer.gov/cancertopics/pdq/treatment/colon/healthprofessional.
3. Karapetis C.S., Khambata-Ford S., Jonker D.J., O'Callaghan C.J., Tu D., Tebbutt N.C., Simes R.J., Chalchal H., Shapiro J.D., Robitaille S., Price T.J., Shepherd L., Au H.J., Langer C., Moore M.J. and Zalcberg J.R., *K-ras mutations and benefit from cetuximab in advanced colorectal cancer.* The New England journal of medicine, 2008. **359**(17): p. 1757-65.
4. Sancho E., Batlle E. and Clevers H., *Signaling pathways in intestinal development and cancer.* Annual review of cell and developmental biology, 2004. **20**: p. 695-723.
5. Radtke F. and Clevers H., *Self-renewal and cancer of the gut: two sides of a coin.* Science, 2005. **307**(5717): p. 1904-9.
6. Barker N., van Es J.H., Kuipers J., Kujala P., van den Born M., Cozijnsen M., Haegebarth A., Korving J., Begthel H., Peters P.J. and Clevers H., *Identification of stem cells in small intestine and colon by marker gene Lgr5.* Nature, 2007. **449**(7165): p. 1003-7.
7. Sangiorgi E. and Capecchi M.R., *Bmi1 is expressed in vivo in intestinal stem cells.* Nature Genetics, 2008. **40**(7): p. 915-920.
8. May R., Riehl T.E., Hunt C., Sureban S.M., Anant S. and Houchen C.W., *Identification of a novel putative gastrointestinal stem cell and adenoma stem cell marker, doublecortin and CaM kinase-like-1, following radiation injury and in adenomatous polyposis coli/multiple intestinal neoplasia mice.* Stem cells, 2008. **26**(3): p. 630-7.
9. Morrison S.J. and Kimble J., *Asymmetric and symmetric stem-cell divisions in development and cancer.* Nature, 2006. **441**(7097): p. 1068-74.
10. Li L. and Clevers H., *Coexistence of quiescent and active adult stem cells in mammals.* Science, 2010. **327**(5965): p. 542-5.
11. Haramis A.P., Begthel H., van den Born M., van Es J., Jonkheer S., Offerhaus G.J. and Clevers H., *De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine.* Science, 2004. **303**(5664): p. 1684-6.
12. Kopan R., *Notch: a membrane-bound transcription factor.* Journal of cell science, 2002. **115**(Pt 6): p. 1095-7.
13. Nakamura Y., Sakakibara S., Miyata T., Ogawa M., Shimazaki T., Weiss S., Kageyama R. and Okano H., *The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2000. **20**(1): p. 283-93.
14. Hitoshi S., Alexson T., Tropepe V., Donoviel D., Elia A.J., Nye J.S., Conlon R.A., Mak T.W., Bernstein A. and van der Kooy D., *Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells.* Genes & development, 2002. **16**(7): p. 846-58.
15. Jensen J., Pedersen E.E., Galante P., Hald J., Heller R.S., Ishibashi M., Kageyama R., Guillemot F., Serup P. and Madsen O.D., *Control of endodermal endocrine development by Hes-1.* Nature Genetics, 2000. **24**(1): p. 36-44.
16. Yang Q., Bermingham N.A., Finegold M.J. and Zoghbi H.Y., *Requirement of Math1 for secretory cell lineage commitment in the mouse intestine.* Science, 2001. **294**(5549): p. 2155-8.
17. Sansom O.J., Reed K.R., Hayes A.J., Ireland H., Brinkmann H., Newton I.P., Batlle E., Simon-Assmann P., Clevers H., Nathke I.S., Clarke A.R. and Winton D.J., *Loss of Apc in vivo immediately*

perturbs Wnt signaling, differentiation, and migration. Genes & development, 2004. **18**(12): p. 1385-90.
18. Fearon E.R. and Vogelstein B., *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.
19. Kinzler K.W. and Vogelstein B., *Lessons from hereditary colorectal cancer.* Cell, 1996. **87**(2): p. 159-70.
20. Smith G., Carey F.A., Beattie J., Wilkie M.J., Lightfoot T.J., Coxhead J., Garner R.C., Steele R.J. and Wolf C.R., *Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(14): p. 9433-8.
21. Jones S., Chen W.D., Parmigiani G., Diehl F., Beerenwinkel N., Antal T., Traulsen A., Nowak M.A., Siegel C., Velculescu V.E., Kinzler K.W., Vogelstein B., Willis J. and Markowitz S.D., *Comparative lesion sequencing provides insights into tumor evolution.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(11): p. 4283-8.
22. Vogelstein B., Fearon E.R., Kern S.E., Hamilton S.R., Preisinger A.C., Nakamura Y. and White R., *Allelotype of colorectal carcinomas.* Science, 1989. **244**(4901): p. 207-11.
23. Korinek V., Barker N., Morin P.J., van Wichen D., de Weger R., Kinzler K.W., Vogelstein B. and Clevers H., *Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma.* Science, 1997. **275**(5307): p. 1784-7.
24. Morin P.J., Sparks A.B., Korinek V., Barker N., Clevers H., Vogelstein B. and Kinzler K.W., *Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.* Science, 1997. **275**(5307): p. 1787-90.
25. Friend S.H., Bernards R., Rogelj S., Weinberg R.A., Rapaport J.M., Albert D.M. and Dryja T.P., *A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma.* Nature, 1986. **323**(6089): p. 643-6.
26. Fung Y.K., Murphree A.L., T'Ang A., Qian J., Hinrichs S.H. and Benedict W.F., *Structural evidence for the authenticity of the human retinoblastoma gene.* Science, 1987. **236**(4809): p. 1657-61.
27. Lee W.H., Bookstein R., Hong F., Young L.J., Shew J.Y. and Lee E.Y., *Human retinoblastoma susceptibility gene: cloning, identification, and sequence.* Science, 1987. **235**(4794): p. 1394-9.
28. Groden J., Thliveris A., Samowitz W., Carlson M., Gelbert L., Albertsen H., Joslyn G., Stevens J., Spirio L., Robertson M. and et al., *Identification and characterization of the familial adenomatous polyposis coli gene.* Cell, 1991. **66**(3): p. 589-600.
29. Kinzler K.W., Nilbert M.C., Su L.K., Vogelstein B., Bryan T.M., Levy D.B., Smith K.J., Preisinger A.C., Hedge P., McKechnie D. and et al., *Identification of FAP locus genes from chromosome 5q21.* Science, 1991. **253**(5020): p. 661-5.
30. Nishisho I., Nakamura Y., Miyoshi Y., Miki Y., Ando H., Horii A., Koyama K., Utsunomiya J., Baba S. and Hedge P., *Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients.* Science, 1991. **253**(5020): p. 665-9.
31. Powell S.M., Zilz N., Beazer-Barclay Y., Bryan T.M., Hamilton S.R., Thibodeau S.N., Vogelstein B. and Kinzler K.W., *APC mutations occur early during colorectal tumorigenesis.* Nature, 1992. **359**(6392): p. 235-7.
32. van Es J.H., Giles R.H. and Clevers H.C., *The many faces of the tumor suppressor gene APC.* Experimental cell research, 2001. **264**(1): p. 126-34.
33. Rubinfeld B., Souza B., Albert I., Muller O., Chamberlain S.H., Masiarz F.R., Munemitsu S. and Polakis P., *Association of the APC gene product with beta-catenin.* Science, 1993. **262**(5140): p. 1731-4.

34. Rubinfeld B., Albert I., Porfiri E., Fiol C., Munemitsu S. and Polakis P., *Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly.* Science, 1996. **272**(5264): p. 1023-6.
35. Aberle H., Bauer A., Stappert J., Kispert A. and Kemler R., *beta-catenin is a target for the ubiquitin-proteasome pathway.* The EMBO journal, 1997. **16**(13): p. 3797-804.
36. Orford K., Crockett C., Jensen J.P., Weissman A.M. and Byers S.W., *Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin.* The Journal of biological chemistry, 1997. **272**(40): p. 24735-8.
37. Salomon D., Sacco P.A., Roy S.G., Simcha I., Johnson K.R., Wheelock M.J. and Ben-Ze'ev A., *Regulation of beta-catenin levels and localization by overexpression of plakoglobin and inhibition of the ubiquitin-proteasome system.* The Journal of cell biology, 1997. **139**(5): p. 1325-35.
38. McCrea P.D., Turck C.W. and Gumbiner B., *A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin.* Science, 1991. **254**(5036): p. 1359-61.
39. Behrens J., von Kries J.P., Kuhl M., Bruhn L., Wedlich D., Grosschedl R. and Birchmeier W., *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. **382**(6592): p. 638-42.
40. Neufeld K.L., Zhang F., Cullen B.R. and White R.L., *APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export.* EMBO reports, 2000. **1**(6): p. 519-23.
41. Rosin-Arbesfeld R., Cliffe A., Brabletz T. and Bienz M., *Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription.* The EMBO journal, 2003. **22**(5): p. 1101-13.
42. Sierra J., Yoshida T., Joazeiro C.A. and Jones K.A., *The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes.* Genes & development, 2006. **20**(5): p. 586-600.
43. Hamada F. and Bienz M., *The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF.* Developmental cell, 2004. **7**(5): p. 677-85.
44. He T.C., Sparks A.B., Rago C., Hermeking H., Zawel L., da Costa L.T., Morin P.J., Vogelstein B. and Kinzler K.W., *Identification of c-MYC as a target of the APC pathway.* Science, 1998. **281**(5382): p. 1509-12.
45. Tetsu O. and McCormick F., *Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.* Nature, 1999. **398**(6726): p. 422-6.
46. Shtutman M., Zhurinsky J., Simcha I., Albanese C., D'Amico M., Pestell R. and Ben-Ze'ev A., *The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(10): p. 5522-7.
47. Lustig B., Jerchow B., Sachs M., Weiler S., Pietsch T., Karsten U., van de Wetering M., Clevers H., Schlag P.M., Birchmeier W. and Behrens J., *Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors.* Molecular and cellular biology, 2002. **22**(4): p. 1184-93.
48. Yan D., Wiesmann M., Rohan M., Chan V., Jefferson A.B., Guo L., Sakamoto D., Caothien R.H., Fuller J.H., Reinhard C., Garcia P.D., Randazzo F.M., Escobedo J., Fantl W.J. and Williams L.T., *Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta -catenin signaling is activated in human colon tumors.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(26): p. 14973-8.
49. Jho E.H., Zhang T., Domon C., Joo C.K., Freund J.N. and Costantini F., *Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway.* Molecular and cellular biology, 2002. **22**(4): p. 1172-83.

50. Sansom O.J., Reed K.R., van de Wetering M., Muncan V., Winton D.J., Clevers H. and Clarke A.R., *Cyclin D1 is not an immediate target of beta-catenin following Apc loss in the intestine.* The Journal of biological chemistry, 2005. **280**(31): p. 28463-7.
51. Cadigan K.M. and Nusse R., *Wnt signaling: a common theme in animal development.* Genes & development, 1997. **11**(24): p. 3286-305.
52. Nusse R. and Varmus H.E., *Wnt genes.* Cell, 1992. **69**(7): p. 1073-87.
53. Polakis P., *The many ways of Wnt in cancer.* Current opinion in genetics & development, 2007. **17**(1): p. 45-51.
54. Fearon E.R., *Molecular genetics of colorectal cancer.* Annual review of pathology, 2011. **6**: p. 479-507.
55. Cong F., Schweizer L. and Varmus H., *Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP.* Development, 2004. **131**(20): p. 5103-15.
56. Mao J., Wang J., Liu B., Pan W., Farr G.H., 3rd, Flynn C., Yuan H., Takada S., Kimelman D., Li L. and Wu D., *Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway.* Molecular cell, 2001. **7**(4): p. 801-9.
57. Fearnhead N.S., Britton M.P. and Bodmer W.F., *The ABC of APC.* Human molecular genetics, 2001. **10**(7): p. 721-33.
58. Laken S.J., Papadopoulos N., Petersen G.M., Gruber S.B., Hamilton S.R., Giardiello F.M., Brensinger J.D., Vogelstein B. and Kinzler K.W., *Analysis of masked mutations in familial adenomatous polyposis.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(5): p. 2322-6.
59. Sparks A.B., Morin P.J., Vogelstein B. and Kinzler K.W., *Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer.* Cancer research, 1998. **58**(6): p. 1130-4.
60. Neufeld K.L., Nix D.A., Bogerd H., Kang Y., Beckerle M.C., Cullen B.R. and White R.L., *Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(22): p. 12085-90.
61. Henderson B.R., *Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover.* Nature cell biology, 2000. **2**(9): p. 653-60.
62. Zhang F., White R.L. and Neufeld K.L., *Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(23): p. 12577-82.
63. Wang Y., Azuma Y., Friedman D.B., Coffey R.J. and Neufeld K.L., *Novel association of APC with intermediate filaments identified using a new versatile APC antibody.* BMC cell biology, 2009. **10**: p. 75.
64. Wang Y., Coffey R.J., Osheroff N. and Neufeld K.L., *Topoisomerase IIalpha binding domains of adenomatous polyposis coli influence cell cycle progression and aneuploidy.* PloS one, 2010. **5**(4): p. e9994.
65. Nakamura M., Okano H., Blendy J.A. and Montell C., *Musashi, a neural RNA-binding protein required for Drosophila adult external sensory organ development.* Neuron, 1994. **13**(1): p. 67-81.
66. Okano H., Kawahara H., Toriya M., Nakao K., Shibata S. and Imai T., *Function of RNA-binding protein Musashi-1 in stem cells.* Experimental cell research, 2005. **306**(2): p. 349-56.
67. Hirota Y., Okabe M., Imai T., Kurusu M., Yamamoto A., Miyao S., Nakamura M., Sawamoto K. and Okano H., *Musashi and seven in absentia downregulate Tramtrack through distinct mechanisms in Drosophila eye development.* Mechanisms of development, 1999. **87**(1-2): p. 93-101.

68. Okabe M., Imai T., Kurusu M., Hiromi Y. and Okano H., *Translational repression determines a neuronal potential in Drosophila asymmetric cell division.* Nature, 2001. **411**(6833): p. 94-8.
69. Imai T., Tokunaga A., Yoshida T., Hashimoto M., Mikoshiba K., Weinmaster G., Nakafuku M. and Okano H., *The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA.* Molecular and cellular biology, 2001. **21**(12): p. 3888-900.
70. Sakakibara S., Nakamura Y., Yoshida T., Shibata S., Koike M., Takano H., Ueda S., Uchiyama Y., Noda T. and Okano H., *RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(23): p. 15194-9.
71. Sakakibara S., Imai T., Hamaguchi K., Okabe M., Aruga J., Nakajima K., Yasutomi D., Nagata T., Kurihara Y., Uesugi S., Miyata T., Ogawa M., Mikoshiba K. and Okano H., *Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell.* Developmental biology, 1996. **176**(2): p. 230-42.
72. Potten C.S., Booth C., Tudor G.L., Booth D., Brady G., Hurley P., Ashton G., Clarke R., Sakakibara S. and Okano H., *Identification of a putative intestinal stem cell and early lineage marker; musashi-1.* Differentiation; research in biological diversity, 2003. **71**(1): p. 28-41.
73. Kayahara T., Sawada M., Takaishi S., Fukui H., Seno H., Fukuzawa H., Suzuki K., Hiai H., Kageyama R., Okano H. and Chiba T., *Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine.* FEBS letters, 2003. **535**(1-3): p. 131-5.
74. Dekaney C.M., Rodriguez J.M., Graul M.C. and Henning S.J., *Isolation and characterization of a putative intestinal stem cell fraction from mouse jejunum.* Gastroenterology, 2005. **129**(5): p. 1567-80.
75. Fukui T., Takeda H., Shu H.J., Ishihama K., Otake S., Suzuki Y., Nishise S., Ito N., Sato T., Togashi H. and Kawata S., *Investigation of Musashi-1 expressing cells in the murine model of dextran sodium sulfate-induced colitis.* Digestive diseases and sciences, 2006. **51**(7): p. 1260-8.
76. He X.C., Yin T., Grindley J.C., Tian Q., Sato T., Tao W.A., Dirisina R., Porter-Westpfahl K.S., Hembree M., Johnson T., Wiedemann L.M., Barrett T.A., Hood L., Wu H. and Li L., *PTEN-deficient intestinal stem cells initiate intestinal polyposis.* Nature Genetics, 2007. **39**(2): p. 189-98.
77. Samuel S., Walsh R., Webb J., Robins A., Potten C. and Mahida Y.R., *Characterization of putative stem cells in isolated human colonic crypt epithelial cells and their interactions with myofibroblasts.* American journal of physiology. Cell physiology, 2009. **296**(2): p. C296-305.
78. Sureban S.M., May R., George R.J., Dieckgraefe B.K., McLeod H.L., Ramalingam S., Bishnupuri K.S., Natarajan G., Anant S. and Houchen C.W., *Knockdown of RNA binding protein musashi-1 leads to tumor regression in vivo.* Gastroenterology, 2008. **134**(5): p. 1448-58.
79. Moser A.R., Pitot H.C. and Dove W.F., *A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse.* Science, 1990. **247**(4940): p. 322-4.
80. Su L.K., Kinzler K.W., Vogelstein B., Preisinger A.C., Moser A.R., Luongo C., Gould K.A. and Dove W.F., *Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene.* Science, 1992. **256**(5057): p. 668-70.
81. Oshima M., Oshima H., Kitagawa K., Kobayashi M., Itakura C. and Taketo M., *Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(10): p. 4482-6.
82. Neufeld K.L., *Nuclear Functions of APC*, in *Adenomatous polyposis coli protein*, I. Näthke and B. McCartney, Editors. 2008, Landes Bioscience.

83. Wang Y., Azuma Y., Moore D., Osheroff N. and Neufeld K.L., *Interaction between tumor suppressor adenomatous polyposis coli and topoisomerase IIalpha: implication for the G2/M transition.* Molecular biology of the cell, 2008. **19**(10): p. 4076-85.
84. Ashton G.H., Morton J.P., Myant K., Phesse T.J., Ridgway R.A., Marsh V., Wilkins J.A., Athineos D., Muncan V., Kemp R., Neufeld K., Clevers H., Brunton V., Winton D.J., Wang X., Sears R.C., Clarke A.R., Frame M.C. and Sansom O.J., *Focal adhesion kinase is required for intestinal regeneration and tumorigenesis downstream of Wnt/c-Myc signaling.* Developmental cell, 2010. **19**(2): p. 259-69.
85. Rowan A.J., Lamlum H., Ilyas M., Wheeler J., Straub J., Papadopoulou A., Bicknell D., Bodmer W.F. and Tomlinson I.P., *APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits".* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(7): p. 3352-7.
86. Chan T.A., Wang Z., Dang L.H., Vogelstein B. and Kinzler K.W., *Targeted inactivation of CTNNB1 reveals unexpected effects of beta-catenin mutation.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(12): p. 8265-70.
87. Rezza A., Skah S., Roche C., Nadjar J., Samarut J. and Plateroti M., *The overexpression of the putative gut stem cell marker Musashi-1 induces tumorigenesis through Wnt and Notch activation.* Journal of cell science, 2010. **123**(Pt 19): p. 3256-65.
88. Battelli C., Nikopoulos G.N., Mitchell J.G. and Verdi J.M., *The RNA-binding protein Musashi-1 regulates neural development through the translational repression of p21WAF-1.* Molecular and cellular neurosciences, 2006. **31**(1): p. 85-96.
89. Kawahara H., Imai T., Imataka H., Tsujimoto M., Matsumoto K. and Okano H., *Neural RNA-binding protein Musashi1 inhibits translation initiation by competing with eIF4G for PABP.* The Journal of cell biology, 2008. **181**(4): p. 639-53.
90. Ferrell J.E., Jr., *Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability.* Current opinion in cell biology, 2002. **14**(2): p. 140-8.
91. Ferrell J.E., Jr., *How regulated protein translocation can produce switch-like responses.* Trends in biochemical sciences, 1998. **23**(12): p. 461-5.
92. Laurent M. and Kellershohn N., *Multistability: a major means of differentiation and evolution in biological systems.* Trends in biochemical sciences, 1999. **24**(11): p. 418-22.
93. Yao G., Tan C., West M., Nevins J.R. and You L., *Origin of bistability underlying mammalian cell cycle entry.* Molecular systems biology, 2011. **7**: p. 485.
94. Akasaka Y., Saikawa Y., Fujita K., Kubota T., Ishikawa Y., Fujimoto A., Ishii T., Okano H. and Kitajima M., *Expression of a candidate marker for progenitor cells, Musashi-1, in the proliferative regions of human antrum and its decreased expression in intestinal metaplasia.* Histopathology, 2005. **47**(4): p. 348-56.
95. Spears E. and Neufeld K.L., *Novel double-negative feedback loop between adenomatous polyposis coli and Musashi1 in colon epithelia.* The Journal of biological chemistry, 2011. **286**(7): p. 4946-50.
96. Barker N., Ridgway R.A., van Es J.H., van de Wetering M., Begthel H., van den Born M., Danenberg E., Clarke A.R., Sansom O.J. and Clevers H., *Crypt stem cells as the cells-of-origin of intestinal cancer.* Nature, 2009. **457**(7229): p. 608-11.
97. Rosin-Arbesfeld R., Townsley F. and Bienz M., *The APC tumour suppressor has a nuclear export function.* Nature, 2000. **406**(6799): p. 1009-12.
98. Miyanoiri Y., Kobayashi H., Imai T., Watanabe M., Nagata T., Uesugi S., Okano H. and Katahira M., *Origin of higher affinity to RNA of the N-terminal RNA-binding domain than that of the C-terminal one of a mouse neural protein, musashi1, as revealed by comparison of their structures,*

modes of interaction, surface electrostatic potentials, and backbone dynamics. The Journal of biological chemistry, 2003. **278**(42): p. 41309-15.
99. Liu J., Stevens J., Rote C.A., Yost H.J., Hu Y., Neufeld K.L., White R.L. and Matsunami N., *Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein.* Molecular cell, 2001. **7**(5): p. 927-36.
100. Shibamoto S., Higano K., Takada R., Ito F., Takeichi M. and Takada S., *Cytoskeletal reorganization by soluble Wnt-3a protein signalling.* Genes to cells : devoted to molecular & cellular mechanisms, 1998. **3**(10): p. 659-70.
101. Clarke A.R., *Wnt signalling in the mouse intestine.* Oncogene, 2006. **25**(57): p. 7512-21.
102. Kaneko Y., Sakakibara S., Imai T., Suzuki A., Nakamura Y., Sawamoto K., Ogawa Y., Toyama Y., Miyata T. and Okano H., *Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells.* Developmental neuroscience, 2000. **22**(1-2): p. 139-53.
103. Morin P.J., *beta-catenin signaling and cancer.* BioEssays : news and reviews in molecular, cellular and developmental biology, 1999. **21**(12): p. 1021-30.
104. Rabbitts P.H., Watson J.V., Lamond A., Forster A., Stinson M.A., Evan G., Fischer W., Atherton E., Sheppard R. and Rabbitts T.H., *Metabolism of c-myc gene products: c-myc mRNA and protein expression in the cell cycle.* The EMBO journal, 1985. **4**(8): p. 2009-15.
105. Bingham P.M., Chou T.B., Mims I. and Zachar Z., *On/off regulation of gene expression at the level of splicing.* Trends in genetics : TIG, 1988. **4**(5): p. 134-8.
106. Bernstein P. and Ross J., *Poly(A), poly(A) binding protein and the regulation of mRNA stability.* Trends in biochemical sciences, 1989. **14**(9): p. 373-7.
107. Hinnebusch A.G., *Mechanisms of gene regulation in the general control of amino acid biosynthesis in Saccharomyces cerevisiae.* Microbiological reviews, 1988. **52**(2): p. 248-73.
108. Okano H., Imai T. and Okabe M., *Musashi: a translational regulator of cell fate.* Journal of cell science, 2002. **115**(Pt 7): p. 1355-9.
109. Sansom O.J., Meniel V.S., Muncan V., Phesse T.J., Wilkins J.A., Reed K.R., Vass J.K., Athineos D., Clevers H. and Clarke A.R., *Myc deletion rescues Apc deficiency in the small intestine.* Nature, 2007. **446**(7136): p. 676-9.
110. Nesbit C.E., Tersak J.M. and Prochownik E.V., *MYC oncogenes and human neoplastic disease.* Oncogene, 1999. **18**(19): p. 3004-16.
111. Popescu N.C. and Zimonjic D.B., *Chromosome-mediated alterations of the MYC gene in human cancer.* Journal of cellular and molecular medicine, 2002. **6**(2): p. 151-9.
112. Grandori C., Cowley S.M., James L.P. and Eisenman R.N., *The Myc/Max/Mad network and the transcriptional control of cell behavior.* Annual review of cell and developmental biology, 2000. **16**: p. 653-99.
113. Murphy D.J., Junttila M.R., Pouyet L., Karnezis A., Shchors K., Bui D.A., Brown-Swigart L., Johnson L. and Evan G.I., *Distinct thresholds govern Myc's biological output in vivo.* Cancer cell, 2008. **14**(6): p. 447-57.
114. Grandori C. and Eisenman R.N., *Myc target genes.* Trends in biochemical sciences, 1997. **22**(5): p. 177-81.
115. Eisenman R.N., *Deconstructing myc.* Genes & development, 2001. **15**(16): p. 2023-30.
116. Haegebarth A. and Clevers H., *Wnt signaling, lgr5, and stem cells in the intestine and skin.* The American journal of pathology, 2009. **174**(3): p. 715-21.
117. Zimmerman K.A., Yancopoulos G.D., Collum R.G., Smith R.K., Kohl N.E., Denis K.A., Nau M.M., Witte O.N., Toran-Allerand D., Gee C.E. and et al., *Differential expression of myc family genes during murine development.* Nature, 1986. **319**(6056): p. 780-3.

118. Dekaney C.M., Fong J.J., Rigby R.J., Lund P.K., Henning S.J. and Helmrath M.A., *Expansion of intestinal stem cells associated with long-term adaptation following ileocecal resection in mice.* American journal of physiology. Gastrointestinal and liver physiology, 2007. **293**(5): p. G1013-22.
119. Sato T., Vries R.G., Snippert H.J., van de Wetering M., Barker N., Stange D.E., van Es J.H., Abo A., Kujala P., Peters P.J. and Clevers H., *Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche.* Nature, 2009. **459**(7244): p. 262-5.