THE REGULATION OF MUSASHI RNA BINDING PROTEINS AND THE IMPLICATIONS FOR CANCER THERAPY

By

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THE REGULATION OF MUSASHI RNA BINDING PROTEINS AND THE IMPLICATIONS FOR CANCER THERAPY

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Abstract:

RNA-binding protein and stem cell regulator, Musashi-1 (MSI1) is overexpressed in a broad range of human cancers and associated with poor clinical outcome. MSI1 encourages tumorigenesis by promoting Notch and Wnt signaling, along with cell cycle progression. *In vitro* and *in vivo* data have demonstrated that MSI1 is an attractive therapeutic target. Accordingly, there is great interest in identifying and exploiting MSI1 regulating mechanisms as anti-cancer therapy.

We successfully determined that non-coding RNA, miR-137, acts as a negative regulator of MSI1 by binding to the 3′UTR of MSI1 mRNA. Using numerous *in vitro* and *in vivo* studies, we show that miR-137 acts as a tumor suppressive miRNA in colon cancer, in part by inhibiting MSI1. To understand the clinical relevance of our observations, we measured the expression of miR-137 and MSI1 in tissue samples from patients with rectal cancer. miR-137 expression is decreased in approximately 84% of rectal tumor samples as compared to paired normal rectal mucosal samples. Inversely, MSI1 protein was found to be highly expressed in 79% of primary rectal tumors. In addition to this study, we show preliminary data that miR-137 also negatively regulates Musashi-2 (MSI2). We provide a comprehensive review on the expression of MSI1 and MSI2 in human cancers and correlate this expression with clinical outcome and evidence for MSI-induced malignancy.

In addition to using noncoding RNAs to inhibit MSI1, we are also identifying small molecules that inhibit the RNA-binding function of MSI1. Our preliminary studies identified (−)-Gossypol, gossypolone and azaphilone-9 as potent MSI1 inhibitors using a fluorescence polarization-based screening of our in-house library of compounds. In this study, we validated and characterized

the binding event between hit compounds and MSI1 RNA-binding domain 1 (RBD1) using nuclear magnetic resonance. In summary, we discovered two novel means of inhibiting the function of MSI1 in cancer, using miR-137 and small molecule inhibitors. Our study supports the idea of exploiting miRNAs and small molecules as novel cancer therapy targeting the RNA-binding protein, Musashi.

Dedication

I lovingly dedicate this work to my husband Joshua and our son, Ethan.

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To my mentor, Dr. Liang Xu,

Thank you for your guidance and patience; for giving me space to think independently and the opportunities and resources to seek answers to my own questions. I appreciate that you constantly challenged me to think beyond the bench. You gave me so many opportunities to develop skills that are necessary for succeeding in this field; from exposure to grant writing to managing undergraduate students. I feel like I'm ready for the next stage in my career and it's because of you. Also, thank you for encouraging a collaborative and friendly lab environment. I can honestly say that I loved walking into the lab every day. It's something I will cherish forever. Thank you.

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List of Abbreviations:

(–)-G	(–)-gossypol
137-I	miR-137 Inhibitor
137-m	miR-137 mimic
AGO	Argonaute
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
AZA-9	azaphilones-9
BMI1	BMI1 proto-oncogene, polycomb ring finger
CAR	constitutive androstane receptor
CCND1	cyclin D1
CDKN1A	cyclin-dependent kinase inhibitor/p21^{WAF-1}
CDS	coding sequence
CML	chronic myeloid leukemia
CNS	Central nervous system
CRC	Colorectal cancer
CSC	cancer stem cells
DCX	Doublecortin
DGCR8	DiGeorge syndrome critical region 8
DKK3	Dickkopf-3
DLL	Delta-like
DOX	Doxycycline
dsRNA	Double-stranded RNA
ER	estrogen receptor
FP	fluorescence polarization
FRAT1	frequently rearranged in advanced T-Cell lymphomas 1
Gn	Gossypolone
GSK-3β	glycogen synthase kinase 3β
HCC	hepatocellular carcinoma
HES1	hairy and enhancer of split-1

HMGA	high-mobility group AT-hook
HOXA9	homeobox 9
HR	Hazard risk
HSC	hematopoietic stem cell
HTS	high-throughput screening
Hu/ELAV	human embryonic lethal abnormal vision
IGF2	insulin-like growth factor 2
IHC	Immunohistochemistry
LGR5	leucine-rich repeat containing G protein-coupled receptor 5
LNM	lymph node metastasis
m^7G	5′-7-methyl-guanosine
MDR	multi-drug resistance
miRISC	miRNA-induced silencing complex
miRNA	microRNA
mRNA	Messenger RNA
mRNP	messenger ribonucleoprotein particle
MSI1	Musashi-1
MSI1-si	Musashi-1 siRNA
MSI2	Musashi-2
MSI2-si	Musashi-2 siRNA
MYC	v-myc myelocytomatosis viral oncogene homolog
NC	Negative control
NC-I	Negative control Inhibitor/antagomiR
NC-m	Negative control mimic
NC-si	Negative control siRNA
NICD	NOTCH intracellular domain
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
nt	Nucleotide
NUMB	numb homolog

ORF	open reading frame
OS	Overall survival
PABP	poly(A)-binding protein
PABP	Poly(A)-binding protein
PLF	Proliferin
PR	progesterone receptor
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary microRNA
RBD1	RNA binding domain 1
RBP	RNA-binding protein
RIE	Rat intestinal epithelial cells
RIP	RNA immunoprecipitation
RNP	Ribonucleoprotein
RRM	RNA recognition motifs
SELEX	systematic evolution of ligands by exponential enrichment
SP	side population
SPR	surface plasmon resonance
TARBP2	transactivation responsive RNA-binding protein 2
TCF/LEF1	T cell-specific transcription factor/lymphoid enhancer-binding factor 1
TCGA	The Cancer Genome Atlas
TIC	Tumor initiating cells
TMA	Tissue microarray
TTK69	Tramtrack69
UTR	untranslated region
XNR1	exoribonuclease 1
XPO5	exportin 5
YB-1	Y-box binding protein-1

Chapter 1: Introduction

Globally, cancer is an enormous burden. Cancer accounts for nearly 1 in every 7 deaths worldwide [1]. In 2012 alone, approximately 14 million people were newly diagnosed with cancer and over 8 million people died from cancer [1]. Due to population growth and aging, this cancer burden is growing at a disturbing rate. In 2030, it's predicted that approximately 21 million people will be diagnosed with cancer and 13 million will die from the deadly disease [1].

In the United States, cancer is the second leading cause of death. This disease is expected to earn the top spot as cause of death in the near future; surpassing heart disease. In 2015, 1.6 million people are expected to be diagnosed with cancer, nearly 4,500 people per day [1]. Over 500,000 men and women, are expected to die from cancer this year alone, nearly 1,600 people per day [1]. To date, the lifetime risk for being diagnosed with cancer is 43% for men and 38% for women in the United States [1]. The Agency for Healthcare Research and Quality estimate that the direct medical cost for cancer in the United States in 2011 was 88.7 billion dollars. Overall, cancer is an overwhelming burden personally and financially for millions of people every year.

Normal cellular function is mediated by a highly organized and complex circuit known as the Central Dogma of molecular biology. DNA, which contains a unique genetic code is transcribed into messenger RNA (mRNA) which is then translated into proteins. Being highly susceptible to degradation, the RNA message must be protected from powerful RNA degrading enzymes. Cells use sophisticated mechanisms to produce and use messenger RNA with optimal efficiency according to the needs of the cell. The two major types of post-transcriptional regulators are RNA binding proteins (RBPs) and microRNAs (miRNAs) [2]. When RBPs and miRNAs are dysregulated, they produce a myriad of consequences, including tumorigenesis.

RNA binding proteins

Typically, RNA is accompanied by RNA binding proteins. RBPs are essential for orchestrating the processing, localization, lifetime and translation of RNAs (Figure 1) [3, 4]. Therefore, RBPs serve as protective gatekeepers of the genetic message for proper protein translation and normal cell homeostasis. RNA binding proteins are major regulators of gene expression. The human genome contains more than 500 RBPs [5]. Some RBPs recognize the 5′- cap or poly(A) tail of mRNA, however most RBPs recognize specific secondary structures mediated by RNA-binding domains [3].

A large percentage of aberrant protein expression in tumors is dysregulated at the post-transcriptional level. For example, one study found that only 21% of the transcriptome and proteome are positively correlated in lung adenocarcinoma [6]. The differential expression of RBPs is observed in most human cancers and is thought to be either a cause or consequence of tumorigenesis [7, 8]. RBPs regulate the expression of genes implicated in multiple cancer-relevant processes, including proliferation, differentiation, apoptosis and cell cycle [9].

The proper function of cells depend on the dynamic expression of proteins to regulate cell survival, homeostasis, and adaptation to stress in response to environmental signals [10]. Proper expression of genes is globally coordinated by post-transcriptional regulation factors, such as RBPs and microRNAs, termed the messenger ribonucleoprotein particle (mRNP) [11-13]. mRNPs serve to expand the functional repertoire of humans' unexpectedly small genome [11]. Therefore, cells can respond to a myriad of environmental cues by mRNPs ability to exquisitely fine-tune the expression of genes in response to fluctuating conditions.

The post-transcriptional 'RNA-operon' theory suggests that multiple trans-acting factors cooperatively coordinate the processing, stability and translation of RNA, allowing cells to dynamically respond to environmental cues [11, 14]. Recent evidence suggest that functionally related mRNAs are post-transcriptionally regulated together by a large regulatory network including RBPs and non-coding RNAs, such as miRNAs (Figure 1) [11] .

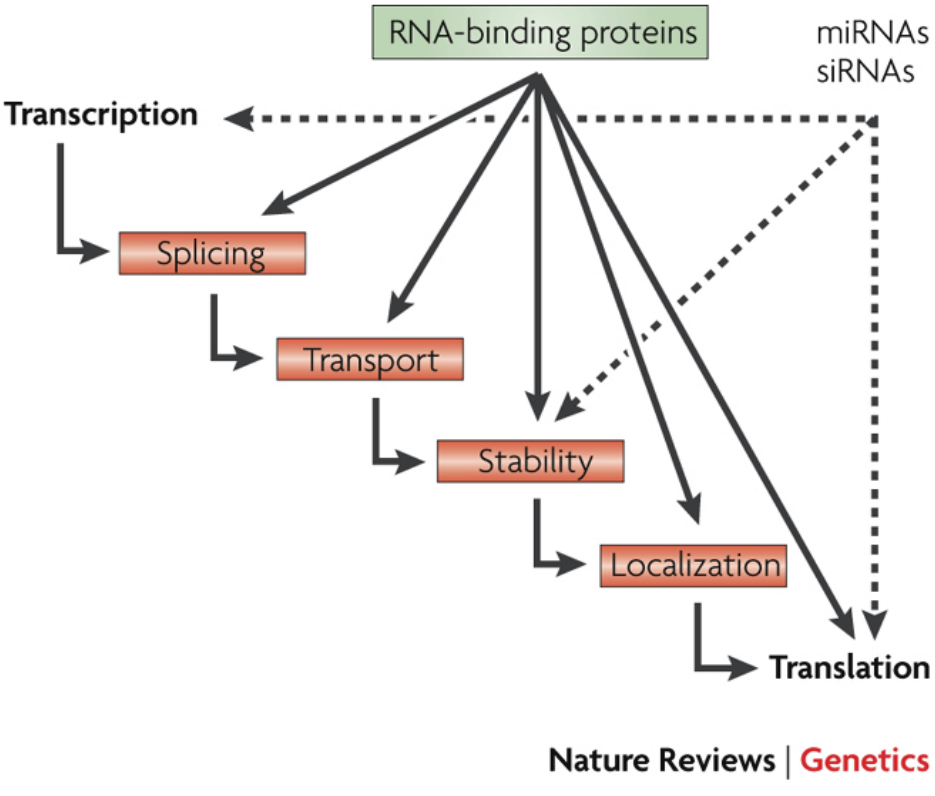

Figure 1. Post-transcriptional regulation by RBPs and miRNAs. RBPs and miRNAs work together to coordinate the splicing, transport, stability, localization and translation of mRNAs. Image is adapted from [4].

MicroRNAs:

miRNAs are ~22 nucleotide-long, non-protein coding RNAs that negatively regulate gene expression. miRNAs function predominately by inhibiting the protein translation of target mRNAs by binding to the 3′-untranslated region (UTR). miRNAs are predicted to regulate over 50% of human genes, giving them significant regulatory power over cellular homeostasis [15]. To date, almost 1,500 evolutionarily conserved miRNAs have been identified in the human genome [16-18].

Most miRNAs are transcribed by specific miRNA gene loci, although 30% are located within the introns of protein encoding genes [19]. miRNAs are transcribed by RNA polymerase II generating a long hairpin structure as a primary microRNA transcript (pri-miRNA) (Figure 2). The primary transcript is further processed with a 5′-7-methyl-guanosine (m^7G) cap and a 3′-poly-adenylated tail. The primary transcript may contain a single miRNA or clusters of miRNAs that are processed together [19]. The RNAse III endonuclease Drosha, cleaves the double-stranded stem into a ~70 nucleotide (nt) precursor miRNA (pre-miRNA). This catalytic event is positioned by DiGeorge syndrome critical region 8 (DGCR8) [20, 21]. The pre-miRNA is recognized by the exportin 5 (XPO5) and RNA-GTP complex and shuttled to the cytoplasm [22]. The terminal loop of the pre-miRNA is cleaved by the double-stranded RNA (dsRNA)-specific RNase III, Dicer into ~22 nt RNA duplex with the help of the human immunodeficiency virus transactivation responsive RNA-binding protein 2 (TARBP2) [23, 24]. TARBP2 recruits an Argonaute (AGO) protein to the guide strand of the miRNA duplex [25]. The guide strand of the mature miRNA is assembled into the miRNA-induced silencing complex (miRISC), along with an Argonaute and GW182 family proteins [25]. The miRISC recognizes complementary

sequences of target RNA for post-transcriptional gene silencing in the processing bodies [26, 27].

miRNAs bind by base-pairing with partial or perfect complementary sites located in target mRNA 3′-UTR [28]. A small percentage of miRNA target sites have been found in open reading frames (ORFs) or the 5′-UTR of target mRNA [29] Perfect complementary base-pairing between the miRNA and target mRNA promotes the catalytic cleavage of mRNA by AGO2 [30]. Out of the four AGO family protein members, only AGO2 is catalytically active [30]. When mRNA cleavage is not possible, additional proteins are recruited to the miRISC to mediate silencing [31]. miRNAs can mediate the silencing of mRNA by inhibiting translation, or promoting mRNA instability through the deadenylation and decapping of the 5′- and 3′-ends therefore accelerating degradation [31]. It's believed that miRNA-mediated degradation of mRNA accounts for approximately 66-90% of miRNA function in cultured cells [32]. The cytoplasmic nuclease 5′-to-3′ exoribonuclease 1 (XNR1) is responsible for degrading the deadenylated and decapped mRNAs. miRNA-mediated translational repression accounts for 6-26% of mRNA silencing, independent of mRNA decay [33].

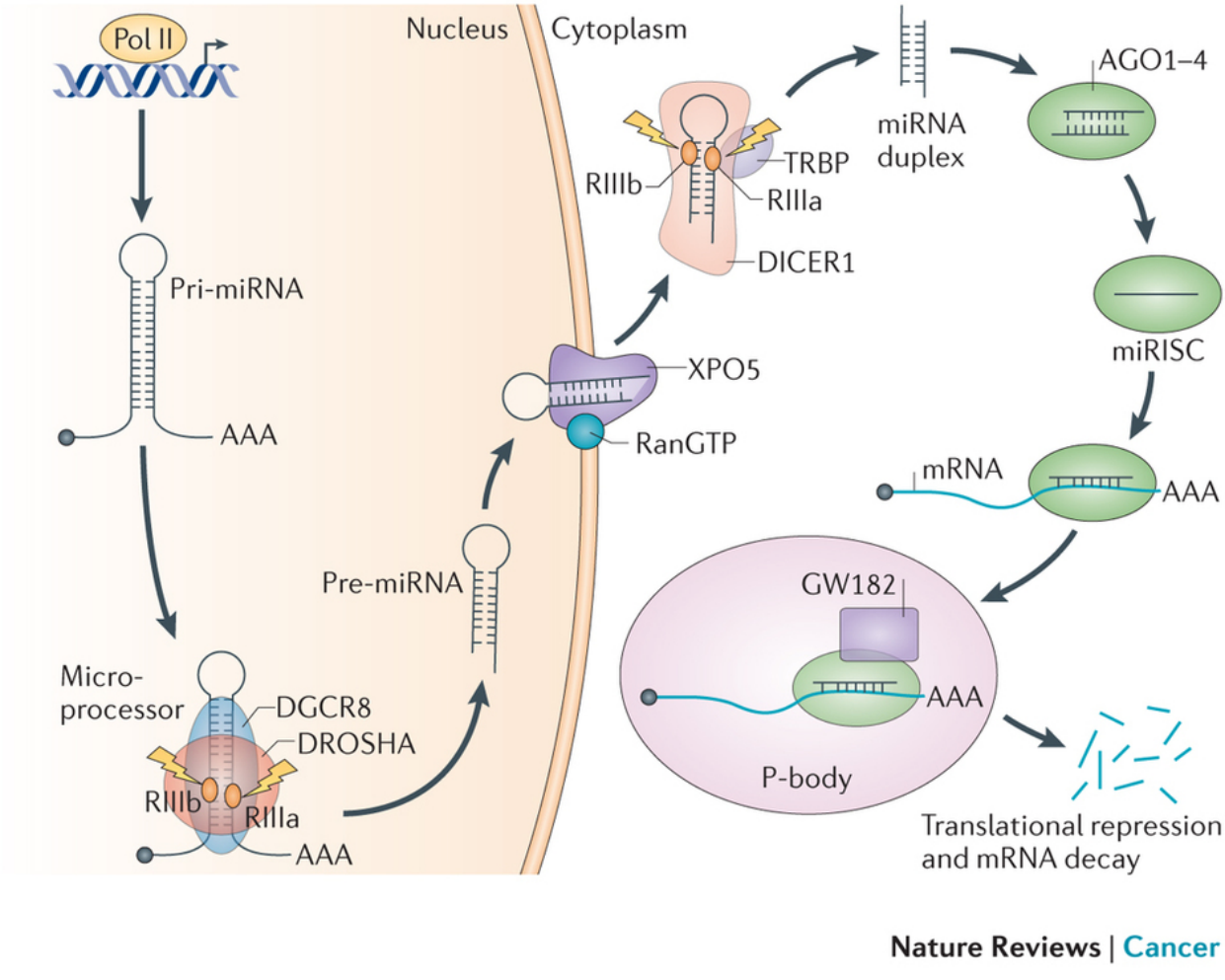

Figure 2. MicroRNA Biogenesis. Figure adapted from [19].

MiRNAs and cancer

The function of miRNAs are critical for the proper function of embryonic development [34]. During differentiation, the global expression of miRNAs increase in order to define tissue-specific gene expression patterns [35]. The global downregulation of miRNAs has been observed in a variety of tumor types [35]. Alterations in miRNA processing and function have been identified in nearly every cancer type [19] and are involved with a variety of cancer promoting processes, including proliferation [36], migration [37], invasion [38], apoptosis [39], and angiogenesis [40]. Disruption of normal miRNA expression by inhibiting any of the critical

miRNA processing proteins was shown to promote tumorigenesis [41]. Furthermore, the aberrant expression of DICER1, AGO2, and XPO5 was identified in multiple cancer types, including breast, ovarian and melanoma [42, 43]. The expression profile of miRNA signatures has been correlated with cancer prognosis, diagnosis, progression, and clinical outcome.

The altered transcriptional expression of specific miRNAs can be mediated by aberrant DNA methylation. Approximately 20% of miRNAs are located within regions of DNA known as sites of methylation, called CpG islands [44]. One particular microRNA of interest is miR-137 which is located within a non-protein-coding gene AK094607, on chromosome 1p22 [45]. miR-137 is embedded in a CpG island, and is silenced in multiple cancer types following hyper-methylation of its promoter region, including colorectal [46], gastric [47], uveal melanoma [48], oral cancer [49], glioblastoma multiforme [50], squamous cell carcinoma of head & neck [51, 52], and breast cancer [53].

In this study, we show evidence that RNA binding proteins Musashi-1 (MSI1) and Musashi-2 (MSI2) are both negatively regulated by miR-137. Additionally, miR-137 acts as a tumor-suppressive miRNA, in part by negatively regulating Musashi-1 and possibly Musashi-2.

Discovery of Musashi-1

The Musashi family of RNA-binding proteins is made up of MSI1 and MSI2. First identified in Drosophila, MSI1 plays a critical role in the asymmetrical division of sensory organ stem cells [54]. During asymmetrical division of the sensory organ precursor cells, the daughter cells split into a non-neural precursor cell and a neural precursor cell. In MSI1 mutants, two non-neural precursor cells are produced giving rise to hair-forming cells and a double-bristle phenotype, as

opposed to the single-hair wild-type phenotype [54]. Musashi was named after a Japanese samurai warrior, Miyamoto Musashi, who fought with two swords [55]. Later studies found that MSI1 promotes the neural differentiation of one daughter cell by repressing the translation of Tramtrack69 (TTK69) protein [56].

Musashi proteins repress the translation by binding to the 3′UTR of target RNA while also binding to the poly(A)-binding protein (PABP) and inhibiting it's interaction with the translational activation protein eIF4G [57, 58]. Collectively, studies indicate the major function of MSI1 is to repress the translation of target mRNA. However a few studies have highlighted a translational activation mechanism for MSI1, although the mechanism controlling the translational activation function of MSI1 is not well understood.

To identify RNA binding motifs of MSI1, researchers used an *in vitro* selection method known as systematic evolution of ligands by exponential enrichment (SELEX) [56]. They discovered that mouse MSI1 preferentially binds to sequences containing two or three repeats of GUU…UAG or GUU…UG. In a separate study, RNA targets of mouse MSI1 was identified using SELEX method. Similarly, this study found that mouse MSI1 preferentially binds to the RNA sequence (G/A)U_nAGU [n = 1-3] [59].

In humans, the mRNA targets for MSI1 were identified using an RNA immunoprecipitation (RIP)-Chip assay [60]. A total of 64 mRNA targets were identified. The genes belong to two main functional categories; 1) cell cycle, proliferation, differentiation and apoptosis, and 2) protein modification. Both functional categories are pertinent to tumorigenesis. From this study,

proteomic analysis revealed that MSI1 can negatively regulate or promote the translation of mRNA targets [60].

Musashi-1 positively regulates Notch and Wnt signaling

Notch and Wnt signaling are two important signaling cascades involved in the initiation and progression of cancer, reviewed in [61, 62]. Notch signaling influences many cell fate decisions, such as; differentiation, apoptosis and proliferation [63]. Notch signaling is mediated through the cleavage of the NOTCH intracellular domain (NICD) and the translocation of NICD to the nucleus where it acts as a transcriptional co-activator to initiate the expression of Notch target genes, such as hairy and enhancer of split-1 *(HES1)* [64]. Notch signaling is negatively regulated by numb homolog (NUMB) binding to and preventing the cleavage of NICD [65]. MSI1 positively regulates Notch signaling by negatively regulating *NUMB* translation by interacting with the cis-acting repressor motif, $GU_{3\text{-}5}$(G/AG), in the 3′UTR [59].

Wnt signaling is another important signaling pathway involved in cancer initiation and progression [62, 66]. During canonical Wnt signaling, β-catenin accumulates in the cytoplasm and translocates to the nucleus. β-catenin acts as a transcriptional co-activator by binding to T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF1) transcription factors to initiate expression of Wnt target genes; such as v-myc myelocytomatosis viral oncogene homolog (*MYC*) and cyclin D1 (*CCND1),* reviewed in [67]. The destruction complex comprised of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK-3β) negatively regulate Wnt signaling by binding to β-catenin and marking it for degradation through ubiquitination (reviewed in [68]). MSI1 promotes Wnt signaling by binding to the

3′UTR and negatively regulating the translation of APC [69]. APC is a well-characterized tumor suppressive protein and an inhibitor of Wnt signaling [70].

In intestinal epithelial primary cultures derived from the small intestine of neonatal mice, MSI1 knock-in increases Wnt signaling by upregulating the expression of frequently rearranged in advanced T-Cell lymphomas 1 (FRAT1), although the exact mechanism is unknown [71]. FRAT1 enhances Wnt signaling by promoting the accumulation of β-catenin [72, 73]. Additionally, MSI1 expression in mammary progenitor cells decreases the expression of the secreted Wnt inhibitor, Dickkopf-3 (DKK3) [74]. However, the mechanism responsible for MSI1-mediated down regulation of DKK3 is unclear. Collectively, MSI1 promotes Wnt signaling by negatively regulating APC and DKK3 and upregulating FRAT1.

Additional functions of Musashi-1

MSI1 promotes the growth of cells by regulating multiple mechanisms. MSI1 promotes cell cycle progression by negatively regulating the translation of $p21^{cip1}$ [75]. Additionally, studies have shown that MSI1 upregulates proliferation by increasing the growth factor, proliferin (PLF1) [74]. However the specific mechanism associated between MSI1 and PLF1 expression has not been studied.

In mouse embryonic brain tissue, MSI1 negatively regulates the protein translation of doublecortin (DCX) by binding to the 3′UTR [76]. DCX is expressed primarily in neuronal precursor cells newly differentiated from neural stem cells. DCX promotes the migration of newborn neurons [77]. Therefore, MSI1 negatively regulates the migration of neural stem cells by inhibiting the translation of DCX.

Localization of Musashi-1

To date, there remains much ambiguity in regards to the precise subcellular localization of MSI1 [78]. Depending on the cell type, MSI1 has been reported to be localized predominantly in the cytoplasm, diffused evenly throughout the cell, or localized in the nucleus. In neuronal stem and progenitor cells, MSI1 is predominately located in the cytoplasm, although, MSI1 can be localized in the nucleus [79, 80]. Using a GFP-tagged, full-length protein, MSI1 was found evenly distributed in the cytoplasmic and nuclear compartments in neural progenitor cell line, PC12. Upon differentiation, MSI1 was predominately expressed in the nucleus. These results suggest that MSI1 is sequestered in the nucleus during differentiation in order to de-repress MSI1 target mRNAs. (unpublished data, mentioned in the review article [81]). The conflicting results highlight an important need to study the localization of MSI1 further. A nuclear localization signal (NLS) sequence is found within the highly conserved MSI1 RNA binding domain 1 (RBD1). Since RBD1 is the major domain for binding to RNA, interaction with target mRNA in the cytoplasm may inhibit the NLS signal and MSI1 localization to the nucleus [82].

The nuclear function of MSI1 is largely unknown. Recently, a study found that MSI1 is responsible for the RNA maturation of *TAU* in the nucleus of neurons, suggesting that MSI1 may have distinct nuclear functions [83]. In neuronal stem/progenitor cells, MSI1 can be localized in the nucleus where it post-transcriptionally regulates miRNAs along with Lin28 [79]. Lin28 is a microRNA binding protein that will bind to pre-miRNAs and prevents maturation [84]. Collectively, the data suggests that the regulatory mechanism responsible for the localization of MSI1 should be an important focal point for future studies.

Expression of Musashi-1 in normal tissues

Adult stem cells have been identified in multiple tissue types and are essential in maintaining proper organ function and response to damage. So far, adult stem cells have been identified in the hematopoietic system, mammary gland, testis, skeletal muscle, skin, intestine, hair follicles, myocardium and the central nervous system (CNS). MSI1 is most famously known for its role in maintaining stem cell function. The expression of MSI1 has been identified in a variety of adult stem cell populations, including the mouse eye [85], intestine [86], stomach [87], mammary gland [88], hair follicle [89], and the CNS [55].

In order to gain a better understanding of the expression of Musashi1 in the developing intestinal tissues, Asai et al., tracked the expression of MSI1 in the developing intestinal organs in chicken and mice [90]. During the early stages of development, MSI1 is broadly expressed in both the developing stomach and intestine and located in endoderm and mesenchymal derivatives. In later morphogenetic stages, MSI1 expression is restricted to the epithelia. After crypt formation in the intestinal tract, MSI1 is restricted to a few cells at the base of the mature crypt [86, 90, 91].

In the intestinal epithelium, stem cells are located at the base of the crypts of Lieberkühn, and responsible for the constant cell renewal, with a single crypt producing approximately 250 new epithelial cells each day [92]. The lining of the intestinal track is renewed every week. As cells migrate up the transit amplifying region of the crypt, the stem progenitor cells begin to differentiate into mature intestinal cells. Once they reach the top, cells are finally shed into the lumen and undergo apoptosis [93]. Two populations of stem cells have been identified in the small intestine; actively cycling stem cells expressing the marker leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) and the quiescent stem cells located at the +4 position at

the base of the crypt, expressing BMI1 proto-oncogene, polycomb ring finger (BMI1) [92, 94]. MSI1 is expressed in both stem cell populations [92].

Musashi: stem cell marker of the intestinal epithelium

Multiple studies have provided evidence that MSI1 is a marker of stem cells in the intestinal epithelium. $MSI1^+$ cells have the ability to retain DNA labeling after several rounds of cell division, a characteristic of stemness [86, 90, 91]. MSI1 cells also express the LGR5 proliferating stem cell marker [95]. Shown in mice, $MSI1^+$ cells express the Notch signaling target gene and stemness regulator, HES1 [86, 90]. After injury to the small-intestine in mice, $MSI1^+/HES1^+$ cells enhanced tissue regeneration, a characteristic of stem cells [96]. Using an elegant reporter mouse model with a GFP knock-in within the LGR5 locus [97], researchers studied the stem cell signature using cell sorting and transcriptomic and proteomic analysis. MSI1 was identified as a stem cell signature gene in the intestinal $LGR5^+$ stem cell populations.

Although there is compelling evidence that MSI1 is associated with stem cell status in intestinal tissues, few studies have shown the function of MSI1 in normal intestinal physiology. In an *in vitro* model, MSI1 was shown to be responsible for the proliferative capability of intestinal epithelial progenitor cells by up regulating both Wnt and Notch signaling pathways [71]. Additionally, MSI1 negatively regulates NUMB, an important inhibitor of Notch signaling. In asymmetric cell division, NUMB and NOTCH1 are heterogeneously expressed in daughter cells, therefore NUMB defines the differentiating daughter cell, while the NOTCH1 daughter cell retains stem cell status [59]. This data suggests that MSI1 is directly involved in inhibiting the differentiation of intestinal stem/progenitor cells by negatively regulating NUMB.

Recently, a study found that MSI2 plays a role in the transformation of the intestinal epithelium [98]. Using an intestinal specific MSI2 knock-in mouse model, they found that MSI2 promotes the expansion of $LGR5^{+}$ stem cells, increases crypt proliferation and fission. This is the first study to report MSI2 function in the intestinal epithelium.

Musashi1 expression in adult stem cells

In the CNS, MSI1 is predominately expressed in undifferentiated neural stem and progenitor cells within the peri-ventricular area of mouse embryos and postnatal mammalian brains [55, 99, 100]. The expression of MSI1 is rapidly shut-down in post-mitotic neurons, although the mechanism is not understood [55]. The proliferation and self-renewal of neural stem and progenitor stem cells is maintained by MSI1-mediated down regulation of NUMB and subsequent activation of Notch signaling [59, 78]. Unlike MSI1, MSI2 is expressed in neural precursor cells and is continuously expressed in mature neurons in the neocortex and basal ganglia [100].

In the stomach, MSI1 was detected in the isthmus/neck region, the putative location of stem cells [87]. In the mammary gland, putative stem cells were identified using MSI1 antibodies [88]. MSI1 is highly expressed in $CD24^{hi}/CD133^{+}$ cells, markers for breast progenitor cells [101]. The proliferation and expansion of mammary progenitor cells is driven by a MSI1-dependent autocrine process [74]. MSI1 increases the secretion of PLF1 and reduces the secretion of Wnt inhibitor, DKK3. This results in the enhancement of Wnt and Notch signaling and increased $CD24^{hi}/Sca\text{-}1^{+}$ and $CD24^{hi}/CD29^{+}$ cells, markers for breast progenitor cells (Figure 3). It's important to note that MSI1 did not drive the expansion of the breast stem cells, but rather

progenitor cells with multipotent differentiation capabilities [74]. $CD24^{hi}/CD29^{+}$ mammary cells are multipotent and capable of developing a mammary gland from a single cell [102].

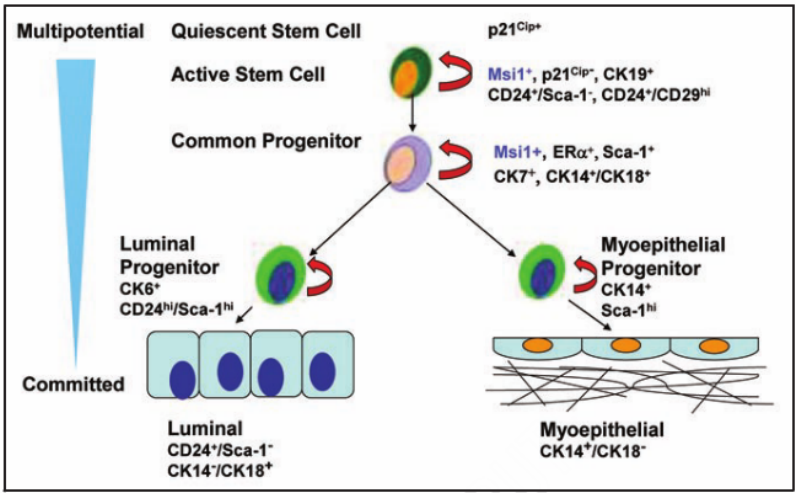

Figure 3. MSI1 maintains the proliferation of breast progenitor stem cells with multipotent differentiation potential [74].

Regulation of Musashi proteins

Multiple studies have shown that MSI1 is positively regulated by the canonical Wnt pathway. MSI1 is expressed in the intestinal tumors of $APC^{min/+}$ mice, suggesting that MSI1 is downstream of Wnt signaling [86]. One study identified a functional Tcf/Lef binding sites 6 kb upstream of mouse MSI1 transcriptional start site [71]. Additionally, MSI1 is transcriptionally upregulated by Wnt signaling stimulation. Using human colonocytes, Wnt-mediated activation of MSI1 was found to be inhibited upon knock-down of APC [69]. Inversely, this study found that MSI1 can negatively regulate APC by binding to its 3′UTR and preventing protein translation. Collectively, these studies provide significant evidence that MSI1 is a Wnt target gene and also a promoter of Wnt signaling.

The mRNA of *MSI1* is stabilized by RNA binding proteins, human embryonic lethal abnormal vision (Hu/ELAV) family orthologs; HuB, HuC, HuD, and HuR, by binding to the AU-rich elements in the *MSI1* 3′UTR [103, 104]. The oncogenic nature of HuR, also known as ELAV1, was shown to be mediated by an upregulation of MSI1 in glioblastoma cells [103]. HuR knock-down HuR reduces the stability of *MSI1* mRNA and protein translation [103].

The expression of insulin-like growth factor 2 (IGF2) increases the number of MSI^+ mammary stem and progenitor cells and promotes tumorigenesis. However, the specific mechanism mediating IGF2-dependent MSI1 expression is currently unknown and needs to be studied further [74]. During neuronal differentiation, MSI1 expression is down regulated, although the specific mechanism mediating the down regulation of MSI1 has not been studied [75, 105]. It's possible that microRNAs are mediating the down-regulation of MSI1 during differentiation of neural, breast and intestinal stem cells.

Stimulation of Notch3 receptor (not Notch1/2) by the notch ligand Delta-like (DLL)-4, increased the expression of MSI1 mRNA and protein in colorectal cancer cell lines, $MICOL\text{-}14^{tum}$ and LoVo along with primary cultures of CRC metastases samples [106]. This study revealed a novel Notch signaling feed-forward loop where Notch 3 activates MSI1, which indirectly activates Notch 1 signaling by down regulating NUMB. However, the mechanism of Notch3-dependent activation of MSI1 is unknown. It is likely that Notch 3 signaling increases the expression of MSI1 in an indirect mechanism. When cells were transfected with a plasmid containing the MSI1 promoter sequence downstream of a luciferase gene, activation of Notch 3 did not stimulate the expression of luciferase. Additionally, in ovarian cancer cells, the MSI1 promoter sequence was not found to be bound to the Notch3 complex in a ChIP-on-ChIP study

[107]. These results suggest that Notch 3 stimulates the expression of MSI1 indirectly, possibly through a microRNA-mediated mechanism.

As for the regulation of MSI2, one study found that MSI2 contains a homeobox 9 (HOXA9)-binding element at -5.7 kb upstream of its transcriptional start site [108]. HOXA9 can bind to MSI2 promoter region and initiate the transcriptional activation of MSI2. This level of regulation is highly relevant for myeloid leukemia. A well characterized oncogenic fusion protein NUP98-HOXA9 is responsible for the progression to blast crisis phase, the advanced and aggressive form of chronic myeloid leukemia [109]. NUP98-HOXA9 activates the transcription of MSI2, leading to an increased expression level of MSI2 in blast crisis phase and a less differentiated phenotype [108].

Since Musashi proteins are so commonly overexpressed and associated with tumorigenesis, metastasis and poor overall survival for patients with a variety of cancer types (reviewed in detail in Chapter 2), the specific regulation of MSI needs to be extensively studied. One advantage of understanding the mechanisms associated with MSI1 overexpression in cancer is the development of potential anti-cancer therapeutics targeting MSI. Additionally, by understanding the mechanisms of MSI regulation, we hope to gain a better understanding of cancer etiology and possibly cancer prevention. For example, one study found that MSI1 expression was increased in response to *helicobacter pylori* infection in the human gut [110]. Maybe the increased expression of MSI1 in cancer is initially caused by an overactive wound-healing response that can be more easily controlled during the early stages of disease. Since APC is so commonly mutated in colon cancer and is known to inhibit Wnt signaling and MSI1 expression [69], maybe

the loss of functional APC is the initiating event that leads to an overexpression of MSI1 in cancer.

MSI1 mRNA contains a long 3′UTR (~1800 nucleotides) consistent with possible post-transcriptional regulation by miRNAs. The average 3′UTR length of miRNA target genes is approximately 1600 nucleotides, as compared to non-miRNA target genes, 1000 nucleotides [111]. Recently, miRNAs negatively regulating *MSI1* mRNA were identified and found to be dysregulated in glioblastoma [112]. Co-transfection of putative targeting miRNAs with the *MSI1* 3′UTR luciferase reporter resulted in decreased luciferase activity indicating miRNA-mediated translational repression. This study, investigating the role of miRNA regulation of *MSI1* in glioblastoma, provides substantial precedent that miRNAs regulate *MSI1*.

In the coming chapters, we provide evidence that Musashi proteins are negatively regulated by microRNAs. Specifically miR-137 acts as a tumor suppressive microRNA, in part by negatively regulating MSI1 and possibly MSI2. We provide a comprehensive review of the expression of Musashi proteins in a variety of cancer types along with the correlation with clinical outcome. Finally, we report preliminary data on the development of novel small molecule inhibitors of MSI1. Using NMR, the binding interaction between MSI1-RBD1 and hit compounds was characterized. Overall, our studies illuminate a novel mode of anti-cancer therapy targeting Musashi RNA binding proteins using either microRNA-based molecular therapy or small molecule inhibitors.

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Chapter 2:
Musashi-1 and Musashi-2: New Therapeutic Targets and Prognostic Biomarkers in Cancer

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Introduction

In the new age of precision medicine, initiatives are being heavily pursued on defining the molecular subtypes of cancer and determining the best therapeutic strategy for a given genomic and molecular tumor landscape [1]. The future of molecular oncology will focus on understanding the molecular attributes of an individual tumor to inform treatment decisions, much like building a map of a person's cancer to better formulate an attack. Precision oncology will require a coupling of molecular profiling with pathological indexes to improve diagnostic, prognostic and therapeutic strategies.

Musashi proteins have been reported to be involved with tumorigenesis in multiple adult tissues: including glioblastoma, colon, breast, lung, ovarian and leukemia. This chapter will provide a comprehensive review of the expression of Musashi in patient tumor samples (summarized in Table 1), the correlation with patient outcome, and evidence for Musashi induced malignancy. Finally, the therapeutic potential of targeting MSI1 and MSI2 for anti-cancer therapy will be discussed.

Colorectal cancer

Using a tissue microarray (TMA) from a cohort of patients with colorectal cancer, researchers used immunohistochemistry (IHC) to measure the expression of MSI1 [2]. They found that MSI1 protein expression gradually increased from normal colon mucosal tissue to advanced stage colorectal adenocarcinoma. MSI1 was detected in 50% (n = 8) of colorectal adenoma samples and was highly expressed in 66.7% (n = 69) of colorectal carcinoma tissues [2]. High expression of MSI1 correlated with advanced TNM stage.

In a separate study, researchers measured the expression of MSI1 in a TMA consisting of 203 primary colon cancer and matching normal colon mucosa and 66 lymph node metastasis (LNM) and correlated the expression level of MSI1 with clinical outcome [3]. Interestingly, they found that MSI1 was strongly expressed in the nucleus of colon cancer cells. MSI1 was highly expressed in 64.5% (n = 203) of primary colon cancer tumors and 87.9% (n = 66) of LNM. High expression of MSI1 positively correlated with invasion depth, distant metastasis, clinical stage and Ki67 staining (a marker for proliferation). Patients with $MSI1^{+}$ tumors developed metastasis more than patients with $MSI1^{-}$ tumors ($P<0.001$). Additionally, patients with $MSI1^{+}$ tumors had an increased risk of developing distant metastasis (HR = 5.1, $P < 0.001$) and poor 5-year overall survival (HR = 3.8) as compared to patients with $MSI1^{-}$ tumors. In RKO cells, an invasive colon cancer cell line, knock-down of MSI1 significantly decreased cell growth, migration and invasion [3].

The mRNA level of *MSI1* was shown to be increased (greater than 2-fold) in 66.7% (n = 15, $P<0.01$) of primary colorectal tumors as compared to adjacent normal mucosal tissue [4]. Additionally, MSI1 knock-down using siRNA, inhibits the xenograft tumor growth of HCT-116 colon adenocarcinoma cells as compared to cells treated with a negative control siRNA.

We recently studied the expression of MSI1 in tissue samples from patients with rectal cancer (discussed at length in Chapter 3) [5]. MSI1 was strongly expressed in 79% of primary rectal tumors (n = 146), 53% of LNM (n = 49), 1% of distant normal rectal mucosa (n = 118), and 8% of adjacent normal rectal mucosa (n = 80). The correlation between expression of MSI1 and clinical outcome was determined using publically available microarray data coupled with clinical parameters using PrognoScan [6]. We found that high expression of MSI1 significantly

correlates with poor overall survival in patients with multiple cancer types, including colorectal, bladder, acute myeloid leukemia (AML), and ovarian cancers [5].

The expression of MSI2 was recently measured in tissue samples from patients with colorectal cancer [7]. Using The Cancer Genome Atlas (TCGA), *MSI2* mRNA was analyzed in 314 healthy colon mucosal tissue samples and 314 colon adenocarcinoma tissue samples (non-matching). The expression of *MSI2* mRNA was consistently increased in tumor samples as compared to normal mucosal tissue. Additionally, the expression of MSI2 protein was measured in tissue samples using IHC. The expression of MSI2 gradually increased with increasing grade of colorectal adenocarcinoma, suggesting that MSI2 may be involved with the progression of colon cancer [7].

Gastric cancer

Biopsy and surgical tissue samples from patients with gastric cancer were obtained and stained for MSI1 [8]. MSI1 protein expression is significantly higher in intestinal-type gastric cancer than normal gastric mucosa ($P < 0.05$). The expression of MSI1 was weak in normal gastric mucosa samples. There was no statistical significance in the expression of MSI1 between low and high-grade intraepithelial neoplasia ($P < 0.05$), suggesting that MSI1 expressing cells may play an early role in the development of gastric cancer. MSI1 expression in gastric cancer positively correlated with lymph node metastasis ($P < 0.01$), suggesting that MSI1 cells may be involved in gastric cancer metastasis.

Hepatocellular carcinoma

In hepatocellular carcinoma (HCC), MSI1 and MSI2 were found to be upregulated in 38% and 49% respectively, in tumor tissue samples as compared to 5% and 8% of adjacent normal hepatic tissues (n = 149) [9]. Interestingly, MSI1 was expressed primarily in the cytoplasm whereas MSI2 was expressed primary in the nucleus of HCC samples. Although MSI1 and MSI2 were both overexpressed in HCC, only MSI2 was correlated with large tumor size, multiple tumor lesions, invasion, stage and early recurrence. Specifically, high expression of MSI2 was correlated with poor overall survival ($P < 0.0001$) and tumor recurrence ($P < 0.0001$). High expression of MSI1 was correlated with solitary lesions.

Breast cancer

In normal breast tissue, MSI1 is expressed in ~1% of ductal epithelial cells in the terminal ductal lobular unit of normal human breast tissue, the site where breast cancer is thought to be initiated [10]. MSI1 was expressed in 55% (n = 20) of human breast cancer cell lines and shows a strong positive correlation with the expression of ErbB2. MSI1 expression was enriched in isolated breast cancer stem cell marker $CD133^+$ cells as compared to $CD133^-$ cells. In patient samples, MSI1 was over-expressed in 51% (n = 140) of primary breast tumor samples and 84% (n = 19) of metastatic lesions in the lymph nodes. Patients with high expression of MSI1 in their tumors survived less than patients with low expression of MSI1 ($P = 0.0402$), suggesting that MSI1 is a negative prognostic marker for patients with breast cancer. Additionally, MSI1 knock-down reduced tumor growth of breast cancer cells MCF-7 and T47D xenografts by approximately 50-60%, indicating its therapeutic potential for treating breast cancer [10].

Medulloblastoma

Using a TMA, the expression of MSI1 was analyzed using IHC in 103 tumor samples from patients with medulloblastoma along with 10 normal cerebellum tissue samples used for control [11]. MSI1 expression was significantly higher in the medulloblastoma samples as compared to the expression of MSI1 in the normal cerebellum tissues [11].

In a separate study using 259 tumor samples from patients with medulloblastoma compiled in a tissue microarray, the expression of MSI1 was studied [12]. MSI1 staining was negative in 23% (n = 59/259) of samples and strongly expressed in 77% (n = 200/259) of tumor samples. High MSI1 was significantly correlated with poor overall survival and progression-free survival. The samples were separated into molecular subtypes of medulloblastoma; SHH, WNT, groups 3 & 4. High MSI1 expression was frequently observed in groups 3 & 4. Specifically, 98% of samples with group 3 molecular subtype had high expression of MSI1. Additionally, in groups 3 & 4, high expression of MSI1 is associated with poor prognosis and overall survival as compared to $MSI1^{-}$ tumors. Daoy (medulloblastoma cell line) xenografts treated with MSI1-siRNA grew significantly less than tumors treated with a negative control siRNA. A RIP-ChIP experiment was done to identify mRNAs associated with MSI1 protein in Daoy cells. The mRNAs bound to MSI1 protein were then subjected to a microarray and analyzed using pathway analysis software. They found that MSI1-associated mRNAs are involved with many cancer relevant processes; such as apoptosis (44%), cell cycle (24%), cell differentiation (40%), cell proliferation (46%), cell survival (36%) and DNA repair (7%) [12].

Myeloid leukemia

In human hematopoietic stem cells, MSI2 is expressed at much higher levels than MSI1 [13]. MSI2 is highly expressed in the blast crisis phase (n = 30), the aggressive form of chronic myeloid leukemia (CML) in which the treatment outcome is extremely poor. Researchers then used a separate microarray study and found that *MSI2* was overexpressed in 100% of patients in blast crisis phase (n = 90) [13]. In patients with chronic phase of CML, high expression of *MSI2* is positively correlated with a high risk of relapse (HR = 4.35, P = 0.07, n = 37). In patients with blast crisis phase, high expression of *MSI2* is positively correlated with high risk of relapse (P = 0.06) and a high risk of death (HR = 6.76, P = 0.08) [13].

In acute myeloid leukemia, MSI2 protein is expressed in the nucleus and cytoplasm of 70% of patients tumor samples (n = 120) [14]. High expression of MSI2 was observed in 33% of patients and was correlated with poor outcome (P < 0.0001). Interestingly, nuclear MSI2 was an independent predictor of poor outcome. This is the first study to show that nuclear MSI2 may be an independent marker for prognosis.

Endometrial carcinoma

The endometrium is the inner lining of the uterus. Growth of endometrial tissue outside of the uterine cavity is referred to as endometriosis [15]. Moderate staining of MSI1 protein was observed in 50% (n = 16) endometriosis cases and 75% (n = 9) of endometrial carcinomas as compared to normal endometrial tissue samples [16]. The cytoplasmic expression level of MSI1 progressively increased in early adenomyosis to endometroid carcinoma samples, suggesting that MSI1 is involved in the initiation and progression of endometrial carcinoma.

Lung carcinoma

MSI1 is enriched in $CD133^{+}$ (cancer stem cell marker) cells, in A549 and NCI-H520, bronchioalveolar carcinoma and squamous cell carcinoma cell lines [17]. Knock-down of MSI1 significantly reduced spheroid colony formation. MSI1 protein is highly expressed in 86% (n= 202) of primary lung carcinomas and rarely detected in adjacent normal lung tissue. In primary lung carcinoma tissue samples, MSI1 is expressed cytoplasmic in a diffuse pattern 50% of the time. In bronchioscopic biopsy samples, *MSI1* mRNA is increased in 80% (n = 188) of malignant samples and rarely detected in non-malignant diseased tissues [17]. Since MSI1 was rarely detected in normal tissue samples, it may serve as an important diagnostic marker for lung cancer.

Cervical carcinoma

The expression of MSI1 was studied using IHC and a TMA made up of tissue samples collected from normal cervix tissue, cervical carcinoma in situ and invasive cervical carcinoma tissues [18]. MSI1 staining was found to be equally localized throughout the nucleus and cytoplasm of normal and cancer tissues. MSI1 positive staining was found in 30% (9/30) of normal cervical tissue, 43.3% (13/30) of cervical carcinoma in situ tissue and 81.4% (48/59) of invasive cervical carcinoma tissue. MSI1 expression was significantly increased in cervical carcinoma [18]. Furthermore, they show that knocking down MSI1 in HeLa cells (cervical cell line), significantly reduced the proliferation *in vitro* and inhibited xenograft tumor growth, suggesting that MSI1 may be a therapeutic target for cervical cancer.

In a separate study, the expression of MSI1 was analyzed using IHC on a TMA comprised of tissue samples from 235 patients with various degrees of cervical cancer [19]. MSI1 expression

was found to be significantly increased in the mild dysplasia and moderate-severe dysplasia as compared to normal cervical epithelia. Additionally, MSI1 expression was even stronger in squamous cervical carcinoma tissue samples. Collectively, the data suggest that MSI1 may be involved in the progression of cervical cancer.

Glioma cancers

One study measured the percentage of $MSI1^{+}$ cells in 73 glioma samples and 3 normal brain tissue samples [20]. The study included samples from glioblastoma multiforme, anaplastic astrocytomas, astrocytomas, oligodendrogliomas, anaplatic oligoastrocytomas and oligoastrocytomas. In normal adult and fetal brain tissue, MSI1 was localized in the ventricular and subventricular zones and within glial and astrocyte cells. The neural stem cell population is thought to reside in the periventricular zone of both adult and fetal brains [21]. MSI1 protein was highly expressed in 73.9% (17/25) of glioblastoma multiforme, 29.4% (5/17) anaplastic astrocytomas, and 18.8% (3/16) astrocytomas tissue samples. MSI1 was not expressed in pure oligodendroglioma tissue samples but was found in oligoastrocytomas and anaplastic oligoastrocytomas. The percentage of MSI1 cells increased in association with increased astrocytoma grade and also correlated with proliferation. This data suggests that MSI1 plays a role in the progression of glioblastoma multiforme, anaplastic astrocytomas and astrocytomas.

Table 1: Expression of Musashi-1 and Musashi-2 in Cancer

Musashi-1					
Cancer Type	**% High**	**% Low**	**n-value**	**Noteworthy Observations**	**Reference**
Rectal Primary Tumors [a]	79	21	146	Determined using TMA and IHC.	[5]
Rectal lymph node metastasis [a]	53	47	49	Determined using TMA and IHC.	[5]
Colon primary tumors [b]	64.5		203	Determined using IHC. Strong Msi1 correlated with stage, metastasis, distant metastasis and + Ki-67 staining, poor overall survival	[3]
Colon lymph node metastasis [b]	87.9		66	Determined using IHC.	[3]
Colorectal adenomas [a, b]	50	50	8	Determined using TMA and IHC. High MSI1 correlated with TNM stage	[2]
Colorectal adenocarcinoma [a, b]	66.7	33.3	69	Determined using TMA and IHC	[2]
Colorectal adenocarcinoma	66.7	33.3	15	MSI1 mRNA. Determined using qRT-PCR	[4]
Primary Breast Cancer	51	49	140	Determined using IHC. High Msi1 correlated with poor survival.	[10]
Breast lymph node metastasis	84	16	19	Determined using IHC.	[10]
Hepatocellular carcinoma [b]	37.6	62.4	149	Correlated with solitary lesions. Not correlated with OS.	[9]
Endometrial carcinoma [a]	75		9	Determined using IHC	[16]
Medulloblastoma	77	23	259	Determined using TMA and IHC. Significantly correlated with molecular subtypes: groups 3 & 4. Correlated with poor survival.	[12]
Cervical carcinoma [a, b]	81.4	18.6	59	Determined using TMA and IHC.	[18]
Lung primary cancer [a]	86	14	202	Determined using TMA and IHC.	[17]
Glioblastoma multiforme	73.9	26.1	25	IHC	[20]
Anaplastic astrocytomas	29.4	70.6	17	IHC	[20]
Astrocytomas	18.8	81.2	16	IHC	[20]
Musashi-2					
Cancer Type	**% High**	**% Low**	**n-value**	**Noteworthy Observations**	**Reference**
Hepatocellular carcinoma [b]	49	51	149	High Msi2 correlated with large tumor burden, invasion, advanced stage, tumor recurrence and poor overall survival.	[9]
Chronic myelogenous leukemia	100		90	Associated with high risk of relapse and death	[13]
Acute myeloid leukemia [b]	33	77	210	High MSI2 was correlated with poor outcome ($P < 0.0001$). Nuclear MSI2 is a predictor of outcome.	[22]

HR = Hazard Risk, OS = Overall survival, TMA = tissue microarray, IHC = Immunohistochemistry

[a] Intensity of cytoplasmic Musashi [b] Intensity of nuclear Musashi

Musashi proteins: potential diagnostic and prognostic biomarkers

In support of the new precision medicine initiative, there is a clear need in identifying diagnostic and prognostic biomarkers. The success of curing cancer patients is greatly increased by early detection along with administering therapy best suited to attack a patient's tumor type. Multiple studies have shown that MSI proteins may serve as diagnostic and prognostic biomarkers for a wide range of cancer types.

Breast cancer is a highly heterogeneous disease and has been organized into multiple molecular subtypes [23]. Patients with high expressing MSI1 primary breast tumors are at greater risk for death as compared to patients with low MSI1 expressing tumors [10]. One weakness of this study is that the expression of MSI1 was not correlated with the molecular subtype of breast cancer. The breast cancer patient cohort included a range of molecular subtypes. Although this study provides significant precedents that MSI1 may be a negative prognostic biomarker for breast cancer, future studies need to examine the expression and function of MSI1 in different subtypes of breast cancer.

In colon cancer tumors, MSI1 was highly expressed in the nucleus [3]. Furthermore, they found that high expression of MSI1 was correlated with poor 5-year survival. This study suggests that nuclear MSI1 may be a negative prognostic biomarker for colon cancer. In rectal cancer, MSI1 was found to be highly expressed in the cytoplasm of rectal cancer cells and very weak staining in adjacent and distant normal rectal mucosal tissue [5]. Additionally, high expression of *MSI1* mRNA in colorectal tumor tissues was correlated with poor overall survival in patients [5]. Since MSI1 is not highly expressed in normal tissues, high expression of MSI1 may also be used as a diagnostic marker for colorectal cancer. In gastric cancer tumor samples, MSI1 was highly

expressed in early epithelial neoplasias and barely detected in normal gastric mucosa samples, suggesting that MSI1 may be used as an early detection marker for gastric cancer [8].

High expression of MSI1 was significantly associated with poor overall survival and progression-free survival for patients with medulloblastoma [12]. More specifically, high expression of MSI1 in molecular subtypes 3 & 4, is associated with poor prognosis and overall survival as compared to $MSI1^{-}$ tumors. This data suggests that MSI1 may be a promising diagnostic marker for subtypes 3 & 4 medulloblastoma and a negative prognostic marker.

In hepatocellular carcinoma, MSI1 and MSI2 were highly expressed in 38% and 49% of tumor tissue samples respectively [9]. However, only high expression of MSI2 was correlated with poor overall survival and tumor recurrence. Interestingly, MSI2 was high expressed in the nucleus of hepatocellular carcinoma cells, suggesting that nuclear MSI2 may be a promising prognostic biomarker.

In CML, high expression of MSI2 is correlated with high risk of relapse and high risk of death [13]. In AML patients, high MSI2 was correlated with poor outcome and more specifically, high expression of nuclear MSI2 was a predictor of poor outcome [14]. Altogether, high expression of MSI2 may serve as a negative prognostic biomarker for patients with CML and AML.

Interestingly, strong expression of nuclear MSI1 and MSI2 has been observed in multiple cancer types, including colon, cervical, hepatocellular carcinoma and acute myeloid leukemia and associated with poor clinical outcome. The nuclear function of Musashi proteins is vastly understudied. In future studies, measuring the expression of Musashi proteins in specific

locations within the cell, along with correlating the expression with molecular subtypes will greatly enhance our current knowledge of using MSI as prognostic and diagnostic biomarkers. Collectively, the over expression of Musashi proteins is associated with poor clinical outcome in multiple cancer types and are promising prognostic markers.

Evidence for Musashi induced malignancy

Musashi proteins may act as oncogenic triggers by inhibiting the differentiation of cells and promoting a more stem-cell like genotype and proliferative phenotype. MSI1 was shown to drive the expansion of mammary progenitor cells by upregulating Wnt and Notch signaling [24]. This process was mediated by a novel autocrine process involving the MSI1 induced expression of PLF and decrease in the Wnt inhibitor, DKK3 and Notch inhibitor, mNUMB. In this study, they found that MSI1 knock-in in mammary epithelial cells increased the abundance of $CD24^{hi}/Sca\text{-}1^{+}$ and $CD24^{hi}/CD29^{+}$ cells, previously identified as breast stem cells. Additionally, MSI1 knock-in enhances the expression of genes involved with proliferation, double-stranded DNA break repair, cell cycle and cell motility. Although MSI1 knock-in increased the proliferation of mammary epithelial cells, it was unable to promote anchorage-independent growth in a 3D culture model, suggesting that over expression of MSI1 does not lead to full transformation independently.

Approximately 80% of all colon cancers are associated with a loss of function mutation in the tumor suppressor, APC [25]. Loss of APC in the small intestine of mice produces an over expression of MSI1 and expansion of undifferentiated cells [26]. Additionally, MSI1 is over expressed in $APC^{min/+}$ intestinal tumors, suggesting an important function in tumorigenesis, downstream of Wnt signaling [27]. Recently, MSI2 was also discovered to be over expressed in

$APC^{min/+}$ intestinal tumors [7]. Collectively, the studies suggest that both MSI1 and MSI2 are involved in the early initiation of colon cancer, downstream of APC and Wnt signaling.

To test if MSI1 acts as a proto-oncogene, the untransformed rat intestinal cell line IEC6 was transduced with a lentiviral MSI1 knock-in vector [28]. IEC6-MSI1 mice grew subcutaneous xenografts *in vivo* while the negative control IEC6 cells were unable to grow xenografts in mice. The MSI1 induced xenografts expressed high levels of nuclear β-catenin. Additionally, xenografts expressed high levels of CCND1 and HES-1; well characterized targets of Wnt and Notch signaling [28]. Overall, this study provides evidence that the over expression of MSI1 has tumorigenic potential in the colon epithelium, mediated by the upregulation of Wnt and Notch signaling.

Using conditional dox-inducible MSI2 knock-in mice, researchers found that MSI2 is involved in the transformation of intestinal epithelium [7]. MSI2 knock-in promotes the expansion of LGR5+ stem cells and increases crypt height, fission and proliferation. Furthermore, they found that MSI2 knock-in mimics the phenotype associated with a loss-of-function APC, suggesting that MSI2 is directly involved in the transformation of the epithelium downstream of APC.

MSI2 is highly expressed in the most aggressive form of CML, known as the blast crisis phase [29]. In CML mice models, MSI2 is expressed at higher levels than MSI1 and is enriched in HSC populations [29]. To understand the role of MSI2 in CML tumorigenesis, researchers produced a doxycycline-inducible MSI2 knock-in mouse model [14]. MSI2 knock-in was shown to expand the HSC population, but did not induce malignant transformation alone. However, when the chronic myeloid leukemia-associated (BCR-ABL) oncoprotein [29] was transduced

into the inducible MSI2 cells, mice quickly developed an aggressive form of chronic myeloid leukemia as compared to BCR-ABL alone [13, 14]. Furthermore, knocking down MSI2 using shRNA induced the differentiation of leukemia cells, inhibited tumor growth and increased survival in mice [13].

Collectively, these studies provide evidence that Musashi proteins are directly involved in the development of cancer. However, most models highlight the need for a second oncogenic event, aside from MSI over expression, in order to fully transform normal cells into malignant cells. This is very consistent with the multi-hit hypothesis, suggesting that malignancy results from accumulating mutations and oncogenic hits [30].

MSI1 and MSI2: therapeutic targets for anti-cancer therapy

MSI1 and MSI2 promote tumorigenesis by upregulating Wnt and Notch signaling pathways; two attractive signaling cascades for anti-cancer therapy [31]. Multiple studies have shown that knocking down MSI1/MSI2 decreases tumorigenesis and prolongs survival in mice. Additionally evidence suggests that knocking down MSI1/MSI2 may sensitize cancer cells to chemo/radiation therapy.

In xenograft models, MSI1 knock-down reduces tumor growth in breast [10], colon cancer [4], medulloblastoma [12], and cervical cancer [18]. Similarly, inhibiting MSI2 was shown to decrease tumor growth in leukemia [13], AML [32], and colorectal cancer [7] xenograft models. Additionally, MSI2 knock-down in hepatocellular carcinoma reduced tumor invasion [9].

Inhibition of MSI1 and MSI2, individually or together, may provide an advantage for overcoming chemo and radiation resistance. MSI1 is highly expressed in side population (SP) cells of the intestinal crypt [33]. SP cells have distinct molecular characteristics and are capable of excluding the nuclear stain Hoechst due to the expression of ABC transporters [34]. The expression of the ABC transporters are implicated in the ability of stem cells to resist drug-based anticancer therapies [33].

MSI1 is overexpressed in both colon and rectal primary tumors [3, 5]. Colorectal tumor cells co-expressing $CD133^{+}/MSI1^{+}$ are resistant to Oxiliplatin and 5-flurouracil [35]. Furthermore, crypts exposed to toxic levels of 5-flurouracil still express MSI1, suggesting that $MSI1^{+}$ cells are resistant [36]. One study found that inhibiting MSI1 in the colon cancer cell line HCT-116, sensitized cells to radiation-induced apoptosis [4].

Knock down of MSI2 sensitized Dami cells (AML cell line) and primary AML cells to daunorubicin-induced apoptosis. Daunorubicin is standard therapy for AML patients [32]. The blast crisis phase of myeloid leukemia is known to be highly aggressive and less responsive to treatment [37]. Since MSI2 is highly expressed in the blast crisis phase in myeloid leukemia patients and associated with poor prognosis, inhibiting MSI2 could be a novel therapeutic strategy for these patients [13].

Altogether, MSI1 and MSI2 are promising prognostic and diagnostic biomarkers for a variety of cancer types. Compelling evidence suggests that MSI1/MSI2 inhibition is a promising therapeutic strategy for anti-cancer therapy and for possibly overcoming chemo and radiation resistance.

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Chapter 3:
Tumor Suppressive MicroRNA-137 Negatively Regulates Musashi-1 and Colorectal Cancer Progression

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Abstract

Stem cell marker, Musashi-1 (MSI1) is over-expressed in many cancer types; however the molecular mechanisms involved in MSI1 over-expression are not well understood. We investigated the microRNA (miRNA) regulation of MSI1 and the implications this regulation plays in colorectal cancer. MicroRNA miR-137 was identified as a *MSI1*-targeting microRNA by immunoblotting and luciferase reporter assays. MSI1 protein was found to be highly expressed in 79% of primary rectal tumors (n=146), while miR-137 expression was decreased in 84% of the rectal tumor tissues (n=68) compared to paired normal mucosal samples. In addition to reduced MSI1 protein, exogenous expression of miR-137 inhibited cell growth, colony formation, and tumorsphere growth of colon cancer cells. Finally, *in vivo* studies demonstrated that induction of miR-137 can decrease growth of human colon cancer xenografts. Our results demonstrate that miR-137 acts as a tumor-suppressive miRNA in colorectal cancers and negatively regulates oncogenic MSI1.

Introduction

Colorectal cancer (CRC) is the 3rd most common cause of cancer-related deaths for men and women in the United States [1]. Worldwide, an estimated 1.4 million people were diagnosed with CRC in 2012 [2]. Although, screening methodologies have reduced the incidence of patients presenting with late stage disease at diagnosis, treating advanced stage colorectal cancer continues to challenge physicians. We are entering a new era of cancer research which depends on understanding the clinicopathological indexes paired with understanding the molecular footprint of cancer for improved tailored therapy, recently termed “precision medicine” [3, 4]. Recent evidence suggests that cancer originates from stem cell-like cells, termed tumor initiating cells (TICs) [5, 6]. It is believed that these stem-cell like cells are responsible for cancer

initiation and also mediate metastasis and chemoresistance [6]. By gaining insight into the mechanisms of these rogue stem cell-like cells, targeted therapies can be designed against TICs.

Recently, a stem cell regulator, Musashi-1 (MSI1), was discovered to be highly expressed in colon primary tumors and metastatic lesions in the lymph nodes as compared to paired adjacent normal colon mucosal tissue [7]. Furthermore, this study identified MSI1 as a novel prognostic biomarker and therapeutic target for treating colon cancer [7]. Knocking down MSI1 in human colon cancer cell lines reduces growth, enhances apoptosis after radiation treatment [8] and reduces colon cancer proliferation, migration and invasion *in vitro* [7].

Normally expressed in stem cells, MSI1 is an RNA binding protein which can inhibit translation of target mRNAs, including that of *APC*, *NUMB* and cyclin-dependent kinase inhibitor/$p21^{WAF-1}$ (*CDKN1A*) [9-11]. MSI1 represses translation by binding to the 3′-UTR of target mRNA, therefore inhibiting formation of the 80S ribosome complex [12]. By down-regulating *NUMB*, *APC* and $p21^{WAF-1}$, MSI1 positively regulates the Notch and Wnt signaling pathways and promotes cell cycle progression [9-11]. Though MSI1 has been identified as a therapeutic target, the molecular mechanisms responsible for overexpression of MSI1 in some colorectal cancers are not well understood. One possibility is a dysregulation of miRNAs that negatively regulate *MSI1* mRNA.

miRNAs are short, 20-22 nucleotide, non-coding RNAs that regulate gene expression by binding to the 3′UTR of target mRNA thereby preventing protein translation or inducing mRNA destabilization [13]. miRNAs are predicted to target approximately 60% of all mRNAs, therefore, providing substantial regulatory power over many cellular processes [14]. The

average 3′UTR length of miRNA target genes is approximately 1600 nucleotides, while non-miRNA target genes average 1000 nucleotides [15]. *MSI1* mRNA contains a long 3′UTR (~1800 nucleotides) consistent with possible post-transcriptional regulation by miRNAs. Recently, miRNAs negatively regulating *MSI1* mRNA were identified and found to be dysregulated in glioblastoma [16]. In that study, an initial list of putative *MSI1* targeting miRNAs was identified using the miRNA prediction program, TargetScan. Only the candidate miRNAs that had previously been reported to have implications in central nervous system tumors were examined for the ability to inhibit *MSI1*. This study provides substantial precedent that miRNAs regulate MSI1. We hypothesized that MSI1-regulating miRNAs are dysregulated in colorectal cancer, producing an over expression of MSI1. The objective of this project was to study the post-transcriptional regulation of MSI1 by miRNAs in colorectal cancer.

In this study, we show that miR-137 directly regulates MSI1. An inverse correlation between miR-137 and MSI1 is revealed in a panel of colon cancer cell lines. Additionally, we found that MSI1 protein expression is more abundant in rectal tumors compared to paired adjacent and distant normal mucosa. Alternatively, miR-137 levels are significantly decreased in rectal tumors compared to paired adjacent and distant normal mucosa. *In vitro* studies demonstrated that miR-137 over-expression decreases MSI1 expression, reduces cell growth, colony formation and tumorsphere growth. The restoration of miR-137 expression in xenograft tumor models also reduced tumor growth *in vivo*. Our work reveals a novel mechanism for MSI1 dysregulation in CRC and demonstrates miR-137 as a tumor suppressor miRNA.

Results

Identification of MSI-targeting miRNAs

MSI1 mRNA and protein is over-expressed in a panel of colon cancer cell lines compared to normal colon cell line, CCD-841 (Figure 1A). An apparent uncoupling of the mRNA and protein levels of MSI1 is revealed in this panel of cell lines, suggesting post-transcriptional regulation of MSI1. The molecular mechanism for MSI1 over-expression in colorectal cancer is not well understood. One possibility is a dysregulation of miRNA regulation. We utilized three miRNA targeting prediction programs (miRanda, TargetScan, PicTar) to identify highly conserved putative miRNA binding sites in *MSI1* 3′UTR. Using a variety of computational algorithms based on seed sequence position, pairing and conservation, these programs predict miRNA sites within target genes 3′UTR [17-19]. Among the three prediction programs, five overlapping miRNAs contained conserved, potential binding sites within *MSI1* 3′UTR; miR-125b, miR-137, miR-144, miR-185, and miR-342-3p (Figure 1B, Appendix Table 1).

In order to determine which miRNAs negatively regulate MSI1 in colon cancer cell lines, miRNA mimics and a negative control (NC) mimic were transfected into high-MSI1 expressing cell lines; HCT-116 and DLD-1. Exogenous expression of miR-137 reduced MSI1 protein levels compared to NC mimic in both HCT-116 and DLD-1 cell lines (Figure 1C). Interestingly, miR-125b and miR-342-3p mimics increased the expression of MSI1 in HCT-116 and DLD-1 respectively, suggesting an alternative mechanism of MSI1 regulation. Although this observation is beyond the scope of our current study, future studies focused on the miR-125b and miR-342-3p regulation of MSI1 may be of interest. Additional colon cancer cell lines HT29 and HCT-116 β/W were used to validate our findings, both of which displayed reduced MSI1 protein expression in cells transfected with miR-137 mimic (Figure 1D).

Since MSI1 is overexpressed in the panel of colon cancer cell lines; we hypothesized that miR-137 is down-regulated. We analyzed the expression of pre and mature-miR-137 in the same panel of colon cancer cell lines. In all five colon cancer cell lines examined, miR-137 expression was significantly decreased compared to the normal colon cell line, CCD-841 (Figure 1D). Normal human lung fibroblast cell line, WI-38, has similar miR-137 expression levels as the normal colon cell line, CCD-841. As expected, miR-137 and MSI1 expression are inversely correlated in cell lines ($P = .04$, Fisher Exact Test).

miR-137 directly regulates MSI1

Since miR-137 significantly decreased MSI1 protein expression in both HCT-116 and DLD-1 compared to the other mimics; we focused this study on understanding the miR-137-mediated regulation of MSI1. miR-137 reduced MSI1 protein expression in a dose-dependent manner (Figure 2A). Furthermore, miR-137 decreased *MSI1* mRNA levels more than cells transfected with NC mimic ($P < .0001$) and similarly as cells transfected with MSI1 siRNA (Figure 2B). Alternatively, inhibiting endogenous miR-137 in HEK-293FT and HCT-116 using antagomiRs increased MSI1 protein expression (Figure 2C and 2D).

Luciferase reporter assays were conducted to determine whether miR-137 inhibits MSI1 via the *MSI1* 3′UTR. HEK-293FT cells were co-transfected with *MSI1* 3′UTR luciferase reporter construct and miR-137 or NC mimic. As expected, miR-137 inhibited the luciferase expression

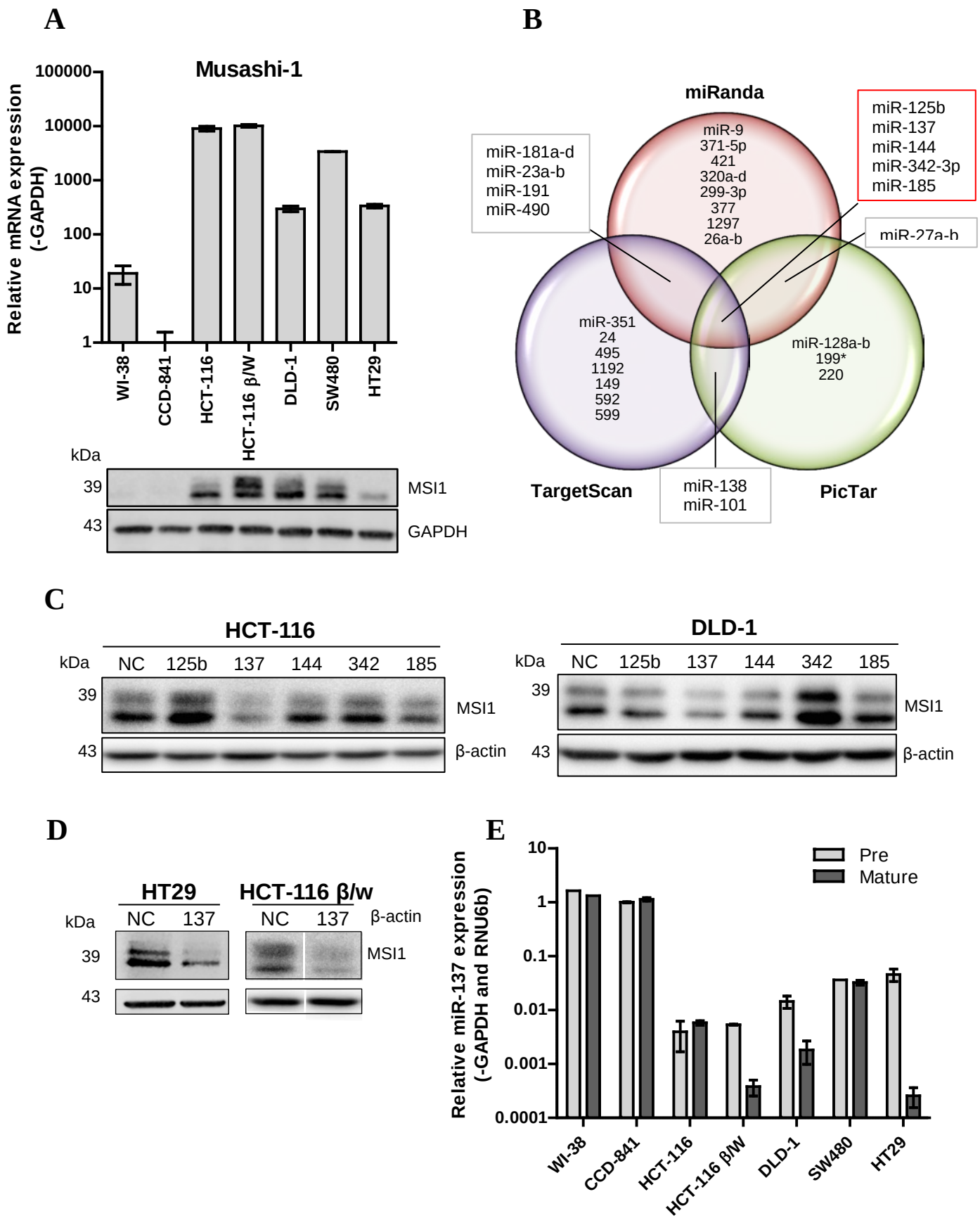
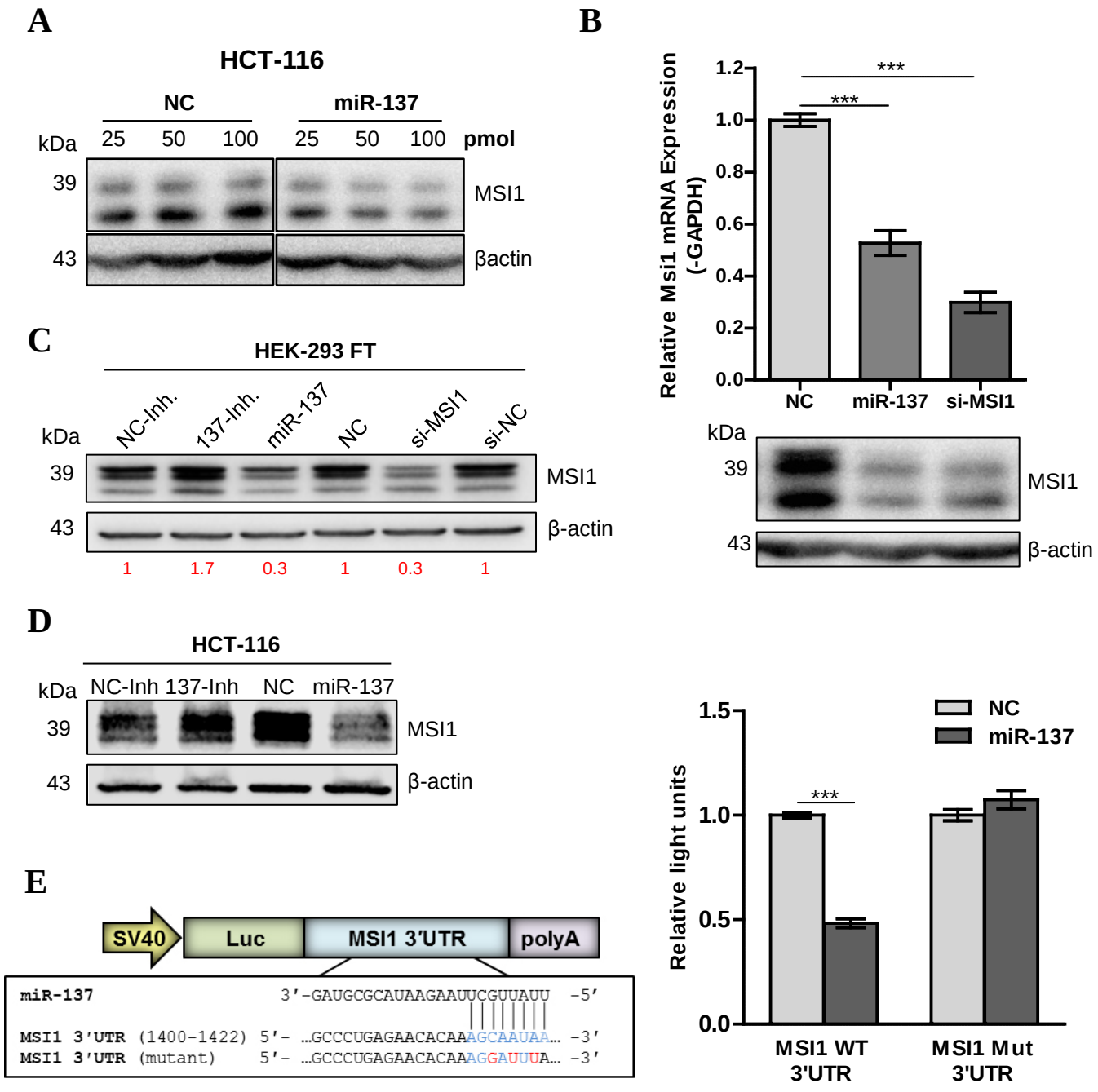

A
Musashi-1
Relative mRNA expression (-GAPDH)
100000
10000
1000
100
10
1
WI-38
CCD-841
HCT-116
HCT-116 β/W
DLD-1
SW480
HT29
kDa
39
MSI1
43
GAPDH
B
miRanda
miR-125b
miR-137
miR-144
miR-342-3p
miR-185
miR-181a-d
miR-23a-b
miR-191
miR-490
miR-9
371-5p
421
320a-d
299-3p
377
1297
26a-b
miR-27a-b
miR-351
24
495
1192
149
592
599
miR-128a-b
199*
220
TargetScan
miR-138
miR-101
PicTar
C
HCT-116
kDa
NC
125b
137
144
342
185
39
MSI1
43
β-actin
DLD-1
kDa
NC
125b
137
144
342
185
39
MSI1
43
β-actin
D
HT29
HCT-116 β/w
kDa
NC
137
NC
137
β-actin
39
MSI1
43
E
Relative miR-137 expression (-GAPDH and RNU6b)
10
1
0.1
0.01
0.001
0.0001
Pre
Mature
WI-38
CCD-841
HCT-116
HCT-116 β/W
DLD-1
SW480
HT29

Figure 1. miRNA regulation of MSI1.

(A) Expression of MSI1 mRNA and protein analyzed in a panel of colon cancer cell lines using quantitative real-time PCR and Western blotting. mRNA normalized to GAPDH and set relative to expression in normal colon epithelial cell line, CCD-841. (B) Venn diagram displaying highly conserved miRNAs predicted to bind to MSI1 3′UTR using miRanda, TargetScan and PicTar prediction software. (C) MSI1 protein expression analyzed in HCT-116 and DLD-1 colon cancer cell lines transfected with a panel of miRNA mimics as compared to cells transfected with a NC miRNA mimic. (D) MSI1 protein expression analyzed in HT29 and HCT-116 β/W colon cancer cells lines transfected with miR-137 mimic as compared to cells transfected with a NC miRNA mimic. (E) Precursor and mature miR-137 expression was analyzed in analyzed in a panel of cell lines using qRT-PCR and Taqman PCR. Pre-miR-137 was normalized to GAPDH and mature miR-137 expression was normalized to RNU6b. Expression data was set relative to CCD-841.


A
HCT-116
NC
miR-137
kDa
25
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39
MSI1
43
βactin
B
Relative Msi1 mRNA Expression (-GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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NC
miR-137
si-MSI1
kDa
39
MSI1
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β-actin
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137-Inh.
miR-137
NC
si-MSI1
si-NC
kDa
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MSI1
43
β-actin
1
1.7
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1
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HCT-116
kDa
NC-Inh
137-Inh
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miR-137
39
MSI1
43
β-actin
Relative light units
1.5
1.0
0.5
0.0
NC
miR-137
***
MSI1 WT 3'UTR
MSI1 Mut 3'UTR
E
SV40
Luc
MSI1 3'UTR
polyA
miR-137
3'-GAUGCGCAUAAGAAUUCGUUAUU -5'
MSI1 3'UTR (1400-1422) 5'- …GCCCUGAGAACACAAAGCAAUAA… -3'
MSI1 3'UTR (mutant) 5'- …GCCCUGAGAACACAAAGGAUUUA… -3'

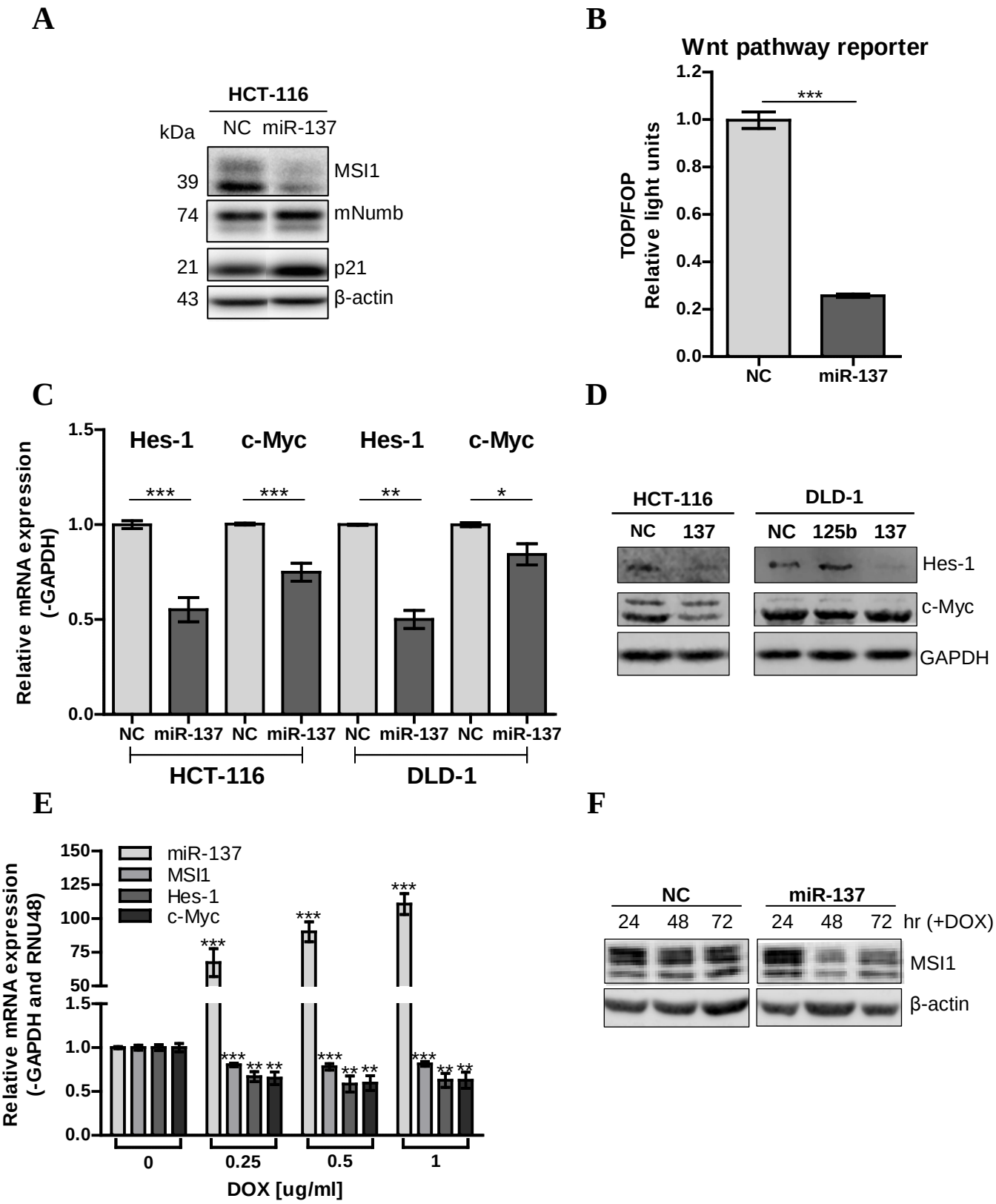
Figure 2. miR-137 negatively regulates MSI1.

(A) MSI1 protein expression in HCT-116 cells transfected with increasing concentrations of miR-137 mimic as compared to cells transfected with NC mimic. (B) *Top,* MSI1 mRNA and protein expression analyzed in HCT-116 cells transfected with NC mimic, miR-137 mimic and positive control, MSI1-siRNA. mRNA expression analyzed using qRT-PCR, normalized to GAPDH and set relative to NC transfected cells. *Bottom,* MSI1 protein analyzed using Western blotting with β-actin as loading control. (C) MSI1 protein expression in HEK-293FT cells transfected with miR-137 antagomiR (137-Inh.), negative control antagomiR (NC-Inh.), miR-137 mimic (miR-137), negative control mimic (NC), MSI1 siRNA (si-MSI1) and a negative control siRNA (si-NC). Intensity of MSI1 protein was normalized to β-actin and set relative to control. Change in MSI1 protein shown in red. (D) MSI1 protein expression in HCT-116 transfected with NC-Inh, miR-137-Inh, NC-mimic and miR-137 mimic. (E) HCT-116 cells were transfected with wild-type (WT) or mutant (mut) pSGG-MSI1 3′UTR luciferase construct with miR-137 or NC mimic. Data are means ± SE; n = 3; *** $P < 0.001$.

of the *MSI1* WT 3′UTR construct ($P < .0001$), which was de-repressed by mutating the miR-137 seed sequence within the *MSI1* 3′UTR (Figure 2E). Our results confirm that miR-137 negatively regulates MSI1 via the *MSI1* 3′UTR.

miR-137 down-regulates Wnt and Notch signaling

If miR-137 successfully knocks down MSI1 levels, we would expect an increase in MSI1 target genes, p21 and NUMB. As expected, in miR-137-transfected HCT-116 cells, NUMB and p21 protein expression is increased compared to cells transfected with NC mimic (Figure 3A). MSI1 promotes cell growth by positively regulating Notch and Wnt signaling [20], therefore, we measured the change in both signaling pathways after miR-137 restoration. As measured by a β-catenin reporter (TOP/FOP) assay, miR-137 significantly reduced Wnt signaling in HCT-116 cells ($P < .0001$) (Figure 3B). We also measured Wnt and Notch signaling target genes, C-MYC and HES-1, in miR-137- and NC-treated HCT-116 and DLD-1 cells. Both C-MYC and HES-1 mRNA and protein expression were significantly reduced in miR-137-treated HCT-116 cells compared to cells treated with NC mimic ($P = 0.0003$ and $P < .0001$) (Figures 3C and 3D). Since the MSI1 target, APC is homozygous mutated in DLD-1 cell line and unable to interact with β-catenin, we expected to see a minimal change in Wnt signaling in this cell line. Notch signaling was reduced in DLD-1 cells, indicated by reduced HES-1 mRNA ($P = 0.002$) and protein expression (Figures 3C and 3D). c-Myc mRNA ($P = 0.0325$) was slightly reduced in miR-137 treated DLD-1 cells; however there was no change in C-MYC protein expression in DLD-1 cells when treated with miR-137 (Figures 3C and 3D). Our data suggests that miR-137 reduces Wnt and Notch signaling pathways, in part by negatively regulating MSI1.


A
HCT-116
kDa
NC miR-137
39
MSI1
74
mNumb
21
p21
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β-actin
B
Wnt pathway reporter
TOP/FOP
Relative light units
1.2
1.0
0.8
0.6
0.4
0.2
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NC
miR-137
C
Relative mRNA expression
(-GAPDH)
1.5
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Hes-1
c-Myc
Hes-1
c-Myc
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*
NC miR-137 NC miR-137 NC miR-137 NC miR-137
HCT-116
DLD-1
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HCT-116
DLD-1
NC 137
NC 125b 137
Hes-1
c-Myc
GAPDH
E
Relative mRNA expression
(-GAPDH and RNU48)
150
125
100
75
50
1.5
1.0
0.5
0.0
miR-137
MSI1
Hes-1
c-Myc
0
0.25
0.5
1
DOX [ug/ml]
F
NC
miR-137
24 48 72 24 48 72 hr (+DOX)
MSI1
β-actin

Figure 3. miR-137 negatively regulates Notch and Wnt signaling in colon cancer cell lines.

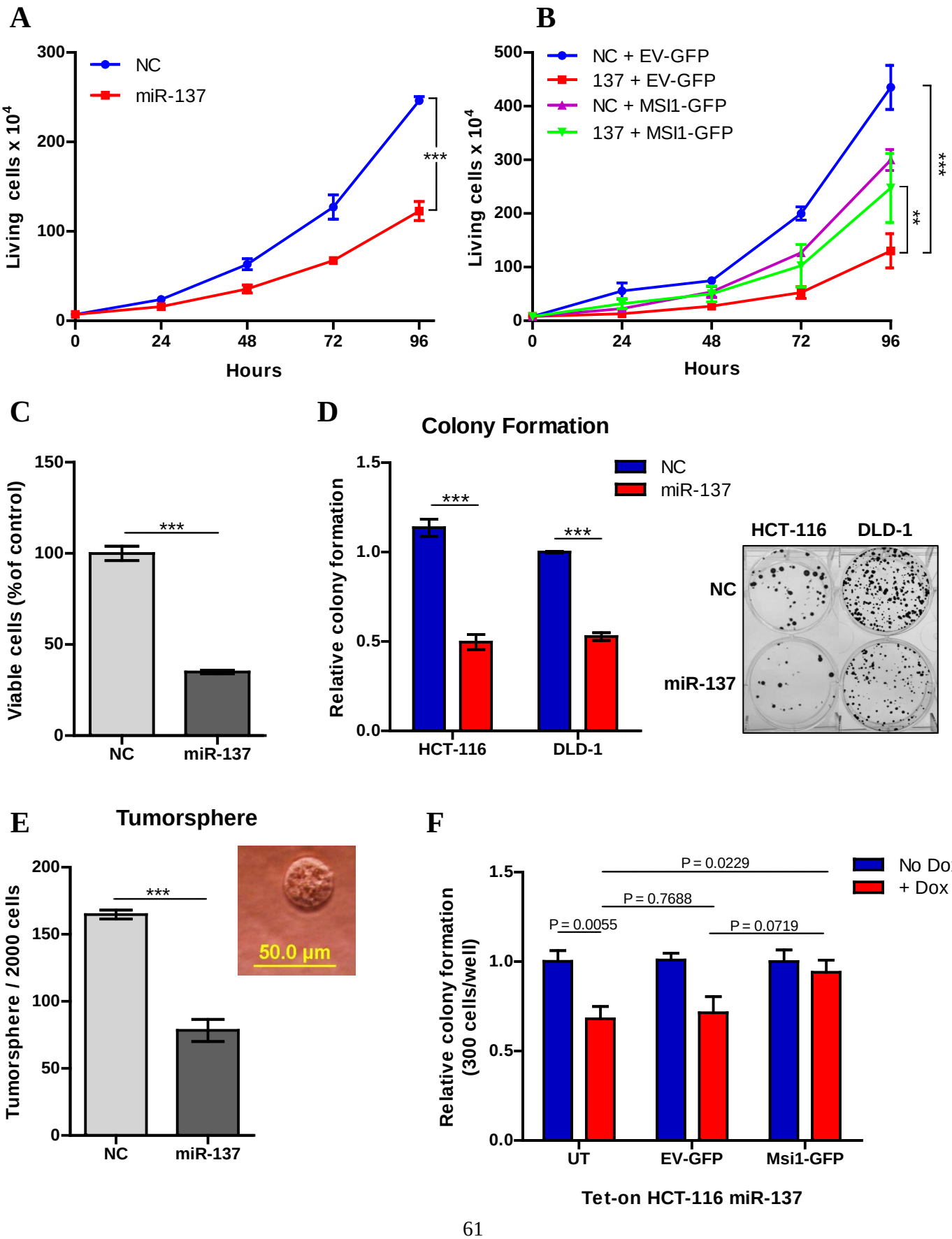
(A) MSI1, mNumb and p21 protein expression was analyzed in HCT-116 cells transfected with miR-137 and NC mimics. (B) HCT-116 cells were transfected with Top or Fop Flash constructs and miR-137 or NC miRNA mimic. Wnt signaling was stimulated with 20 mM LiCl for 16 h. Top Flash luciferase values were normalized to Fop Flash luciferase values. Data are means ± SE; n = 3; $^{**}P < 0.01$. (C) mRNA expression of Hes-1 and c-Myc was analyzed in HCT-116 and DLD-1 cells transfected with miR-137 or NC mimic using qRT-PCR. Expression data was normalized to GAPDH and set relative to NC. Data are means ± SE; n = 3; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. (D) Protein expression of c-Myc and Hes-1 in HCT-116 and DLD-1 cells transfected with NC mimic and miR-137 mimics. GAPDH is loading control. (E) Tet-on miR-137 HCT-116 cells were treated with increasing doses of DOX. Expression of mature miR-137 was analyzed using Taqman qRT-PCR, normalized to RNU48 and set relative to cells treated without DOX. MSI1, Hes1 and c-Myc mRNA was analyzed using qRT-PCR, normalized to GAPDH and set relative to cells treated without DOX. Data are means ± SE; n =3. (F) Tet-on miR-137 and NC HCT-116 cells were treated with 1 ug/ml DOX for 24, 48 and 72 hours. MSI1 protein was analyzed using Western blotting. β-actin used as loading control for Westerns.

To study the effect of constitutively active miR-137, Tet-inducible miR-137 HCT116 stable cells were produced using a pTRIPZ lentiviral expression vector. These stable clones are capable of inducing transcription of miR-137 when treated with doxycycline (DOX) to a physiological relevant level as compared to the expression of mature miR-137 in colon cell line, CCD-841 (Appendix Figure S1). The addition of DOX results in decreased MSI1 expression and downstream Notch and Wnt signaling targets, HES1 and C-MYC (Figure 3E). The decreased MSI1 expression is also observed at the protein level (Figure 3F). In summary, miR-137 restoration in colon cancer cell lines significantly reduced Wnt and Notch signaling; two important oncogenic signaling pathways regulated by MSI1 and involved in colorectal cancer progression.

miR-137 acts as a tumor suppressor miRNA in colon cancer

Based on our preliminary data, we hypothesized that miR-137 acts as a tumor suppressor miRNA by negatively regulating MSI1. To examine the effect of miR-137 restoration on colon cancer cells, *in vitro* cell growth and cell viability assays were utilized. miR-137 mimic transfected HCT-116 cells grew significantly less than cells treated with NC mimic ($P < .0001$) (Figure 4A). To examine whether the miR-137-induced cell growth inhibition was via MSI1 down-regulation, a phenotype rescue experiment was performed. miR-137 mediated inhibition of cell growth was partially restored when HCT-116 cells were co-transfected with a MSI1 cDNA expression vector that lacks the 3′UTR as compared to cells co-transfected with an empty vector (Figure 4B). Surprisingly, MSI1 knock-in did not increase the proliferation of HCT-116. One possibility is that MSI1 is already highly expressed in this cell line, suggesting that maybe too much MSI1 is toxic to the cells, but this needs to be studied in future studies. Overall, our data suggests that miR-137 tumor suppressive function is mediated in part by negatively

regulating MSI1. We also show that cell viability was significantly reduced upon miR-137 restoration as measured by a MTT cell viability assay ($P < .0001$) (Figure 4C).


A
Living cells x 10^4
300
200
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NC
miR-137
***
0
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96
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B
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NC + MSI1-GFP
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1.0
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100
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50.0 µm
NC
miR-137
F
Relative colony formation (300 cells/well)
1.5
1.0
0.5
0.0
P = 0.0229
P = 0.7688
P = 0.0055
P = 0.0719
No Dox
+ Dox
UT
EV-GFP
Msi1-GFP
Tet-on HCT-116 miR-137

Figure 4. miR-137 inhibits colon cancer growth and clonogenic growth by inhibiting MSI1.

(A) Cell growth curve in HCT-116 cells transfected with miR-137 and NC mimic. Cells were collected and counted every day for 4 days. Data are means ± SE; n = 3; *** $P < 0.001$. (B) Cell growth curve in HCT-116 cells co-transfected with miR-137/NC mimic and EV-GFP/MSI1-GFP expression vectors. Cells were collected and counted every day for 4 days. Data are means ± SE; n = 2; ** $P < 0.01$, *** $P < 0.001$. (C) Cell viability was measured using a MTT colorimetric assay in HCT-116 cells transfected with miR-137 and NC mimic. (D) Colony formation assay in HCT-116 and DLD-1 cells transfected with miR-137 and NC mimic. Image of representative colonies are shown in right panel. (E) Tumorsphere assay in HCT-116 cells transfected with miR-137 and NC mimic. Data are means ± SE; n = 3; *** $P < 0.001$. (F) Colony formation on Tet-on miR-137 cells transfected with MSI1-GFP or EV-GFP constructs. Cells were plated in media with and without 1 μg/ml DOX. Data are means ± SE; n = 2; * $P < 0.05$, ** $P<0.01$ and *** $P<0.001$.

Since MSI1 is a regulator of intestinal multipotent stem cells [21], we predicted that miR-137 reduces clonogenic cell growth in colon cancer. miR-137 significantly reduced clonal expansion of HCT-116 cells ($P < .0001$) and DLD-1 ($P < .0001$), as determined by a colony formation assay (Figure 4D). Similarly, tumorsphere growth was also significantly reduced by approximately 52% ($P = .0006$) upon miR-137 restoration in HCT-116 cells (Figure 4E). Tet-inducible miR-137 HCT-116 cells were transfected with either a MSI1 cDNA expression plasmid or control vector, in the presence or absence of DOX. In non-transfected cells, colonies grew significantly less when miR-137 expression was induced using DOX ($P = .0055$) (Figure 4E). However, when cells were transfected with MSI1 cDNA that lacks the 3′UTR, the colony formation capability was nearly completely restored ($P = .0719$) (Figure 4F).

In summary, miR-137 inhibits clonogenic growth, supporting its predicted role as a tumor suppressor miRNA that reduces colon cancer stem cell properties, in part by directly down-regulating MSI1.

miR-137 reduces tumor growth *in vivo*

To study the effect of constitutively active miR-137 expression on tumor progression, HCT-116-miR-137 tet-on stable clones were subcutaneously injected into flanking sides of athymic nude mice. Ten mice (mean weight = 18.68 g ± 0.563) were randomly separated into two groups. One group of mice received 10 mg/ml DOX in the drinking water immediately following injection, while the second group of mice did not receive DOX. The induction of miR-137 decreased tumor growth by approximately 75% (n = 8, $P = .003$, day 33) compared to the tumor growth in mice not fed DOX (n=10) (Figure 5A).

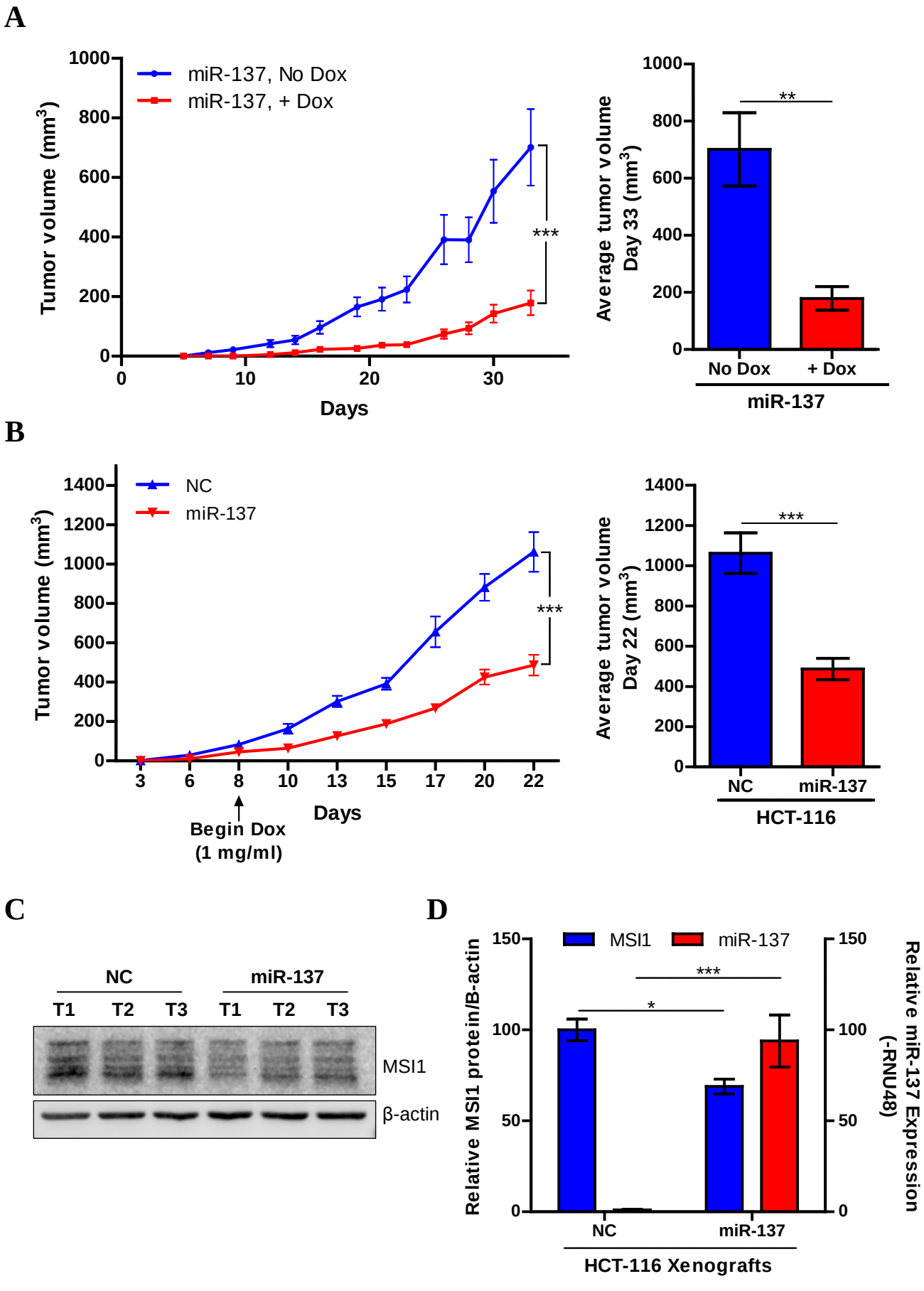

A
miR-137, No Dox
miR-137, + Dox
Tumor volume (mm3)
Days
***
Average tumor volume Day 33 (mm3)
**
No Dox
+ Dox
miR-137
B
NC
miR-137
Tumor volume (mm3)
Days
Begin Dox (1 mg/ml)
***
Average tumor volume Day 22 (mm3)
***
NC
miR-137
HCT-116
C
NC
miR-137
T1
T2
T3
T1
T2
T3
MSI1
β-actin
D
MSI1
miR-137
Relative MSI1 protein/B-actin
Relative miR-137 Expression (-RNU48)
***
*
NC
miR-137
HCT-116 Xenografts

Figure 5. miR-137 inhibits human colon cancer xenografts growth.

(A) Tet-on miR-137 HCT-116 cells were injected into subcutaneously into mice. 10 mg/ml DOX was administered to the drinking water of one group of mice immediately following injections. Average tumor size was calculated at the end of the study and averaged for each group (on right). (B) Tet-on miR-137 and NC HCT-116 cells were injected into mice. After tumors grew to be approximately 50 mm^3 in size, both groups of mice were given 1 mg/ml DOX in their drinking water. Average tumor size was calculated at the end of the study and averaged for each group (on right). (C) MSI1 protein expression was analyzed in three tumor samples from Tet-on miR-137 and NC HCT-116 xenograft samples. β-actin was used as loading control. (D) Relative mature miR-137 expression and relative MSI1 protein expression in xenografts tumor samples. Data are means ± SE; n = 3; **P* < 0.05, ***P*<0.01 and ****P*<0.001.

DOX was recently shown to inhibit breast CSCs by reducing energy metabolism and DNA repair [22]. To control for DOX's effect on tumor growth in our studies, a separate animal experiment was performed using HCT-116 NC and miR-137 stable cells. Ten mice (mean weight = 22.1 g ± 0.458) were randomly separated into two groups. After cells were injected subcutaneously, both groups of mice were fed DOX in their drinking water once tumor volumes reached approximately 50 mm^3. Similar to the previous animal study, the induction of miR-137 significantly inhibited colon cancer xenograft tumor growth by 55% (n=10, $P < .0001$, day 22), as compared to mice inoculated with inducible NC mimic (n=10) (Figure 5B).

Tumors were excised at the end of the study to analyze the expression of miR-137 and MSI1. The protein expression of MSI1 was decreased in the miR-137-treated tumors as compared to NC tumors ($P = .0124$) (Figure 5C and 5D). Additionally, miR-137 expression was significantly increased in the tet-on miR-137 tumors compared to NC xenografts ($P < .0001$) (Figure 5D). In summary, induction of miR-137 significantly inhibited the human colon cancer xenograft tumor growth. Collectively the data supports our hypothesis that miR-137 acts as a tumor suppressor miRNA by down-regulating the oncogenic MSI1 that subsequently leads to tumor growth inhibition.

Expression of MSI1 and miR-137 in patient tumor samples

In order to understand the clinical relevance of our study, we used PrognoScan [23], a database used to correlate gene expression with patient prognosis, to determine the correlation between MSI1 expression with overall survival in patients with a variety of cancer types. Patients with high MSI1 expression was significantly correlated with an increased hazards risk for poor overall survival in patients with bladder cancer (n = 165, $P = 0.0150$), AML (n = 34, $P = 0.0002$), colorectal cancer (n = 55, $P = 0.0151$) and ovarian cancer (n = 133, $P = 0.0019$) (Figures 6A). In

the colorectal dataset (GSE17537), high MSI1 significantly correlates with increased hazard risk, analyzed using a Kaplan-Meier curve and Cox proportional hazards regression test (Figure 6B).

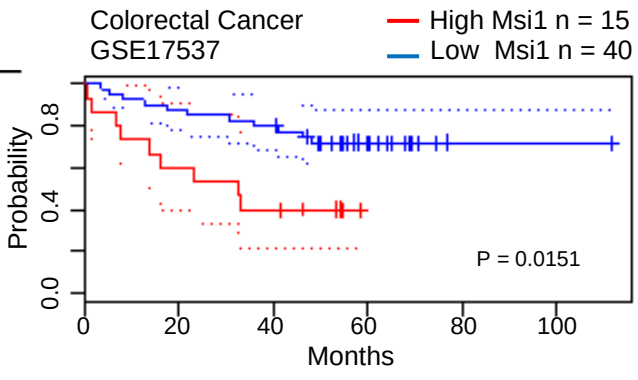
We also examined the expression of MSI1 and miR-137 in available tissue samples from patients with rectal cancer. MSI1 protein expression was analyzed using immunohistochemistry in distant normal mucosa, adjacent normal mucosa, primary tumor and lymph node metastasis tissue samples collected from patients with rectal cancer (Figure 6C). The average score for MSI1 intensity was significantly higher in tumor samples collected from primary tumor ($P <$ .0001) and metastatic lesions ($P < .0001$) (Appendix Table 3) as compared to distant normal and adjacent normal mucosal tissue samples. For analysis, samples were categorized into high MSI1 expressing with intensity scores greater than two, while scores equal to or less than two were defined as low MSI1 expressing. MSI1 was highly expressed in 79% of primary tumor samples, and 53% of metastatic lesion samples. Inversely, MSI1 was highly expressed in 1% of distant normal samples and 8% of adjacent normal samples (Figure 6D and Appendix Table 4).

The expression of miR-137 was measured in 68 paired normal mucosal and primary tumor tissue samples using Taqman qRT-PCR. miR-137 was significantly decreased in 84% of primary tumor tissue samples when compared to the paired adjacent normal tissues (n = 68, $P < .0001$) (Figure 6E and Appendix Table 5). The expression of miR-137 did not statistically correlate with MSI1 expression in tissue samples ($P = 0.54$, Appendix Table 6), although a clear trend is revealed in our patient data analysis and provides important precedence that analyzing more

A

Cancer type	Dataset	n	P-value	ln (HR_{high}/HR_{low})
Bladder	GSE13507	165	0.0150	1.26
AML	GSE8970	34	0.0002	3.12
Colorectal	GSE17537	55	0.0151	1.14
Ovarian	DUKE-OC	133	0.0019	0.99

B



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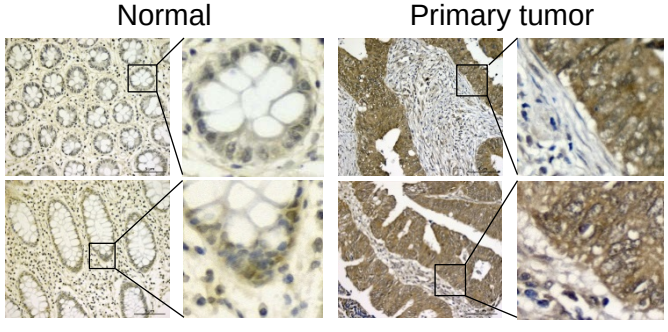

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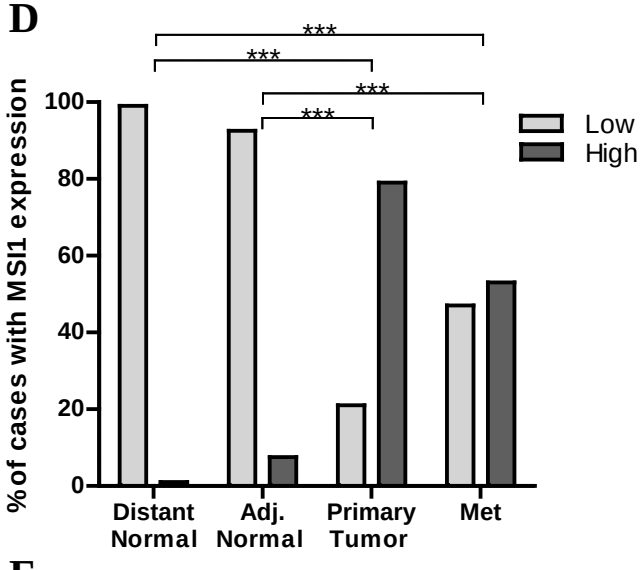

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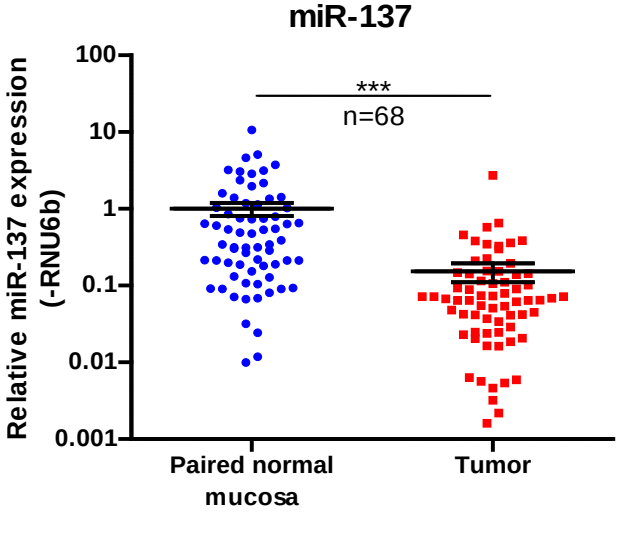

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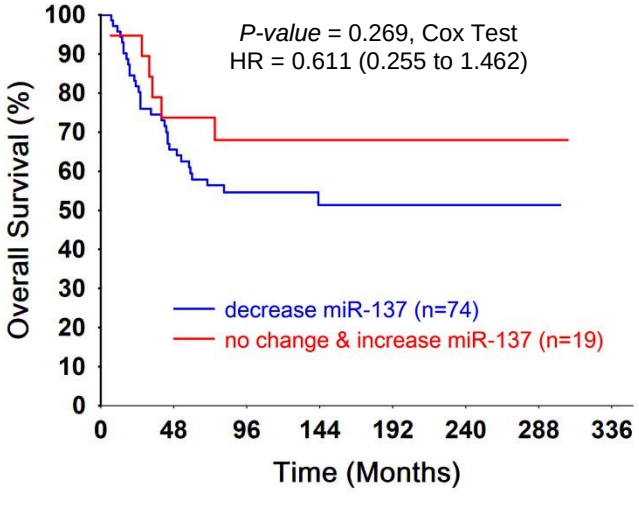

Figure 6. Musashi-1 is over expressed and miR-137 is decreased in rectal cancer tissue samples.

(A) Expression of MSI1 in a variety of cancer types is correlated with survival using PrognoScan database. (B) Kaplan-Meier curve associated with the colorectal dataset GSE17537. High MSI1 correlates with poor overall survival. Analyzed using PrognoScan database, Log-rank test, P = 0.0151. (C) Representative images of MSI1 immunohistochemistry in distant normal rectal mucosal tissues (left panel) and rectal primary tumor tissue (right panel). Bar = 5 μm. Magnification: 40x. (D) MSI1 intensity scores were categorized into low expressing (TMA scores of 0+1+2) and high expressing (TMA scores of 3). The percentage of low and high MSI1 expression was calculated for each tissue type. (E) Mature miR-137 expression was analyzed in 68 pairs of normal and primary rectal tumor tissues using Taqman PCR. miR-137 was normalized to RNU6b and set relative to matching normal. *** $P < 0.001$. (F) The overall survival of 93 patients was analyzed comparing decreased and increased expression of miR-137 using Kaplan-Meier survival analysis.

patients for the expression of MSI1 and miR-137 is needed in order to obtain statistical significance. Correlating miR-137 expression with patient survival demonstrated that patients with decreased miR-137 had an increased hazard of death (HR = 0.61) as compared to patients with no change or increased expression of miR-137, although this did not reach statistical significance (P = .269) (Figure 6F). In conclusion, our results show that miR-137 expression is decreased in colon cancer cell lines and rectal cancer tissues, and supports our overall hypothesis that loss of miR-137 promotes the overexpression of MSI1 in colorectal cancer.

Discussion

In the current study, we show that miR-137 can regulate MSI1 in colon cancer cell lines. We revealed an inverse correlation between miR-137 and MSI1 in a panel of colon cancer cell lines as compared to a normal epithelial cell line, and in primary rectal tumor tissues as compared to paired normal rectal mucosal tissue. miR-137 restoration in colon cancer cell lines, reduces MSI1 mRNA and protein levels, and inhibits cell growth, colony formation and tumorsphere growth. Furthermore, expressing miR-137 in HCT-116 significantly reduces xenografts tumor growth. In conclusion, our data suggest that miR-137 acts as a tumor suppressive miRNA and when down-regulated promotes tumorigenesis through the up regulation of MSI1.

Multiple studies have found an overexpression of MSI1 in a broad range of cancer types, including breast cancer, lung and colon cancer [7, 8, 24, 25]. We are the first to report an overexpression of MSI1 specifically in rectal cancer in a large patient cohort. Although colon and rectal cancer are often grouped together under the category of colorectal cancer, their treatment strategies differ significantly. For colon cancer, typically surgical resection combined with chemotherapy is the primary treatment strategy [26]. For rectal cancer, radiation therapy

combined with surgical resection is commonly used [26]. Previous studies have found that MSI1 knockdown sensitizes colon cancer cells to radiation therapy [8]. Collectively, this suggests that future therapeutic strategies targeting MSI1 may be relevant for both colon and rectal cancer patients and possibly in combination with conventional treatment strategies, such radiation therapy. We hope to address these possibilities in future studies.

One aim of our study was to understand why MSI1 is overexpressed in colorectal cancer. As shown in the small intestine and colon tissue of adult and developing mice, MSI1 is predominately expressed in the base of crypts and less so towards the top of the transit amplifying region of the crypt, before reaching the villus location.[21] Stem cell genes are often tightly regulated by miRNAs upon differentiation [27, 28]. We propose that a lack of miRNA regulation during proliferation and subsequent differentiation of colon stem cell progeny causes MSI1 overexpression in colorectal cancer. The purpose of this study was to identify miRNAs capable of negatively regulating MSI1 in hopes of learning more about the potential cause of MSI1 overexpression in colorectal cancer. We successfully identified miR-137 as a negative regulator of MSI1 in colon cancer cells. Our findings are consistent with a previous study that discovered miR-137 as a negative regulator of MSI1 in a glioblastoma cell model [16].

Taken together, the data suggests that in normal cells, MSI1 is negatively regulated by miR-137, possibly during differentiation, and this level of regulation has been dismantled in colorectal cancer cells, thus leading to the MSI1 overexpression. Previous studies have shown that miR-137 expression increases upon differentiation of neural stem cells [29, 30] and mouse embryonic stem cells [31]. In cancer, miR-137 has been shown to be decreased in glioblastoma [30, 32], melanoma [33], gastric cancer [34], and colorectal cancer [35-37].

According to our results, loss of miR-137 appears to be at the transcriptional level since both pre and mature forms of miR-137 were decreased in colon cancer cell lines. Our data is consistent with previous studies that discovered loss of miR-137 occurs early in the progression of colon cancer due to hypermethylation of the promoter region [35], which prevents binding of the transcription factor, high-mobility group AT-hook (HMGA)1 [37]. This observation strongly supports our results and overall hypothesis that in colon cancer, miR-137 expression is silenced during differentiation due to hypermethylation, resulting in MSI1 over-expression. However this specific mechanism needs to be studied further.

Overall, we describe an important tumor-suppressive mechanism of miR-137 through the negative regulation of MSI1 and Notch/Wnt signaling, outlined in our working model (Figure 7). miR-137 is a promising candidate for future miRNA-based molecular therapy for treating a variety of cancer types, including colorectal cancer. Our findings provide insight into the mechanism of dysregulation of colon cancer stem cells and eventually colorectal cancer initiation and progression. By understanding the molecular mechanisms of colorectal cancer biology, therapies may be developed to better combat this deadly disease.

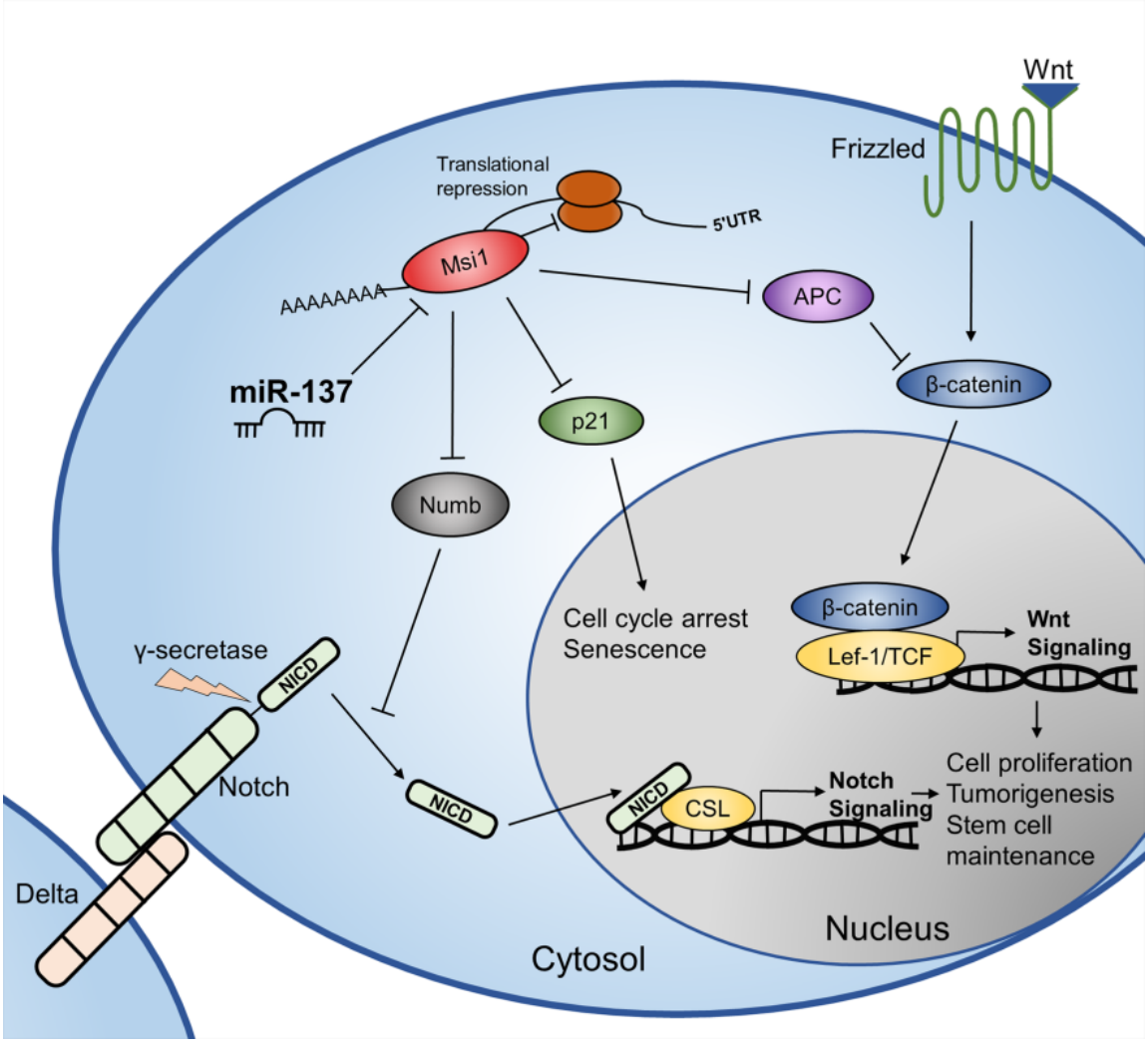

Figure 7. Working model.

RNA binding protein and stem cell regulator, MSI1, positively regulates the Wnt and Notch signaling pathways by binding to and inhibiting the translation of target mRNA; *APC*, $p21^{WAF-1}$, and *NUMB*. In this study, we show that miR-137 acts as a tumor suppressive microRNA, in part by negatively regulating MSI1 and subsequently Wnt and Notch signaling pathways.

Material and methods

Patient Samples and Tissue Microarray

Tissue microarray (TMA) slides were prepared from formalin-fixed paraffin embedded tissue blocks and contained tissue specimens from 146 primary rectal adenocarcinoma samples, 116 distant normal mucosa samples, 80 adjacent normal mucosa specimens, and 49 lymph node metastases. Tissue samples were collected from patients enrolled in a randomized clinical trial testing preoperative radiation therapy in the Southeast region of Sweden [38]. Distant normal mucosa was taken from proximal or distal margin (4-35 cm from the primary tumor) of the resected rectum and adjacent normal mucosa was taken from the mucosa tissue adjacent to the primary tumor. Both normal sections were histologically free from tumor. Additional details regarding the patient cohort and tumor characteristics are presented in Appendix Table 2. Each patient was provided detailed information about the study aims and protocol, and gave their written informed consent prior to enrollment. The study was approved by the Institutional Review Board of the Linköping University, Sweden.

PrognoScan Analysis

The correlation between MSI1 expression and patient prognosis was evaluated using the PrognoScan database (http://www.abren.net/PrognoScan/) [23]. Using the minimum P-value approach, patients samples are grouped into high and low MSI1 expressing groups and correlated with survival using a Kaplan-Meier curve and log-rank test. Hazard risk is tested using a cox proportional hazards regression analysis.

Immunohistochemistry of TMA

Immunohistochemistry was performed on TMA slides according to our previous publication [39]. Anti-Musashi-1 rabbit monoclonal antibody was used at a dilution of 1:50 (EMD Millipore, Billerica, MA, United States). A negative control experiment was performed using tissue samples from primary rectal cancer, incubated with PBS rather than anti-Musashi-1 primary antibody and probed with secondary anti-rabbit antibody to detect background signal produced by the secondary antibody (data not shown). The specificity of the anti-MSI1 antibody (Millipore, 04-1041) was tested in HEK-293FT cells treated with a MSI1-siRNA and NC-siRNA using Western blotting (data not shown). Specific details of the reagents and material used for this study are outlined in Appendix Table 7.

Since MSI1 was homogeneously expressed in the epithelial cells among the tumor tissue sections, only MSI1 intensity was scored. Scoring was performed by two trained scientists independently without knowledge of clinicopathological information and reviewed by a pathologist. Each tissue sample had three replicate tissue cores included in the TMA. The highest TMA score for the three replicates was used as a representative score and the average intensity score between the two independent scorers was used as a final representative TMA score for each tissue sample. Scoring was based on MSI1 intensity: scores 0 displayed no visible MSI1 staining (negative), scores 1, 2 and 3 were weak, moderate and strong MSI1 staining.

Taqman microRNA Analysis in Patient Tissue Samples

MicroRNA was isolated from paraffin-embedded tissue sections from rectal cancer patients using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, Stockholm, Sweden). The microRNA quality was assessed by studying amplification efficiencies of

microRNAs after serial dilutions of cDNA and measuring CT values of microRNAs using real time PCR. miR-137 expression was determined using Taqman microRNA assay and normalized to control small RNA, RNU6b. The expression of miR-137 in the tumor samples was normalized to RNU6b and set relative to the expression of miR-137 in normal mucosal samples.

Cell Culture and Reagents

The following cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States); HCT-116, DLD-1, SW480, HT29, 293 FT, 293 WT, CCD-841 CoN and passaged according to ATCC protocol. HCT-116 β/W cell line was a generous gift from Bert Vogelstein (The Johns Hopkins University School of Medicine) [40]. Cells were cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, St. Louis, MO, United States), supplemented with 10% fetal bovine serum (GE Healthcare HyClone, Logan, Utah, United States), and 1% Penicillin-Streptomycin antibiotic (Life Technologies, Grand Island, NY, United States).

***In Vitro* Studies**

miRNA mimics, siRNAs, NC mimics, NC siRNA, miR-137 antagomiR and antagomiR-NC were purchased from Dharmacon (GE Dharmacon, Lafayette, CO, United States). Transfections were carried out as previously described [41]. Briefly, cells plated in a 6-well plate were transfected with 100 pmol (50nM) miRNA mimics, siRNAs or antagomiRs using Lipofectamine 2000 (Invitrogen, Life Technologies). For cell growth assays, HCT-116 cells were transfected with miR-137 mimic and negative control (NC) mimic and re-seeded 24 hours later in a 24-well plate. Cells were collected every day for 4 days and living cells were counted using trypan blue staining and a hemocytometer as previously described [42]. For the cell growth rescue

experiment, cells were co-transfected with miR-137 and NC mimics (50nM) and the MSI1-GFP/EV-GFP expressing constructs (1 µg) for 24 hours and re-seeded in a 24-well plate. Cells were collected and counted as described above every day, for 4 days. Cell viability of cells was determined using a MTT colorimetric assay. Briefly, cells were transfected with miR-137 and NC mimics, 24 hours later cells were re-seeded into a 96-well plate in triplicate. After 4-6 days (or until ~90% confluency of NC group), cell medium was replaced with WST-8 (Sigma) dye for 1-5 hours. The absorbance was quantified using a microplate reader (BioTek, Winooski, VT, United States) at 450 nm. The absorbance of miR-137-treated cells was set relative to cells treated with NC mimic.

Western Blot Analysis

After 48-72 hours of transfection with mimics, antagomiRs, or siRNAs, cells were collected for Western blotting as previously described [43]. Antibodies used for our studies are detailed in Appendix Table 7.

Luciferase Reporter Assays

The pSGG-MSI1 3′UTR luciferase construct was a gift from Luiz O.F. Penalva and previously described [44]. Cells were transfected with 200 ng of pSGG-MSI1 3′UTR luciferase construct and 20 nM miR-137 mimic or NC mimic using Lipofectamine 2000. Three nucleotides within the miR-137 seed sequence in the pSGG-MSI1-3′UTR construct were mutated using the QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, United States). After 48 hours, cells were harvested and assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States). Wnt signaling was measured using the TOP/FOP Flash reporter constructs (Millipore). Cells were transfected with 200 ng of TOP or FOP Flash

constructs and 20 nM miR-137 mimic or NC mimic using Lipofectamine 2000. Transfected cells were stimulated with 20 mM LiCl for 16 hours prior to harvest at 48 hours post-transfection. A renilla luciferase construct was used as a normalizing control for all luciferase assays. Experiments were conducted in triplicate.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using gene specific primers (Appendix Table 8), SYBR® Select master mix (Applied Biosystems, Life Technologies) and normalized to GAPDH. The expression of mature miR-137 was quantified using microRNA TaqMan® Assays (Life Technologies) and normalized to housekeeping small RNAs, RNU6b or RNU48 (Appendix Table 7). Expression of genes were normalized using the equation $2^{-\Delta Ct} = 2^{-(Ct\ (housekeeping\ gene) - Ct\ (gene\ of\ interest))}$ [45].

Colony Formation and Tumorsphere Assay

Colony formation assays were performed using HCT-116 cells transfected with 100 pmol mimics (miR-137 and NC) in a 6-well plate for 24 hours and then re-seeded (300 cells/well) in a 6-well plate in triplicate according to our previous publication [41]. For the tumorsphere assay, HCT-116 cells were transfected as described above and re-seeded into 24-well ultra-low attachment plates in triplicate at 2000 cells per well according to our previous publication [46]. The number of colonies and tumorspheres grown in miR-137 treated cells was set relative to the number of tumorspheres grown in NC treated cells. A rescue experiment was performed using a colony formation assay and a pCMV6-MSI1-GFP construct purchased from Origene (Rockville, MD, United States). A pCMV6-EV-GFP construct was produced by removing the MSI1 coding

sequence from Sgf1/Xho1 sites and replacing it with a 24 base pair linker; 5′-TCACAACCTCCTAGAAAGAGTAGA-3′.

Production of Tet-on HCT-116 Stable cell lines

The mature miR-137 or NC mimic sequence was cloned into the Tet-inducible pTRIPZ expression vector (Dharmacon) according to manufacturer's instructions. Stable cell lines were generated by lentiviral transduction of HCT-116 cells and selected with 1.2 μg/ml puromycin for 1 week. Cells were treated with 0.25, 0.5 or 1 μg/ml of doxycycline (DOX) to induce the transcription of miR-137 in HCT-116.

Animal Studies

Animal experiments were done according to the Institutional Animal Care and Use Committee (IACUC) protocol approved by the University of Kansas Guidelines for Use and Care of Animals. HCT-116 stable clones with inducible miR-137 and NC miRNA were injected (0.5 x 10^6) into sub-cutaneous regions 5-6 week old, female athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu}, Harlan). Mice were separated into control and experimental groups randomly (N=5/group) for each animal study. In the tumor progression experiment, after the tumors reached approximately 50 mm^3 in size, mice were fed 1 mg/ml Doxycycline Hyclate (Sigma) in the drinking water to induce the expression of miR-137 or NC miRNA. In the tumor initiation study, DOX (1 mg/ml) was administered immediately after cell injections. Tumor growth was measured using calipers, 2-3 times per week. To avoid biased interpretations, scientists measuring tumor volume were unaware of treatment groups. Tumor volume was calculated using the following equation: (length x width^2)/2.

Statistical Analysis

Chi-square test was used to examine the significance of the differences in MSI1 expression in distant or adjacent normal mucosa, primary cancer and lymph node metastasis, as well as the correlation of MSI1 expression and miR-137 expression with clinicopathological variables. miR-137 expression with overall survival was tested using Log-Rank and Cox proportional hazards regression analysis. Survival curves were computed according to Kaplan-Meier estimates. Two-way ANOVA test was utilized to study the significance of the cell growth curve and xenograft tumor growth studies. All tests were two sided and a P-value (P) of $< .05$ was considered statistically significant. Data represent average results from at least three independent experiments and shown as the mean ± SE. $*P < .05$; $**P < .01$; and $***P < .001$.

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Chapter 4:
miR-137 Negatively Regulates Musashi-2

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Introduction

The RNA binding protein, Musashi-2 has been identified as a critical regulator for hematopoietic stem cell (HSC) maintenance, specifically by negatively regulating NUMB [1, 2]. When overexpressed, MSI2 impairs the ability of HSC to differentiate into mature blood cells [1]. The over expression of MSI2 has been identified in multiple cancer types and is most commonly studied in cancers of the blood [1, 3, 4]. However, the function and expression of MSI2 in solid tumors has not been extensively studied. Recently, MSI2 was shown to be overexpressed in colorectal cancer and identified as a driver of transformation in the intestinal epithelium [5]. Although the expression of MSI2 and its oncogenic nature has been studied in multiple cancer types, the regulation of MSI2 remains largely under studied.

We recently identified miR-137 as a tumor suppressor microRNA, in part by negatively regulating Musashi-1 [6]. Additionally, we discovered that miR-137 was significantly decreased in rectal tumor samples as compared to adjacent normal mucosa tissues. Musashi proteins share similar RNA binding domains and are thought to be functionally redundant [7]. Therefore, we speculated that miR-137 negatively regulates both Musashi RNA-binding proteins.

In this study, we identified a miR-137 seed sequence in exon 12 of *MSI2* coding sequence. Exogenous miR-137 negatively regulates MSI2 in colon and breast cancer cell lines. Inhibiting endogenous miR-137 in normal colon cells, kidney fibroblast, and rat intestinal epithelial cells increases the expression of MSI2 mRNA and protein. We are the first to report the overexpression of MSI2 in breast cancer cell lines and the miR-137-mediated regulation of MSI2.

In conclusion, our study provides preliminary evidence that MSI2 is a novel target of miR-137 mediated by a non-canonical mechanism. Our study suggests that miR-137 may play an important role in the differentiation of breast and colon stem cells by negatively regulating the stem cell regulators and RNA-binding proteins, MSI1 and MSI2.

Results

Musashi-2 is overexpressed in breast and colon cancer cell lines

We first set out to characterize the expression of MSI2 in breast and colon cancer cell lines. In a recent study, MSI2 was found to be highly expressed in colorectal cancer, however that study did not measure the expression of MSI2 in cell lines [5]. We recently published a paper in *Oncotarget* showing that miR-137 was significantly downregulated in colorectal cancer and colon cancer cell lines [6]. To our knowledge, the expression of MSI2 in breast cancer has not been reported. A previous study reported a significant decrease in miR-137 expression in cell lines; MCF-7, T47D SKBR-3, BT-474 and MDA-MB-231 as compared to breast epithelial cell line MCF-10a [8].

We characterized the expression of MSI2 and miR-137 in a panel of breast and colon cancer cell lines. In breast cancer cell lines, the expression level of *MSI2* mRNA was set relative to expression of *MSI2* in HMEC, a primary mammary epithelial cell line. Multiple non-tumorigenic breast cell lines were included in the study to serve as additional controls; MCF-10a, MCF-12a and MCF-15a. Out of the 13 breast cancer cell lines, 9 (69%) had overexpressed *MSI2* mRNA and 8 (62%) had overexpressed MSI2 protein (Figure 1A and 1C). To our knowledge, this is the first study to show that MSI2 is overexpressed in breast cancer. Our results are consistent with a previous study that showed that miR-137 is decreased in multiple breast cancer cell lines (Figure

1B) [8]. Our study expands upon the current knowledge by including expression data for miR-137 in a larger panel of breast cancer cell lines. Interestingly, not every cell line had low miR-137, suggesting that the mechanism for controlling miR-137 expression is heterogeneous in breast cancer, such as DNA methylation [9, 10].

In the panel of colon cancer cell lines, *MSI2* mRNA was slightly overexpressed in multiple cell lines as compared to the expression of *MSI2* in the normal colon cell line, CCD-841. Inversely, miR-137 was negatively regulated in all colon cancer cell lines tested (Figure 1D). We measured the expression of the precursor and mature form of miR-137 to assess the processing efficiency of miR-137. With the exception of 293FT, the transcription of miR-137 appears to be inhibited. This observation is consistent with previous observations that mR-137 in silenced in colon cancer due to hypermethylation of its promoter [9]. The expression of MSI2 protein in the panel of colon cancer lines needs to be analyzed in future studies.

Exon 12 of Musashi-2 contains a single miR-137 seed sequence

In previous studies, we found that Musashi-1, the sister protein for Musashi-2, is negatively regulated by miR-137 [6]. Since the RNA binding proteins have been shown to be functionally redundant, we wondered if miR-137 negatively regulates both Musashi proteins. A seed sequence for miR-137 was not found in the 3′UTR of *MSI2* using a variety of miRNA prediction programs such as TargetScan, miRanda and PicTar [11-13]. However, using DIANA microT-CDS miRNA prediction program [14], a miRNA prediction program that will identify predicted miRNA seed sequences in the 3′UTR and coding sequence (CDS) of target mRNA, a single miR-137 putative binding site was found within exon 12 of *MSI2* coding region (Figure 2A).

miR-137 is predicted to bind between nucleotides 358-386 of the *MSI2*-CDS-3′UTR sequence; corresponding to nucleotides 531-559 of the full length sequence of *MSI2* (5′UTR-CDS-3′UTR).

Additionally, we used the MIRZA-G program to predict the interaction between miR-137 and *MSI2* based upon the predicted secondary structure of target mRNA, the accessibility of the miRNA seed sequence and the biophysical properties [15]. This program predicted that a binding event between *MSI2* and miR-137 was most likely located between nucleotides 531-559 of *MSI2*-CDS (Figure 2B). This binding site corresponds to the predicted miR-137 seed sequence predicted by DIANA microT-CDS program, thus providing additional evidence that miR-137 may negatively regulate *MSI2* by binding to exon 12 of the CDS.

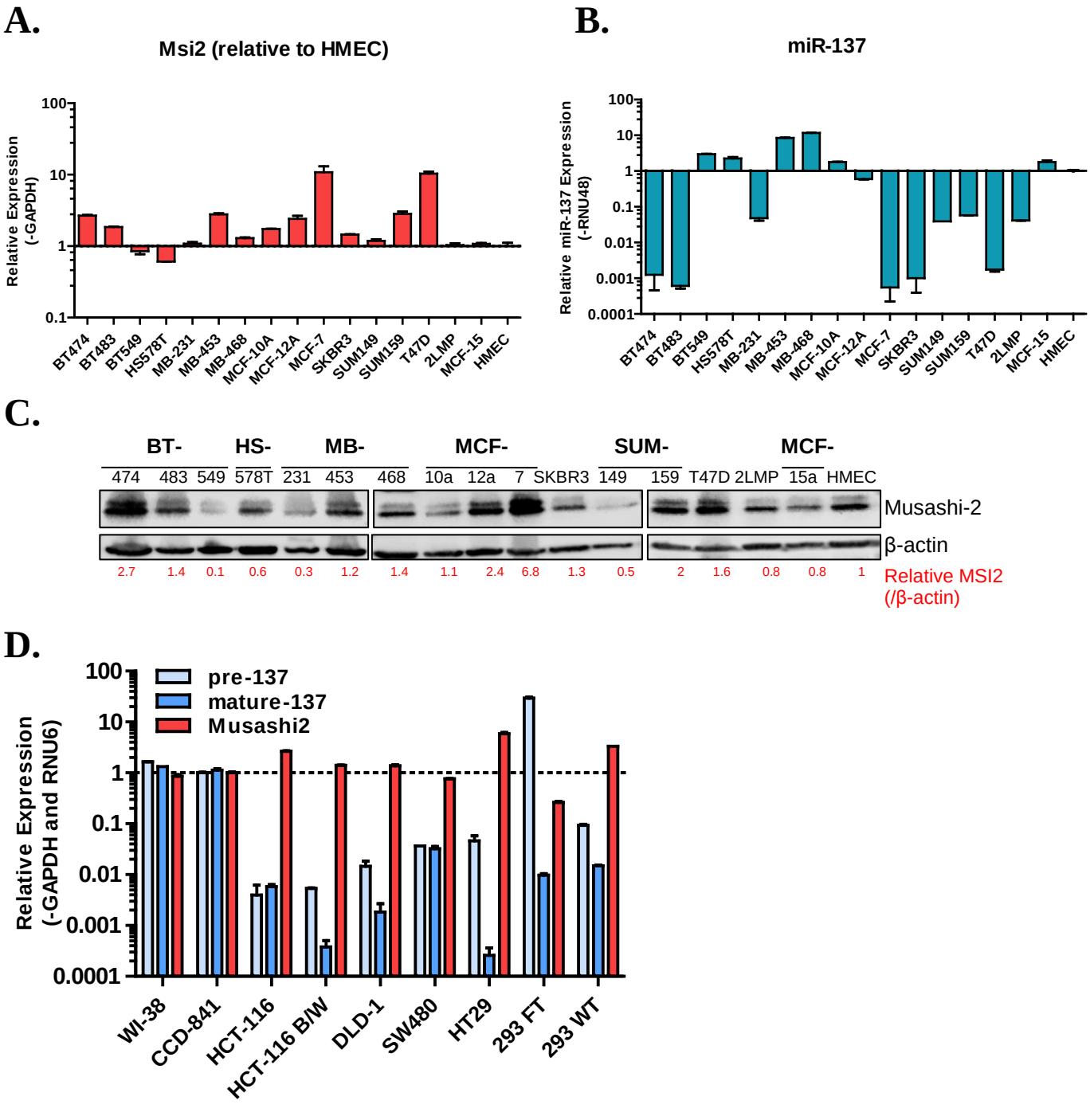

Figure 1. **MSI2 is overexpressed in breast and colon cancer cell lines**.

(A) *MSI2* mRNA was measured in a panel of breast cancer cell lines, normalized to GAPDH and set relative to the primary human mammary epithelial cell line (HMEC). **(B)** The expression of mature miR-137 was measured using Taqman qRT-PCR, normalized using RNU48 and set relative to HMEC. **(C)** MSI2 protein expression was measured using Western blotting. The expression of MSI2 was normalized to β-actin and set relative to HMEC (values displayed in red). **(D)** The expression of precursor/mature miR-137 and *MSI2* mRNA was measured in a panel of colon cancer cell lines using qRT-PCR and Taqman qRT-PCR, normalized to RNU6b and GAPDH and set relative to normal colon epithelial cell line CCD-841.

A.

CDS	8mer	358-386	0.032701050547477

Position on chromosome: 17:55478786-55478814
Conserved species:

```
(Transcript) 5'ACACAGUAGUGGAAGA                U3'
                          UGUA     AAGCAAUA
Binding area:             .|||     ||||||||
                          GCAU     UUCGUUAU
(miRNA)      3'           C    AAGAA       U5'
```

B.

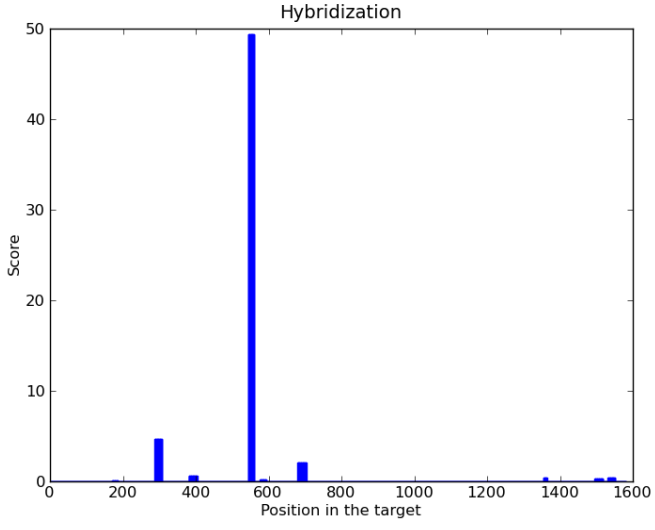

Figure 2. miR-137 contains a seed sequence within exon 12 of Musashi-2 **(A)** A seed sequence for miR-137 was identified in exon 12 of *MSI2* using DIANA microT-CDS program. **(B)** The location of miR-137 binding sequence is predicted based on biophysical parameters using MIRZA-G.

Exogenous miR-137 negatively regulates Musashi-2 in multiple breast and colon cancer cell lines

To determine if miR-137 negatively regulates MSI2 expression, miR-137 mimic (137-m), negative control (NC-m), negative control siRNA (NC-si) and positive control MSI2-siRNA (MSI2-si) were transfected into high MSI2 expressing colon and breast cancer cell lines; HT29, DLD-1, MCF-7 and SUM-159. Exogenous expression of miR-137 negatively regulated MSI2 protein in all four cell lines **(Figure 3)**. Interestingly, in colon cancer cell lines HT29 and DLD-1, miR-137 knock-in reduced the expression of *MSI2* mRNA **(Figure 3A and 3B)**, but not in breast cancer cell lines MCF-7 and SUM-159 **(Figure 3C)**. Our data suggests that in colon cancer cell lines, miR-137 promotes the degradation/destabilization of *MSI2* mRNA, whereas in breast cancer cell lines, miR-137 only prevents the protein translation of *MSI2*. The difference between these two mechanisms of miR-137 mediated regulation of MSI2 needs to be studied in future studies.

Knockdown of miR-137 increases Musashi-2 in multiple cell lines

Colon cell line, CCD-841, was transfected with a miR-137 antagomiR, an inhibitor of mature miR-137 (137-I). The expression of *MSI2* was measured using qRT-PCR and Western blotting. Knocking down miR-137 increased the expression of *MSI1* and *MSI2* mRNA as compared to a negative control antagomiR (NC-I) **(Figure 4A)**, however, only MSI2 protein expression was significantly increased **(Figure 4B and 4C)**. In this cell line, MSI2 is more highly expressed than MSI1, suggesting that MSI2 is more transcriptionally activated. This may explain why the change in protein level is more easily observed for MSI2 rather than MSI1. This experiment was repeated in HEK-293WT and RIE cells. In both cell lines, knocking down miR-137 **(Figure 4D)** increased MSI2 protein **(Figure 4D)**. Inversely, knocking in miR-137 decreased MSI2 in both HEK-293 WT and RIE cells **(Figure 4E)**.

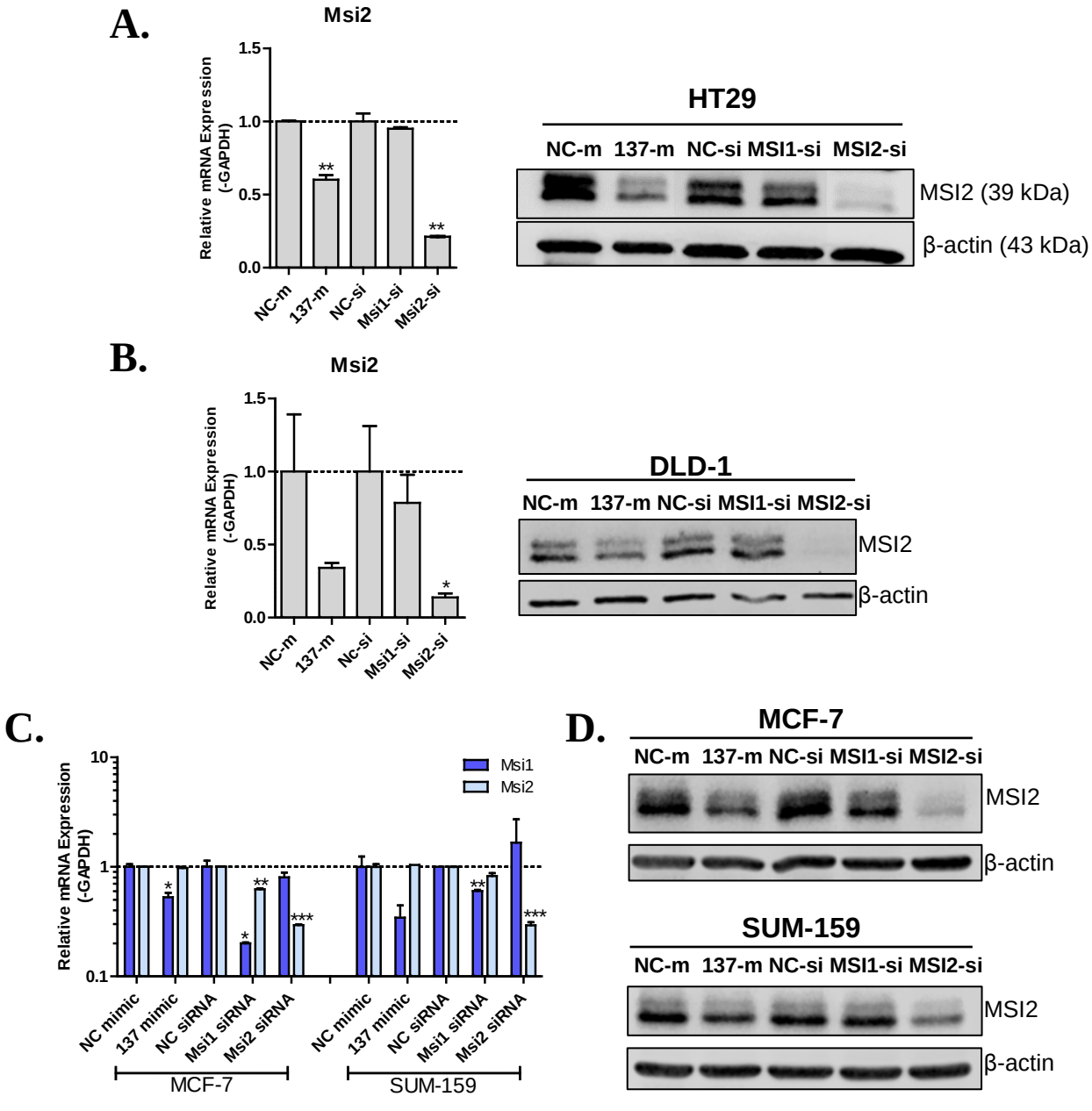

Figure 3. Exogenous miR-137 negatively regulates MSI2 in colon and breast cancer cell lines.

MSI2 mRNA and protein expression was measured in colon cancer cell lines HT29 **(A)** and DLD-1 **(B)** hours after transfection with microRNA mimics and siRNAs. **(C)** *MSI1* and *MSI2* mRNA was measured in transfected MCF-7 and SUM-159 breast cancer cell lines. **(D)** MSI2 protein expression in MCF-7 and SUM-159 after transfection with mimics and siRNAs. β-actin was used as loading control for Western blots. The expression of MSI1 and MSI2 mRNA was normalized to GAPDH and set relative to appropriate control.

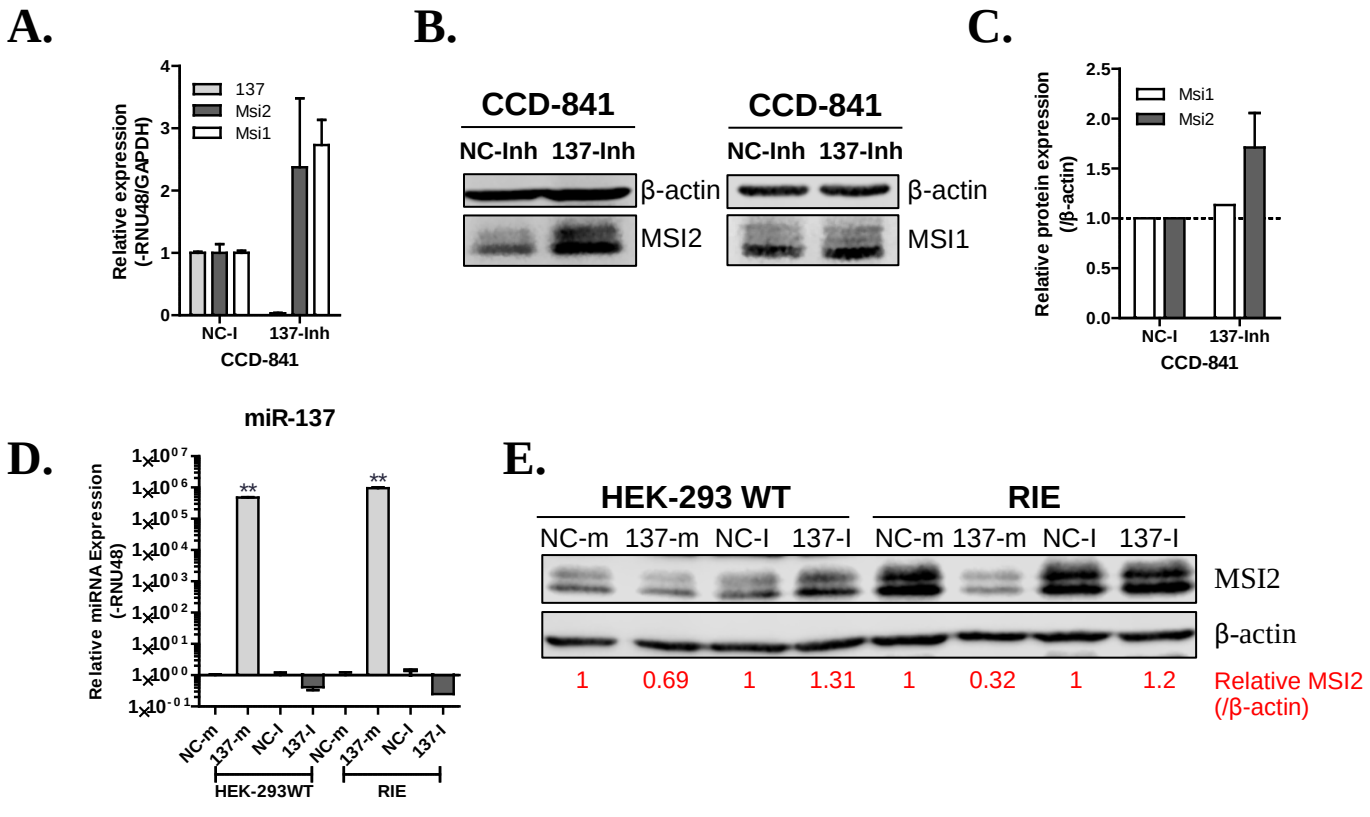

Figure 4. Knock-down of endogenous miR-137 increases MSI2 in multiple cell lines

(A) Expression of mature miR-137, *MSI2* and *MSI1* mRNA in CCD-841 cells transfected with a negative control inhibitor (NC-I) and a miR-137-inhibitor (137-I). miR-137 was normalized to RNU48 and *MSI1/MSI2* mRNA was normalized to GAPDH and set relative to NC-I. **(B)** Expression of MSI1 and MSI2 protein in transfected CCD-841. **(C)** The relative expression of MSI1 and MSI2 in transfected CCD-841 from multiple experiments. Data is normalized to β-actin and set relative to NC-I control. **(D)** Expression of mature miR-137 in transfected HEK29WT and RIE cells. Data is normalized to RNU6b and set relative to appropriate control. **(E)** Expression of MSI2 protein in transfected HEK293WT and RIE cells. B-actin is used as loading control. Relative MSI2 protein shown in red.

miR-137 directly regulates Musashi-2

To understand if miR-137 directly regulates MSI2 by binding to the coding sequence of MSI2, we utilized a cmv-MSI2 cDNA expression vector (does not contain a 3′UTR) for transfections. Kidney fibroblast cells (HEK293WT) and rat intestinal epithelial cells (RIE) were co-transfected with a cmv-empty vector/cmv-MSI2 vector with NC-mimic and 137-mimic. *MSI2* mRNA was measured 72 hours after transfections using qRT-PCR. Consistently, exogenous miR-137 negatively inhibits endogenous *MSI2* mRNA (Figure 5a). Additionally, we saw a significant decrease in the expression of cmv-MSI2 mRNA in HEK293WT cells co-transfected with miR-137-mimic as compared to cells co-transfected with cmv-MSI2 and NC-mimic (Figure 5a). This data suggests that miR-137 inhibits *MSI2* by directly binding to MSI2-CDS.

Interestingly, miR-137 knock-in does not inhibit the expression of endogenous *MSI2* mRNA in RIE cells (Figure 5b), although we previously showed that miR-137 negatively regulates MSI2 protein in RIE (Figure 4E). The miR-137 seed sequence is nearly perfect conserved between rats and humans, with only 1 nucleotide difference, and is predicted to be involved in the complementary base pairing between miR-137 and *MSI2* mRNA. This suggests that this 1 nucleotide directly influences the miR-137 mediated regulation on MSI2. When co-transfected with cmv-MSI2 and miR-137 mimic in RIE cells, *MSI2* mRNA was significantly decreased as compared to cells co-transfected with cmv-MSI2 and NC-mimic (Figure 5b). Overall, our data suggests that miR-137 directly inhibits MSI2 expression; however, the mechanism of the miRNA-mediated repression varies in cell lines and needs to be dissected further.

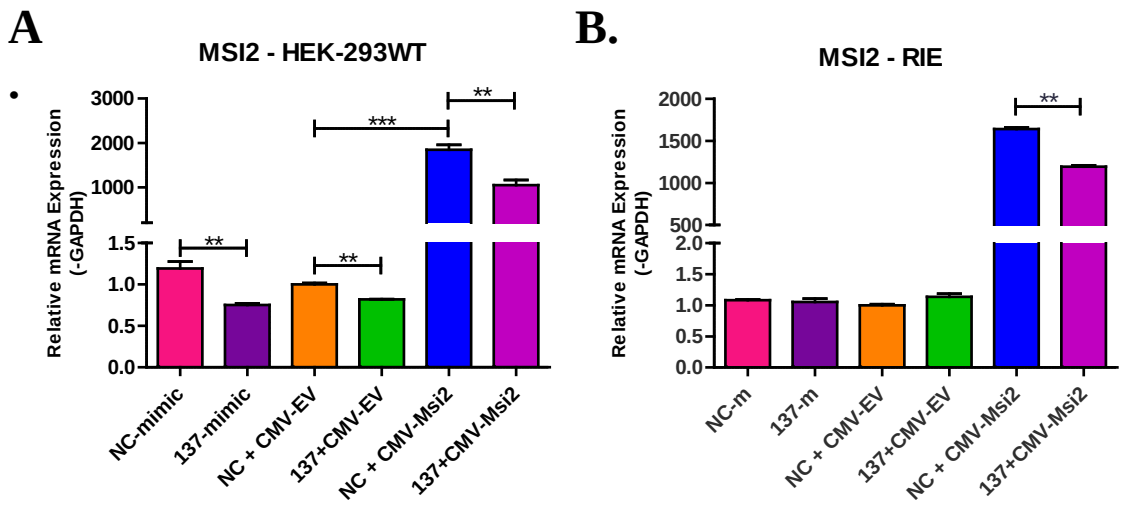

Figure 5. Exogenous miR-137 inhibits exogenous MSI2 in HEK-293WT and RIE cells.

Expression of *MSI2* mRNA in HEK-293WT cells **(A)** and RIE cells **(B)**. Data was normalized to GAPDH and set relative to appropriate control.

Discussion

RNA binding protein MSI2 has been well studied in hematopoietic cancers and is well known for its oncogenic function and maintenance of leukemia stem cells [1, 16]. The function of MSI2 in solid tumors is vastly understudied. One recent study found that MSI2 was overexpressed in colorectal tumor samples as compared to adjacent tissues [5]. Furthermore, they found that doxycycline-inducible MSI2 knock-in lead to an expansion of the $LGR5^+$ stem cells, proliferation, crypt fission and growth, and overall encouraged transformation of the intestinal epithelium. The mechanism associated with MSI2 overexpression in colorectal cancer has not been studied.

Our study is the first to report an overexpression of MSI2 in breast cancer cell lines. Interestingly, when comparing the expression of MSI2 with the molecular subtype of breast cancer, we discovered that all estrogen receptor (ER^+) and progesterone receptor (PR^+) breast cancer cell lines had high expression of MSI2 and low expression of miR-137. However, not all high-MSI2 expressing cells were ER^+ and PR^+. Out of the 7 luminal breast cancer cell types, 5 of the luminal cell lines had high expression of MSI2. Our data suggests that MSI2 expression may correlate with ER^+/PR^+ breast cancer from luminal origin, but needs to be studied further. Additionally, characterizing the expression of MSI2 and miR-137 in clinical breast cancer tumor samples would provide a great deal of value.

We recently discovered that miR-137 is a negative regulator of MSI1 and is lost in colon cancer cell lines and rectal tumor tissue samples as compared to adjacent normal mucosal tissue [6]. miR-137 restoration decreased the mRNA and protein expression of MSI1. Since MSI1 and

MSI2 have been shown to work in redundant pathways, we wondered if miR-137 was capable of negatively regulating both Musashi proteins.

In this study, we discovered that *MSI2* contains a single seed sequence for miR-137 in exon 12 of its coding sequence. Restoration of miR-137 in colon and breast cancer cell lines reduced the protein expression of MSI2. Additionally, exogenous miR-137 negatively regulates the expression of a cmv-MSI2 cDNA vector, suggesting that miR-137 directly inhibits MSI2. Future miR-137 seed sequence mutational studies would provide additional evidence of the direct binding event and the specific location of the miR-137 seed sequence within MSI2 CDS.

Although miR-137 appears to negatively regulate both Musashi proteins, the mechanism by which miR-137 regulates these sister proteins may be entirely different. In breast cancer cell lines MCF-7 and SUM-159, exogenous miR-137 reduces the amount of *MSI1* mRNA in cells, but not *MSI2* mRNA, even though MSI2 protein was knocked-down after 72 hours. This data suggests that miR-137 promotes the cleavage of *MSI1* mRNA, but not *MSI2* in breast cancer. In colon cancer cell lines HT29 and DLD-1, exogenous miR-137 reduces *MSI2* mRNA and protein, suggesting that the miR-137 mediated regulation of MSI2 is a very dynamic mechanism across cell types. More studies are needed to tease out the different miR-137-mediated regulation of MSI1/MSI2.

Altogether, our data provides evidence that miR-137 negatively regulates both RNA-binding proteins Musashi-1 and Musashi-2. Our findings may provide an explanation for the overexpression of MSI1 and MSI2 in colorectal and breast cancer [5, 6, 17, 18], although additional studies need to be carried out.

Materials and methods

Cell Lines and Reagents

Cell lines used for the studies were purchased from American Type Culture Collection (ATCC) and cultured according to their recommendations. Rat intestinal epithelial (RIE) cells were a kind gift from Dr. Kristi Neufeld (University of Kansas).

MicroRNA Target Prediction

DIANA-microT-CDS is a microRNA target prediction program that predicts miRNA seed sequences within the 5′UTR, coding sequence and 3′UTR [14]. MIRZA-G predicts canonical and non-canonical miRNA binding sites, along with siRNA off-target sites [15]. This program considers the biophysical interaction between miRNA and target mRNA, such as the nucleotide composition surrounding the miRNA seed sequence, the predicted secondary structure of the target RNA, the accessibility of the miRNA target site and the location within the 3′UTR.

In Vitro Studies

Reagents used for the *in vitro* studies are detailed in Appendix Table 7. Transfections were carried out in 6-well plates according to our previous studies [6]. For co-transfection studies, 50 pmol or [25 nM] of mimic and 0.5 µg DNA (CMV-EV and CMV-MSI2) were transfected into cells using 5 µl of Lipofectamine 2000. Control co-transfection experiments were first performed using a Cy3-labeled negative control mimic and CMV-EV DNA to track the transfection of efficiency and to optimize the transfection conditions.

Western Blot Analysis

Cell lysates were collected 72 hours after transfections using RIPA lysis buffer (50 mM Tris-HCl

pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 25x protease inhibitor cocktail (Sigma-Alrich) and 1mM PMSF). Protein concentration was determined using the Bradford protein assay (Bio-Rad). Proteins were prepared using 6x loading dye (0.375 M Tris-HCl, pH 6.8, 12% SDS, 30% β-mercaptoethanol, 60% glycerol, 0.012% bromophenol blue) at a concentration of 0.5 – 2 ug/ul, and degraded at 95°C for 10 minutes. Proteins were electrophoresed using 10-12% tris-glycine gels and transferred onto PVDF membranes. Membranes were blocked in Odyssey® blocking buffer (LI-COR) for 1 hour and then probed with primary antibodies overnight. The following primary antibodies were used for our studies; anti-Musashi-2, 1:1000 (AbCam #ab76148), anti-Musashi-1 1:1000 (Millipore, 04-1041), β-actin 1:5000 (Sigma #A5316). Membranes were washed using PBST and probed for 1 hour using anti-mouse, IRDye® 680RD and anti-rabbit, IRDye® 800CW secondary antibodies (LI-COR) protected from light. Membranes were imaged using an Odyssey® Fc dual-mode imaging system (LI-COR).

Quantitative Real-Time PCR

RNA from cells was collected using TRIzol® (Invitrogen) and isolated using Direct-zol™ RNA isolation kit (Zymo). Total cDNA was synthesized using reverse transcriptase polymerase and random hexamers (Applied Biosystems). Expression of mRNA was measured using quantitative real-time PCR using gene specific primers and SYBR® master mix (Applied Biosystems, Life Technologies). Expression of mRNA was normalized to control GAPDH and set relative to appropriate control. The primers used for this study are outlined in the appendix, Table 8. MicroRNA TaqMan® Assay (Life Technologies) was used to quantify the expression of mature miR-137. The expression of miR-137 was normalized to housekeeping small RNA, RNU48 and set relative to appropriate control. The TaqMan primers used for this study are listed in the

appendix, Table 7. The expression of genes were normalized using the equation $2^{-\Delta Ct} = 2^{-(Ct\ (housekeeping\ gene) - Ct\ (gene\ of\ interest))}$ [19].

Statistical Analysis

Unpaired, two-tailed, T-Test was used to test the significance of two unmatched groups. The data represented are the average results from at least three independent experiments and shown as the mean ± SE. **P* < .05; ***P* < .01; and ****P* < .001.

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Chapter 5:
NMR Characterization of Small Molecule Inhibitors Bound to Musashi-1

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(Some of the data described in this chapter were previously published in *Molecular Oncology* and reformatted for this dissertation)

Introduction

In 2012, over 8 million people worldwide died from cancer and nearly 14 million people were diagnosed [1]. The estimated direct cost for cancer in the United States in 2011 was nearly 90 billion dollars. It is imperative that new methods for combating this deadly and expensive disease are actively pursued. The RNA-binding protein Musashi-1 presents an emerging new target for cancer therapeutics.

Mouse Musashi-1 contains 362 residues and two evolutionarily conserved ribonucleoprotein (RNP)-like RNA-binding domains (RBD), RBD1 and RBD2 [2, 3]. Mouse Musashi-1 and Human Musashi-1 share 99% amino acid homology. The RNP-like RNA-binding domain is a common motif found in eukaryotes [4]. Both RNA binding domains share similar folds, containing 4 anti-parallel β-sheets packed against two α-helices [5].

Miyanori et al., determined that the first RNA binding domain (RBD1) has a higher affinity for RNA than RBD2. All of the residues located at the surface of the anti-parallel β-sheet of RBD1 are positively charged, providing the necessary electrostatic energy to bind to negatively charged RNA. The residues located at the surface of the anti-parallel β-sheet of RBD2 are largely neutral in charge. Additionally, RBD1 has a flexible backbone, allowing the necessary dynamics to bind to RNA, whereas, RBD2 backbone structure is rigid in nature. Using a variety of methods, including gel mobility assays, scientist concluded that RBD1 is the major RNA binding domain for MSI1, although, the presence of RBD2 increases the binding affinity to RNA [5, 6]. The binding affinity of an RNA binding domain to RNA is not governed by protein fold alone, rather a combination of electrostatic potential and backbone flexibility, with RBD1 being more fit to bind to RNA.

The three-dimensional solution structure of mouse Musashi1 (MSI1) RBD1 and RBD2 have been determined using nuclear magnetic resonance (NMR) [3, 5]. *In vitro* selection methods were used to determine the RNA binding sequence of mouse MSI1, identified as (G/A)U_nAGU (n = 1-3) [7]. Multiple studies have shown that phenylalanines are important for the direct interaction between the RNA binding domain and target RNA [3, 5]. Substitutions of these phenylalanine residues with alanine residues (F63L, F65L, F68L) inhibits the ability of MSI1 RBD1 to bind to *NUMB* RNA [7]. Five MSI1 target mRNAs contain this sequence in their 3′UTR: *NUMB* [7], $p21^{WAF-1}$ [8], *C-MOS* [9], *DCX* [10] and *APC* [11].

Ohyama et al., determined the minimal RNA binding sequences of mouse Musashi1 RBD1 and RBD2 using NMR-based binding and structural studies [12]. The consensus binding sequence for RBD1 is r(GUAG) and for RBD2 is r(UAG). Using NMR, the binding characteristics of RBD1:r(GUAG) was revealed. Specifically, the adenine of target RNA is sandwiched between conserved residue F23 and F96. The fourth guanine is stacked on the conserved F65. The first guanine is stacked on W29 located within the loop between α1 and β1 [12]. Additionally, NMR revealed that this architecture is highly conserved among MSI1 proteins.

A study done by Abreu et al., found that MSI1 target sequences are generally located in hairpin structures, suggesting that RNA secondary structure is an important consideration for MSI1 recognition [13]. Taken together, the ability of Musashi-1 to bind to target RNA is mediated by a variety of characteristics, including protein structure, dynamics, electrostatic potential and the secondary structure of target RNA. These characteristics provide an important foundation for the future design of small molecule inhibitors of MSI1.

MSI1 functions as a translational repressor by binding to the 3′UTR of target RNA and blocking the Poly(A)-binding protein (PABP) interaction with eIF4a, thus inhibiting the formation of the 80S ribosome complex [14]. Key targets of MSI1 translational inhibition include NUMB, an inhibitor of the Notch pathway [15], $p21^{WAF-1}$ a cyclin-dependent kinase inhibitor [8] and the tumor suppressor APC, an inhibitor of Wnt signaling [16]. Both Notch and Wnt signaling are crucial for normal and cancer stem cell homeostasis. Because MSI1 stimulates both Notch and Wnt signaling and is also over-expressed in a wide variety of cancers, MSI1 inhibition is an attractive target for novel cancer therapy. In a collaborative effort at the University of Kansas, our focus is on the development of small molecule inhibitors of MSI1 as a possible novel therapeutic approach.

Screening for MSI1-inhibitors

Our overall goal is to identify novel compounds which can specifically disrupt unique MSI1/RNA interactions, thus leading to MSI1 inhibition. Such compounds would be expected to compromise the viability of cancer cells that depend on MSI1 and Notch/Wnt signaling for their survival (Figure 1). RBPs such as MSI1 are considered "*undruggable*" due to the lack of a well-defined binding pocket for target RNA. In an effort to "drug the undruggable", a pilot project was funded from NIH COBRE Center for Cancer Experimental Therapeutics (CCET) at the University of Kansas to set up a fluorescence polarization (FP)-based binding assay for high-throughput screening (HTS) to find small molecule inhibitors of MSI1. Hit compounds were then validated using surface plasmon resonance (SPR). After validation, the specific binding interaction between hit compounds and MSI1-RBD1 was studied using nuclear magnetic resonance (NMR). The screening workflow is outlined in Figure 2.

From our in-house library of compounds, we identified 3 hit compounds; (–)-gossypol ((–)-G), gossypolone (Gn) and azaphilones-9 (AZA-9). To validate our results and to provide a better understanding of the binding event between our hit compounds and MSI1-RBD1, we used NMR to characterize the compounds binding activity with MSI1-RBD1. The NMR characterization of the binding interaction of MSI1-RBD1 with lead compounds (–)-G, Gn, and AZA-9 will be described in detail in this chapter.

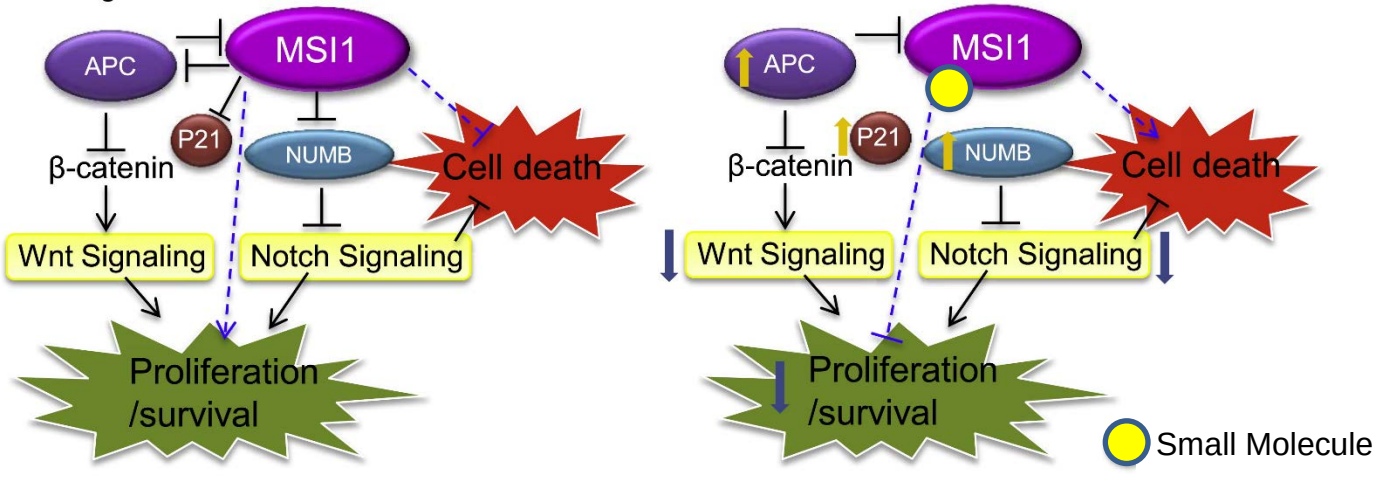

Figure 1: Project Working Model

Working model: (Left) In cells with MSI1 overexpression, MSI1 blocks the translation of *NUMB*, *p21* and *APC* mRNA, which leads to the up-regulation of both Notch /Wnt signaling pathway and cell cycle progression. Thus MSI1 indirectly promotes proliferation/survival of the cells and inhibits apoptosis; (Right) Designed small molecules (yellow circle) will bind to the RBD1 of MSI1, presumably releasing *NUMB*, *APC* and *P21* mRNA from their translational repression. Increased level of Numb and APC protein will block Notch and Wnt signaling respectively. Increased P21 will block cell cycle progression. This figure was adapted from our recent publication in *Molecular Oncology* [17].

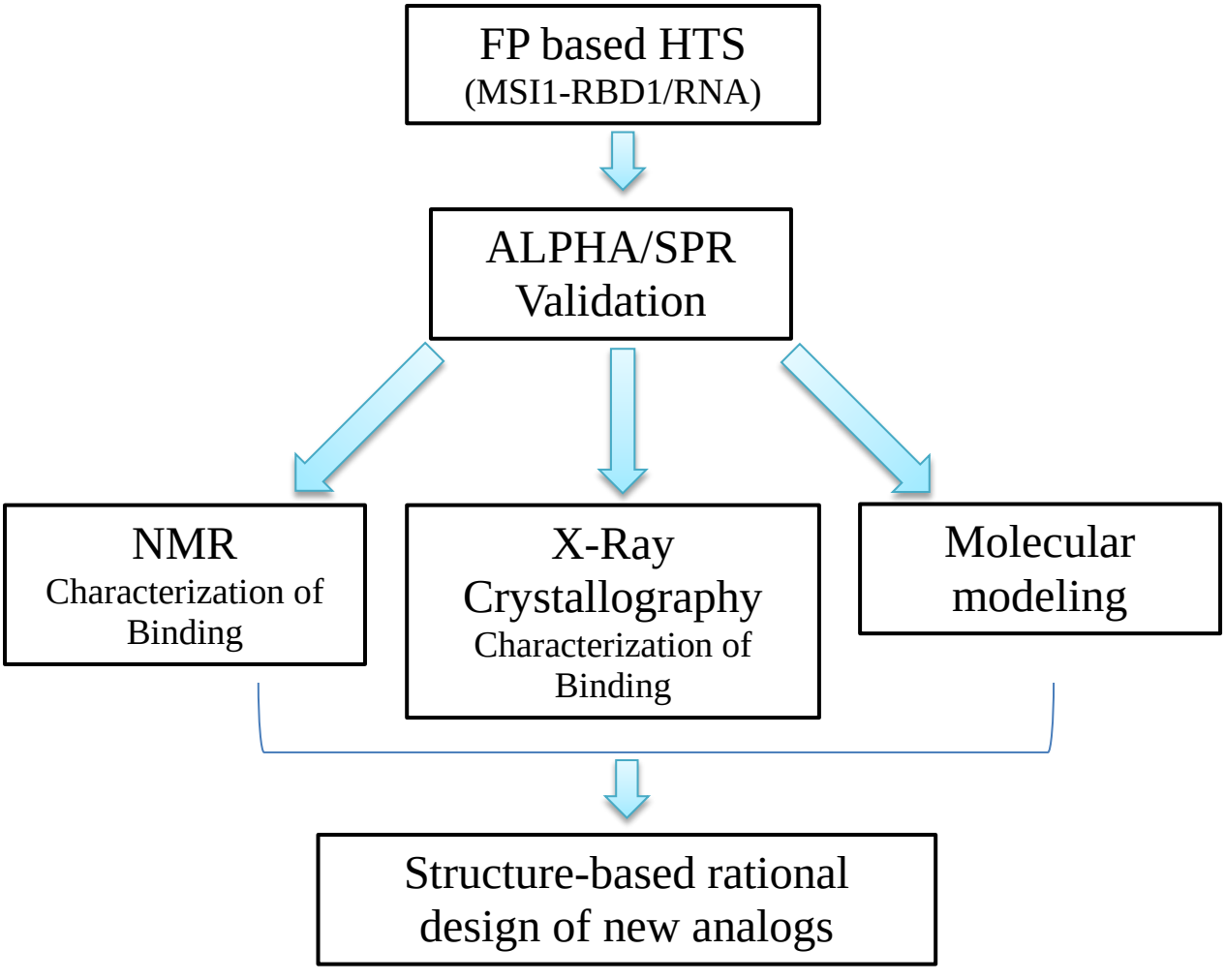

Figure 2. Screening Workflow.

Compounds that inhibit the RNA binding activity of MSI1 are identified using a FP-based competition assay. Hit compounds are then validated using ALPHA and SPR assays. The binding and structural characteristics of MSI1-RBD1 bound to lead compounds are then studied using a variety of techniques, including NMR, X-ray crystallography and molecular modeling. The information collected is used for structure-based rational design of new analogs to improve potency and target specificity.

Results:

NMR validation of (–)-gossypol binding to MSI1:

(The following results were recently published in *Molecular Oncology* and reformatted for this dissertation [17].)

To confirm the binding of (–)-gossypol to Msi1 and to determine if this binding event affects the RNA binding residues, we employed an NMR assay. First, we reproduced the previously published spectra data for ^{15}N-Msi1-RBD1 bound to a synthetic RNA corresponding to the MSI1 consensus target site (GUAGU) with consistent results, as shown in Figure 3A left panel [5]. We identified known RNA binding residues W29, K93, F23 and F65 Figure 3A right panel, consistent with published peak assignments of MSI1 [18]. Specifically, residue F65 is required for Msi1-RBD1 to bind to target RNA [7]. In response to increasing doses of (–)-gossypol, residues W29, K93, F23 and F65 peaks exhibited line-broadening, as shown in Figure 3B left panel, suggesting that the compound bound most closely to these residues. Thus (–)-gossypol interacts with the same binding pocket that Msi1 uses to bind target RNA. *In silico* docking analysis confirms a feasible binding mode for (–)-gossypol in the lower portion of the RBD1 binding pocket, with aromatic stacking interactions that mimic the cognate RNA and include three of the four shifted residues (F23, K93, F65; Figure 3B right panel). Based on the NMR line-broadening of the remote position W29, (–)-gossypol (Figure 3B) may also bind to more than one region of the protein or induce a conformational change upon binding.

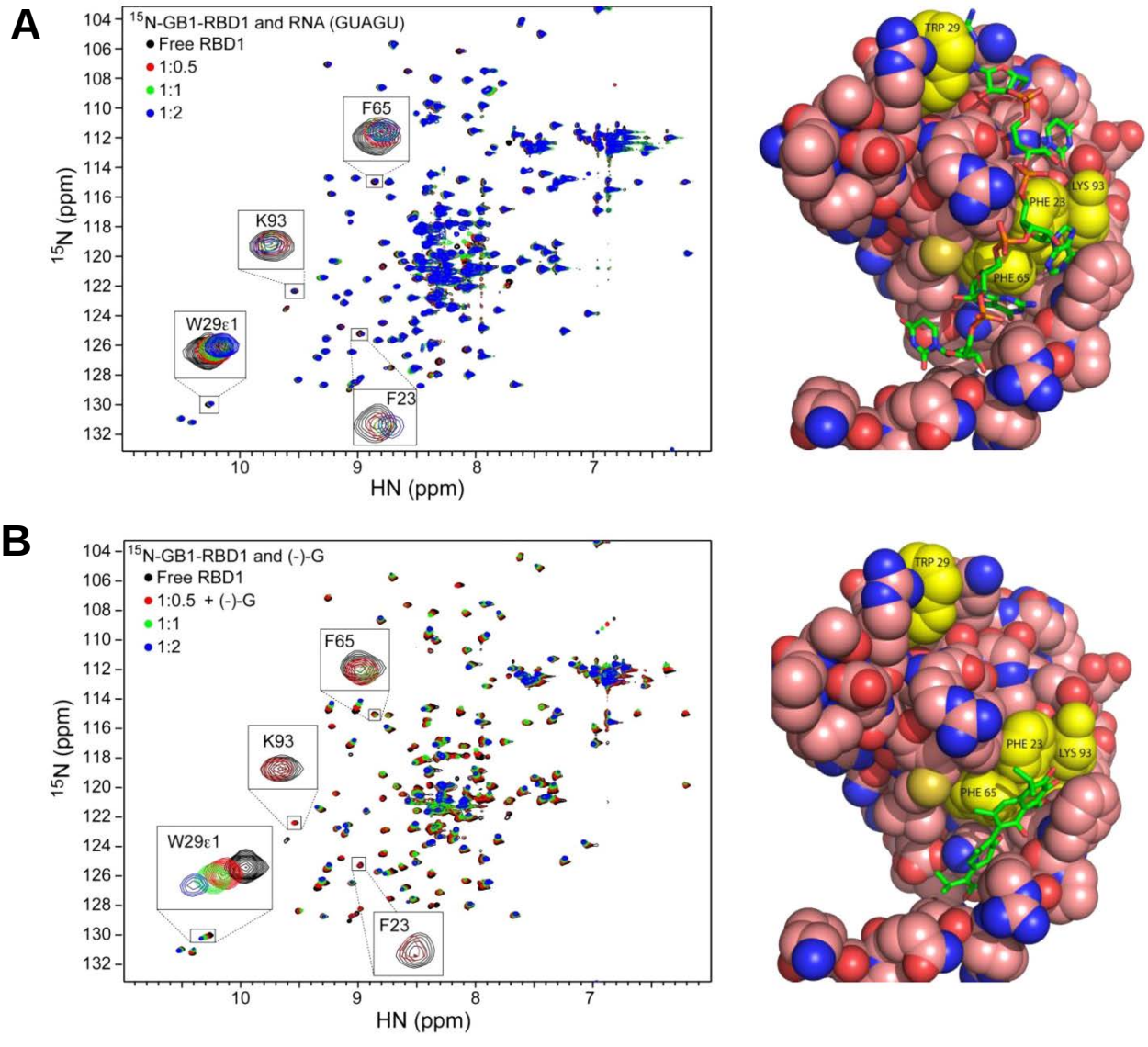

Figure 3. Validation of (–)-gossypol binding to RBD1 of MSI1 **A**. (left) Overlay of 2D-HSQC spectra sections of ^{15}N-MSI1-RBD1 (black) titrated with RNA oligo (GUAGU). Four of the MSI1-RBD1 RNA binding residues were identified (highlighted in box) and undergo significant line broadening and peak shifts upon addition of RNA. (right) The structure of RBD1 of MSI1 bound with RNA (PDB 2RS2). The protein atoms are show as spheres. The RNA is shown as sticks. Four of the MSI1-RBD1 RNA binding residues (F23, W29, F65, K93) that undergo significant shifts as shown in 2B were highlighted in yellow color. **B**. (left) Overlay of 2D-HSQC spectra sections of ^{15}N-MSI1-RBD1 (black) titrated with (–)-gossypol. RNA binding residues (highlighted in box) undergo significant line broadening upon addition of (–)-gossypol indicating micro molar affinity between (–)-gossypol and RBD1. The residues that undergo line broadening in RBD1 indicates either they are directly involved in binding or they are in close proximity of the binding site. (right) The model of RBD1 of MSI1 bound with (–)-gossypol. The protein atoms are colored as described in 2C and the (–)-gossypol structure was shown as sticks. Figure was prepared using PyMOL. This figure was recently published in *Molecular Oncology [17]*.

NMR validation of gossypolone binding to MSI1:

(Unpublished data)

Gossypolone (Gn) is a major metabolite of gossypol [19], and is oxidized in the liver by the P450 enzyme [20]. It has been shown to share similar biological activity of gossypol [21-27], including binding to and inhibiting BCL-2 family member BCL-xL protein, with a Ki of 0.28 μM [24]. Gn was identified as a potent inhibitor of MSI1s RNA binding activity with a Ki of 13.5 nM. The binding activity of Gn to MSI1-RBD1 was confirmed using NMR. A decrease in peak intestity of RNA binding residues K93, F23, W29 and W29ε1 (side chain) with increasing titration of Gn suggests that these residues are involved in the binding to Gn (Figure 4). The majority of non-RNA binding residues were unaffected when titrated with Gn, suggesting that the interaction between Gn and RBD1 tends to be more localized near the RNA binding pocket.

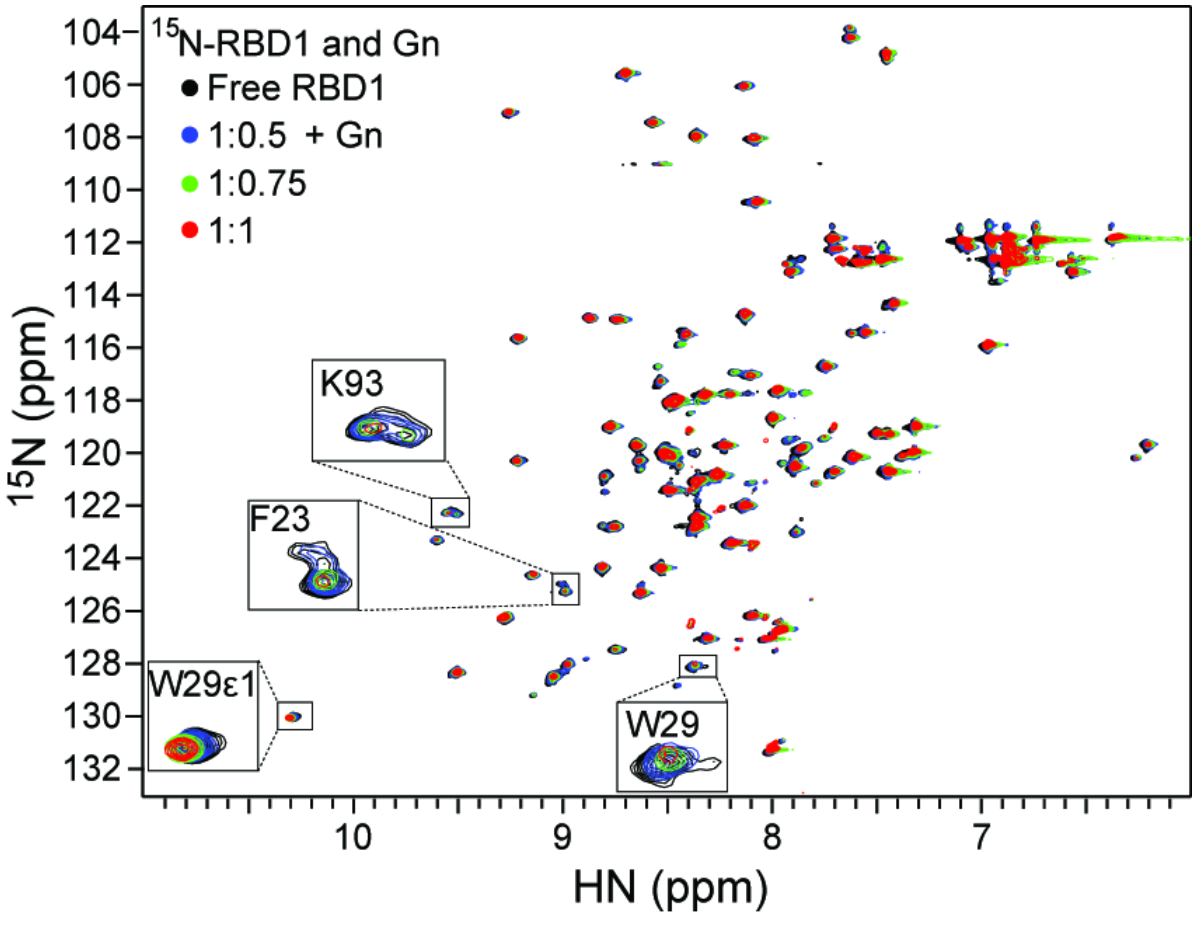

Figure 4. Validation of Gn binding to RBD1 of MSI1. Overlay of 2D-HSQC spectra sections of ^{15}N-MSI1-RBD1 (black) titrated with Gn. Three RNA binding residues (highlighted in box) undergo line broadening upon addition of Gn indicating that they are in close proximity of the binding site or are directly involved in binding to Gn.

NMR validation of AZA-9 binding to MSI1:

(Unpublished data)

Within our in-house library of compounds was a collection of secondary metabolite analogues produced by *Aspergillus nidulans* [28]. Surprisingly, we found that one particular metabolite, AZA-9 showed potent binding affinity to MSI1-RBD1 in FP binding assays with a Ki of 1.18 μM. To investigate the mechanism of Aza-9 binding to MSI1-RBD1, we carried out NMR studies. Upon addition of AZA-9 compound, MSI1-RBD1 residues W29, F23, R61, F65, K93 and M22 experienced significant line broadening at the 1:4 titration, indicating a binding event (Figure 5A). When mapped onto the structure of MSI1-RBD1, many of the residues known to bind to RNA (W29, F23, F65 and K93) are directly manipulated upon AZA-9 addition, along with RNA-binding neighboring residues (M22 and R61) (Figure 5B). The NMR studies confirm that AZA-9 directly binds to MSI1-RBD1 and that this binding event affects the RNA binding pocket.

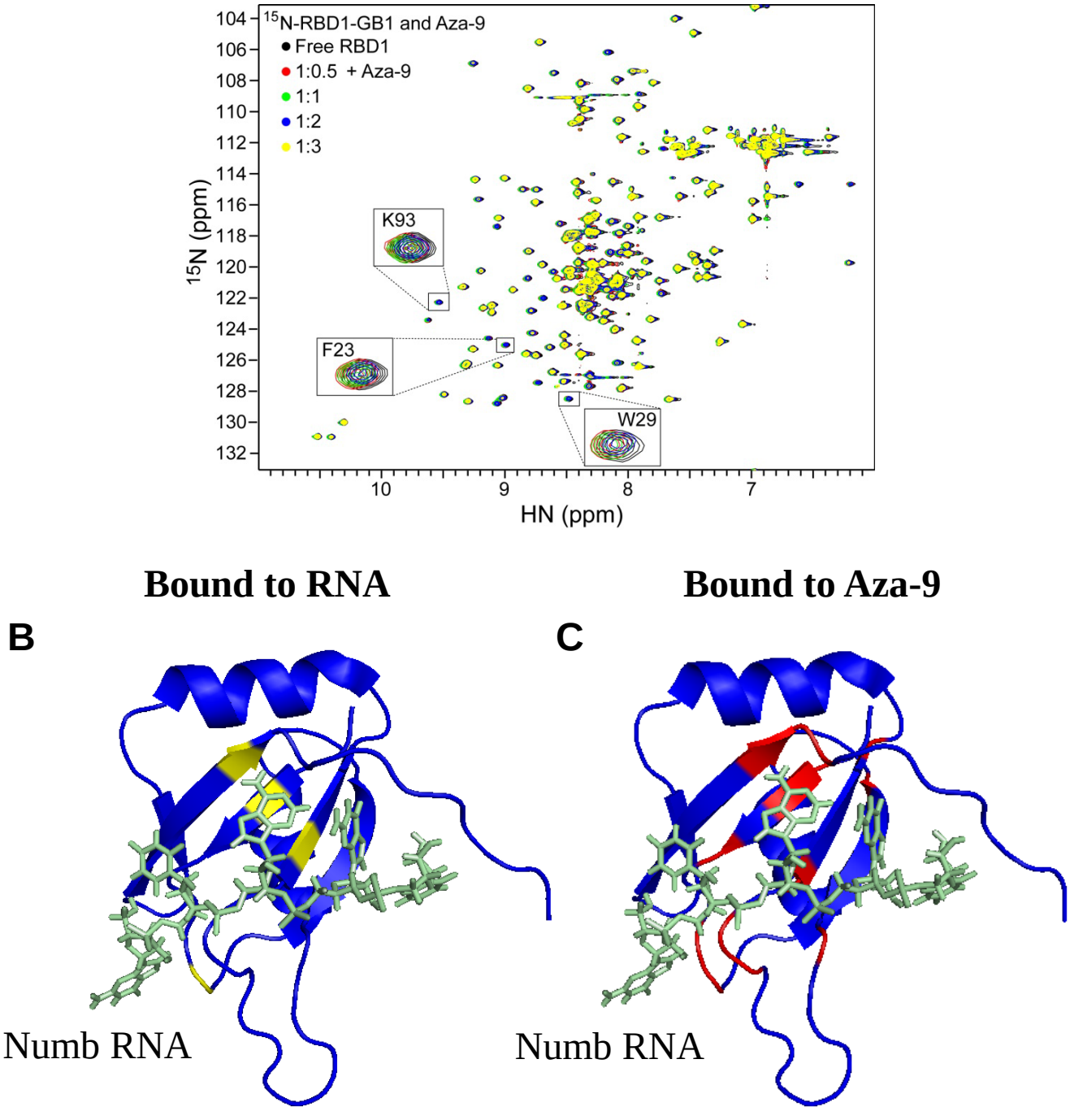

Figure 5. **(A)** Overlay of 2D-HSQC spectra of ^{15}N-MSI1-RBD1 titrated with increasing molar ratios of AZA-9. RNA binding residues W29, F23 and K93 undergo significant line broadening upon addition of AZA-9 indicating micro molar binding affinity. **(B)** Structure of RBD1 bound to NUMB RNA with RNA binding residues highlighted in yellow. **(C)** Structure of RBD1 bound to NUMB RNA with RNA binding residues affected by AZA-9 titration highlighted in red.

Discussion

RNA binding proteins are considered “undruggable”, due to lack of a well-defined binding pocket for target RNA. There are a couple of reasons that RNA binding proteins are difficult to drug. Unlike many enzymes that have a defined pocket employing the “lock and key” scheme, regulatory RNA/DNA binding proteins have shallow binding pockets, and the binding events are not tight and less specific. Targeting protein-protein interaction has yielded a list of small molecule compounds that are in clinical trials or in the clinic [29], while inhibitors of protein-RNA/DNA interaction are fairly new to the arena.

Multiple studies have characterized the binding event between MSI1-RBD1 and target RNA. Using site-directed mutagenesis, three phenylalanine residues were substituted for alanine residues within MSI1 RBD1 (F63L, F65L, F68L) and shown to inhibit the ability to bind to *NUMB* RNA [7]. In a separate study, researchers used NMR to identify the residues responsible for binding to RNA as F23, W29, F65, K93 and F96 [12]. Collectively, these data suggest that specifically F65 is required for RNA binding. In order to inhibit the function of MSI1 RNA binding activity, we aimed to identify small molecules that bind to MSI1 and interfere with these essential RNA binding residues.

In previous studies, our lab identified three hit compounds that potently inhibit the RNA binding activity of MSI1 using FP-based HTS, (–)-gossypol, Gn, and AZA-9. We used NMR assays to study the specific interaction between hit compounds and MSI1-RBD1. Our study is the first to identify small molecule inhibitors of the RNA binding protein Musashi-1 and provide structural evidence of binding.

(–)-Gossypol has completed Phase a IIb multicenter clinical trial for treating prostate cancer (e.g., NCT00286806, NCT00286793, NCT00666666) and a variety of other cancers (e.g., CT00275431, NCT00397293). These clinical trials stemmed from previous work including studies from our lab, which showed that (–)-gossypol induces autophagy and apoptosis in prostate and other cancer cell lines [23, 25, 30]. (–)-Gossypol has been shown to bind to BCL-2 family proteins and inhibit their anti-apoptotic and/or anti-autophagic function in various cancer cells and to inhibit tumor growth *in vivo* [30-33]. (–)-Gossypol binds MSI1 with similar affinity as it binds BCL-2 family members, raising the possibility that the drug may also have utility in the treatment of cancers associated with high levels of MSI1 expression and Notch/Wnt signaling [17]. Repurposing of an existing promising compound such as (–)-gossypol will provide a novel mechanism of action for the compound, and it will also guide the design of new clinical trials with new criteria for selecting patient populations that will benefit the most from the drug.

Our NMR studies indicate that (–)-gossypol may compete with target RNAs for binding to MSI1 by directly interfering with the RNA binding pocket. The observed shift at W29 in the upper portion of the binding pocket could be due to a second, cryptic (–)-gossypol binding site or an allosteric effect of binding to the lower portion of the pocket. More biochemical studies and/or X-ray crystallography need to be carried out to distinguish between these possibilities.

In terms of target specificity, (–)-gossypol appears to be a promiscuous compound. Although the NMR results showed that (–)-gossypol affects the known RNA-binding residues; multiple residues were also affected outside of the binding pocket. Our results indicate that (–)-gossypol may bind to multiple regions of MSI1-RBD1 or through allosteric interactions affect the overall

protein confirmation. Additionally, (–)-Gossypol has already been shown to be a potent BCL-2 family protein inhibitor. In order to move this compound forward as a specific MSI1 inhibitor, more detailed structural characteristics using X-ray crystallography to determine the structure of (–)-G and MSI1-RBD1 is critical in order to design (–)-G derivatives with the hope of improving potency and target specificity.

One derivative of (–)-G that was identified as a hit from our screening pursuits was Gn. Gn had a higher binding affinity to MSI1-RBD1 than (–)-G with a Ki of 13 nM (unpublished data) [17]. After titration with Gn, a small collection of residues experienced shifts and/or loss of intensity, including RNA binding residues W29, F23 and K93, suggesting that the binding event between Gn and MSI1-RBD1 is more localized as compared to the binding event between (–)-Gossypol and MSI1-RBD1. Future crystallography studies would greatly enhance our knowledge of the specific binding event between RBD1 and hit compounds.

In colon cancer cells, the same concentration of Gn is less potent than that of (–)-gossypol in all of the assays tested [17]. Gn, a reduced and oxidized form of gossypol, has poor water-solubility that can result in compromised cell permeability. In future studies, the Gn will be delivered to cells using a formulated lipid-based nanocarrier. Liposomes have long been used as nanocarriers for targeted cancer therapy and have shown good biocompatibility and controlled drug release in some previous studies [34-37].

Surprisingly, we found that one particular secondary metabolite analogue (AZA-9) produced by the fungus, *Aspergillus nidulans*, potently inhibits the RNA binding function of MSI1 (unpublished data) [28]. We further validated the binding event between AZA-9 and RBD1

using NMR. Our results show that Aza-9 directly binds MSI1 and specifically interferes with RNA binding residues W29, F23, F65 and K93 and neighboring residues of the RNA binding pocket, M22 and R61. Altogether, it appears that the RNA binding β-sheet of RBD1 is greatly affected by AZA-9, indicating that this compound has a great deal of potential in the development of an inhibitor of MSI1.

In summary, we discovered three small molecules capable of binding to MSI1-RBD1 and manipulating the RNA binding residues. Our research suggests that RNA binding proteins are druggable even though they tend to lack a well-defined binding pocket. Our study also provides substantial evidence that fungus metabolites should be considered more for drug discovery studies. Future studies will focus on characterizing additional hit compounds along with designing AZA-9 analogs for increased binding efficiency and selectivity.

Materials and Methods

Protein expression and purification

Protein used for these studies were produced and purified by the University of Kansas Protein Production Group. pGEX4T-1-MSI1 and pET21a-GB1-RBD1 plasmids encoding full length MSI1 and the RNA binding domain 1 (RBD1) of MSI1, were constructed with *Mus musculus* cDNAs under Tac and T7 promoter, respectively. GB1-RBD1 proteins were expressed in *E. coli* and purified as previously described [38, 39]. Protein concentrations were determined using the Bradford assay (Bio-Rad).

Nuclear Magnetic Resonance

The oligonucleotide sequence for MSI1 RNA binding studies was 5'-GUAGU-3', corresponding to MSI1 minimal recognition and binding sequence as previously described [15]. For NMR analyses, ^{15}N-labeled GB1-RBD1 protein (residues 20-103) was produced as previously described [40]. The ^{15}N-labeled GB1-RBD1 was prepared at the concentration of 200 μM in NMR buffer (50mM sodium phosphate pH 7.0, 100 mM NaCl, 10mM β-mercaptoethanol and 10% D_2O). Labeled 200 μM GB1-RBD1 was titrated with increasing amounts of (–)-gossypol to obtain molar protein:drug ratios of 1:0, 1:0.5, 1:1, and 1:2. Labeled 200 μM GB1-RBD1 was titrated with increasing amounts of gossypolone to obtain molar protein:drug ratios of 1:0, 1:0.5, 1:0.75, and 1:1. Labeled 200 μM GB1-RBD1 was titrated with increasing amounts of AZA-9 to obtain molar protein:drug ratios of 1:0, 1:0.5, 1:1, 1:2, and 1:4.

2D-^{1}H-^{15}N HSQC spectras were recorded for each titration at 298K on a Bruker Avance 800 MHz NMR instrument, equipped with a triple resonance (^{1}H/^{13}C/^{15}N) cryoprobe. NMR

backbone assignments for MSI1-RBD1 were obtained from the BMRB (entry 11450). Data was processed using NMRPipe [41] and analyzed with NMRView [42].

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Chapter 6.
Concluding Remarks

The intricate and sophisticated dance between DNA, RNA and protein is regulated in a tightly choreographed fashion, dictating cellular function. Messenger RNAs provide the blueprint for protein synthesis and therefore require a great deal of regulation. Two major post-transcriptional regulators of the RNA message are RNA-binding proteins (RBPs) and microRNAs (miRNAs). The aberrant regulation of miRNAs and RBPs produces a variety of consequences, including the initiation and progression of cancer.

One particular miRNA frequently dysregulated in cancer is miR-137. Silencing of miR-137 can be caused by hypermethylation of its promoter region [1, 2]. Low miR-137 due to hypermethylation has been identified in colorectal [2], gastric [10], uveal melanoma [12], oral cancer [1], glioblastoma multiforme [15], squamous cell carcinoma of head & neck [16, 17], and breast cancer [6]. Furthermore, the methylation and silencing of miR-137 occurs early in the progression of colorectal cancer [2].

In colon cancer, miR-137 was found to be hypermethylated in 81% of primary tumors (n = 113), 82% of early adenoma (n = 68) and only 14% of adjacent normal colon mucosa tissue (n = 113). Interestingly, miR-137 was hyper-methylated in only 4% of normal colon mucosa tissue (n = 21) collected from patients without colorectal cancer. This data suggests that patients with normal methylation status and expression level of miR-137 are less susceptible to developing colorectal cancer, but needs to be studied further.

In colon cancer cell lines HCT-116, SW480, and HT29, silencing of miR-137 is due to hypermethylation [2]. In our study, we found that MSI1 and MSI2 are overexpressed in these cell lines [3]. Additionally, we show that knocking down endogenous miR-137 in multiple cell

types, including normal colon cells, increases the mRNA and protein expression of MSI1/MSI2. Collectively, the data suggests that hypermethylation and silencing of miR-137 produces an overexpression of MSI1/MSI2.

The mechanism responsible for hypermethylation and silencing of miR-137 is not well understood. There is compelling evidence that miR-137 becomes activated during differentiation of stem cells in multiple tissue types, but to date, the methylation status of miR-137 in stem cells specifically has not been studied. Since tumor initiation and progression has been shown to arise from stem-cell like cells, termed cancer stem cells or tumor initiating cells [18], it is possible that the hypermethylation status of miR-137 in tumors is due to the expansion of cancer stem/progenitor cells with silenced miR-137.

In this body of work, we expand upon the current knowledge of miR-137 by showing evidence that the tumor suppressive function of miR-137 in colon cancer is in part by negatively regulating the RNA-binding protein, Musashi-1 [3]. We also show preliminary evidence that miR-137 also negatively regulates Musashi-2. However future studies are needed to properly understand this mechanism.

In the context of the colon epithelium, MSI1 expression is typically restricted to the base of the crypt, marking the $LGR5^+$ and BMI^+ stem cell populations [19]. MSI2 is expressed in the base of the crypt and transit amplifying region, but not in differentiated intestinal cells [20]. The interaction and relevance of miR-137 mediated regulation of MSI1/MSI2 in normal intestinal physiology is not understood. One study found that miR-137 is located in the transit amplifying region and base of the crypts in normal colonic mucosa using *in situ* hybridization [2], but it is

not clear if miR-137 is expressed in the stem cell population of the crypts. One possibility is that miR-137 negatively regulates the expression of MSI1/MSI2 during differentiation of the intestinal stem/progenitor cells.

Multiple studies have found that miR-137 is expressed during differentiation of stem cells from a variety of tissue types [15]. In neural stem cells, miR-137 is upregulated during differentiation and negatively regulates LSD1 [14]. In mouse embryonic stem cells, miR-137, miR-100, and miR-34a are upregulated during differentiation [13]. Blocking all three miRNAs inhibits the ability of embryonic stem cells to differentiate. Collectively, the preliminary studies provide compelling evidence that miR-137 plays an important role in differentiation. The role of miR-137 in the differentiation of intestinal stem cells has not been studied, but would be worth investigation in the future.

The mRNA expression of MSI1 is also tightly regulated by the RNA binding protein HuR [21]. Our lab recently discovered that MSI2 is also regulated by HuR (unpublished data). HuR is a member of the Hu/ELAV embryonic lethal abnormal vision family of proteins [22]. Containing three RNA recognition motifs, HuR functions by binding to AU- or U-rich regions within the 3′UTR of target RNA [23]. MSI1's 3′UTR contains multiple AU- and U-rich sequences, therefore allowing HuR to bind to and stabilize MSI1 mRNA. HuR increases the rate of MSI1 turnover from mRNA to protein in glioblastoma [21].

The miR-137 seed sequence within MSI1's 3′UTR is located in close proximity to four putative HuR binding sites; the closest HuR location resides 76 nucleotides from the miR-137 seed sequence. We show that miR-137 negatively regulates MSI1 in multiple colon cancer cell lines;

HCT-116, DLD-1, and HT29. HuR protein expression is overexpressed in these same cell lines (unpublished data). Therefore, the miR-137-mediated repression of MSI1 seems to trump the HuR mediated stabilization of MSI1 mRNA. The interaction between these two post-transcriptional regulators of MSI1/MSI2 needs to be studied further. One possibility is that although HuR is overexpressed in HCT-116, DLD-1, and HT29, its expression tends to be largely localized to the nucleus. Therefore, suggesting that the miR-37 mediated regulation of MSI1 is possible because HuR does not have access to MSI1 mRNA. Returning to the context of the colon epithelium and normal physiological functions, the regulation of MSI1/MSI2 mRNA by HuR and miR-137 is largely understudied.

The signaling pathways mediating wound healing and tumorigenesis are extremely similar and thought to be interconnected [24, 25]. The expression of MSI1 seems to be connected with a wound healing response. During times of injury either by an infection, UV radiation or ethanol induced tissue damage, the expression of MSI1 increases [26-28]. MSI1 response to tissue damage is likely due to its important role in maintaining the stem cells and proliferation. During times of injury, stem cells are busy regenerating and repairing damaged tissue [25]. One possibility is that miR-137 negatively regulates the expression of MSI1 enough to encourage differentiation, but during times of stress, the miR-137 mediated regulation of MSI1 is aborted, allowing alternative mechanisms, such as HuR, to upregulate MSI1 to promote stem cell expansion and tissue regeneration. Supporting this hypothesis, HuR is enriched in the cytoplasm during times of stress [29]. Gaining a better understanding of the miR-137/HuR mediated regulation of MSI1/MSI2 in wound healing may help us understand the initiation of $MSI1^{+}/MSI2^{+}$ cancer.

Precision medicine

President Obama recently announced a bold Precision Medicine Initiative in an effort to revolutionize health care and biomedical research. With the support of this initiative set to be released in 2016, new treatments will be tailored to the specific genomic landscape of an individual's tumor. Using the advancement in our understanding of basic cancer biology coupled with specific therapeutic strategies, physicians can select the most appropriate treatment to improve the chances of survival while reducing harsh side effects.

One therapeutic target of interest in our lab is the RNA binding protein and stem cell regulator, MSI1. As previously discussed in detail in Chapter 2, MSI1 is overexpressed in a variety of cancers types and associated with poor clinical outcome [3, 30, 31]. Knockdown of MSI1 leads to reduced tumor progression in multiple cancer types [32, 33]. Preliminary data suggests that inhibition of MSI1 will sensitize cancer to chemo/radio therapy [34] [35]. As an oncogenic protein and regulator of stem cell self-renewal, MSI1 makes an attractive target for novel molecular therapy targeting cancer stem cells. We investigated two means of therapeutic intervention: microRNA-based molecular therapy and designing small molecule inhibitors.

miR-137 based molecular therapy

Since the tumor suppressor miR-137 has been shown to negatively regulate multiple cancer-promoting genes, signaling pathways and biological functions (Figure 1), we believe miR-137 is an attractive agent for microRNA-based molecular therapy. MicroRNA-therapeutics is an exciting new field within the cancer therapy arena. MicroRNAs make attractive therapeutic agents for multiple reasons. First, microRNA-based molecular therapy will utilize a multi-hit approach by targeting a subset of genes with oncogenic functions, therefore, enhancing the therapeutic effect and hopefully reducing the ability of cancer clones to develop resistance.

Additionally, miRNAs are fairly stable in the blood and tissues, enhancing their attraction for tumor diagnosis and molecular therapy [36].

Multiple studies have provided the proof-of-principle that microRNAs can be used as anti-cancer therapy, reviewed in [37]. Mirna Therapeutics is sponsoring a first in class clinical trial with SMARTICLES® (NCT01829971); a miR-34 liposome based therapeutics. The group recently released interim Phase I data describing good safety profiles for patients with liver cancer.

miR-137 has been shown to sensitize neuroblastoma, colon and hepatocellular carcinoma cells to doxorubicin by negatively regulating constitutive androstane receptor (CAR), a key player in multi-drug resistance (MDR) [7]. In breast cancer cells, miR-137 sensitizes cells to a variety of chemotherapy (adriamycin, vincristine, and paclitaxel) by negatively regulating Y-box binding protein-1 (YB-1) [38].

In this study, we show that the tumor suppressive function of miR-137 is in part due to negatively regulating MSI1 and possibly MSI2. In colorectal cancer, cells expressing $CD133^{+}$ and $MSI1^{+}$ are resistant to Oxiliplatin and 5-flurouracil [35, 39]. miR-137 may sensitize cells to Oxiliplatin and 5-flurouracil by negatively regulating MSI1. We are currently conducting studies to explore the therapeutic potential of miR-137.

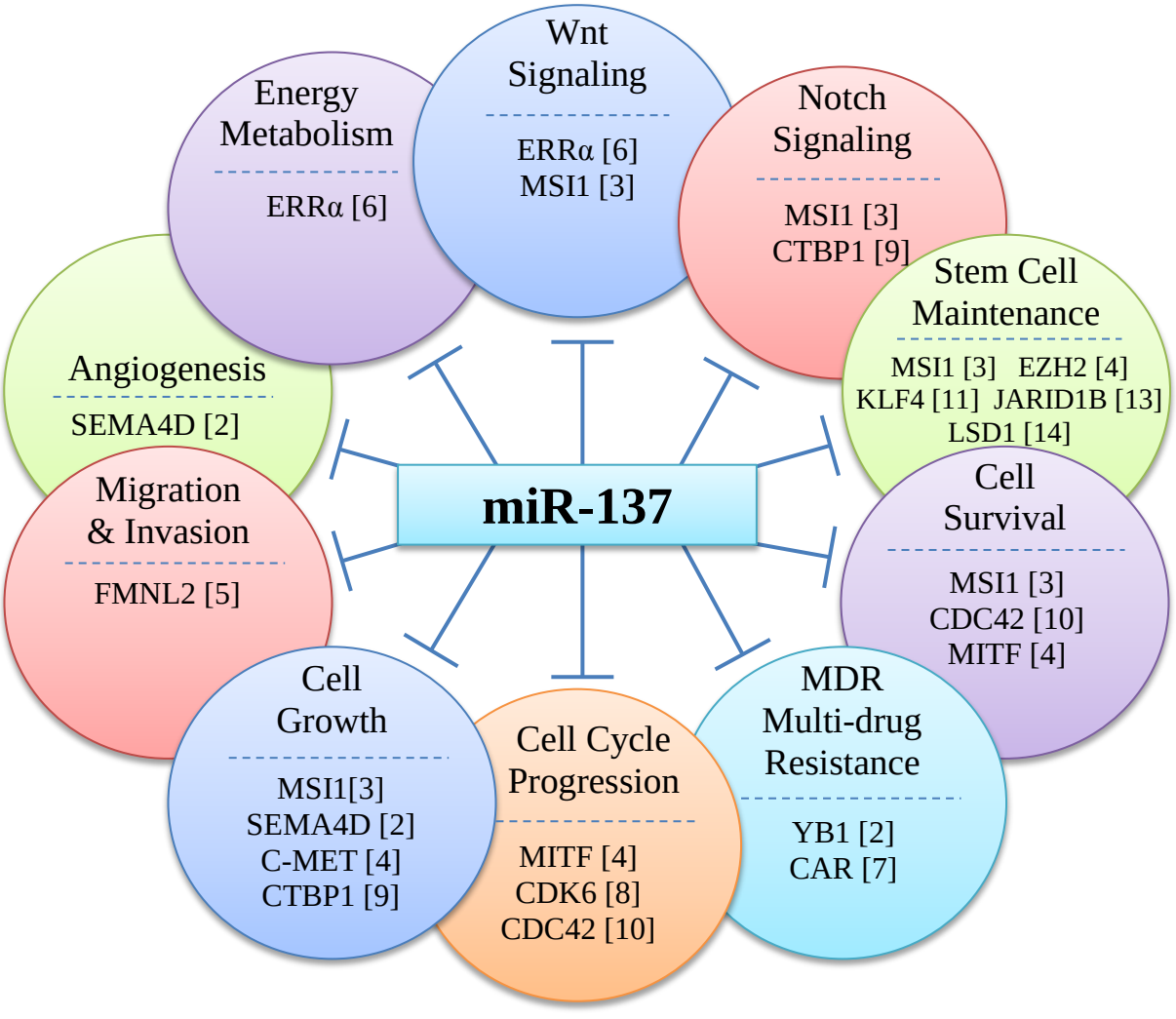


Figure 1. miR-137 negatively regulates multiple tumor promoting genes, signaling pathways and biological functions.

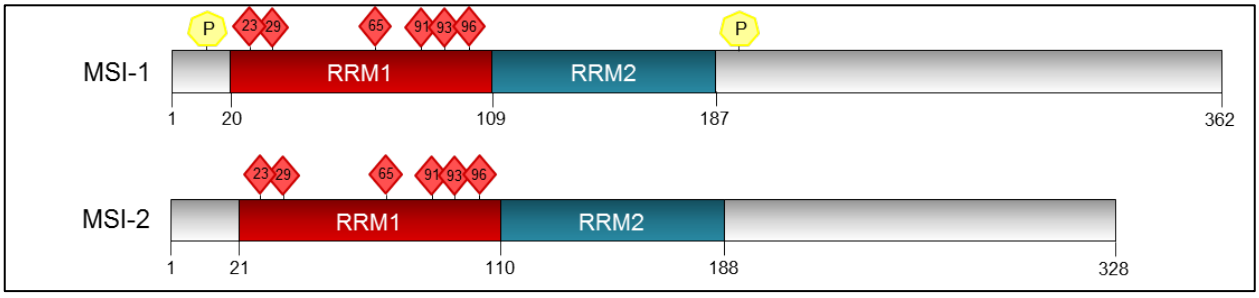
MSI1 small molecule inhibitors

In this work, we provided a comprehensive review of the expression and function of MSI1 and MSI2 in a variety of cancer types, along with describing the therapeutic potential of targeting the Musashi RNA-binding proteins as novel anti-cancer therapy. In a highly collaborative effort at KU, we identified three hit compounds that bind to and inhibit the RNA binding pocket of MSI1-RBD1. In the future, additional hit compounds will be screened and studied for binding affinity

and target specificity. The NMR structure data of hit compounds binding MSI1-RBD1 will be used to aid in the development of optimized analogs. Crystal structures of MSI1-RBD1 bound with hit compounds are currently ongoing.

In future studies, a dual MSI1/MSI2 small molecule inhibitor may be considered. The Musashi family of RNA binding proteins is characterized by two RNA recognition motifs (RRMs) [40, 41]. Structurally, the MSI1 and MSI2 full-length proteins share 69% amino acid homology (Figure 2A). MSI1 and MSI2 RRM1 shares 81% amino acid homology and 93% homology in RRM2. The known RNA binding residues (red diamonds in Figure 2a) are perfectly conserved in MSI1 and MSI2 RRM1. The NMR solution structure of MSI1-RBD1 is shown in Figure 2B (left model), however the solution structure of MSI2 RBD1 has not been solved. A model of MSI2-RBD1 was predicted using the I-TASSER protein structure prediction program (Figure 2B, right model) [42]. Although the structure of MSI2-RBD1 needs to be experimentally determined by NMR or crystallography, the predicted structure of MSI2-RBD1 is similar to MSI1-RBD1.

A.



B.

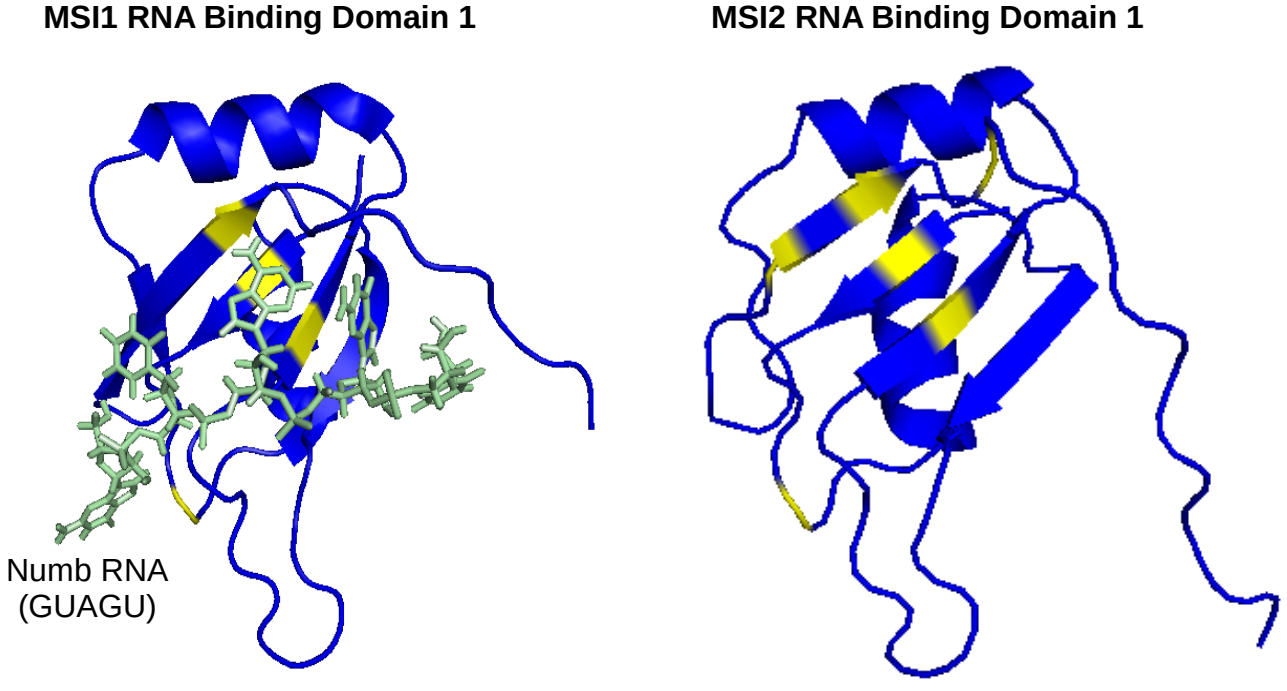


Figure 2: **Characterization of MSI1 and MSI2 RNA Binding Domains.**

A) Cartoon visual of MSI1/2 RNA recognition motifs. Red diamonds highlight identified RNA-binding residues. **B)** Conserved RNA-binding residues are highlighted in yellow. Target Numb RNA (GUAGU), in green, is modeled with MSI1-RBD1.

The RNA-binding specificity of MSI1 and MSI2 are similar [43] and both have been shown to bind to and negatively regulate mNUMB [44, 45]. Since the RNA binding proteins MSI1 and MSI2 have been shown to work in redundant manners, it is possible that they compensate for one another if one becomes dysregulated or inhibited. Double knock-in and knock-out studies of MSI1 and MSI2 in the colon epithelium and other tissues would greatly enhance our current understanding in this regard. In neurons, a double knock-out of MSI1/MSI2 greatly inhibited neurosphere formation, whereas, a single knockout did not [46]. In order to understand the dual function of MSI1/MSI2 on tumor growth, we aim to generate a double MSI1/MSI2 knockout cell line either by stable transduction or transient transfections. We hope to gain a better understanding of how these conserved Musashi proteins work together to promote stem cell maintenance and tumorigenesis.

Conclusion

Musashi RNA binding proteins are critical regulators of stem cell fate and tumorigenesis. When overexpressed in cells, they promote cell proliferation and clonogenic growth by upregulating Wnt and Notch signaling pathways and cell cycle progression. By understanding the regulation of MSI1 and its function in cancer, we aimed to develop therapeutic strategies targeting MSI1. One goal of this study was to discover if MSI1 was regulated by miRNAs and if this mechanism could be exploited as a therapeutic strategy. In this dissertation, we identified miR-137 as a tumor suppressive microRNA in part by negatively regulating MSI1 and possibly MSI2. We provide compelling evidence that miR-137 is an attractive agent for miRNA-based molecular therapy, in part by its ability to negatively regulate MSI1/2.

In a collaborative effort, we identified hit compounds that inhibit the RNA binding activity of MSI1. In this study, we characterized the specific binding event between hit compounds and MSI1-RBD1 using NMR. The results from our studies will be used to design more potent and specific inhibitors of MSI1. Overall, we hope to provide a therapeutic option for patients with Musashi driven cancers.

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Appendix:

Table 1: Alignment of Predicted miRNAs in MSI1 3′UTR

miRNA	Sequence Alignment	
miR-125b (1090-1097)	5′ ...GAAAGCAAAGGGCGUCUCAGGGA...	Msi1 3′UTR
	3' AGUGUUCAAUCCCAGAGUCCCU	miR-125b
miR-137 (1415-1422)	5' ...GCCCUGAGAACACAAAGCAAUAA...	Msi1 3′UTR
	3' GAUGCGCAUAAGAAUUCGUUAUU	miR-137
miR-144 (779-785)	5' ...CCCCUCAGACACCGUUACUGUAA...	Msi1 3′UTR
	3' UCAUGUAGUAGAUAUGACAU	miR-144
miR-342-3p (1378-1384)	5' ...GAGAGUUAAUUGUCUGUGUGAGG...	Msi1 3′UTR
	3' UGCCCACGCUAAAGACACACUCU	miR-342-3p
miR-185 (943-949)	5' ...CUGGACAGGAAUUAACUCUCCAA...	Msi1 3′UTR
	3' AGUCCUUGACGGAAAGAGAGGU	miR-185
miR-185 (1722-1729)	5' ...UCCUCCGGUGUCUCUUCUCUCCA...	Msi1 3′UTR
	3' AGUCCUUGACGGAAAGAGAGGU	miR-185

Table 2. Patient and Tumor Characteristics

Characteristics	Msi1 TMA (%)	P-value [a]	miR-137 (%)	P-value [a]
Sex				
Male	87 (60)	0.20	60 (65)	0.36
Female	59 (40)		33 (35)	
Age				
≤ 70	89 (61)	0.24	57 (61)	0. 59
> 70	57 (39)		36 (39)	
TNM				
I	42 (29)	0.57	30 (32)	0.39
II	41 (28)		24 (26)	
III	54 (37)		32 (34)	
IV	9 (6)		7 (8)	
Growth Pattern				
Expansive	87 (69)	0.22	53 (65)	0.57
Infiltrative	40 (31)		29 (35)	
Differentiation				
Good	3 (2)	0.39	2 (2)	0.72
Med	113 (77)		70 (75)	
Poor (poor + mu + sig)	30 (21)		21 (23)	
Local-Recurrence				
No	124 (85)	0.30	76 (82)	0.66
Yes	22 (15)		17 (18)	
Distant-Recurrence				
No	88 (60)	0.44	53 (57)	0.44
Yes	58 (40)		40 (43)	

[a] Determined using Chi-Square test.

Table 3. Musashi-1 Staining Intensity in Distant Normal, Adjacent Normal, Biopsy, Primary Tumor and Metastatic Rectal Cancer Tissue Samples

	MSI1 Staining Intensity[a]				
Tissue Type	**-**	**+**	**++**	**+++**	**Total N**
Normal	22 (18.6%)	65 (55%)	30 (25.4%)	1 (0.8%)	118
Adjacent Normal	8 (10%)	32 (40%)	34 (42.5%)	6 (7.5%)	80
Primary	0	3 (2%)	28 (19%)	115 (78.7%)	146
Met	0	9 (28.6%)	14 (28.6%)	26 (53%)	49

[a] MSI1 TMA scores of 0,1,2,3 are defined as -, +, ++, +++.

Table 4. Musashi-1 Expression in Distant Normal, Adjacent Normal, Biopsy, Primary Tumor and Metastatic Rectal Cancer Tissue Samples (low vs. high)

	MSI1 Expression[a]		
Tissue Type	**Low**	**High**	**Total N**
Normal	117 (99%)	1 (1%)	118
Adjacent Normal	74 (92.5%)	6 (7.5%)	80
Primary	31 (21%)	115 (79%)	146
Met	23 (47%)	26 (53%)	49

[a] TMA scores of 0+1+2 are defined as low expression and TMA scores of 3 are defined as high expression

Table 5. miR-137 Expression in Normal Rectal Tissues and Primary Tumor Rectal Tissue Samples

	miR-137 Expression[a]			
Tissue Type	**No Change**	**Decreased**	**Increased**	**Total N**
Matched Primary[a]	2 (3%)	57 (84%)	9 (13%)	68
Unmatched Primary[b]	0	22 (73%)	8 (27%)	30
Total Primary[c]	2(2%)	79 (81%)	17 (17%)	**98**

[a] miR-137 expression in primary tumor tissue sample was normalized to RNU6b and set relative to the expression of miR-137 in matching normal tissue sample.

[b] miR-137 expression in primary tumor tissue sample was normalized to RNU6b and set relative to the average expression of miR-137 in unmatched normal tissue samples.

[c] Combined change in miR-137 expression in paired and un-paired primary tumor tissues as compared normal mucosal tissues.

Table 6. Correlation of miR-137 and Musashi-1 Expression in Primary Rectal Tumor Tissue Samples

	miR-137 Expression[a]				
MSI1	**No Change**	**Decreased**	**Increased**	**Total N**	**P-value[d]**
Low[b]	1 (6%)	13 (76%)	3 (18%)	17	0.54
High[c]	1 (1%)	57 (81%)	12 (17%)	70	

[a] miR-137 expression in primary tumor tissue sample was normalized to RNU6b and set relative to the expression of miR-137 in normal tissue sample.

[b] MSI1 TMA scores of 0+1+2.

[c] MSI1 TMA scores of 3.

[d] Determined using Chi-Square test, χ^2, df = 1.224, 2

Table 7. Antibodies and Reagents

Product	Catalog #	Supplier	Comments
Antibodies			
Rabbit anti-Musashi1	5663	Cell Sign.	1:500
Rabbit anti-Musashi1	04-1041	Millipore	1:1000
Rabbit anti-Musashi2	Ab76149	AbCam	1:1000
Mouse anti-GAPDH	sc-51905	Santa Cruz	1:500
Rabbit anti-mNumb	2756	Cell Sign.	1:500
Rabbit anti-p21	sc-397	Santa Cruz	1:500
Rabbit anti-Hes1	Ab71559	Abcam	1:500
Rabbit c-Myc	5605	Cell Sign.	1:1000
Mouse anti-β-actin	A5316	Sigma	1:5000
Mouse anti-GAPDH	Sc-51095	Santa Cruz	1:500
Goat anti-mouse, peroxidase conjugated	31430	Fisher	1:8000
Goat anti-rabbit, peroxidase conjugated	31460	Fisher	1:8000
Goat anti-mouse, IRDye® 680RD	926-68070	LI-COR	1:15000
Goat anti-rabbit, IRDye® 800CW	926-32211	LI-COR	1:15000
Rabbit anti-Musashi-1 (for IHC)	04-1041	Millipore	1:50
Anti-Rabbit, peroxidase conjugated (for IHC)	K4010	Dako	
Reagents			
Hematoxylin	S3302	Dako	
EnVision+ System-HRP (for IHC)	K4010	Dako	
Doxycycline Hyclate	D9891	Sigma	
SYBR® Select Master Mix	4472908	AB	
Lipofectamine 2000	11668019	LT	
TRIzol	15596	LT	
Plasmids			
pTRIPZ		Dharmacon	
pCMV6-MSI1-GFP	RG215992	Origene	
pCMV-MSI2	SC123160	Origene	
CMV-EV-GFP		Origene	Modified
Renilla control vector	E2241	Promega	
TOP/FOP vectors	21-170/21-169	Millipore	
Mimics/siRNAs/AntagomiRs			
hsa-miR-125b miRIDIAN mimic	C-300595-03	Dharmacon	100 nM
hsa-miR-137 miRIDIAN mimic	C-300604-07	Dharmacon	100 nM
hsa-miR-144 miRIDIAN mimic	C-300612-05	Dharmacon	100 nM
hsa-miR-342-3p miRIDIAN mimic	C-300696-05	Dharmacon	100 nM
hsa-miR-185 miRIDIAN mimic	C-300636-07	Dharmacon	100 nM
NegCtl. #1, miRIDIAN mimic	CN-001000-01	Dharmacon	100 nM
hsa-miR-137, miRIDIAN Hairpin Inhibitor	IH-300604-08-005	Dharmacon	100 nM
NegCtl. #1, miRIDIAN Hairpin Inhibitor	IN-001005-01	Dharmacon	100 nM
Human Msi1 siRNA	J-011338-08	Dharmacon	100 nM
Non-targeting-siRNA #1	D-001810-01	Dharmacon	100 nM
Primers			
Taqman hsa-miR-137	000593	LT	
Taqman RNU6b	001093	LT	
Taqman RNU48	001006	LT	

Abbreviations: AB = Applied Biosystems, LT = Life Technologies, IHC = Immunohistochemistry

Table 8. Sequences for Oligonucleotide Primers

Genes	Accession #	Sequence (5′-3′)
Musashi-1	NM_002442	F: TTGGCAGACTACGCAGGAAG R: TGGTCCATGAAAGTGACGAAGC
Musashi-2	NM_138962.2	F: CAACGACTCCCAGCACGAC R: GTCAATCGTCTTGGATCTAACTC
GAPDH	NM_002046	F: ATGTTCGTCATGGGTGTGAA R: GGTGCTAAGCAGTTGGTGGT
Hes-1	NM_005524	F: CTGGAGAGGCGGCTAAGGTGTTT R: GTGCCGCTGTTGCTGGTGTAGA
C-Myc	NM_002467	F: GCCACGTCTCCACACATCAG R: TCTTGGCAGCAGGATAGTCCTT
Pre-miR-137	MI0000454 (miRbase)	F: CGGTGACGGGTATTCTTGGGTGG R: TGCCGCTGGTACTCTCCTCG

S1

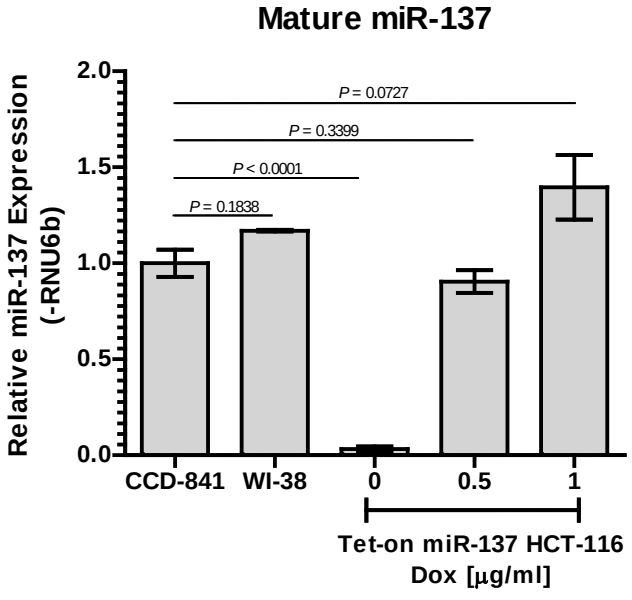

Figure S1. Expression of miR-137 in HCT-116 stable clones

(A) Tet-on miR-137 HCT-116 cells were treated with increasing doses of DOX [µg/ml]. Expression of mature miR-137 was analyzed using Taqman qRT-PCR, normalized to RNU6b and set relative to the expression of miR-137 in CCD-841. Expression of miR-137 in WI-38 (lung fibroblast) was also included in the study.