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Determining the Roles that DICER1 and Noncoding RNAs Play in Endometrial Tumorigenesis

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ABSTRACT OF THE DISSERTATION

Cancer is both a genetic and epigenetic disease. Changes in DNA methylation, histone modifications, and microRNA processing promote tumorigenesis, just as mutations in coding sequences of specific genes contribute to cancer development. In my thesis work I sought to determine the role that noncoding RNAs play in endometrial tumorigenesis. Aberrant methylation of the promoter region of the *MLH1* DNA mismatch repair gene in endometrial cancer is associated with loss of *MLH1* expression and a "mutator phenotype" in endometrial and other cancers. The molecular and cellular processes leading to aberrant methylation of the *MLH1* promoter region are largely unknown. I tested the hypothesis that the *EPM2AIP1* antisense transcript at the *MLH1* locus could be involved in *MLH1* transcriptional silencing. I characterized the *MLH1/EPM2AIP1* bidirectional promoter region in endometrial cancer and normal cell lines and found an abundance of forward and reverse transcripts initiating from a large region of nucleosome-free DNA in expressing cells. The DICER1 protein, which is necessary for processing small RNAs involved in post-transcriptional silencing, is downregulated in many cancers, including endometrial cancer. I used genomic methods (RNA-Seq and MeDIP/MRE) to characterize the transcriptome and methylome of endometrial cancer cells depleted of DICER1. Using a combination of computational and wet lab methods I showed that reduced DICER1 triggers an interferon response in cancer cells because of accumulation of pre-microRNAs that activate immune sensors of viral dsRNA. The methylome of DICER1 knockdown cells revealed subtle changes in methylation, including decreased methylation at the Alu family of repetitive elements.

Small RNAs processed by DICER1 may thus be involved in silencing repetitive regions. Non-coding RNA has effects on endometrial cancer cells that may contribute to tumorigenesis, such as influencing the active state of the *MLH1* gene and modulating the immune response.

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ABBREVIATIONS USED IN THE TEXT

bp: base pairs
chIP: chromatin immunoprecipitation
chIP-Seq: chromatin immunoprecipitation combined with Next-Generation Sequencing
cDNA: complementary DNA
COBRA: combined bisulfite and restriction analysis
DNA: deoxyribonucleic acid
dsRNA: double-stranded RNA
ELISA: Enzyme-linked immunosorbent assay
H3K27me3: histone 3 lysine 27 trimethylation
H3K9me3: histone 3 lysine 9 trimethylation
HDAC: histone deacetylase
ISG: interferon stimulated gene
MeDIP: methylated DNA immunoprecipitation
miRNA: microRNA
MMR: mismatch repair
mRNA: messenger RNA
MRE: methylation-sensitive restriction enzymes
MSI: microsatellite instability
MSPA: methylase-based single promoter analysis
PCR: polymerase chain reaction
PEV: position effect variegation
Pre-miRNA: pre-microRNA
Pri-miRNA: primary microRNA transcript
PTGS: post-transcriptional gene silencing
ncRNA: noncoding RNA
nt: nucleotide
q-RT-PCR: quantitative reverse transcriptase polymerase chain reaction
RACE: rapid amplification of cDNA ends
RISC: RNAi induced silencing complex
RITS: RNAi induced transcriptional silencing
RNA: ribonucleic acid
RNA-Seq: sequencing the transcriptome using Next-Generation Sequencing
RNAi: RNA interference
RRBS : reduced representation bisulfite sequencing
RT-PCR: reverse transcriptase polymerase chain reaction
shDcr: DICER1 knockdown (by shRNA)
shRNA: short hairpin RNA
siRNA: small interfering RNA
SINE: short interspersed element
TE: transposable element
TGS: transcriptional gene silencing
UTR: untranslated region

“I am one of those that think like Nobel; that humanity will draw more good than evil from new discoveries.”

-- Marie Curie

“In our adventures, we have only seen our monster more clearly and described his scales and fangs in new ways - ways that reveal a cancer cell to be, like Grendel, a distorted version of our normal selves. May this new vision … inspire our band of biological warriors to inflict much greater wounds tomorrow.”

-- Harold Varmus

CHAPTER ONE: Introduction

Epigenetic mechanisms

Epigenetics, the non-Mendelian regulation of gene expression, is crucial to normal development and often altered in disease states. Epigenetic mechanisms involve DNA packaging and other forms of transcriptional regulation along with posttranscriptional regulation. One such example of epigenetic regulation is the *agouti* locus in mice. Transcription of the A^{vy} (*Agouti viable yellow)* allele during development produces a protein that changes mouse coat color from black to yellow. However, mouse littermates with the same A^{vy} allele can have different coat colors. The IAP retrotransposon is responsible for this variation: by inserting itself proximal of the A^{vy} promoter, it induces transcription of this gene and changes the coat color of the mice. This is due to a loss of DNA methylation, an epigenetic silencing mark (Morgan et al. 1999), at the A^{vy} promoter. Thus two mice with the same allele of A^{vy} have different phenotypes due to differential expression of the allele. This epigenetic control is determined by the packaging of DNA in chromatin.

The nucleosome is the key building block of chromatin. It consists of about 147 base pairs of DNA wrapped around an octamer of histone proteins, two copies each of histones H2A, H2B, H3, and H4. The basic histones associate with the acidic DNA to create a stable nucleosome for packaging DNA (Allis et al. 2007). Nucleosomes are dynamic in that they rapidly move on and off of the DNA during DNA replication and transcription (Schones et al. 2008). Chemical modifications to the histones of the nucleosome make DNA more or less accessible to the transcription machinery. Chromatin modifications include posttranslational additions of specific chemical groups (including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination) to

the four histones that make up the nucleosome core. Modifications to the N-terminal tails of these histones either open up the chromatin, making it more accessible to transcription factors and transcription machinery (euchromatin), or create a more condensed chromatin state, repressing transcription (heterochromatin) (Allis et al. 2007).

Cytosine methylation is a critical epigenetic modification that results in transcriptional silencing. The DNA methyltransferases DNMT3A and DNMT3B establish methylation by adding methyl groups to the cytosine residues of DNA at CpG dinucleotides. DNA methylation is a heritable modification because as methylated DNA replicates, the maintenance DNMT1 methyltransferase adds methyl groups at CpG hemimethylated sites in the new strand of DNA as it is synthesized. This methylation typically recruits specific Methyl-CpG-binding proteins that combine to create a chromatin structure that represses transcription. Genes with such a closed chromatin structure at their promoter regions are less likely to be transcribed, as access to DNA by transcription factors and transcription machinery is restricted (Allis et al. 2007). Many genes have stably positioned nucleosomes and/or DNA methylation at their gene bodies. The function of gene body methylation is presently unclear, but it is often associated with highly expressed genes (Ball et al. 2009). DNA methylation is necessary to silence one of two X chromosomes in mammalian females. X inactivation is initiated by the long noncoding RNA (ncRNA) *Xist*, leading to packaging of almost the entire chromosome with repressive histone marks and DNA methylation (Lyon 1961; Penny et al. 1996). Imprinting is another function in mammals that requires DNA methylation. This is the selective expression of either a maternal or paternal allele. For example, the *Igf2r, Kcnq1, Pws*, and *Gnas* genes have methylation on the maternal allele, while *Igf2* and *Dlk2* are

methylated on the paternal allele. In each of these cases a ncRNA is expressed from the allele that is not methylated (Regha, Latos and Spahn 2006). Imprinting is crucial to normal development; the debilitating Prader-Willi and Angelman Syndromes are caused, respectively, by deletion of a section of the paternal chromosome 15 at a position at which the maternal copy is silenced, and by deletion of a section of the maternal chromosome 15 at a position at which the paternal copy is silenced (Jiang, Bressler and Beaudet 2004). Thus DNA methylation is important during mammalian development, and inappropriate methylation can lead to disease. My work focuses on the changes in DNA methylation occurring in cancer. My rotation project in the Goodfellow Laboratory involved characterization of methylation at the promoter of the *DUSP6* gene. DUSP6 is a phosphatase that negatively regulates the MAP kinase pathway. While methylation and silencing of this gene is common in pancreatic cancer, another cancer type in which ERK signaling is frequently activated, I found that it is quite uncommon in endometrial cancer (Appendix A).

While some chromatin modifications (*i.e.* H3K27me3, H3K9me3) and DNA cytosine methylation repress genes transcriptionally, microRNA (miRNA) regulation is an example of post-transcriptional epigenetic regulation. Most miRNAs, small ncRNAs, bind to the 3' untranslated region (UTR) of their target transcript and repress target genes either by inhibiting translation or stimulating degradation of the mRNA (Ambros 2001; Lagos-Quintana et al. 2001; Lau et al. 2001). MiRNA biogenesis begins with RNA polymerase II or III transcription of miRNA genes into long primary transcripts. The mature miRNA is derived from a stem-loop secondary structure within the primary transcript. The RNase III enzyme DROSHA cleaves the RNA to cut out the precursor

stem loop (60-100 nucleotides), which is then brought out of the nucleus by the RAN GTPase XPO-5. In the cytoplasm, the PAZ and RNase III domains of DICER1 cut out the mature miRNA, which is about 22 nucleotides long. The newly cleaved dsRNA is then processed by the RNAi induced silencing complex (RISC) (Hammond 2005). In humans, the Argonaute proteins AGO1 or AGO2 associate with the miRNA and mRNA to inhibit translation or cleave the mRNA. Recent work shows that miRNAs preferentially inhibit the initiation of translation in flies and zebrafish (Bazzini, Lee and Giraldez 2012; Djuranovic, Nahvi and Green 2012) (left panel of Figure 7 in Chapter 3). The miRNA in the RISC enables the complex to associate with the 3' UTR of the target gene through imperfect complementarity between miRNA and mRNA (Sontheimer 2005). Because miRNAs are not perfectly complementary to their targets, they may regulate more than one target gene, or several miRNAs may cooperate to regulate a single target. miRNAs are crucial for developmental and tissue-specific regulation at the translational level, and deregulation of specific miRNAs has been implicated in several diseases, including cancer.

Cancer epigenetics

Cancer is a genetic and epigenetic disease. A cancer (a malignant neoplasm) is defined simply as an overproliferation of abnormal cells, which eventually spread throughout the body, infiltrating other organs. Humans have had cancer for over five thousand years; a breast tumor is mentioned in the Edwin Smith Papyrus from Ancient Egypt, from around 3000 B.C. (Hajdu 2010). The Greek physician Hippocrates gave cancer its name, from the Greek word *carcinos* (crab), as he thought a malignant tumor

appeared crablike, with a solid mass in the middle and veins feeding into it. Scientists and physicians have sought to understand and treat cancers for as long as they have existed, but an acceleration in the progress of cancer research occurred in the past forty years with the focus on molecular biology. President Nixon declared a "War on Cancer" in 1971; subsequently the United States has spent $200 billion on cancer research, resulting in better understanding of the disease and novel therapies. Molecular biology and genomic research have enabled researchers to implicate specific genes in the development of cancer.

A cancer cell must be able to evade apoptosis, attain self-sufficiency in growth signals as well as insensitivity to anti-growth signals, replicate infinitely, sustain angiogenesis, and invade tissue and metastasize (Hanahan and Weinberg 2011; Hanahan and Weinberg 2000). These properties may be attained by mutations in specific genes. Cells with mutations in key regulatory genes (*i.e.* genes in developmental pathways or genes that regulate the cell cycle) may replicate continually. Oncogenes, genes that promote cancer, were first characterized in tumor-promoting viruses. *BRAF* is an example of an oncogene; a common mutation in this gene makes it constitutively active, activating the RAF/RAS/ERK pathway and driving cellular proliferation. Tumor suppressor genes such as *P53*, on the other hand, are necessary for control of cell division. When these genes are disabled by a mutation, cellular replication proceeds without bound. Another class of tumor suppressor gene is genes involved in maintaining genome stability, such as the mismatch repair protein MLH1. Mutations in these genes cause an increased rate of mutation throughout the genome, allowing for increased

activation of oncogenes and disabling of tumor suppressors (Vogelstein and Kinzler 2004).

Epigenetic events also play a large role in tumorigenesis. In a cancer cell, the normal and highly controlled epigenetic regulation of gene expression is disrupted and the packaging of genes in chromatin is changed. Cancer cells exhibit global hypomethylation (loss of methylation at normally silenced regions such as repetitive elements) and a gain of methylation (hypermethylation) at specific CpG islands, including those of tumor suppressor genes. CpG islands are long stretches of CpG dinucleotides that remain unmethylated to protect the promoter region of a gene from genetic mutations or epigenetic silencing. Methylation predisposes cytosine to deamination to thymine, so keeping CpGs unmethylated reduces the amount of mutations (Bird 1986). Aberrant methylation of CpG islands in promoter regions of genes silences gene expression (Jones et al. 1999). Hypermethylated, stably silenced genes have been shown to colocalize in the nucleus (Berman et al. 2012; Easwaran and Baylin 2010). Cancer cells undergo a global decrease in DNA methylation, turning on genes that are silenced in normal cells, de-repressing transposable elements, and contributing to genomic instability. Histone modifications and microRNA profiles are fundamentally different in cancer cells, leading to altered gene expression (Lujambio and Esteller 2009).

Epigenetic mechanisms in endometrial cancer

A number of the key molecular lesions that contribute to the progression of endometrial (uterine) cancer have been identified, making it a good tumor model for studying cancer genetics and epigenetics. Endometrial cancer is the most common

gynecological malignancy in the United States, with more than 47,000 new cases predicted to occur in 2012 (Siegel, Naishadham and Jemal 2012). Type 1 (endometrioid) cancer is associated with exposure to high levels of estrogen, promoting hyperplasia of the uterine lining. Activation of MAPK and AKT signaling and loss of DNA mismatch repair are frequent events in endometrial cancer (Dedes et al. 2010). Mutations in the *KRAS* and *FGFR2* genes, which feed into the MAPK-ERK developmental pathway, have been documented in endometrioid endometrial cancers. In addition, 20% of endometrioid endometrial cancers are characterized by a defect in DNA mismatch repair. Loss of DNA MMR leads to a microsatellite instability (MSI) phenotype. This phenotype is a measurement of the increased mutations (often insertions or deletions) observed in repetitive microsatellite DNA (Hecht and Mutter 2006). This "mutator phenotype" and resulting microsatellite instability are caused by epigenetic silencing of the *MLH1* gene, one of the best understood examples of epigenetic silencing of a tumor suppressor gene in cancer.

The *MLH1* gene encodes a highly conserved protein necessary for DNA mismatch repair. Mutations in *MLH1* have been shown to cause Lynch Syndrome, a cancer predisposition syndrome that confers a 50% lifetime risk for developing endometrial cancer. Unsurprisingly, sporadic endometrial carcinomas also have defects in *MLH1*. Endometrial carcinomas deficient in DNA mismatch repair often exhibit hypermethylation of the *MLH1* promoter CpG island and transcriptional silencing of *MLH1*. Methylation of *MLH1* can be inherited (Hitchins et al. 2007) and there is evidence that inherited *cis* variation contributes to risk for epigenetic silencing of *MLH1* in both endometrial and colon cancer. *MLH1* promoter methylation is significantly associated

with a single nucleotide polymorphism (G/A; rs1800734) in the *MLH1* regulatory region, -93 from the transcription start site (Allan et al. 2008; Chen et al. 2007). *MLH1* is thus a *bona fide* example of a cancer gene with a genetic association with epigenetic regulation. DNA methylation silences *MLH1* transcription and has an effect functionally equivalent to a mutation or deletion in the *MLH1* gene. As MLH1 is an essential component of DNA mismatch repair, cancers that do not express *MLH1* have a mutator phenotype. The mutation rate in these tumors is drastically increased and tumors lacking normal DNA mismatch repair have an MSI tumor phenotype. Cells that lack the DNA mismatch repair system also are deficient in an S phase checkpoint that promotes apoptosis, allowing cells with methylated *MLH1* to evade apoptosis and promote tumorigenesis (Brown et al. 2003).

The mechanism by which transcriptional silencing of *MLH1* is initiated has not been determined. Methylation of the *MLH1* promoter region recruits methyl CpG binding proteins as well as repressive histone modifications, packaging the chromatin into a "closed" state that prevents access by transcription factors and the transcription machinery (Xiong et al. 2006). When the DNA is methylated, additional nucleosomes (relative to the active state) are present at the *MLH1* promoter region, creating a more compact chromatin state (Lin et al. 2007). However, neither of these studies addresses the underlying question of what process establishes *MLH1* methylation.

My thesis research focuses on RNA-mediated transcriptional silencing in endometrial cancer. Long noncoding antisense RNAs are one class of RNA-mediated silencing molecules. Antisense RNAs are transcribed from the DNA strand opposite the protein-coding strand and may be complementary to the mRNA for a specific gene; they

can act in *cis* or *trans* to silence a gene. A long overlapping antisense RNA is sufficient for transcriptional silencing of the *P15* (*CDKN2B*) gene involved in the initiation of cell cycle arrest (Yu et al. 2008). Like *P15 (CDKN2B),* the *MLH1* locus is characterized by bidirectional transcription. A promoter on the opposite strand of DNA to *MLH1* and about 200 bp upstream of the *MLH1* start site contains the start site for the antisense transcript *EPM2AIP1*. This antisense RNA could function to silence the *MLH1* gene in cancer, as the *P15* antisense RNA does. In Chapter Two I describe my work elucidating the function of *EPM2AIP1* and whether it can induce transcriptional silencing of *MLH1*. In addition, I describe extensive characterization of transcription, both sense and antisense, at the *MLH1/EPM2AIP1* bidirectional promoter, and mapping of nucleosomes at this locus.

Small RNA transcriptional gene silencing (TGS) is an extensively characterized epigenetic mechanism in plants, yeast, and protozoa. Genes and regions of repetitive DNA, such as the pericentromeric repeats, are transcriptionally silenced by complementary small RNAs, processed by DICER1 and targeted to the DNA by the Argonaute proteins. Recent findings suggest that this process may be conserved in humans. Evidence for DICER1 and small RNA involvement in mammalian TGS includes the nuclear localization of proteins involved in small RNA processing and targeting. The AGO2 protein was previously thought to be localized to the cytoplasm, where it guides miRNAs to their targets and aids in translational repression. The presence of AGO2 in the nucleus implies a role for small RNAs in the nucleus of mammalian cells (Weinmann et al. 2009). Research from the Goodfellow laboratory has shown that DICER1 localizes to the nucleus. ERK phosphorylates DICER1, prompting nuclear localization (Rimel et

al. 2012). At present phosphorylated DICER1's function in the nucleus is unknown; the phosphorylation and nuclear localization could reduce DICER1's function in the cytoplasm, or phosphorylated DICER1 could have a novel function in the nucleus. The relocalization/ nuclear shuttling seen when DICER1 is phosphorylated by ERK could be especially relevant to endometrial cancer because an estimated 40% of endometrioid endometrial cancers have activating mutations in the MAPK/ERK pathway, either in FGFR2 or KRAS (Byron et al. 2008). Furthermore, downregulation of DICER1 is associated with transformation and tumorigenesis (Bahubeshi, Tischkowitz and Foulkes 2011; Heravi-Moussavi et al. 2011; Kumar et al. 2007; Kumar et al. 2009; Melo et al. 2010; Melo et al. 2009; Merritt et al. 2008; Sand et al. 2010; Slade et al. 2011). Lower levels of DICER1 in endometrial cancer predict worse outcomes (Zighelboim et al. 2011).

DICER1 has been implicated in the control of CpG island methylation in mammalian cells. In HCT116 colon cancer cells with a mutation in the helicase domain of DICER1, a set of genes that normally had hypermethylated CpG islands showed demethylation and expression. Levels of the DNA methyltransferase proteins were unaffected (Ting et al. 2008). The effect on CpG island methylation could be a direct or indirect effect of altered DICER1 activity. A recent paper described a loss of telomere methylation upon *DICER1* mutation, providing evidence that the change in methylation was secondary to reduced DICER1 activity. The mutation in *DICER1* led to failed processing of the *miR-290* cluster, which normally targets the retinoblastoma-like 2 (RBL2) protein. Thus RBL2 was upregulated, and repressed the DNA methyltransferases, causing a loss of methylation (Benetti et al. 2008).

Recent work implies that small RNAs can target transcriptional silencing to complementary DNA in mammalian cells. Transfecting human cells with dsRNA complementary to the promoter regions of specific genes can epigenetically silence or activate, depending on the gene. This process requires AGO2 (Hawkins et al. 2009; Li et al. 2006; Morris et al. 2004). This work implies that the function of small RNAs in human cells is analogous to their function in *S. pombe*, but more work must be done to determine whether it is a common mechanism or an exception in the case of several genes. In addition, the microRNA *miR-320* has been shown to initiate transcriptional gene silencing (TGS) of the *POLR3D* gene. The authors of this study identified 1200 genes with possible miRNA target sites at their promoter regions (Kim et al. 2008). This study demonstrates a novel role for miRNAs, which were previously thought to be solely involved in post-transcriptional gene silencing (PTGS): either degrading mRNA or inhibiting translation. Instead, the miRNAs may bind to the 5' region of the gene and initiate transcriptional silencing. I contacted the authors to determine whether *MLH1* was among these 1200 genes that had possible miRNA targets in their promoters; it was not (D. Kim, personal correspondence, 2009).

To begin to determine whether small RNAs are involved in transcriptional silencing of *MLH1*, I studied the effects of reduced DICER1, the master regulator of small RNAs, in endometrial cancer. In Chapters Three and Four I describe knocking down the DICER1 protein in endometrial cancer cell lines and testing for changes in methylation of *MLH1*. I assessed changes in global methylation and transcription to determine the role of DICER1 in genomic transcriptional regulation in endometrial cancer. The biggest signal from the transcriptome of DICER1 knockdown cells was an

upregulation of interferon response genes. The interferon response is the cell's innate immune response wherein specialized cytoplasmic sensors recognize foreign molecules such as bacteria or viruses. I validated the interferon response in DICER1 knockdown cells and showed that it was triggered by a buildup of pre-miRNAs, 60-100 nt double-stranded RNA, in the cytoplasm in the absence of DICER1 processing. These structures can be recognized by cytoplasmic sensors which usually detect viral dsRNA and set off the interferon response (Alexopoulou et al. 2001; Li and Tainsky 2011; Platanias 2005). This response may contribute to the tumor phenotypes I observed in DICER1 knockdown (shDcr) cells, including increased cell migration and increased growth in soft agar.

I analyzed the “methylome” (analogous to the transcriptome; levels of methylation across the whole genome) of shDcr endometrial cancer cells. The patterns and extent of methylation overall were not affected by DICER1 knockdown. There were few validated changes in DNA methylation in gene regulatory regions and gene bodies. This finding was unexpected given an earlier report in colon cancer cells with a DICER1 hypomorph (Ting et al. 2008) However, we did observe a decrease in methylation at the Alu family of transposable elements (TEs) in shDcr cells. TEs, first described by Barbara McClintock in maize (McClintock 1950), make up at least 30% of the human genome (Lander et al. 2001; Weiner 2002). These elements are characterized by their ability to transpose themselves into different places in the host genome ("jumping genes"). Many of the TEs that are integrated into the human genome have been stably silenced and are no longer able to transpose themselves into different locations. Alu elements are a type of SINE (short interspersed element); these elements are typically 282 bp in length and are transcribed by RNA Polymerase III (Deininger et al. 2003). They use reverse

transcriptase (encoded by the L1 transposable elements) to create a DNA copy that inserts into a different part of the genome. As frequent Alu insertion would create significant genome instability, there is strong selection to stably silence these elements. They are silenced by chromatin modifications and DNA methylation in normal human tissues (Gama-Sosa et al. 1983; Kochanek, Renz and Doerfler 1993), but may lose silencing and be expressed in cancer or other disease states (Belancio, Hedges and Deininger 2008). Our finding that reduced DICER1 levels cause a loss of methylation at Alu elements is consistent with a recent report showing that low DICER1 levels in macular degeneration cause an accumulation of Alu transcripts (Kaneko et al. 2011). DICER1 may thus be involved in transcriptional silencing of Alu transcripts. As DICER1 is generally reduced and Alu transcription is generally increased in tumorigenesis, this may be an important component of the genomic instability associated with cancers. The following Chapters 2-4 make up my thesis work on the role of noncoding RNA in endometrial cancer.

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CHAPTER TWO: The active *MLH1/EPM2AIP1* bidirectional promoter is characterized by multiple transcripts and an absence of stably positioned nucleosomes

Foreword

My Ph. D. research began with a series of experiments focused on the epigenetic silencing of the DNA mismatch repair gene, *MLH1*, in endometrial cancer. I received a Siteman Cancer Center Cancer Biology Pathway Fellowship award to study *MLH1* epigenetic silencing and specifically to determine what, if any, role an antisense transcript, *EPM2AIP1*, plays in *MLH1* expression and epigenetic silencing.

Cancer cells exhibit hypermethylation of the CpG islands of tumor suppressor genes, silencing transcription (Jones et al. 1999). Active CpG islands have an open chromatin structure with loosely positioned nucleosomes to allow the transcription machinery access to the DNA, but maintain a closed chromatin structure with stable nucleosomes once they become methylated (Deaton and Bird 2011). The *MLH1* gene encodes a highly conserved protein necessary for DNA mismatch repair. Endometrial carcinomas that are deficient in DNA mismatch repair often exhibit hypermethylation of the *MLH1* promoter CpG island that is associated with transcriptional silencing of the gene. Methylation of the *MLH1* regulatory region is thus functionally equivalent to a mutation or deletion in the *MLH1* gene. Cancers lacking *MLH1* have a mutator phenotype, exhibited by microsatellite instability (MSI). DNA methylation of *MLH1* recruits methyl CpG binding proteins and is associated with repressive histone modifications, packaging the chromatin into a "closed" state that prevents access by transcription factors and the transcription machinery (Xiong et al. 2006). Additional nucleosomes are present at the methylated *MLH1* promoter region, creating a more compact chromatin state (Lin et al. 2007). However, the mechanism by which transcriptional silencing of *MLH1* is initiated has not been established.

EPM2AIP1, a long noncoding RNA transcribed from the opposite strand of DNA about 200 bp upstream of the canonical *MLH1* transcription start site, could be a player in transcriptional silencing of *MLH1*. Long noncoding RNA has been shown to be involved in transcriptional silencing of several loci, as described in Chapter One. A long overlapping antisense RNA is implicated in transcriptional silencing of the *P15* gene involved in the initiation of cell cycle arrest. The antisense transcript (referred to as *P15AS*) is present at high levels in leukemia cells but at low levels in normal cells, while the sense transcript has the opposite expression pattern. The antisense transcript *P15AS* decreases *P15* expression at the mRNA level and recruits silencing histone modifications to the *P15* promoter region. Transfecting cells with *P15AS* causes accelerated proliferation, implicating the *P15* antisense transcript in a cellular function relevant to tumorigenesis (Yu et al. 2008). The *EPM2AIP1* antisense RNA could function to silence the *MLH1* gene in cancer, as *P15AS* silences *P15* in cancer. In this chapter, I explore the function of *EPM2AIP1*. I first demonstrate the existence of overlapping *EPM2AIP1* and *MLH1* transcripts, establishing the possibility that a dsRNA could lead to silencing. Then I determine whether *EPM2AIP1* could induce transcriptional silencing of *MLH1*, as another example of antisense RNA silencing of a tumor suppressor gene.

Contrary to the *P15* and *P15AS* transcripts, the *MLH1* and *EPM2AIP1* transcripts are concordantly expressed. There is significant overlap between the transcripts in all cell lines in which they are expressed and about a third of primary tumors, creating a potential dsRNA structure. In addition, I identified several novel transcripts for each gene, some initiating in the middle of the region previously described as having stably positioned nucleosomes (Lin et al. 2007). After helpful discussions with my thesis research advisory

committee chair, Dr. Sarah Elgin, I decided to determine the nucleosome positioning at this locus in endometrial cancer cells. I showed that in endometrial cells expressing *MLH1*, the *MLH1/EMP2AIP1* bidirectional promoter is devoid of nucleosomes and that there are multiple transcripts for both genes. The “open chromatin” state for endometrial cells is in contrast to previous research (Lin et al. 2007) describing a region with three stably placed nucleosomes when *MLH1* is being transcribed that gains additional nucleosomes and DNA methylation when the gene is silenced. The nucleosome-free region could be the result of RNA polymerase landing on open regions of chromatin and creating novel transcripts, or transcription from multiple start sites (other than the coding transcript) could serve to keep this important locus open for transcription of the canonical coding *MLH1* transcript. The following manuscript (submitted to *Epigenetics)* details the characterization of the *MLH1* bidirectional promoter in endometrial cancer.

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The active *MLH1/EPM2AIP1* bidirectional promoter is characterized by multiple transcripts and an absence of stably positioned nucleosomes

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Abbreviations: bp: base pairs, cDNA: complementary DNA, COBRA: combined bisulfite and restriction analysis, dsRNA: double-stranded RNA, MSI: microsatellite instability, MSPA: methylase-based single promoter analysis, RACE: rapid amplification of cDNA ends, RT-PCR: reverse transcriptase polymerase chain reaction, qRT-PCR: quantitative RT-PCR, UTR: untranslated region

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Abstract

The *MLH1* gene is frequently epigenetically silenced in endometrial cancer. The silenced state is associated with DNA methylation and the presence of stably positioned nucleosomes in the 5' regulatory region. Characterization of the *MLH1/ EPM2AIP1* bidirectional promoter revealed multiple transcripts in both the forward and reverse directions, with overlap and potential dsRNA in 40% of specimens investigated, including primary endometrial tumors. Several of the novel transcripts identified appear to initiate within the region previously described as including stably positioned nucleosomes. We demonstrated that the active *MLH1/ EPM2AIP1* regulatory region in endometrial cancer and normal cell lines is not characterized by the presence of stable nucleosomes when the genes are active and the region is unmethylated. We conclude that when unmethylated, a 569 bp region including the start sites for the *MLH1* and *EPM2AIP1* transcripts is free of nucleosomes in endometrial cells. Our finding suggests greater variability in how nucleosomes are positioned in the shared *MLH1/ EPM2AIP1* regulatory region than has been previously appreciated.

Introduction

The nucleosome, the fundamental unit of chromatin, consists of 147 base pairs of DNA wrapped around an octamer of histone proteins. Nucleosomes are dynamic and are positioned differently at active and silent loci. Transcriptional events such as the binding of RNA polymerase have been shown to change nucleosome positioning (Schones et al. 2008). The structure of the nucleosome provides opportunities for chemical modifications

to make DNA more or less accessible to the transcription machinery. Modifications to the N-terminal tails of specific histones either open up the chromatin, making it more accessible to transcription factors and the transcription machinery, or create a more condensed chromatin state, repressing transcription (Allis et al. ; Schones et al. 2008).

DNA methylation is a critical epigenetic modification that may cause transcriptional silencing when added to the promoter regions of genes (Jones and Baylin 2002). Methylation recruits methyl-CpG-binding proteins, creating a condensed chromatin structure and preventing access to DNA by transcription machinery (Allis et al.). Cancer cells exhibit hypermethylation of CpG islands, long stretches of CpG dinucleotides that normally remain unmethylated. Aberrant methylation of CpG islands silences tumor suppressor genes in cancer (Jones and Laird 1999). Recent evidence has shown that epigenetically silenced genes may be colocalized in the nucleus (Berman et al. 2012).

Endometrial (uterine) cancer is the most common gynecologic malignancy in the United States. A significant fraction of endometrial cancers have defective DNA mismatch repair and tumor microsatellite instability (MSI) (Hecht and Mutter 2006; Zighelboim et al. 2007a). Epigenetic silencing of the *MLH1* locus accounts for the vast majority of MSI-positive endometrial tumors. Cells that lack the DNA mismatch repair system lose an S phase checkpoint that promotes apoptosis, allowing cells with methylated *MLH1* to evade apoptosis and promote tumorigenesis (Brown et al. 2003). In addition to somatic epigenetic silencing of *MLH1*, germline *MLH1* epimutations have been identified in patients with multiple primary Lynch-associated cancers including endometrial cancer (Hitchins et al. 2007). There is evidence that inherited *cis* variation

contributes to risk for epigenetic silencing of *MLH1* in both endometrial and colon cancer. *MLH1* promoter methylation is significantly associated with a single nucleotide polymorphism (G/A; rs1800734) in the *MLH1* regulatory region, 93 bp upstream of the translation start site (Allan et al. 2008; Chen et al. 2007). *MLH1* is thus the first example of a cancer gene with a genetic association with epigenetic regulation.

The mechanism by which transcriptional silencing of *MLH1* is initiated has not been established. When the *MLH1* locus is methylated, additional nucleosomes are present at the *MLH1* promoter region, creating a more compact chromatin state (Lin et al. 2007; Xiong et al. 2006).We sought to determine the changes in transcription at the methylated *MLH1* locus versus the unmethylated locus. A promoter on the opposite strand of DNA to *MLH1* starts ~30 bp from the longest reported *MLH1* start site (Lin et al. 2007). We hypothesized that this antisense RNA could function to silence the *MLH1* gene in cancer, as has been shown for the tumor suppressor gene *P15* (Yu et al. 2008). We explored the transcriptional status of the *MLH1* gene in endometrial cancer cell lines and found that an abundance of transcripts were expressed when the locus was unmethylated. We also found there to be a nucleosome-free region of at least 569 bp around this gene when it was actively transcribed. We conclude that antisense transcripts do not silence the *MLH1* region and that, in contrast to previous studies, the *MLH1/ EPM2AIP1* bidirectional promoter is nucleosome free when unmethylated in endometrial cells.

Results and Discussion

Overlapping *MLH1* and *EPM2AIP1* transcripts

5' RACE and RT-PCR analyses for endometrial cancer cell lines, an immortalized normal endometrial epithelial cell line, and primary tissues revealed the 5' untranslated regions of *MLH1* and *EPM2AIP1* were longer than previously described. *MLH1* and *EPM2AIP1* are a bidirectional gene pair transcribed head-to-head on opposite strands of the DNA. Overlapping transcripts were seen in > 40% of tissues investigated, with a maximum overlap of 455 bp based on the RACE findings (Figure 1, Table 1). It is possible that there are even longer, low abundance transcripts for either or both genes that were not detected in our RACE analyses. Prior reports on *MLH1* and *EPM2AIP1* expression did not suggest that the transcripts overlapped (Lin et al. 2007) (Ensembl ENST00000231790, NCBI NM_000249.3). Query of the NCBI EST database did, however, reveal several sequences mapping to the region between the published start sites of *MLH1* and *EPM2AIP1*, suggesting potential overlap (Accessions DB278367, DB282952, DA097961, EB388804).

RT-PCR confirmed the longer 5' transcripts for *MLH1* and *EPM2AIP1* in cell lines and primary tissues (Table 2). As previously reported (Lin et al. 2007), *MLH1* expression was seen only when the shared 5' region was unmethylated. Typically the *EPM2AIP1* and *MLH1* transcripts were not detectable in cell lines in which the promoter was methylated. Low levels of *EPM2AIP1* were, however, seen in the AN3CA cell line, which does not express *MLH1* and is methylated at the shared promoter region (Figure S1). Four tumors with methylation of the shared promoter region expressed both transcripts (Table 2). It is likely that the *MLH1* and *EPM2AIP1* transcripts detected were

from the non-neoplastic components of the primary tumors (i.e., stroma, lymphocytes and/or blood vessels). The pattern of expression in three other primary tumors that had methylation of the shared promoter was noteworthy. Those three cancers expressed *EPM2AIP1* but not *MLH1*. We excluded DNA contamination of the RNA specimens, a possible explanation for the discordant expression observed (*EPM2AIP1* active and *MLH1* silenced), by performing a "no reverse transcriptase" control for each RT-PCR reaction (Figure S1). Other possible explanations for the unexpected pattern of expression are that *EPM2AIP1* transcription could be activated when *MLH1* is not, or that the relative levels of the two transcripts could be different (*EPM2AIP1* more abundant). However, qRT-PCR in endometrial cancer cell lines showed that these transcripts were expressed at approximately the same level and that they were concordantly expressed (data not shown).

We observed four different forward (*MLH1*) and four reverse (*EPM2AIP1)* transcription start sites associated with the unmethylated, active shared 5' region (Figure S2). The length of the 5' *MLH1* and *EPM2AIP1* UTRs varied within a given cell line or tissue type. *MLH1* and *EPM2AIP1* transcripts overlapped in all cell lines that expressed the two transcripts and about one third of primary tissues investigated (N=29).

A transcript initiating 260 bp 5' of the *MLH1* ATG and 43 bp upstream from the longest reported transcript was expressed concordantly with a shorter 5' *EPM2AIP1* transcript in KLE tissue (344 bp 5' of the *MLH1* ATG). The "long" MLH1 start site was associated with two transcripts. One was the canonical MLH1 transcript with a longer 5' UTR, and the other was an alternately spliced RNA species lacking *MLH1* exon 1 and giving rise to a long noncoding RNA. It is unclear whether the noncoding transcript is of

functional significance. It was seen in all tissues that express the "long" *MLH1* transcript (approximately one third of all specimens investigated). Overlapping *MLH1* and *EPM2AIP1* transcripts generating dsRNA were present in 3/5 endometrial cancer cell lines, 1 immortalized normal endometrial cell line, 10/18 primary tumors, 2/4 normal endometrium samples, and testis tissue (Table 2). The overlap using RT-PCR assays ranged from 47 to 202 bp (data not shown).

Absence of nucleosomes at the *MLH1/ EPM2AIP1* bidirectional promoter region

Nucleosomal occupancy and its potential role in silencing of the *MLH1/ EPM2AIP1* CpG island was previously assessed by Lin and colleagues (Lin et al. 2007) using DNAse footprinting, chromatin immunoprecipitation (ChIP) and methylase-based single-promoter analysis assays (MSPA). They reported that the *MLH1/ EPM2AIP1* promoter has two stably positioned nucleosomes in expressing cell lines (colon cancer and fibroblasts). The two nucleosomes are positioned 3' of the canonical *MLH1* and *EPM2AIP1* transcription start sites (the -60 *MLH1* transcription start site and the -244 *EPM2AIP1* transcription start site on the opposite strand) (Figure 2A, upper panel). The RKO colon cancer cell line that has epigenetic silencing of *MLH1* has additional nucleosomes covering the entire promoter region, suggesting that epigenetic silencing may be accomplished by the stable placement of nucleosomes into previously vacant positions (Lin et al. 2007).

We identified novel, more 5', start sites for *MLH1* and *EPM2AIP1* that if active in the same cell could give rise to dsRNAs. Lin and colleagues mapped three transcriptional start sites at the *MLH1/EPM2AIP1* bidirectional promoter as well as

nucleosomes after the transcription start sites (Lin et al. 2007) (Figure 2A, upper panel). The functional significance of the four additional transcripts we observed (3 *EPM2AIP1* and one *MLH1;* Figure 2A, lower panel, in grey) is unknown (the -216 *MLH1* transcript was previously characterized- ENST00000231790). These novel transcripts may represent exogenous transcription or could have an important regulatory role. Noncoding RNA sense and antisense transcripts for well characterized genes are quite common in mammalian genomes (Core and Lis 2008; Guttman et al. 2009; Preker et al. 2008; Seila et al. 2008). The four noncoding transcripts at the *MLH1/EPM2AIP1* bidirectional promoter could have a causal or consequential relationship to transcription of the coding transcript. A region of DNA maintained in an "open" chromatin state (largely devoid of nucleosomes) could be generally accessible to the transcriptional machinery. In the open chromatin it is possible multiple start sites combined with alternative splicing would result in multiple distinct transcripts. Alternatively, the transcriptional machinery binding to DNA and initiating transcripts over a relatively broad region could serve to keep the chromatin open and thereby ensure transcriptional potential for an important gene.

The longer 5' UTRs/more 5' transcription start sites we identified for both *MLH1* and *EPM2AIP1* in endometrial tissues were unexpected and inconsistent with the nucleosomal positioning for the active promoter region described by Lin and colleagues (Lin et al. 2007). Methylase-based single promoter analysis assays (MSPA) in three endometrial cell lines suggested a very different pattern of nucleosome positioning (Fig 2A). We observed no nucleosome footprinting over a 569 bp region (-464 to +105) in the normal endometrial cell line EM-E6/E7/TERT and the endometrial cancer cell lines Ishikawa and KLE (Figure 2A) based on sequence analysis of multiple cloned PCR

products from each of the three cell lines. Of the 35 CpGs in the bisulfite PCR amplicon, we found only 4 to be protected (CpGs 11-14, Figure 2A, lower panel). We did not observe the protection at CpGs 1-4 and 24-35 that Lin and colleagues observed (Lin et al. 2007) and proposed to be associated with the presence of nucleosomes (Figure 2A). The methylase protection at CpGs 11-14 we saw (Figures 2A and B) is, however, consistent with what was reported by Lin *et al.(Lin et al. 2007).* As shown in Figure 2B, CpG 10 was methylated (M.SssI-treatment) but the following four CpGs (11-14) were converted to TGs in the EM-E6/E7-TERT cell line, and as such were protected. The genomic DNA control (shown in the bottom half of Figure 2B) was on the other hand methylated at all CpGs. COBRA similarly showed protection (no methylation) at the region of protection observed by sequencing (-269 BsrBI digestion) but methylation (no protection) at a downstream CpG (-250 MluI digestion), confirming the sequencing results (Figure 2C). Restriction enzymes that digest at CGs only cut at the -269 CG in Figure 2B (left panel of Figure 2C) and not at the next TG at -250 (right panel of Figure 2C). A smaller PCR amplicon covering the *MLH1* transcription start site confirmed the absence of nucleosome footprinting (data not shown). Lin *et al.* performed nucleosome positioning in colon cancer cells. Review of the publically available ENCODE data for a leukemia and a lymphoblastoid cell line genome-wide study (ENCODE/Stanford/BYU 2011) indicated that as we saw in endometrial cells, nucleosomes were absent from the 569 bp region by micrococcal nuclease digestion. This could mean that either the region is nucleosome free, or nucleosomes are not stably positioned, rapidly moving on and off the DNA. Taken together, these studies suggest that nucleosome positioning at the *MLH1/EPM2AIP1* bidirectional promoter may vary significantly between cell types.

The four CpGs protected from M.SssI methylation span 20 bp and the distance between CpG 10 and 15 (both unprotected) in only 42 bp. The maximum size of the protected region is too small for a nucleosome (147 bp) but could be protected by the presence of a transcription factor. Search of the TRANSFAC database (Wingender 2008) for transcription factors that could bind to this region previously characterized as a "footprinting region" (Arita et al. 2003) produced only the liver-specific transcription factors HNF-1B and HNF-1C. Search of the Geo database showed that these factors were unlikely to be present in the endometrium. The most frequently observed 5' start site of the *EPM2AIP1* transcript is within the protected region and an *MLH1* transcription start site is nearby (Figure 2A). As such, it is possible the transcription factor(s) for *MLH1* or the transcription machinery for *EPM2AIP1* are giving a footprint. Given our data confirm the protein-binding regions but do not support the nucleosome positioning established by Lin *et al.* (Lin et al. 2007), we used COBRA to detect the presence of nucleosomes at the estrogen receptor (*ESR1*) as a positive control. We investigated Ishikawa cells for which nucleosome positioning has been previously reported (Rocha et al. 2005). As expected, M.SssI was not able to methylate this region, consistent with the presence of stably positioned nucleosomes protecting the region from methylation (Figure S3).

The generally concordant expression of the *MLH1* and *EPM2AIP1* sense and antisense transcripts observed is consistent with previous reports (Lin et al. 2007). Unlike the *P15* antisense transcript (Yu et al. 2008), *EPM2AIP1* is not involved in transcriptional silencing of *MLH1*. Our observation that at least a 569 bp region in the shared *MLH1/EPM2AIP1* promoter region is not characterized by stably positioned

nucleosomes is at odds with an earlier report (Lin et al. 2007). This large nucleosome-free region may in part explain the multiple start sites for both the sense and antisense transcripts.

Materials and Methods

Cell culture

The AN3CA, KLE, and RL952 endometrial cancer cell lines were purchased from the American Type Culture Collection. The Ishikawa and MFE296 cell lines were gifts from Dr. Stuart Adler (Washington University School of Medicine, Department of Internal Medicine) and Dr. Pamela Pollock (Queensland University of Technology, Brisbane), respectively. The EM-E6/E7/TERT immortalized normal endometrial cell line was originally reported by Mizumoto and colleagues (Mizumoto et al. 2006) and provided by Dr. Pamela Pollock. Cell lines were grown as previously described (Byron et al. 2008; Dewdney et al. 2011).

Preparation of Nucleic Acids

Primary endometrial tumors and normal endometrium specimens were collected as part of IRB-approved studies (Washington University Medical Center Human Research Protection Office protocols HRPO-91-0507, -93-0828 and -92-0242). Genomic DNA from tumor tissues, normal endometrium, and cell lines was extracted using the DNeasy Tissue kit (Qiagen, # 69506). Total cellular RNA was prepared using the Trizol reagent (Invitrogen, # 10296-010). Human testes RNA was obtained from Stratagene (Agilent Technologies, Inc., # 540049).

5' RACE and RT-PCR

5' RACE was performed using the Roche 5'/3' RACE reagent, according to the manufacturer's instructions (Roche Applied Science, # 03353621001). Complementary DNA (cDNA) was generated using 1 µg total RNA and the QuantiTect Reverse Transcription Kit (Qiagen, # 205311). Conventional RT-PCR was performed using the primers below.

RT-PCR Primers	Forward	Reverse
MLH1	5' CTGGACGAGACAGTGGTGAA 3'	5' AGGGGCTTTCAGTTTTCCAT 3'
"Long" MLH1	5' AGGGACGAAGAGACCCAGCA 3'	5' GATCCCGGTGCCATTGTCT 3'
EPM2AIP1	5' TGTGGATGACGCCCAAAAGA 3'	5' CCTGCACGAGCAGCTCTCTCT 3'
"Long" EPM2AIP1	5' AGGTGCTTGGCGCTTCTCAG 3'	5' CCTGCACGAGCAGCTCTCTCT 3'
GAPDH	5' TGCACCACCAACTGCTTAGC 3'	5' GGCATGGACTGTGGTCATGAG 3'

Quantitative RT-PCR of transcripts and the GAPDH control was performed using SYBR Green (BioRad) methods and the same primers as for conventional RT-PCR. Relative expression levels were calculated using the delta-delta C_t method (Chiappinelli et al. 2010). All qPCR assays were performed in triplicate and analyses were repeated with new cDNA syntheses. Minus RT controls (reverse transcriptase negative cDNA synthesis reactions) were carried out for at least one sample per plate.

Nucleosome Positioning

Nucleosome positioning at the *MLH1/EPM2AIP1* bidirectional promoter was assessed by the methylase-based single promoter analysis assay (MSPA) as previously described in (Lin et al. 2007). Briefly, isolated nuclei were treated with M.SssI, an

enzyme that methylates all unprotected DNA. DNA was prepared from the treated cells and then bisulfite converted using a commercially available kit (EZ DNA Methylation Gold™ Kit, Zymo Research, # D5006). Cloning and sequencing was performed using standard methods.(Zighelboim et al. 2007b) Bisulfite PCR products were cloned using the PCR-2.1TOPO TA vector (Invitrogen, # K4510-20). A minimum of 5 clones for each cloning experiment sequenced using ABI Prism BigDye Terminator chemistry v1.1 (Applied Biosystems, # 4337451).

COBRA Assays

COBRA (Combined Bisulfite Restriction Analysis) was performed as previously described (Xiong and Laird 1997), using two rounds of amplification (nested PCR). PCR primers, amplicon sizes, and restriction digestions used were as follows.

Assay	Primers*	Amplicon Size	Restriction Digest Products
MLH1/EPM2AIP1	Rd1 For 5' gggaggTTaTaagagTagggT 3'		
promoter	Rd1 Rev 5' aAttctcaatcatctctttAataA 3'		
	Rd2 For 5' ggaggTTaTaagagTagggTTa 3'	569 bp	BsrBI- 374, 195 bp
	Rd2 Rev 5' catctctttAataAcattaActAAcc 3'		MluI- 357, 212 bp
ER promoter	Rd1 For 5' aggaggggggaatTagagaT 3'		
	Rd1 Rev 5' ccaAAAactAttAccttAccctA 3'		
	Rd2 For 5' gggggaatTagagaTaaaTagag 3'	235 bp	AciI- 147, 88 bp
	Rd2 Rev 5' cccaaaAaAcaActtccc 3'		

*Uppercase T indicates cytosine converted to thymine by bisulfite treatment.

Restriction fragments were resolved on 10% polyacrylamide gels, stained with ethidium bromide, and photoimaged with a UV camera (ImageStore 500 Version 7.12, White/UV Transilluminator; UVP, Inc.).

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Jones, P. A., P. W. Laird, et al. (1999). "Cancer epigenetics comes of age." Nat Genet 21(2): 163-167.

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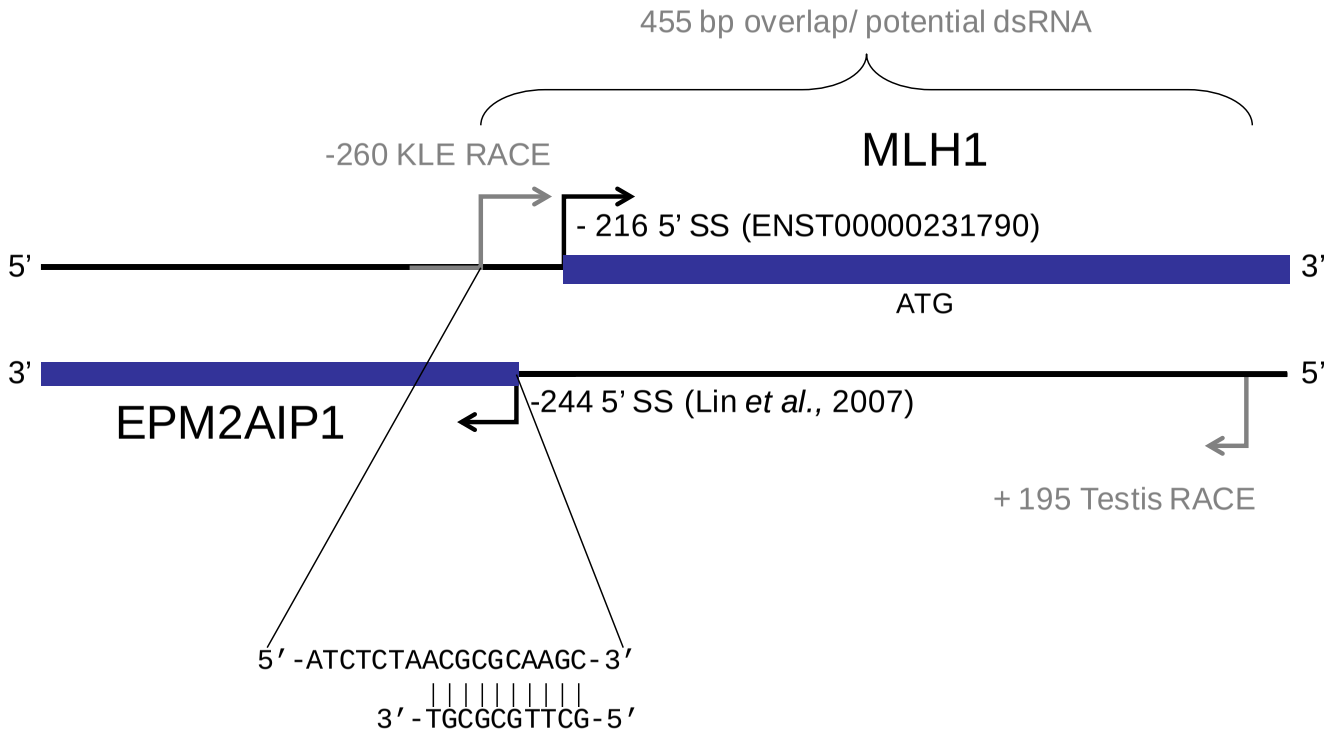

Figure 1. The shared promoter region of the *EPM2AIP1* and *MLH1* genes. Previously published transcription start sites are shown with black arrows. Grey arrows indicate the longest transcription start sites found by RACE. Positions of transcript start sites relative to the *MLH1* translation start site (ATG- 3:37035039-41,hg37.1- 2009) are given. The overlap between the longest *MLH1* start site based on RACE (-260) and the canonical *EPM2AIP1* transcript is shown.

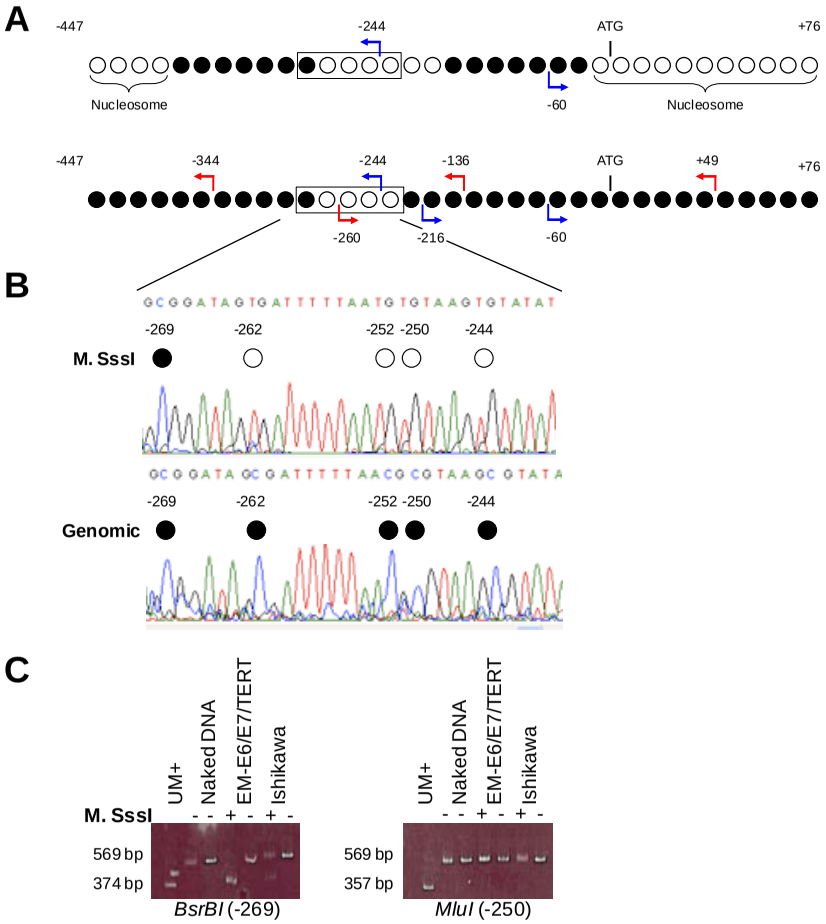

Figure 2. Absence of stably positioned nucleosomes at the *MLH1/EPM2AIP1* bidirectional promoter.

A. Nucleosome protection assay. The results of Lin *et al.* (top half) and our results (bottom half) are shown. The 35 CpGs in the 569 bp amplicon are shown as circles. Filled in circles represent methylated (unprotected) CpGs and white circles represent unmethylated (protected) CpGs. Canonical transcription start sites are shown as blue arrows. Novel transcription start sites are shown as red arrows. *MLH1* transcripts are shown on the bottom strand and *EPM2AIP1* transcripts are shown on the top strand. Positions of transcripts relative to the *MLH1* translation start site (ATG-3:37035039-41 in UCSC Genome Browser 37.1- 2009) are indicated above each site. All five clones for each of three cell lines (EM-E6/E7/TERT, Ishikawa, and KLE) gave the same result. Boxed region is expanded in Figure 2B.

B. Nucleosome positioning results for the boxed region in Figure 2A. Representative sequencing in M.SssI-treated EM-E6/E7/TERT cells compared to genomic DNA. Black circles represent methylated (unprotected) CpGs and white circles represent unmethylated (protected) CpGs. Position relative to the *MLH1* ATG is noted above each circle.

C. COBRA. Restriction enzyme digest with BsrBI *(CCGCTC)* and MluI *(ACGCGT)* of 569 bp amplicon from -464 to + 105 relative to the *MLH1* translation start site. UM+ (universally methylated DNA control), naked DNA, and DNA isolated from treated nuclei of three endometrial cancer cell lines (EM-E6/E7/TERT, Ishikawa, and KLE) are shown. +/- indicate whether the nuclei were treated with M.SssI.

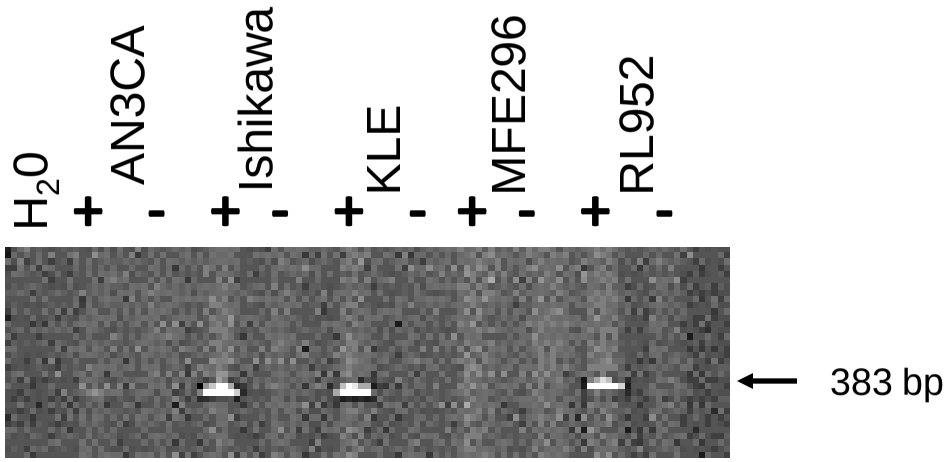

Figure S1. RT-PCR detection of *EPM2AIP1* in endometrial cancer cell lines.

H_20: PCR control, +/- : reverse transcriptase.

Figure S2: The *MLH1/ EPM2AIP1* bidirectional promoter.

A. Sequence showing transcription start sites and initiator codons. The *MLH1* start codon (ATG, chr3:37035039-37035041 hg37.1-2009) and *EPM2AIP1* start codon (TAC:ATG chr3:37034566-37034568) are shown in bold. *MLH1* 5' and *EPM2AIP1* 5' UTRs are highlighted in yellow and gray respectively. *EPM2AIP1* start sites (+49, -136, -244, and -344 relative to the *MLH1* ATG) are underlined. *MLH1* start sites (-60, -216, -260 relative to the ATG) are italicized. The four novel transcription start sites are indicated in blue text.

B. Location of primers used for bisulfite PCR. Round 1 Primers are italicized. Round 2 Primers are shown in red text. Upper case Ts indicate converted unmethylated cytosines and upper case CGs mark the location of methylatable Cs evaluable by bisulfite conversion.

Supplemental Figure 2: The *MLH1/ EPM2AIP1* bidirectional promoter.

A. The MLH1/ EPM2AIP1 bidirectional promoter chr3:37034381-37035154

TCGTGCTCAGCCTCGTAGTGGCGCCTGACGTCGCGTTCGCGGGTAGCTACGATGAGGCG

GCGACAGACCAGGCACAGGGCCCCATCGCCCTCCGGAGGCTCCACCACCAAATAACGCT

GGGTCCACTCGGGCCGGAAAACTAGAGCCTCGTCGACTTCCATCTTGCTTCTTTTGGGCG

EPM2AIP1 start codon (-471)

TCATCCA**CAT**TCTGCGGGAGGCCACAAGAGCAGGGCCAACGTTAGAAAGGCCGCAAGGG

GAGAGGAGGAGCCTGAGAAGCGCCAAGCACCTCCTCCGCTCTGCGCCAGATCACCTCAG

alternate EPM2AIP1 transcription start site (-344)

CAGAGGCACACAAGCC**CGG**TTCCGGCATCTCTGCTCCTATTGGCTGGATATTTCGTATTCC

alternate MLH1 transcription start site (-260)

CCGAGCTCCTAAAAACGAACCAATAGGAAGAGCGGACAGCG***ATC***TCTAACGCGCA**AGC**GC

EPM2AIP1 transcription start site (-244)

ATATCCTTCTAGGTAGCGGGCAGTA***GCC***GCTTCAGGGAGGGACGAAGAGACCCAGCAACC

MLH1 transcription start site (-216)

CACAGAGTTGAGAAATTTGACTGGCATTCAAGCTGTCCAATC**AAT**AGCTGCCGCTGAAGGG

alternate EPM2AIP1 transcription start site (-136)

TGGGGCTGGATGGCGTAAGCTACAGCTGAAGGAAGAACGTGAGCACGAGGCACTGAGGT

MLH1 transcription start site from Lin et al. (-60)

G***ATT***GGCTGAAGGCACTTCCGTTGAGCATCTAGACGTTTCCTTGGCTCTTCTGGCGCCAAA

MLH1 start codon (0) **alternate EPM2AIP1 transcription start site (+49)**

ATGTCGTTCGTGGCAGGGGTTATTCGGCGGCTGGACGAGACAGTGGT**GAA**CCGCATCGC

GGCGGGGGAAGTTATCCAGCGGCCAGCTAATGCTATCAAAGAGATGATTGAGAACTG

B. The bisulfite converted MLH1/ EPM2AIP1 bidirectional promoter chr3:37034381-37035154

tCGtgTtTagTTtCGtagtggCGTTtgaCGtCGCGttCGCGggtagTtaCGatgaggCGgCGaTagaT
TaggTaTagggTTTTatCGTTTtTCGgaggTtTTaTTaTTaaataaCGTtgggtTTaTtCGggTCGga
aaaTtagagTTtCGtCGaTttTTatTttgTttTttttgggCGtTatTTa**Tat**tTtgC*G**ggaggTTaTaagagTag***
*ggTT*aCGttagaaaggTCGTaaggggagaggaggagTTtgagaagCGTTaagTaTTtTTtTCGTtTtg
CGTTagatTaTTtTagTagaggTaTaTaagTTCGgttTCGgTatTtTtgTtTTtattggTtggatatttCGtatt
TTTCGagTtTTtaaaaaCGaaTTaataggaagagCGgaTagCGatTtTtaaCGCGTaagCGTatatTT
ttTtaggtagCGggTagtagTCGTttTagggagggaCGaagagaTTTagTaaTTTaTagagttgagaaatttg
aTtggTattTaagTtgtTTaatTaatagTtgTCGTtgaagggtggggTtggatggCGtaagTtaTagTtgaagga
agaaCGtgagTaCGaggTaTtgaggtgattggTtgaaggTaTttTCGttgagTatTtagaCGtttTTttggTtTtt
TtggCGTTaaa**atg**tCGttCGtggTaggggttattCGgCGgTtggaCGagaTagtggtgaaTCGTatCGC
GgCGggggaagttatTTagC*GgTTagTtaatgTtatTaaagagatgattgagaaTt*g

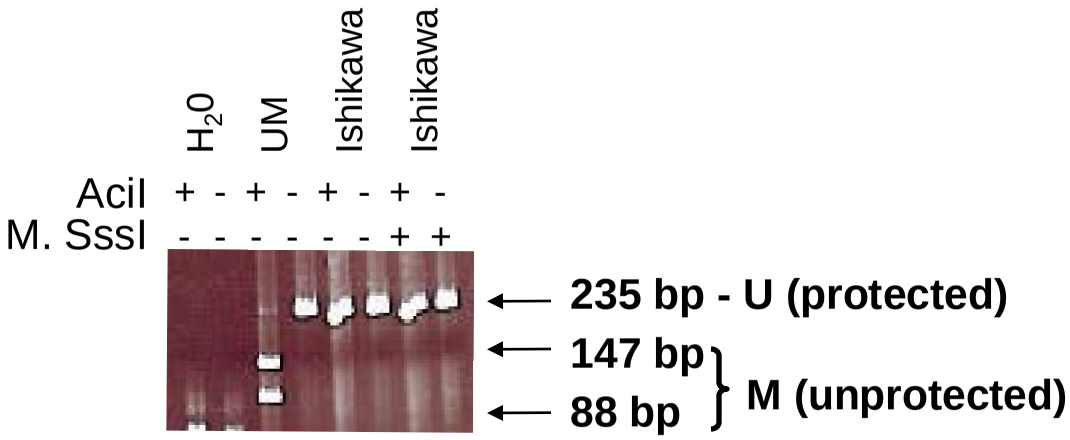

Figure S3. Demonstration of nucleosome protection at the *ESR1* promoter (Rocha *et al.*, 2005). AciI COBRA was performed for Ishikawa cells, with or without M. SssI. The 235 bp PCR product was digested with AciI; if methylated, this produced 147 and 88 bp bands.

M: methylated; U: unmethylated; UM : universally methylated control.

Table 1. MLH1 and EPM2AIP1 transcription start sites by 5' RACE.

	Ishikawa	KLE	Testis
EPM2AIP1 TSS	-244	-344	+195
MLH1 TSS	-60	-260	-60
Overlap	0 bp	0 bp	255 bp

Most abundant transcripts by 5' RACE in RNA isolated from the endometrial cancer cell lines Ishikawa and KLE and testis. Positions of transcription start sites (TSS) relative to the *MLH1* translation start site (ATG- 3:37035039-41, hg37.1-2009) are given.

Table 2. Summary of *MLH1* and *EPM2AIP1* transcripts in primary tumors and normal tissues.

	Transcripts			Promoter
	MLH1	**EPM2AIP1**	**Overlap**	**Methylation***
Endometrial cell lines				
AN3CA	-	-	No	M
MFE296	-	-	No	M
Ishikawa	+	+	Yes	U
KLE	+	+	Yes	U
RL952	+	+	Yes	U
EM E6/E7	+	+	Yes	U
Primary Tissues				
Endometrioid endometrial carcinomas				
1900T	-	+	No	M
2141T	-	-	No	M
2180T	-	-	No	M
2194T	-	+	No	M
2258T	-	+	No	M
1859T	+	+	Yes	U
2160T	+	+	No	U
2212T	+	+	Yes	U
2213T	+	+	Yes	U
2238T	+	+	Yes	U
2247T	+	+	Yes	M
2252T	+	+	Yes	M
2281T	+	+	Yes	U
2283T	+	+	No	U
2293T	+	+	Yes	M
2306T	+	+	Yes	U
2308T	+	+	Yes	U
2310T	+	+	No	M
Normal endometrium				
N-2018	+	+	Yes	ND
N-26	+	+	No	U
N-27	+	+	Yes	ND
N-28	+	+	No	ND
Testes				
normal tissue	+	+	Yes	ND

*COBRA
EC: endometrial carcinoma; M: methylated; U: unmethylated;
ND: not determined.

CHAPTER THREE: Reduced DICER1 elicits an interferon response in endometrial cancer cells

Foreword

Although DICER1's role as a tumor suppressor has been firmly established, the mechanisms by which loss of DICER1 contributes to tumorigenesis remain mostly unknown. I undertook a series of experiments to investigate the effect that reduced DICER1 has on short noncoding RNAs, microRNAs (miRNAs), and mRNA levels in tumorigenesis. I knocked down the DICER1 protein in endometrial cells using shRNAs and evaluated miRNA and mRNA expression levels as well as growth properties of cells with intermediate and long-term DICER1 knockdown.

DICER1 is essential for miRNA biogenesis. MiRNAs are first processed in the nucleus where the RNase III enzyme DROSHA cleaves the primary transcript (pri-miRNA) to cut out the precursor stem loop of about 70 nucleotides (pre-miRNA), which is then brought out of the nucleus by the RAN GTPase EXPORTIN-5. In the cytoplasm, the PAZ and RNase III domains of DICER1 cut out the mature miRNA, which is about 22 nucleotides long. One strand of the cleaved dsRNA enters the RISC (RNA-induced silencing complex) (Hammond 2005), where the Argonaute protein AGO2 associates with the miRNA and mRNA to inhibit translation or cleave the mRNA. The miRNA in the RISC enables the complex to associate with the 3' UTR of the target gene through imperfect complementarity between miRNA and mRNA (Sontheimer 2005). MiRNAs target up to 60% of human genes and are essential to development and normal biology. MiRNA profiles are fundamentally different in cancer cells, leading to altered gene expression (Lujambio and Esteller 2009).

Downregulation of DICER1 and other RNA interference (RNAi) pathway components is associated with transformation and tumorigenesis. In humans, germline

loss-of-function DICER1 mutations are associated with the inherited pleuropulmonary blastoma tumor susceptibility syndrome (Hill et al. 2009). Dicer1 acts as a haploinsufficient tumor suppressor in a Kras-driven mouse model of lung cancer (Kumar et al. 2007; Kumar et al. 2009). Two other key components of miRNA biogenesis, TARBP2 and XPO5, coding, respectively, for the TRBP and Exportin-5 proteins, are mutated in human tumors and affect tumorigenic properties of cancer cells (Melo et al. 2010; Melo et al. 2009). Although this chapter is focused on DICER1 function in endometrial cancers, it is worth noting that I did attempt to corroborate the report of frequent *TARBP2* mutation in endometrial cancer and contrary to the published report (Melo et al. 2009) failed to observe *TARBP2* mutations in primary tumors (Appendix B). Another group failed to replicate the reported rate of *TARBP2* mutations (Melo et al. 2009) in hereditary nonpolyposis colorectal cancer (Garre et al. 2010). At the Endometrial Cancer TCGA meeting April 9/10 2012 hosted at Washington University in St. Louis, the preliminary report for exome sequencing did not include *TARBP2* among the list of significantly mutated genes. *DICER1*, on the other hand, was frequently mutated with 12 of 49 grade 3 endometrioid cancers carrying somatic mutations. Low levels of DICER1 and DROSHA are associated with worse outcome in ovarian cancer (Merritt et al. 2008) and work from our group shows that low levels of DICER1 are associated with decreased time to recurrence in endometrial cancer (Zighelboim et al. 2011). In addition, conditional deletion of both Dicer1 and Pten in mouse fallopian tube causes an ovarian cancer that closely resembles human serous ovarian cancer (Kim et al. 2012).

The current data point to a haploinsufficient tumor suppressor role for DICER1 in solid tumors. However, except for isolated examples such as regulation of miR-200 in metastatic breast cancer (Martello et al. 2010), it is unclear why and how DICER1 acts as a tumor suppressor. Prompted by the clinical data on DICER1 levels in endometrial cancer patients (Zighelboim et al. 2011), I knocked down the DICER1 protein in endometrial cancer cell lines using shRNA delivered via lentiviral infection and stable selection. To determine the complete effects of DICER1 knockdown, I performed mRNA-sequencing (RNA-Seq) on control and DICER1 knockdown cells. This work, performed in collaboration with Dr. Michael Brent's laboratory (Center for Genome Sciences & Systems Biology, Washington University in St. Louis) allowed me to capitalize on contemporary genomic methods to characterize DICER1 knockdown cells. The most striking and somewhat unexpected result was a dramatic upregulation of transcripts involved in the interferon response. I validated the changes in interferon response gene transcript levels and showed that DICER1 knockdown causes a type I interferon response in endometrial cells. I went on to show that the interferon response is due to accumulation of pre-miRNAs in the cytoplasm. The precise relationship between reduced DICER1 activity, the type I interferon response and tumorigenesis remains to be determined. Possible mechanisms include alterations in STAT signaling, inflammatory responses and the activation of tumorigenesis via inflammation.

The following is a manuscript published in Molecular Cancer Research (March 2012) characterizing the interferon response in DICER1 knockdown endometrial cancer cells.

Reduced DICER1 elicits an interferon response in endometrial cancer cells

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Running title: Reduced DICER1 elicits an interferon response

Keywords: Endometrial cancer, DICER1, Interferon response, microRNA, RNA-Sequencing

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The authors declare that there are no conflicts of interest.

Abstract

DICER1 is essential for the generation of mature microRNAs (miRNAs) and other short noncoding RNAs. Several lines of investigation implicate DICER1 as a tumor suppressor. Reduced DICER1 levels and changes in miRNA abundance have been associated with aggressive tumor phenotypes. The global effects of reduced DICER1 on mRNA transcript abundance in tumor cells remain largely unknown. We used shRNA to stably knock down DICER1 in endometrial cancer cell lines to begin to determine how reduced DICER1 activity contributes to tumor phenotypes. DICER1 knockdown did not affect cell proliferation but caused enhanced cell migration and growth in soft agar. miRNA and mRNA profiling in KLE cells revealed overall decreases in miRNA levels and changes in the relative abundance of many mRNAs. One of the most striking changes in mRNA levels was the upregulation of interferon stimulated genes (ISGs), the majority of which lack known miRNA target sequences. IFNβ, a key upstream regulator of the interferon response, was significantly increased in DICER1 knockdowns in the AN3CA, Ishikawa, and KLE endometrial cancer cell lines and in the normal endometrial cell line EM-E6/E7/TERT. IFNβ secreted in media from KLE and EM-E6/E7/TERT shDcr cells was sufficient to activate an interferon response in HT29 cells. The reduced miRNA processing in DICER1 knockdowns was associated with increases in pre-miRNAs in the cytoplasm. Our findings suggest elevated pre-miRNA levels trigger the interferon response to double-stranded RNA. We thus report a novel effect of reduced DICER1 function in cancer cells.

Introduction

Endometrial cancer is the most common gynecological malignancy in the United States and approximately 15% of patients suffer from recurrent disease (Creutzberg et al. 2000; Siegel et al. 2011). Discovery of the molecular lesions contributing to endometrial tumorigenesis will provide opportunities for targeted therapies.

DICER1 is an RNASE III helicase necessary to process double-stranded RNA (dsRNA) in mammalian cells, the predominant form of which is microRNA (miRNA). Primary miRNAs (pri-miRNAs) are cleaved by the enzyme DROSHA into pre-miRNAs. Pre-miRNAs are transported out of the nucleus by EXPORTIN-5 and processed in the cytoplasm by DICER1 and accessory proteins. Mature miRNAs go with AGO proteins to pair imperfectly with the 3' UTRs of target mRNAs and either impede translation or degrade the mRNAs (Kim, Han and Siomi 2009). About 60% of human genes may be regulated post-transcriptionally by miRNAs (Friedman et al. 2009; Lewis, Burge and Bartel 2005). Given the key role of miRNAs in gene regulation it is not surprising DICER1, DROSHA, and other RNAi components have been implicated as “tumor suppressors” in solid tumors (Hill et al. 2009; Kumar et al. 2009; Melo et al. 2010; Melo et al. 2009; Merritt et al. 2008). Germline loss-of-function mutations in DICER1 are associated with the pleuropulmonary blastoma tumor susceptibility syndrome (Hill et al. 2009). The penetrance of inherited DICER1 mutations is, however, modest and it has been proposed that DICER1 is a haploinsufficient tumor suppressor (Slade et al. 2011). A recent report on somatic DICER1 mutations in nonepithelial ovarian tumors further supports the notion DICER1 is a haploinsufficient tumor suppressor (Heravi-Moussavi et al. 2011). DICER1 is an essential gene. The Dicer1 homozygous knockout mouse is

embryonic lethal (Bernstein et al. 2003; Kanellopoulou et al. 2005). Conditional deletion of Dicer1 in a mouse Kras lung cancer model caused homozygous knockout cells to die, but heterozygous tumors to be more aggressive than wild type tumors (Kumar et al. 2009), consistent with Dicer's role as a haploinsufficient tumor suppressor. Our group previously showed lower DICER1 mRNA levels in endometrial cancer are associated with recurrence and accelerated disease progression (Zighelboim et al. 2011).

The interferon response is a component of the innate immune response to pathogens such as RNA viruses. Viral dsRNA binding to Toll-like receptor 3 (TLR3) on the cell membrane or IFIH1 (MDA5), PKR, or RIG-1 in the cytoplasm triggers IRF3 and NFκB translocation to the nucleus and transcription of early genes, specifically IFNβ. Secreted IFNβ activates cell surface receptors by autocrine and paracrine means to induce activation of STAT1 and expression of interferon stimulated genes (ISGs). Next, IFNα genes are transcribed leading to downstream effects including global inhibition of translation and apoptosis (Alexopoulou et al. 2001; Li and Tainsky 2011; Platanias 2005; Wang and Carmichael 2004). Innate immunity and interferon responses in malignancies are context dependent and often paradoxical. An immune response may mediate tumor cell killing; interferons have been used to treat a variety of human cancers (Caraglia et al. 2009; Krejcova et al. 2009). However, inflammatory cytokines downstream of the interferon response have been linked to cellular transformation (Iliopoulos, Hirsch and Struhl 2009). Cellular senescence can trigger an interferon response (Novakova et al. 2010), but increases in ISGs such as ISG15 and IFI44 are prognostic for breast and lung cancer recurrence, respectively (Bektas et al. 2008; Lee et al. 2008). The interferon

response and how it impacts tumor behavior is likely determined by a complex and context dependent interaction of tumor cell specific effects and humoral responses.

Using short hairpin RNAs (shRNAs) we reduced DICER1 levels in endometrial cancer and normal cell lines by greater than 50%. mRNA and miRNA profiling studies revealed global perturbations in RNA levels. The most striking change observed was an increase in transcription of IFNβ and ISGs characteristic of an interferon response. We demonstrate that the interferon response in endometrial cells with reduced DICER1 results from accumulation of pre-miRNAs in the cell cytoplasm.

Materials and Methods

Cell culture

Four endometrioid endometrial cancer cell lines were investigated. AN3CA and KLE were purchased from the American Type Culture Collection. The Ishikawa cell line was a gift from Dr. Stuart Adler (Washington University School of Medicine, Department of Internal Medicine). The MFE296 cell line was kindly provided by Dr. Pamela Pollock (Queensland University of Technology, Brisbane) and the HT29 cell line was kindly provided by Dr. Loren Michel (Washington University). The EM-E6/E7/TERT cell line was originally reported by Mizumoto and colleagues (Mizumoto et al. 2006) and kindly provided by Dr. Pamela Pollock. Cell lines were grown as previously described (Byron et al. 2008) and authenticated as reported in (Dewdney et al. 2011).

Lentiviral transduction to create stable knockdowns

DICER1 and GSK3β knockdowns were created in AN3CA, EM-E6/E7/TERT, Ishikawa, KLE, and MFE296 cell lines as previously described (Ramsingh et al. 2010). Virus production and infections were carried out according to established methods (Stewart et al. 2003). DROSHA knockdown was created with virus kindly provided by Michael Kuchenreuther in Dr. Jason Weber's laboratory (Washington University). The short hairpin sequences used were:

shDcrA 5'-GCTCGAAATCTTACGCAAATA-3'

shDcrC 5'-GCCAAGGAAATCAGCTAAATT-3'

shDro2 5'- CGAAGCTCTTTGGTGAATAAT-3'

shDro4 5'- CCAGCGTCCATTTGTACTATT-3'

shGSK3β 5'-AGCAAATCAGAGAAATGAAC-3'

shLuc 5'CCCTCTGAACATGAGCATCAA-3'

shRFP 5'-TGCTAAGGAGTTTGGAGACAA-3' (Moffat et al. 2006)

The shDcr3 hairpin construct was designed by Sigma-Aldrich (St. Louis, MO).

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted utilizing the Trizol® method (Invitrogen, Carlsbad, CA). Nuclear and cytoplasmic fractions were prepared using the Norgen Biotek Cytoplasmic and Nuclear Purification Kit, according to the manufacturer's instructions (Norgen Biotek, Thorold, Ontario, Canada). RNA concentration was determined with the NanoDrop machine and software (Thermo Fisher Scientific, Wilmington, DE). Complementary DNA (cDNA) was generated using 1 µg total RNA and the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Quantitative RT-PCR of pre-miRNAs

and the DUSP6 control was performed using SYBR Green (BioRad) methods. The primers used are listed below.

Pre-microRNA Primers	Forward	Reverse
PRELET7D	5' TTTAGGGCAGGGATTTTGC 3'	5' TAAGAAAGGCAGCAGGTCGT 3'
PREMIR183	5' CGCAGAGTGTGACTCCTGTT 3'	5' TCGTGGATCTGTCTCTGCTC 3'
PREMIR450A	5' AAACTATTTTTGCGATGTGTTCC 3'	5' TGCAAAATGTCCCCAATACA 3'
DUSP6	5' CCCCTTCCAACCAGAATGTA 3'	5' TGCCAAGAGAAACTGCTGAA 3'

Expression of DICER1, DROSHA, IFI44, IFI44L, IFI6, IFIH1, IFNβ1, MX1, and OAS3 mRNAs, and LET7B, LET7D, MIR107, MIR183, MIR450A, MIR542 pri-miRNAs was assessed by quantitative RT-PCR TaqMan® assays (Applied Biosystems, Foster City, CA) and the Applied Biosystems 7500 Fast real-time PCR system and software. Human β-actin was used as the endogenous control as previously described (Poliseno et al. 2010). Expression of let-7c, miR-10a, miR-16, miR-29b, and miR-126b mature miRNAs was assessed by quantitative TaqMan® microRNA assays (Applied Biosystems, Foster City, CA) and the Applied Biosystems 7500 Fast real-time PCR system and software. U6 was used as the endogenous control (Melo et al. 2009). Relative expression levels were calculated using the delta-delta C_t method (Chiappinelli et al. 2010).

All qPCR assays were performed in triplicate and then repeated with new cDNA synthesis. Minus RT controls (reverse transcriptase negative cDNA synthesis reactions) were carried out for at least one sample per plate.

MicroRNA profiling

KLE and AN3CA cell lines were subjected to global microRNA profiling with Nanostring™ technology (Seattle, WA). 749 miRNAs were evaluated using the nCounter Human miRNA Panel CodeSet®.

RNA-Sequencing

PolyA+ RNA was purified from total RNA using the Dynabeads mRNA Purification Kit (Invitrogen, Carlsbad, CA). Each sample was resuspended in 2 µl of 100 mM zinc acetate and heated at 60°C for 3 minutes to fragment the RNA by hydrolysis. The reaction was quenched by the addition of 2 µl volumes of 200 mM EDTA and purified with an Illustra Microspin G25 column (GE Healthcare). First strand cDNA was made using hexameric random primers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), and the product was treated with *E. coli* DNA ligase, DNA polymerase I, and RNase H to prepare double stranded cDNA using standard methods. cDNA libraries were end-repaired with a Quick Blunting kit (New England BioLabs, Ipswich, MA) and A-tailed using Klenow exo- and dATP. Illumina adapters with four base barcodes were ligated to cDNA and fragments ranging from 150-250 bp were selected using gel electrophoresis. Libraries were enriched in a 10-cycle PCR with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) and pooled in equimolar ratios for multiplex sequencing. Single read, 36-cycle runs were completed on the Illumina Genome Analyzer IIx.

Sequenced reads were aligned to the human reference sequence (hg19 / NCBI Build 37.1) using Tophat (Trapnell, Pachter and Salzberg 2009). Reads that aligned

uniquely to the reference sequence were considered for gene expression quantification with Cufflinks (Trapnell et al. 2010). Gene expression was normalized using the Cufflinks provided option for quartile normalization.

Western blots

Western blot analysis of DICER1 was performed as previously described (Byron et al. 2008; Chiappinelli et al. 2010). GAPDH was used as a loading control. Antibodies used were as follows: rabbit anti-DICER1 H212 (sc-30226, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:200), goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, 1:2500), rabbit anti-DROSHA (ab12286, Abcam, 1:750), mouse anti-GAPDH (NB615, Novus Biologicals, Littleton, CO, 1:4000), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:5000), rabbit polyclonal anti-STAT3 H-190 (sc-7179, Santa Cruz Biotechnology, 1:200), rabbit anti-phospho-STAT3 Ser727 (9134, Cell Signaling Technology, 1:500), rabbit anti-phospho-STAT3 Tyr705 EP2147Y (04-1059, Millipore, 1:500). Band intensities were quantified using the program ImageJ (National Institutes of Health).

ELISA

ELISA was performed with the *Verikine-HS*^{TM} Human Interferon Beta Serum ELISA kit (PBL Interferon Source).

Cell proliferation, wound healing and colony formation assays

For cell proliferation assays 100,000 cells were plated in 6-well plates in triplicate. Cells were trypsinized and counted using trypan blue staining and a hemocytometer every 24 hours for 120 hours.

Wound healing assays were performed using AN3CA and KLE cells. Cells were grown to confluency then scratched down the middle of the plate. Cells were photographed every 4-6 hours for up to 96 hours (GE Healthcare IN Cell Analyzer 2000). The area of the "scratch" (area not filled in) was determined for each time point.

Growth of endometrial cancer cell lines in soft agar was determined as follows: First a base layer of 0.5% agar was plated in media, then a top layer of 0.3% agar in media with 30,000 cells per well was plated in 6-well dishes. After 4 weeks, cells were stained with crystal violet and imaged. Colonies were counted.

Interferon stimulation

PolyI:C (Invitrogen, Carlsbad, CA) was diluted into the media of cells or transfected using the Dharmafect reagent (Thermo Fisher Scientific, Waltham, MA).

Let-7 inhibition

Let-7 inhibition was performed as previously described (Robertson et al. 2010). The CHECK-2 vector with the let-7b target site cloned into the 3' UTR was a kind gift from Annaleen Vermeulen (Thermo Fisher Scientific).

Results and Discussion

Stable knockdown of DICER1

We used shRNA and lentiviral infection to stably knock down DICER1 in four endometrial cancer cell lines and a transformed normal endometrial epithelium cell line; AN3CA, Ishikawa, KLE, MFE296, and EM-E6/E7/TERT. Of five hairpins tested, two (shDcrA and shDcrC) resulted in substantial reductions in DICER1 protein levels (Figure 1A). Knockdowns were generated with shDcrA and shDcrC hairpins and shLuc and shRFP controls. Stable knockdown of DICER1 (<50% of controls) persisted for up to 30 passages for all cell lines, with the exception of MFE296, for which knockdown was unstable (Figure 1B and data not shown). In KLE, DICER1 was reduced to ~10% of controls, suggesting that sufficient shRNA processing can occur with substantially reduced DICER1 activity (Figure 1B). An additional shRNA targeting the DICER1 3' UTR (shDcr3) was used in KLE cells leading to greater than 50% reduction in DICER1 protein levels (Supplemental Figure 1).

Cell doubling times were similar in DICER1 knockdowns and control cells (Supplemental Figure 2). Cell migration was increased in AN3CA shDcr cells (Figure 1C) but no difference was seen in KLE shDcr cells. The Ishikawa and EM-E6/E7/TERT cells could not be evaluated in the cell migration assay because they did not grow as monolayers on glass slides (Supplemental Figure 2). In both KLE and EM-E6/E7/TERT, shDcr cells formed more colonies in soft agar than control cells (Figure 1D and Supplemental Figure 2). These *in vitro* assays for cancer-associated phenotypes suggest that reduced DICER1 in endometrial cancer cells can result in increased cell motility and

anchorage independence. This increased cell motility was previously shown in breast cancer cell lines and attributed to a reduction in miR-200 and upregulation of genes involved in epithelial mesenchymal transition (Martello et al. 2010).

We profiled miRNAs globally in shDcr cells to identify reductions in particular miRNAs that might contribute to cancer-associated phenotypes. Nanostring^{TM} miRNA profiling studies in AN3CA cells as well as KLE knockdowns and controls revealed 133 of 749 miRNAs interrogated were expressed at appreciable levels. When the average levels of miRNA expression in the two KLE knockdowns were compared with the KLE shLuc control, 64% of the 133 miRNAs showed reduced levels in the knockdowns (Supplemental Table 1 and Figure 2A). miR-200 was not expressed in endometrial cancer cell lines (Supplemental Table 1) so could not be responsible for the cancer-associated phenotypes mentioned above. We observed clear increases in a subset of miRNAs (Figure 2A) as previously described in colon cancer cells with reduced DICER1 protein (Melo et al. 2009). Similar effects on miRNA abundance were seen with both knockdowns in the KLE cell line; however, the magnitude of changes in miRNA levels seemed greater in the shDcr3 knockdown than in the shDcrA knockdown. For the shDcrA knockdown, 76/133 miRNAs were less than in shLuc control (average $\log_2$ fold change -.502). With the shDcr3 knockdown, 95/133 miRNAs were less abundant than in the shLuc control with an average -.828 fold change ($\log_2$). KLE shDcrA cells were evaluated at passage 15 and shDcr3 cells at passage 5. The more pronounced effect on miRNA levels seen with the shDcr3 knockdown could be attributable to more efficient targeting of DICER1 with the shDcr3 construct, greater reduction in DICER1 protein

levels at earlier passages, or compensation for DICER1 as shDcrA cells were passaged (*e.g.* stabilization of miRNAs).

qRT-PCR of five miRNAs previously shown to be expressed in normal and cancerous endometrium (Lu et al. 2005) confirmed the relative abundance reported by Nanostring™ profiling in AN3CA and KLE cells. qRT-PCR in pooled endometrial cancers confirmed the rank order of five miRNAs reported by Nanostring™ (Supplemental Table 1 and Figure 2B). miR-16 was the highest expressed of the five miRNAs by profiling and qRT-PCR. miR-29b was the lowest expressed by both profiling and qRT-PCR. qRT-PCR confirmed the Nanostring™ profiling and the functional reduction of DICER1 processing, as five mature miRNAs were significantly decreased in KLE shDcr cells (Figure 2C). pri-miRNAs, the initial miRNA transcripts that are processed by DROSHA, were not significantly altered, showing that effects on mature miRNAs are due to a defect in miRNA processing, not transcription (Figure 2D).

DICER1 knockdown effects on mRNA expression: upregulation of interferon response genes

To further assess the functional consequences of DICER1 knockdown, we profiled mRNA expression using RNA-Sequencing (RNA-Seq) in KLE cells (Trapnell et al. 2010). Out of 9935 genes expressed in KLE by RNA-Seq, 584 were upregulated more than twofold in shDcr cells (Supplemental Table 2). Gene Ontology analysis showed enrichment for functions associated with response to virus or other pathogens when the upregulated gene set was analyzed (Supplemental Table 3). A striking number of

interferon stimulated genes (ISGs) were upregulated (17 of the 28 present in the RNA-Seq data set) (Figure 3A). The probability of 17 genes at random being upregulated in this set is quite low ($p<1.2 * 10E-14$). qRT-PCR confirmed upregulation of six out of seven ISGs tested (Figure 3B). Similar increases in six ISG transcripts were seen in independent knockdowns, providing biologic validation of the effect of reduced DICER1 in KLE cells (shDcrA and shDcr3, Figure 3B; shDcrC, data not shown). To explore a possible mechanism for interferon response activation in shDcr cells, we evaluated mRNA levels of transcription factors that might target ISGs. No transcription factors predicted to bind upstream of the activated ISGs were overexpressed in shDcr cells by RNA-Seq (data not shown). Direct miRNA effects on ISG transcript levels were ruled out as the ISGs have no known targets in their 3' UTRs for miRNAs expressed in KLE (Supplemental Table 4).

This increase in ISGs appeared to be a canonical interferon response (Platanias 2005; Wang and Carmichael 2004). To determine if the upstream IFNβ gene was upregulated and activating ISGs, we assessed IFNβ mRNA and protein levels in DICER1 knockdowns. RNA-Seq did not detect expression of *IFNβ1* in any of the cell lines investigated, as would be expected for a low abundance transcript. *IFNβ1* transcript was, however, detectable using qRT-PCR. Two shDcr hairpins caused upregulated *IFNβ1* transcript compared to shLuc (Figure 3C). The control shRFP hairpin did not significantly upregulate *IFNβ1* while the shDcrC hairpin did (data not shown). shRNA alone does not trigger the interferon response (Gondai et al. 2008; The RNAi Consortium (TRC) 2010). We tested the possibility that knockdown of a cell-essential gene might

activate the interferon response by measuring *IFNβ1* transcript levels in KLE shGSK3β cells. Because neither the shGSK3β nor the control shLuc and shRFP hairpins activate the interferon response, we conclude that the interferon response seen is a DICER1-specific effect. The *IFNβ1* transcript was upregulated at least twofold in DICER1 knockdowns in two additional endometrial cancer cell lines, AN3CA and Ishikawa, and an immortalized normal endometrial cell line, EM-E6/E7/TERT (Figure 3C). The increase in *IFNβ1* transcript due to reduced DICER1 led to increased IFNβ protein levels in the media of KLE shDcr cells (Figure 3D). A similar increase in IFNβ protein was observed in EM-E6/E7/TERT shDcr cell media (Figure 3E), showing that reduced DICER1 leads to increased IFNβ expression in both normal and cancer endometrial cell lines.

DICER1 knockdown causes a canonical interferon response

As some cancer cell lines have abrogated interferon responses (Li and Tainsky 2011), we postulated that activation of the interferon response in KLE might be an artifact of a mutated interferon response pathway. However, the interferon response is intact in the KLE endometrial cancer cell line. Transfection with polyI:C, a dsRNA analog, activated the interferon response (Figure 4). *IFNβ1* transcript levels rose rapidly and peaked at six hours, with concomitant increases in ISGs (Figure 4B). In addition, a cytoplasmic receptor sensing dsRNA (IFIH1) was overexpressed in KLE cells with low DICER1 (Figure 3B). The interferon response in KLE shDcr cells upregulates the same

genes as that in KLE cells transfected with polyI:C, albeit with a smaller magnitude (Figure 3B, 4B).

We used media transfer to determine the biological activity of secreted IFNβ protein in the media of shDcr cells. HT29 colon carcinoma cells exhibit a strong interferon response (Chelbi-Alix et al. 1991; The RNAi Consortium (TRC) 2010), activating IFNβ and ISGs when polyI:C is either added to the cell culture media or transfected (Figure 5A, Supplemental Figure 3). Transfer of media from KLE shDcr3 cells to HT29 cells stimulated an interferon response, while shRFP cell media did not (Figure 5B). Media from EM-E6/E7/TERT shDcr cells similarly stimulated 4/6 ISGs (Figure 5C), indicating DICER1 knockdown causes an interferon response in both normal and cancerous endometrial cells. When media from KLE shDcr3 cells was transferred to KLE shRFP cells, no appreciable interferon response was seen (Supplemental Figure 4). This difference could be due to the relative strength of interferon responses in KLE and HT29 cells (Figure 4B, 5A). KLE shDcr3 cells in culture reflect long-term, continual IFNβ stimulation and KLE controls may not respond to a short stimulus with conditioned medium as HT29s do.

Pre-miRNAs build up in the cytoplasm and may cause an interferon response

To determine a mechanism for activation of the interferon response, we focused on a candidate miRNA. Members of the let-7 miRNA family, known for their tumor-suppressive roles (Johnson et al. 2005; Kumar et al. 2008), were significantly reduced in shDcr cells (Figure 2C and Supplemental Table 1). The let-7 family downregulates the cytokine IL6, which when activated leads to phosphorylation of STAT3 by NFκB,

resulting in an inflammatory response linked to cellular transformation (Iliopoulos, Hirsch and Struhl 2009). To determine whether let-7 was responsible for the interferon response, we inhibited let-7 in KLE cells (Supplemental Figure 5A). No increase in *IFNβ1* was observed when let-7 was inhibited (Supplemental Figure 5B). Thus, let-7 alone is not responsible for the activation of the interferon response.

dsRNA (usually viral) activates the interferon response in mammalian cells. Our studies suggest a possible mechanism for interferon response upregulation by reduced DICER1. Mature miRNAs are too short (averaging 22 nt) to elicit the interferon response through viral dsRNA sensors (Kumar and Carmichael 1998; Wang and Carmichael 2004). Their precursor molecules, pre-miRNAs, are larger (~70 nt) and as such could be recognized by cytoplasmic dsRNA sensors IFIH1, PKR, or RIG-1 (Yang et al. 2001). We determined the subcellular location of pre-miRNAs in control and shDcr cells (Figure 6A). Pre-let7d, pre-miR183, and pre-miR450a were increased in the cytoplasmic fraction of shDcr cells. The corresponding mature miRNAs were decreased in shDcr cells (Supplemental Table 5), reflecting reduced DICER1 processing that results in buildup of pre-miRNAs and reduction of processed, mature miRNAs. Buildup of pre-miRNAs in the cytoplasm may elicit the canonical interferon response. To determine the specificity of this effect, we knocked down DROSHA in the KLE cell line (Figure 6B). Knockdown of DICER1 or DROSHA causes a reduction in mature miRNAs because of reduced processing. However, only DICER1 knockdown results in a buildup of pre-miRNAs. Lower levels of DROSHA did not trigger an interferon response as indicated by *IFNβ1* levels (Figure 6C). Interestingly, DROSHA knockdown appeared to decrease *IFNβ1* transcript levels. This could point to a role for pre-miRNAs in modulating the interferon

response. Because DROSHA knockdown results in fewer pre-miRNAs (due to reduced pri-miRNA processing), this finding demonstrates that pre-miRNA buildup, rather than a decrease in mature miRNAs, causes the interferon response.

Our data point to the accumulation of pre-miRNAs in the cytoplasm as the trigger for the interferon response we observed in cells with reduced DICER1 activity (Figure 7). This is unlikely to be an effect of the system used; while siRNAs may activate the interferon response in mammalian cells (Sledz et al. 2003), shRNAs do not (The RNAi Consortium (TRC) 2010). The immune response to dsRNA is highly conserved, with organisms such as plants and fungi enacting an RNAi-based response to viral RNA (Choudhary et al. 2007). Prior studies demonstrating that overexpression of pre-miRNAs can activate the interferon response in zebrafish (Dang et al. 2008) and that so-called "long hairpin RNAs" similarly activate the innate immune response (Gantier, Baugh and Donnelly 2007) are consistent with our findings that build-up of pre-miRNAs elicits an interferon response. It remains unclear whether or how the interferon response is related to cancer phenotypes such as increased cell migration and growth in soft agar we observed in our DICER1 knockdown cells.

While DICER1 homologs are required for the immune response in many eukaryotes including *D. melanogaster* (Ding 2010), several lines of investigation indicate DICER1 may not be necessary for the interferon response in mammals (Wang and Carmichael 2004). Li and Tainsky evaluated the effects of increased DICER1 in Li-Fraumeni fibroblasts with and without an intact interferon response and showed that overexpression of DICER1 can activate the interferon response (Li and Tainsky 2011). The difference in responses seen in fibroblasts in which DICER1 levels were increased

and epithelial cells with reduced DICER1 could reflect cell-type specific differences or potentially opposing functional consequences of excess and deficient DICER1 activity in mammalian cells. DICER1 knockdown cell lines have increased susceptibility to influenza virus infection, implying DICER1 is necessary for recognizing viral dsRNA (Matskevich and Moelling 2007). However, the cancer cell lines we studied were not challenged by virus. In the absence of viral infection, pre-miRNAs have a stimulatory effect on the interferon response. The relationship between alterations in the miRNA processing machinery and the mammalian interferon response may point to a previously unrecognized role for DICER1 in tumorigenesis.

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Supplemental Information

Supplemental Tables S1 and S2 are available online at http://mcr.aacrjournals.org/.

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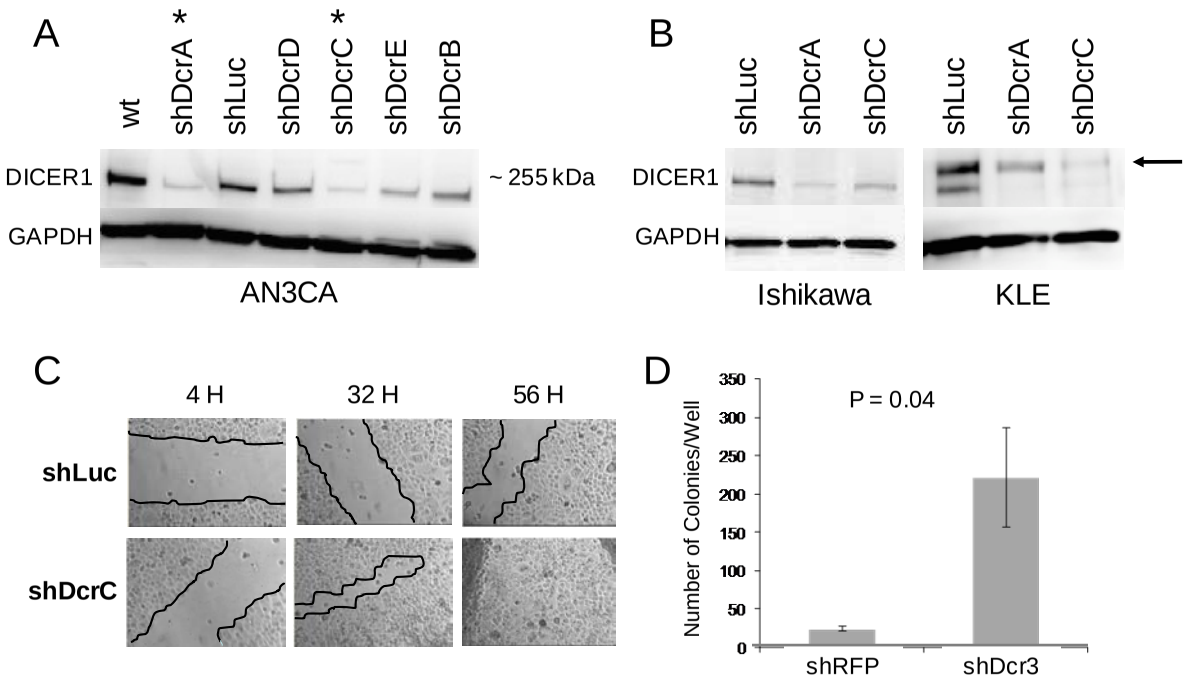

Figure 1. Knockdown of DICER1 in endometrial cell lines. Western blot analysis of DICER1 expression in A) AN3CA (passage 5) with shRNA A-E against DICER1 or Luciferase control. * denotes hairpins showing greatest degree of knockdown. B) Representative knockdowns in additional cell lines, Ishikawa (passage 16) and KLE (passage 26). Arrow denotes nonspecific band of higher molecular weight above the ~255 kDa DICER1 band in KLE. C) Representative wound healing assay in AN3CA cells. shLuc control and shDcrC knockdown at 4, 32, and 56 hours. Black lines denote wound outlines. D) Increased soft agar colony formation in KLE cells with DICER1 knockdown. Results are one representative experiment of two performed in triplicate. KLE cells were plated and growth in soft agar was assessed by staining with crystal violet after 30 days.

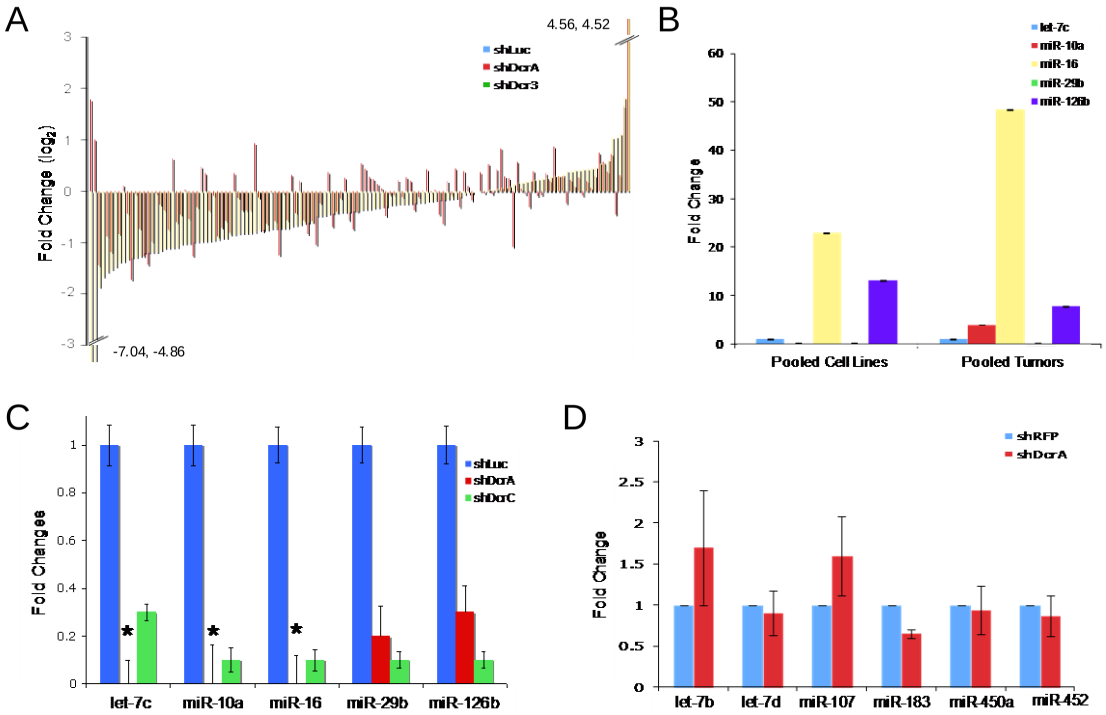

Figure 2. miRNA expression in DICER1 knockdown cells. A) Nanostring™ miRNA profiling of 133 expressed miRNAs in the KLE cell line. B) Relative abundance of 5 miRNAs in endometrial cancer cell lines and tumors by Taqman qRT-PCR assays, normalized to U6 reference gene. RNA from 4 cell lines (AN3CA, KLE, Ishikawa, MFE296) and 4 tumors was pooled and converted to cDNA. C) Levels of 5 miRNAs in control and shDcr KLE cells by Taqman qRT-PCR assays, normalized to U6 reference gene. * denotes miRNAs that were undetectable in shDcrA cells. D) Pri-miRNAs in KLE cells measured by Taqman qRT-PCR and normalized to β-Actin reference gene. Fold change shDcrA/shRFP is plotted on the y-axis. For all qRT-PCR experiments, error bars are SD (data shown is average of two experiments performed in triplicate).

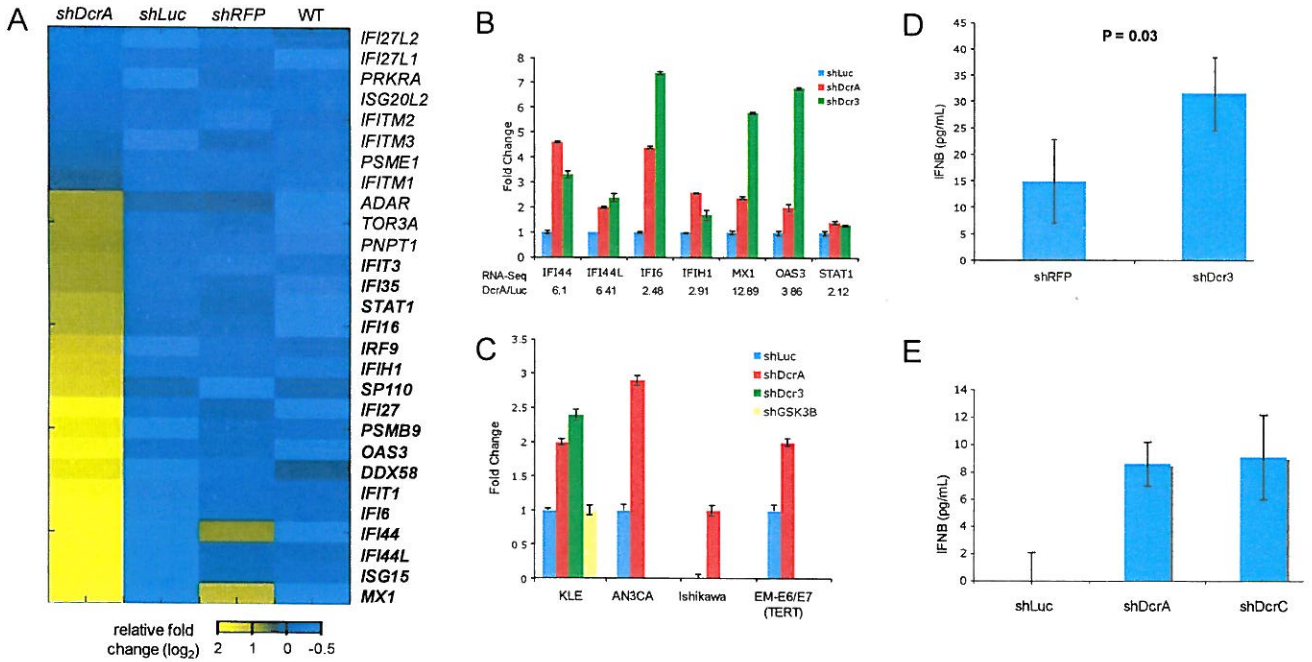

Figure 3. Upregulation of interferon stimulated genes (ISGs) in shDcr cells. A) RNA-Seq heat map for ISGs. Genes indicated in bold revealed a fold change greater than two relative to the average expression of the three controls, shLuc, shRFP, and WT (untransfected KLE cells). B) qRT-PCR validation of ISGs upregulated in RNA-Seq. Fold change shDcr/shLuc is plotted on y-axis. C) *IFNβ1* transcript levels are increased in shDcr cells in four cell lines. shDcr3 and shGSK3β were only performed in the KLE cell line. For all qRT-PCR experiments, mRNA levels were assessed using TaqMan qRT-PCR assays, normalized to β-Actin reference gene. Error bars are SD (data shown is average of two experiments performed in triplicate). IFNβ protein levels in cell culture media are increased in KLE (D) and EM-E6/E6/TERT (E) shDcr cells. IFNβ was assessed by ELISA. One representative experiment of three performed in triplicate is shown. Error bars represent SEM. Significance was determined by t-test.

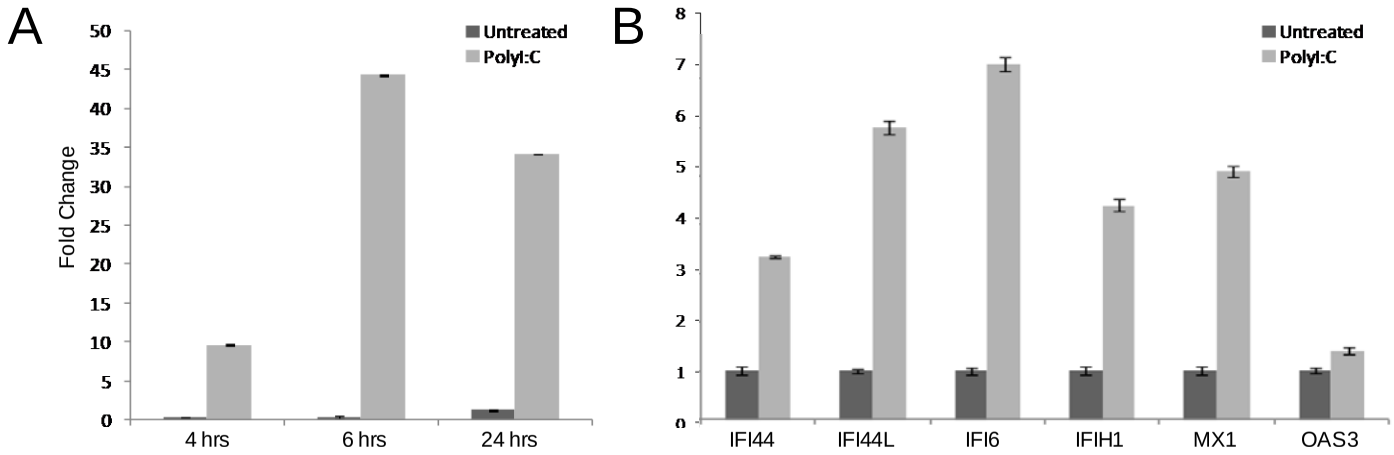

Figure 4. Interferon response in the KLE cell line. (A) Upregulation of *IFNβ1* in polyI:C-treated KLE 4, 6 and 24 hours post transfection. (B) ISGs upregulation 24 hours post polyI:C transfection. Transcript abundance was measured by Taqman qRT-PCR assays, normalized to β-Actin reference gene. Error bars are SD (data shown is average of two experiments performed in triplicate).

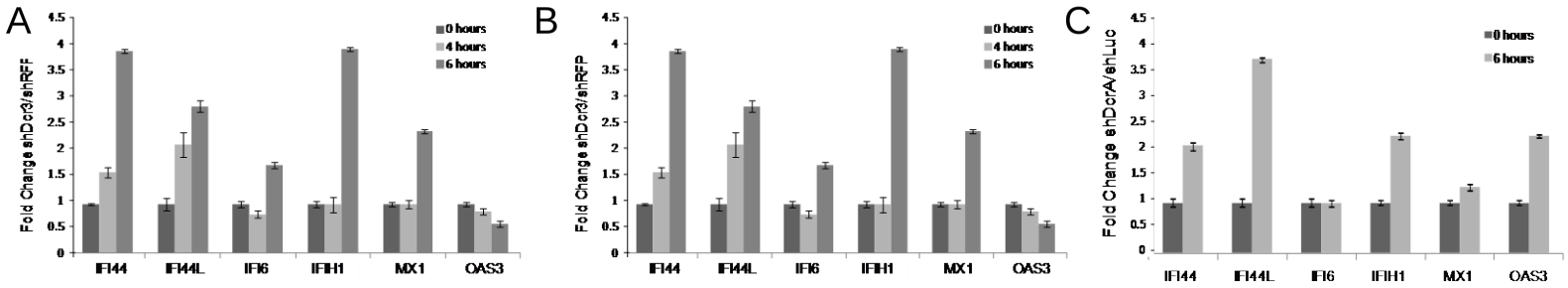

Figure 5. KLE and EM-E6/E7/TERT shDcr media stimulate an interferon response in HT29 cells. A) PolyI:C stimulates a canonical interferon response in HT29 cells. PolyI:C was diluted into media (PolyI:C M) or transfected (PolyI:C T) into HT29 cells and RNA was isolated. B) shDcr3 but not shRFP media stimulates a canonical interferon response in HT29 cells. Media was transferred from KLE shRFP and shDcr3 cells to HT29s and RNA was isolated. Y-axis represents fold change of shDcr3/shRFP media. C) shDcrA but not shLuc media stimulates a canonical interferon response in HT29 cells. Media was transferred from EM-E6/E7/TERT shLuc and shDcrA cells to HT29s and RNA was isolated. Y-axis represents fold change of shDcrA/shLuc media. For all qRT-PCR experiments, transcript abundance was measure by Taqman qRT-PCR assays, normalized to β-Actin reference gene. Error bars are SD (data shown is average of two experiments performed in triplicate).

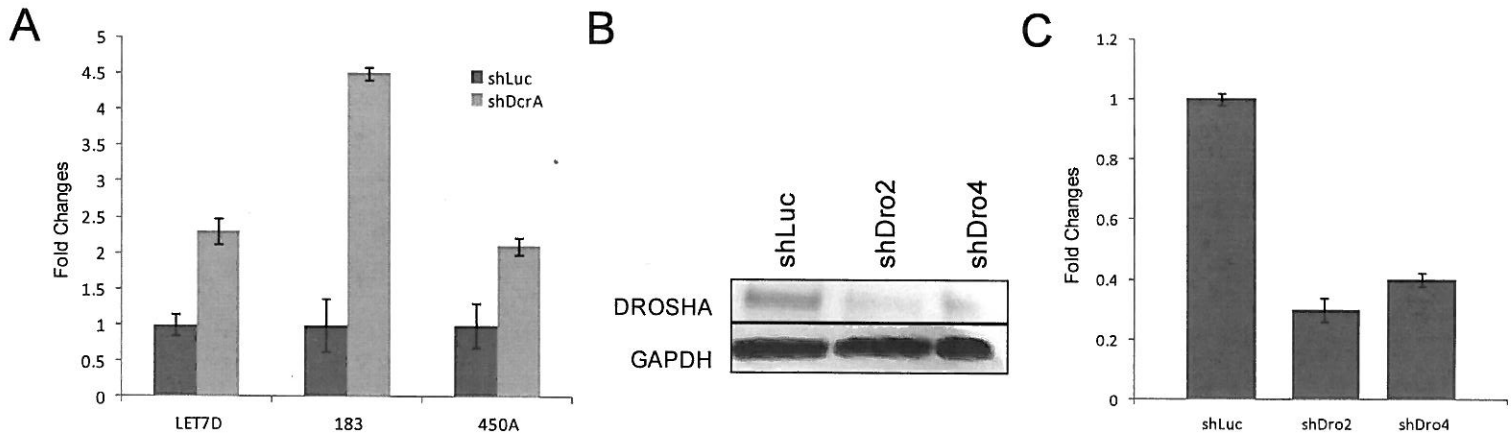

Figure 6. Pre-miRNAs build up in the cytoplasm of shDcr cells. A) Pre-miRNAs are increased in shDcr cytoplasm. qRT-PCR was performed on LET7D, MIR183, and MIR450A pre-miRNAs in shLuc and shDcrA cytoplasmic RNA, using DUSP6 mRNA as an endogenous control. B) KLE whole cell lysates were probed for DROSHA using GAPDH as a reference gene. One representative experiment (of two). C) *IFNβ1* transcript is not increased in shDrosha cells. *IFNβ1* was measured by Taqman qRT-PCR and normalized to β-Actin reference gene. For all qRT-PCR experiments, error bars are SD (data shown is average of two experiments performed in triplicate).

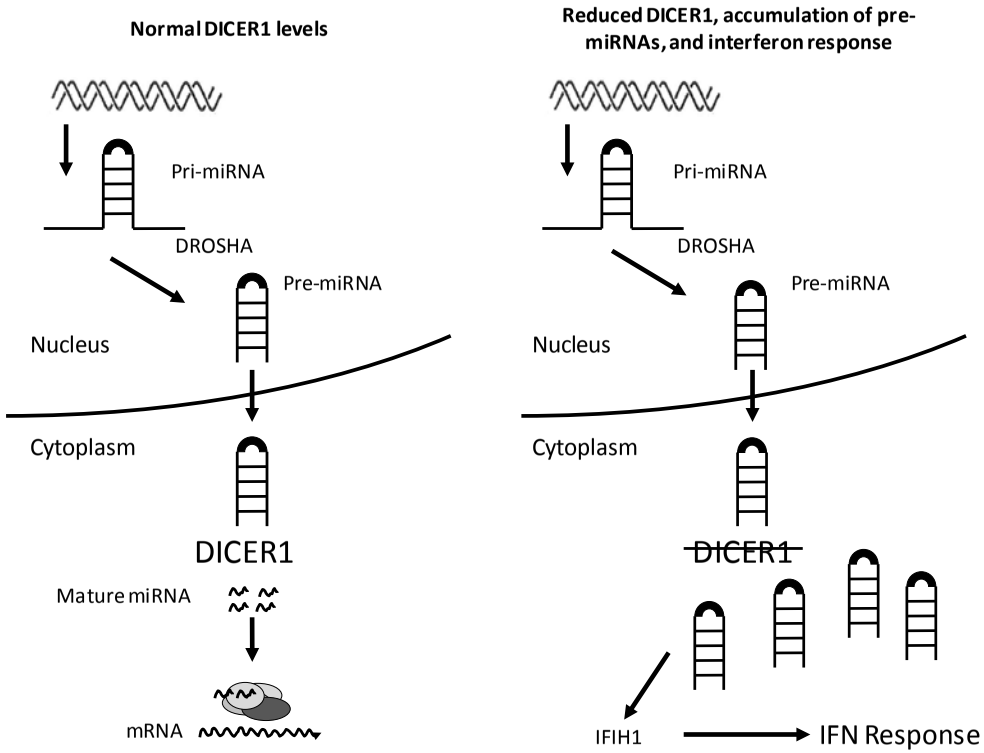

Figure 7. Model for pre-miRNA buildup and interferon stimulation. In cells with normal levels of DICER1 (left panel), pre-miRNAs are processed to mature miRNAs and mRNAs are targeted for silencing. In cells with reduced DICER1 (right panel), pre-miRNA processing is inhibited and pre-miRNAs build up in the cytoplasm. This dsRNA can be sensed by cytoplasmic sensors such as IFIH1 and activate the interferon response.

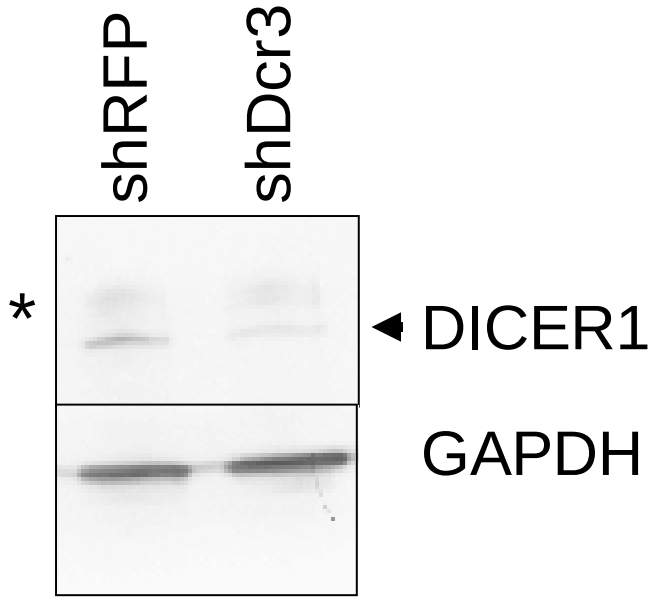

Figure S1. Western blot analysis of DICER1 expression in KLE with shDcr3 shRNA against DICER1 or shRFP control. *Nonspecific band of higher molecular weight above the ~255 kDa DICER1 band in KLE.

A.

	Cell Doubling	Wound Healing	Soft Agar
EM E6/E7/TERT	ND	*	Increased
AN3CA	No effect	Increased	No effect
Ishikawa	No effect	*	**
KLE	No effect	No effect	Increased

B.

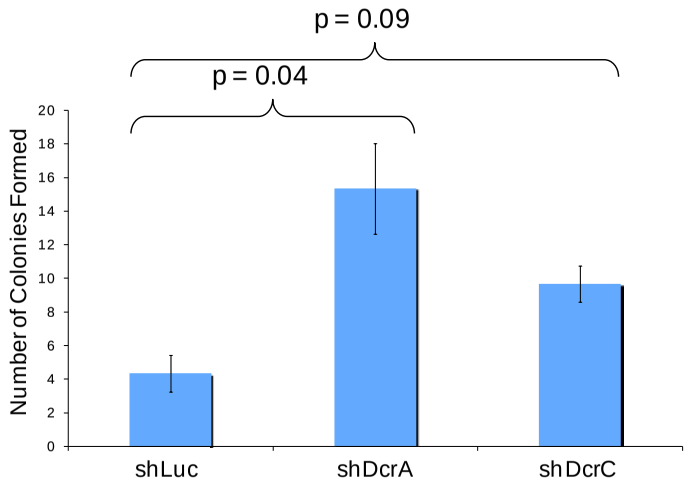

Figure S2. A) Summary of results for cell doubling, wound healing and soft agar assays. ND = no data. *Cells do not form appropriate monolayers on plates for wound healing analysis. ** Cells do not form colonies in soft agar. **B)** Increased soft agar colony formation in EM-E6/E7/TERT cells with DICER1 knockdown. Cells were stained and colonies counted 30 days after plating.

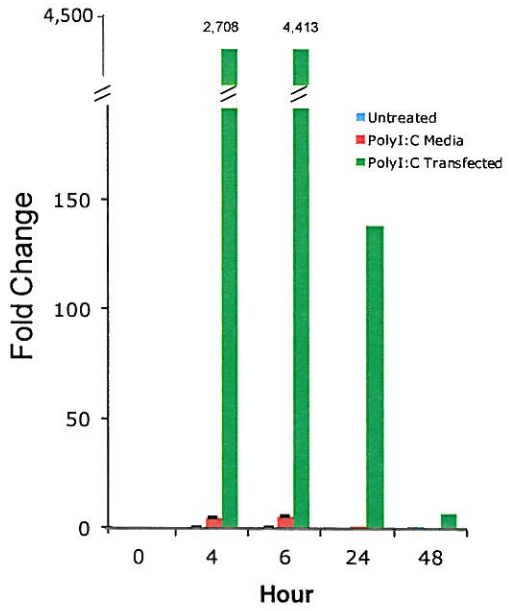

Figure S3. The interferon response in HT29 cells. PolyI:C was diluted into the media or transfected into HT29 cells. RNA was isolated and Taqman qRT-PCR performed for IFNβ1, using β-Actin as a reference gene. Error bars are SD (data shown is the average of two experiments performed in triplicate).

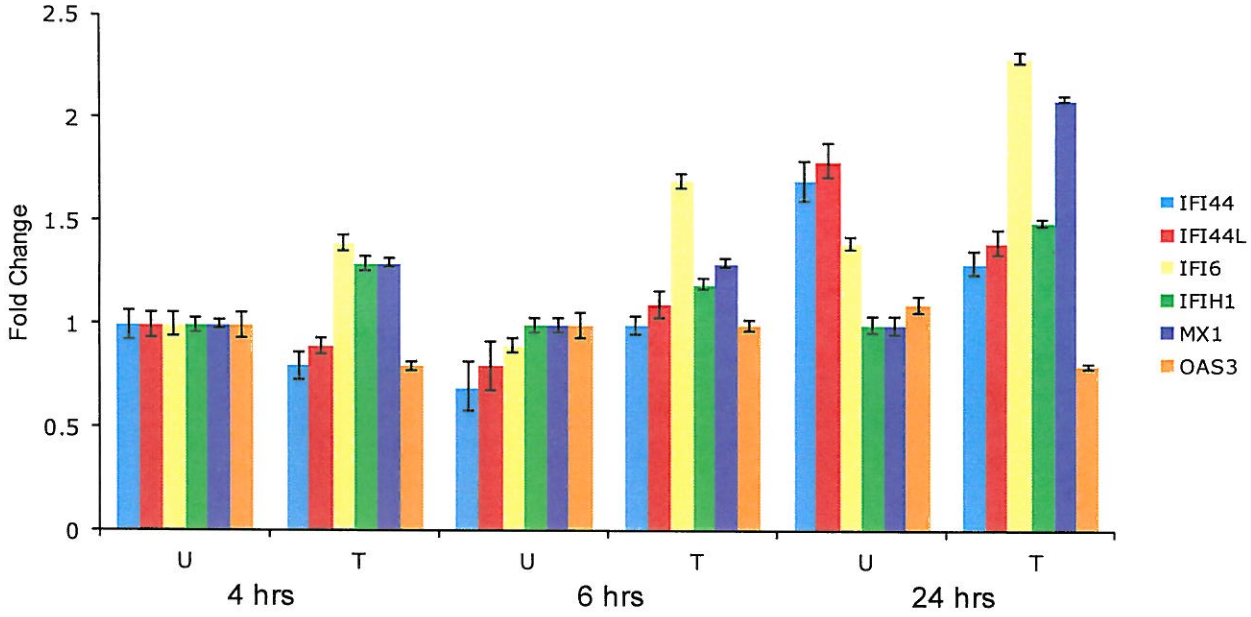

Figure S4. shDcr3 media does not stimulate the interferon response in shRFP KLE cells. RNA was isolated and Taqman qRT-PCR performed for six genes, using β-Actin as a reference gene. Error bars are SD (data shown is the average of two experiments performed in triplicate). U = untreated, T = treated.

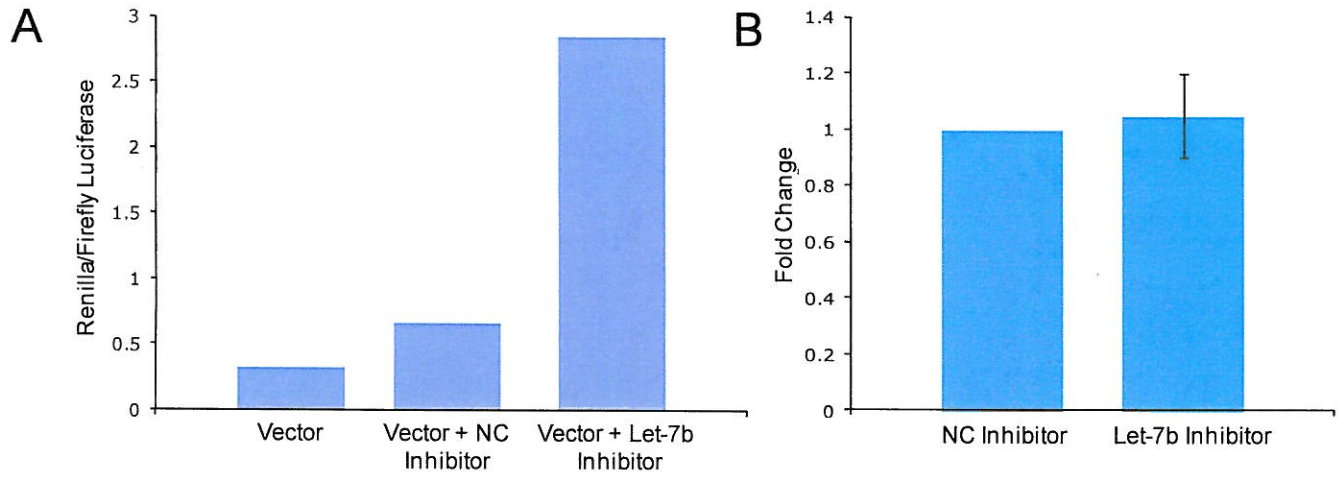

Figure S5. Reduced let-7 family miRNA is unlikely to explain the interferon response. A) To determine the effectiveness of the let-7b inhibitor, the let-7b luciferase vector was transfected into KLE cells along with either negative control (NC) or let-7b inhibitors. The ratio of firefly to renilla (control) luciferase was measured for each combination. B) Negative control or Let-7b inhibitor was transfected into KLE cells. RNA was isolated and Taqman qRT-PCR performed for *IFNβ1*, using β-Actin as a reference gene. Error bars are SD (data shown is the average of two experiments performed in triplicate).

Supplemental Table 3. Gene Ontology enrichment for genes with increased expression in shDcr cells (from Table S2).

GO Category	Function	P value	Genes
GO:0009615	response to virus	2.95E-05	(ifi16 mx1 irf9 ifi44 isg15 ddx58 stat1 ifih1)
GO:0051707	response to other organism	0.0053	(ifi16 mx1 irf9 ifi44 isg15 ddx58 stat1 ifih1)
GO:0009607	response to biotic stimulus	0.00837	(dnajb2 ifi44 isg15 ddx58 stat1 ifih1 mx1 ifi16 irf9)
GO:0005740	mitochondrial envelope	0.00837	(c3orf31 bid sdha vdac2 bad htra2 fahd1 sfxn5 kmo ndufa5 timm8b)
GO:0031966	mitochondrial membrane	0.0154	(c3orf31 bid sdha vdac2 bad fahd1 sfxn5 kmo ndufa5 timm8b)
GO:0005739	mitochondrion	2.33E-05	(bid sdha mrpl54 bad htra2 gstz1 timm8b decr1 c3orf31 vdac2 casp8 aass oxr1 fahd1 sfxn5 rsad1 kmo ndufa5 ifi6 pecr)
GO:0005062	signal transducer activity	0.0144	(sp110 stat1)
GO:0006351	transcription, DNA-dependent	0.0144	(zkscan5 sap30l tarbp2 meox1 elf1 sp100 slc2a4rg c10orf137 e2f5 tsc22d1 znf673 snapc3 ybx2 zhx3 znfx1 l3mbtl2 znf687 xrcc6 parp14 psip1 znf684 gon4l polr3h stat1 arntl2 sp110 meis1 znf143 scmh1 pms1 ssx1 epas1 znf33a itgb3bp rnf2 ifi16 irf9 twist1 gtf2e1)
GO:0008632	apoptotic process	0.0144	(bad bid casp8 ifi6 stat1)
GO:0008219	cell death	0.0144	(bid casp8 atg12 stat1 ifih1 itgb3bp bcar1 bad htra2 mx1 ifi16 pla2g6 pik3r2 ifi6 pecr)
GO:0051704	multi-organism process	0.0154	(ifi44 isg15 ddx58 stat1 ifih1 mx1 ifi16 irf9 col16a1)
GO:0006355	regulation of transcription, DNA-dependent	0.0154	(zkscan5 sap30l tarbp2 meox1 elf1 sp100 slc2a4rg c10orf137 e2f5 tsc22d1 znf673 snapc3 ybx2 zhx3 znfx1 l3mbtl2 znf687 xrcc6 parp14 psip1 znf684 gon4l stat1 arntl2 sp110 meis1 znf143 scmh1 pms1 ssx1 epas1 znf33a itgb3bp ifi16 rnf2 irf9 twist1 gtf2e1)
GO:0043170	macromolecule metabolic process	0.019	(abhd5 sdha psmb9 h2afy2 ngly1 tarbp2 xpc meox1 fgfr2 poll c10orf137 ephb1 stk19 e2f5 znf673 ybx2 zhx3 svil man2b1 nudt1 l3mbtl2 xrcc6 rps12 psip1 rnps1 znf684 b3gnt1 aarsd1 camk2b ap4m1 znf143 rplp1 scmh1 dnajb2 rpusd2 pstk ppil6 csk aass epas1 anapc1 p4ha2 znf33a hist1h2bc itgb3bp ifi16 ap2m1 herc6 ehmt1 rnf123 zkscan5 sap30l elf1 dnajb14 sp100 slc2a4rg c1r dtl ppil2 tsc22d1 casp8 atg4b snapc3 trpt1 nagk znf687 znfx1 parp14 cpvl ptp4a3 rpl38 polr3h gon4l isg15 amtl2 atg12 stat1 fkbp9 rec8 cdc14a sp110 htra2 meis1 map4k2 adpgk rpl19 pms1 galt ssx1 rnf2 irf9 vrk3 gapdh ppap2a twist1 naga gtf2e1)

There were no GO categories significantly ($p < 0.05$) enriched for genes with decreased expression in shDcr cells (from Table S2). Very general categories (i.e., "cytoplasm" or "biological process") were omitted.

Analyzed using GOstat: Find statistically overrepresented Gene Ontologies within a group of genes (Beissbarth T and Speed TP. Bioinformatics, 6.2004; 20(9): 1464-1465.)

Supplemental Table 4: MiRNAs predicted to target IFNβ and six interferon response genes

Gene	Prediction Algorithm		
	MIRanda	**PicTar**	**TargetScanHuman**
IFNβ	4266	None	No conserved
IFI44	9, 27a, 27b, 133a, 133b, 204, 211	None	4753-3p
IFI44L	1, 19a, 19b, 23a, 23b, 31, 98, 124, 129-5p, 132, 133a, 133b, 136, 140-5p, 143, 145, 185, 186, 192, 200b, 200c, 203, *205*, 206, 212, 215, 219-5p, 326, 370, 429, 494, 495, 505, 506, 544, 599, 613	None	*205**
IFI6	330-5p, 371-5p, 485-5p, 590-3p, let7a, let7b, let7c, let7d, let7e, let7f, let7g, let7I	None	4778-5p
IFIH1	186, 223	None	No conserved
MX1	141, 197, 200a, 204, 205, 211, 223	None	No conserved
OAS3	9, 24, 103, 107, 124, 138, 140-5p, 148a, 148b, 149, 182, 183, 185, 185, 199a-5p, 199b-5p, 203, 204, 211, 214, 217, 299-3p, 301a, 329, 342-3p, 361-5p, 362-3p, 378, 384, 422a, 454, 485-5p, 491-5p, 506, 542-3p	*138*	*138**

* miRNAs common to two or more algorithms are italicized. Neither miR-138 nor miR-205 are expressed at detectable levels in KLE or AN3CA endometrial cancer cell lines.

CHAPTER FOUR: Effect of Reduced DICER1 on DNA Methylation in Endometrial Cancer Cells

Foreword

Having established a stable DICER1 knockdown in endometrial cancer cell lines that reduced DICER1 protein to levels comparable to what is seen in primary tumors and had physiological effects (Chapter Three), I undertook a series of experiments to assess the effect of DICER1 depletion on transcriptional silencing, specifically DNA methylation. The RNAi system is involved with heterochromatin formation in the yeast *S. pombe*; this is conserved in other organisms such as flies and worms. Although the role of RNAi in chromatin structure has not been fully established in vertebrates, several components of the RNAi machinery (AGO2, DICER1) have been localized to the nucleus in mammalian cells (Rimel et al. 2012; Weinmann et al. 2009). Small RNAs complementary to the promoter regions of genes can silence or activate transcription, depending on the gene (Hawkins et al. 2009; Li et al. 2006; Morris et al. 2004), and miRNAs complementary to promoter regions can induce transcriptional silencing (Kim et al. 2008). HCT116 colon cancer cells with mutated DICER1 exhibit a loss of methylation and a gain of expression at methylated promoters of genes (Ting et al. 2008).

Targeted analysis of CpG islands frequently methylated in endometrial cancer

One of the first experiments undertaken was to determine if reduced DICER1 and changes in small RNAs could be involved in *MLH1* transcriptional silencing. I used a highly quantitative approach (Pyrosequencing) to assess methylation in the MLH1 5' region, comparing DICER1 knockdowns and controls. Two cell lines in which *MLH1* is heavily methylated and *MLH1* is not expressed (AN3CA and MFE-296) and two in which *MLH1* is unmethylated and expressed (Ishikawa and KLE) were evaluated. No

change in methylation was observed in the four endometrial cancer cell lines when DICER1 was knocked down (Figure 1). Figure 1 presents an average of methylation at four different CpG sites in the *MLH1* regulatory region. Although not entirely conclusive, the stable methylation pattern at the *MLH1* regulatory region in DICER1 knockdowns after many cell doublings (>15 passages) did not support a role for DICER1 and small RNAs in determining *MLH1* promoter methylation and transcriptional silencing.

Evaluation of the methylation status of four other CpG islands (*RSK4, SESN3, SFRP1, TITF1*) revealed no changes based on COBRA, with the possible exception of *RSK4* for which subtle variation was evident (Figures 2-3). Note that in Figure 3, the digestion pattern of unmethylated (higher) and methylated (lower bands) is the same in control and shDcr cells. However, in Figure 2, there does seem be more DNA in the unmethylated (higher band) for shDcr cells in the Ishikawa and KLE cell lines. This is a subtle difference but is interesting as *RSK4* is located on the X chromosome, one copy of which is completed methylated in females (i.e. in uterine cancer cell lines). *RSK4* methylation is frequently altered in cancers (Dewdney et al. 2011). There are contrasting reports on DICER1's role in X inactivation; DICER1 may be involved in X chromosome silencing (Nesterova et al. 2008; Ogawa, Sun and Lee 2008), but other research shows that the X chromosome can be inactivated in the absence of Dicer (Kanellopoulou et al. 2009).

Genome-wide methylation analysis

I moved on to a series of experiments to assess the effects of DICER1 depletion on DNA methylation throughout the genome. Next-Generation Sequencing methods for

profiling DNA methylation continue to evolve. However, when I began the genome-wide methylation studies I chose the MBD-Seq method (Invitrogen MethylMiner^{TM}), in which a methyl-binding-domain protein is used to pull down methylated DNA. This DNA from the pull down is used to generate libraries for Next-Generation Sequencing. Again, this work was a collaboration with Dr. Michael Brent's laboratory (The Center for Genome Sciences & Systems Biology, Washington University in St. Louis). Prior to library construction I demonstrated that I was able to greatly enrich for methylated DNA by assessing a test region (the *MLH1* promoter) using DNA from one cell line with methylation at the *MLH1/EPM2AIP1* bidirectional promoter, and one lacking methylation at this region (Figure 4). In Figure 4, AN3CA is completely methylated, Ishikawa is unmethylated, and a 50/50 mix is about half methylated, as would be expected. When I performed Methylminer^{TM} on the 50/50 mix, only the methylated DNA was recovered (far right of Figure 4, "Cap"). However, sequencing of the isolated KLE DNA (three controls and two DICER1 knockdowns, the same group of cell lines subjected to RNA-Sequencing in Chapter Three) did not provide deep enough coverage to make conclusions about the amount of DNA methylation in control and DICER1 knockdown cells. I moved on to a different method, MeDIP, collaborating with Dr. Ting Wang's laboratory (The Center for Genome Sciences & Systems Biology, Washington University in St. Louis). All of the library preparation data analyses were performed by Dr. Wang and his laboratory members.

MeDIP is similar to chIP-Seq in that it uses an antibody specific to methylated DNA to pull it down, then subjects the DNA to Next-Generation Sequencing. From the MeDIP analysis by Ting Wang, I attempted to validate methylation changes at several

CpG islands that showed a loss of methylation in the shDcr cells and a gain of expression in the mRNA-Sequencing (Chapter Three). However, none of these were methylated by COBRA (Table 1). Several interesting findings came from the initial analysis of the KLE shLuc and shDcrA MeDIP libraries. First, the 20 miRNAs that showed the greatest decreases in abundance based on miRNA profiling (see Chapter Three) appeared to have lost methylation at their gene bodies in shDcr cells relative to controls (Figure 5). While promoter methylation is associated with loss of transcription, gene body methylation is associated with active transcription. If small RNAs were targeted back to their complementary DNA to initiate chromatin silencing and DNA methylation, the gene body is the region that would be affected. I undertook studies to validate the MeDIP data using alternative methods, focusing on six miRNAs that were decreased in the miRNA profiling as well as one control miRNA, and assessed methylation at miRNA gene bodies (Table 2). Unfortunately, while MeDIP showed a decrease of methylation at their gene bodies in shDcr cells, I could not confirm this with several methods, including COBRA, bisulfite cloning, and pyrosequencing. In addition to assessing the methylation status of the miRNAs in the DICER1 knockdown and control lines, I quantified expression, focusing on the primary transcripts (pri-miRNAs). The expression levels for all seven of these were unchanged, implying that any changes in methylation did not affect transcription (Chapter 3, Figure 2). Figures 6 and 7 show representative COBRA, bisulfite cloning, and pyrosequencing at the *MIRLET7D* gene. The second CpG, assessed by both COBRA and bisulfite cloning, is 100% methylated in both cell types, but the first shows a very small decrease in shDcr cells. This decrease was within the margin of error, so I concluded that these results were false positives.

To obtain results with fewer false positives, I used the MRE/MeDIP combination method for sequencing the methylome with Dr. Ting Wang's laboratory. The following details my observations on DNA methylation in shDcr and control cells using this method, considered a "state-of-the-art" approach. This work is currently in progress, and my involvement will be limited to the next two months. However, what follows is a draft of our observations thus far.

First draft for consideration for submission to PloS One

Effects of reduced DICER1 on DNA methylation in endometrial cancer cells

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The authors declare that there are no conflicts of interest.

Abstract

Background/Aims: DNA methylation is a stable epigenetic silencing mark in mammalian cells. Cytosine methylation is associated with transcriptional silencing at gene promoters, but with transcriptional activation at gene bodies. In addition, DNA methylation serves to silence much of the repetitive DNA in the human genome. The RNA interference (RNAi) machinery is involved in establishment and maintenance of chromatin and transcriptional silencing, specifically at repetitive elements, in *S. pombe* and other organisms. There is some evidence for RNAi involvement in transcriptional silencing in mammalian cells. The DICER1 protein, a master regulator of RNAi in mammals, as it is necessary to process microRNAs, is downregulated in many solid tumors and has been characterized as a tumor suppressor. As DNA methylation also changes in cancers, including a global loss of methylation (especially at repetitive regions), there could be a connection between the RNAi system and DNA methylation in human cells. We sought to determine the effect of DICER1 depletion on DNA methylation in endometrial cancer cells.

Methods: A stable knockdown of DICER1 was established in the KLE endometrial cancer cell line. MRE and MeDIP were used to map methylation in shLuc (control) and shDcrA (knockdown) cells. Findings were validated by COBRA, pyrosequencing, bisulfite cloning, and reduced representation bisulfite sequencing.

Results: We found that genome-wide, the effects of DICER1 on DNA methylation were minimal. However, DICER1 depletion led to a specific loss of methylation genome-wide at the Alu family of repetitive elements. Along with other recent evidence, this finding points to a role for DICER1 in processing and perhaps silencing of Alu elements.

Introduction

The epigenetic modification DNA methylation is critical for transcriptional silencing of tissue-specific genes, repressing repetitive regions in the genome, silencing the second X chromosome in mammalian females, and regulating imprinted genes. As methylated DNA replicates, the maintenance methyltransferase enzyme DNMT1 adds methyl groups to the new strand of DNA as it is synthesized. Methyl-CpG-binding proteins bind methylated DNA and establish a closed chromatin structure. This limits access to DNA by transcription factors or transcription machinery and results in transcriptional silencing (Allis et al. 2007).

A hallmark of cancer is the disruption of normal regulation of epigenetic processes and the packaging of genes in chromatin. Cancer cells exhibit hypermethylation of certain CpG islands, long stretches of CpG dinucleotides that remain unmethylated to protect the promoter region of a gene from genetic mutations or epigenetic silencing. Aberrant methylation of these CpG islands may silence tumor suppressor genes (Jones et al. 1999). Cancer cells undergo a global decrease in DNA methylation, turning on genes that are silenced in normal cells, de-repressing transposable elements, and contributing to genomic instability. However, it is unclear what mediates these changes (Lujambio and Esteller 2009).

The DICER1 protein controls another aspect of epigenetic regulation, small RNAs. DICER1 is required for miRNA processing (Hammond 2005; Kim, Han and Siomi 2009). miRNAs are crucial for developmental and tissue-specific regulation at the translational level, and deregulation of specific miRNAs has been implicated in several diseases. Downregulation of DICER1 and other RNA interference (RNAi) pathway

components is associated with transformation and tumorigenesis (Bahubeshi, Tischkowitz and Foulkes 2011; Grelier et al. 2009; Heravi-Moussavi et al. 2011; Karube et al. 2005; Sand et al. 2010; Slade et al. 2011). The current data point to a haploinsufficient tumor suppressor role for DICER1 in solid tumors. However, except for isolated examples such as regulation of miR-200 in metastatic breast cancer (Martello et al. 2010), it is unclear why and how DICER1 acts as a tumor suppressor.

Small RNAs may also be involved in transcriptional gene silencing (TGS). In plants, yeast, and protozoa, genes are transcriptionally silenced by complementary small RNAs, processed by DICER1 and targeted to the DNA by the Argonaute proteins. Recent findings suggest that this process may be conserved in humans. Evidence for DICER1 and small RNA involvement in mammalian TGS includes the nuclear localization of proteins involved in small RNA processing and targeting, such as AGO2 (Weinmann et al. 2009). Recent work suggests that DICER1 miRNA processing may also be at work in the nucleus (Giles, Ghirlando and Felsenfeld 2010; Sinkkonen et al. 2010). Research from our group has shown that DICER1 localizes to the nucleus. ERK phosphorylates DICER1 in *C. elegans* (Arur et al. 2009) and human cells, prompting nuclear localization (Rimel et al. 2012). At present phosphorylated DICER1's function in the nucleus is unknown.

Data from mammalian cells implicates DICER1 in the control of CpG island methylation. In HCT116 colon cancer cells, a set of genes that normally had hypermethylated CpG islands showed demethylation and expression when the cells were transfected with a DICER1 hypomorph (Ting et al. 2008). Transfecting human cells with dsRNA complementary to the promoter regions of specific genes can epigenetically

silence or activate, depending on the gene. This process requires AGO2 (Hawkins et al. 2009; Li et al. 2006; Morris et al. 2004). This work implies that the function of small RNAs in human cells is analogous to their function in plant cells, but more work is necessary to determine whether it is a common mechanism or an exception. In addition, the miRNA *miR-320* has been shown to initiate transcriptional gene silencing (TGS) of the *POLR3D* gene (Kim et al. 2008). This study demonstrated a novel role for miRNAs, which were previously thought to be involved in post-transcriptional gene silencing (PTGS): either degrading mRNA or inhibiting translation.

We sought to determine DICER1's effect on methylation and thus whether small RNAs are involved in transcriptional silencing in human cells. We knocked down DICER1 in endometrial cancer cell lines using shRNAs and assessed changes in global methylation using Next Generation Sequencing methods, MeDIP and MRE. shRNA to DICER1 was delivered via lentiviral infection and stable selection. Sustained, long-term reduction in DICER1 levels achieved using lentivirus short hairpin RNAs should mimic the reduced expression that is a feature of many human cancers and is associated with adverse outcomes (Chiappinelli et al. 2012; Zighelboim et al. 2011). We found that while DICER1 knockdown had little effect on genic regions, repetitive elements were affected differently, with the Alu family in particular losing methylation.

Methods

Cell culture

Five endometrioid endometrial cancer cell lines were investigated. AN3CA, KLE, and RL952 were purchased from the American Type Culture Collection. The Ishikawa cell line was a gift from Dr. Stuart Adler (Washington University School of Medicine, Department of Internal Medicine). The MFE296 cell line was kindly provided by Dr. Pamela Pollock (Queensland University of Technology, Brisbane). The EM-E6/E7/TERT immortalized normal endometrial cell line was originally reported by Mizumoto and colleagues (Mizumoto et al. 2006) and kindly provided by Dr. Pamela Pollock. Cell lines were grown as previously described (Byron et al. 2008) and authenticated as reported in (Dewdney et al. 2011).

Preparation of Nucleic Acids

All primary endometrial tumors and normal endometrium specimens analyzed were collected as part of IRB-approved studies (Washington University Medical Center Human Research Protection Office protocols HRPO-91-0507, -93-0828 and -92-0242). Histologic grading and typing were performed by gynecologic pathologists. Staging was determined using 1988 criteria from the International Federation of Gynecology and Obstetrics. Tissue specimens and blood were obtained at the time of surgery and stored at -70° C until nucleic acids were extracted. Genomic DNA from tumor tissues, normal endometrium, and cell lines was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA). Total cellular RNA was extracted from tumors and cell lines using the Trizol reagent (Invitrogen).

M&M, a Statistical Framework to Detect Differentially Methylated Regions (DMRs) Using MeDIP-seq and MRE-seq Data

MeDIP-Seq and MRE-Seq were performed as described in (Harris et al. 2010; Maunakea et al. 2010). Previous methods for MeDIP-seq and MRE-seq analysis treated each data type independently and did not take full advantage of the complementary nature of the data. The new statistical framework (M&M) used in this publication was developed by Ting Wang's laboratory. M& M assumes that the proportion of MeDIP reads expected in any given genomic location is equal to the proportion of methylated CpGs in that location, while the proportion of MRE reads expected in any given genomic location is equal to the proportion of unmethylated CpGs, and the observations of MeDIP reads and MRE reads each follow a Poisson distribution. The sum of methylated CpGs and unmethylated CpGs is the total number of CpGs which is a constant for any given genomic location. Under these constraints, DMR detection is transformed into a modified T-statistic test that integrates both MeDIP and MRE. Results thus far strongly suggest that M&M outperforms existing tools. A manuscript describing M&M is in preparation by the Wang laboratory.

Bisulfite Conversion

DNA bisulfite conversion was performed using a commercially available kit (EZ DNA Methylation Gold™ Kit, Zymo Research, Orange, CA). 200 ng of gDNA were converted for each sample.

Methylminer™ Preparation

DNA from AN3CA and Ishikawa cell lines was subjected to Methylminer™ preparation (Invitrogen) according to the manufacturer's instructions. DNA isolated before, during, and after the prep was assessed using *MLH1* COBRA as previously described (Chiappinelli et al. 2010). The primers used were as follows: Outer F 5'-tttTtTaaTtTtgtgggttgTtggg-3', Outer R 5'-AAaAAccacaaAaAcaAAAccaa-3', Nested F 5'-TtgTTCgTtaTTtagaaggata-3', Nested R 5'-tctActcctattAActAAatatttc-3'. The resulting 115 bp amplicon was digested with BstUI and MboI (New England Biosystems) to produce 83 and 32 and 76 and 39 bp bands, respectively.

COBRA

COBRA was performed as previously described (Chiappinelli et al. 2010). The primers used for the *RSK4* gene were as follows: Outer F 5'-tggaTttgagagggTTtgTtg-3', Outer R 5'-tcaatAAaActtAAAAaAattcccc-3', Nested F 5'-gagggTTtgTtgagTatgtgtga-3', Nested R 5'-AaAattccccaActtAAAAtAaaAA-3'. The resulting 156 bp amplicon was digested with ZraI (New England Biosystems) and if methylated produced 123 and 33 bp fragments. The primers used for the *PY2B4* (Sestrin-3) gene were as follows: Outer F 5'-ggTagaTttgaTtggggaa-3', Outer R 5'-cataatAcacaAtcctAtAAccaC-3', Nested F 5'-gggtaggggagTTaggtTt-3', Nested R 5'-ctAAActccaAtAaAcacaAaAct-3'. The resulting 218 bp amplicon was digested with BstUI and HinfI (New England Biosystems) to produce 178 and 40 and 172 and 46 bp bands, respectively. The primers used for the *SFRP1* gene were as follows: Outer F 5'-gggaTCGggTagTagTttg-3', Outer R 5'-AcaAcaccatcttcttAtaAcc -3', Nested F 5'-GgaggtTTTtggaagttt-3', Nested R 5'-

caCGcactAaAAtAActtAAtA-3'. The resulting 265 bp amplicon was digested with BstUI and EcoRV (New England Biosystems) to produce 214 and 51 and 110, 56, 35, 31, 29, and 2 bp bands, respectively. The primers used for the *TITF1* gene were as follows: Outer F 5'-TTGTTAGTTTTTTTTTGTGGT-3', Outer R 5'-AAACTCTTACTCCCTCAATACA -3', Nested F 5'-TTTGGGAAGGAAGGGTAA-3', Nested R 5'-AAAACCAACTTCTATAATAACATTC-3'. The resulting 225 bp amplicon was digested with BstUI and MboI (New England Biosystems) to produce 100, 63, and 62, and 115 and 110 bp bands, respectively. The primers used for the *MIRLET7D* gene were as follows: Outer F 5'-gaaaTaaaaTtTaaagaaTatgaTTt-3', Outer R 5'-catttAaaaaaacctacaAaaa-3', Nested F 5'-aaaatgggttTTtaggaagagg-3', Nested R 5'-cttacaccaaaAcaaaAtaAcaaAAa-3'. The resulting 149 bp amplicon was digested with TaqI (New England Biosystems) to produce 111 and 38 bp bands. Primers and restriction digests used for COBRA assays to validate putative M+M differentially methylated regions are listed below.

Assay	Primers*	Amplicon Size	Restriction Digest Products
NDUFA6	Rd1 For 5' gtttggagTtttttTTtgaT 3' Rd1 Rev 5' ctaaActAtttccaAAAtAacaAa 3' Rd2 For 5' ggagTtttttTTtgaTtTt 3' Rd2 Rev 5' AcaAAtctAaAaattAttccc 3'	265 bp	*AciI* (*CCGC*) 149, 91, & 25 bp *BstBI* (*TTCGAA)* 192 & 73 bp
SVIL	Rd1 For 5' gaagggagagaggaTatTtT 3' Rd1 Rev 5' AaAccaAAAaAccctAaAc 3' Rd2 For 5' GtTTTTtgTTTaagTtggTtT 3' Rd2 Rev 5' AAAAccctacaattaatacC 3'	189 bp	*TaqI (TCGA)* 106, 61, & 22 bp

Quantitative RT-PCR

Expression of BAD, IFI44, NDUFA6, RNF123, SLC2A4RG, and SVIL mRNAs was assessed by quantitative RT-PCR TaqMan® assays (Applied Biosystems, Foster City, CA) and the Applied Biosystems 7500 Fast real-time PCR system and software as

previously described (Chiappinelli et al., 2012). If the transcript upregulation was validated by qRT-PCR, COBRA was performed to determine if the promoter regions exhibited DNA methylation. N.D. = not determined.

Pyrosequencing

Pyrosequencing was performed as described in (Shearstone et al. 2011). Briefly, each amplicon was amplified by primers specific to bisulfite converted DNA. Amplified DNA was resolved on 10% polyacrylamide gels, stained with ethidium bromide, and photoimaged with a UV camera (ImageStore 500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA). The amplicons were then subjected to Pyrosequencing using sequencing primers inside the amplicon. To check for bisulfite conversion of the PCR product gDNA, cytosines were dispensed in non-CpG positions. Each locus was analyzed for methylation as a C/T SNP using QCpG software (Qiagen). Primers used for Pyrosequencing are listed below:

Assay	**Primers**
	For 5'-TGGGGAGGAGGGTAGGATATTA-3'
	Rev 5'-ACCCAACAAACTTTTCCTTTT-3'
OAT	Seq 5'-GGGAGGAGGGTAGGATATTAAT-3'
	For 5'-TGGGGTAAAGGAGAGGGTTATA-3'
	Rev 5'-ACACCCTCCCCTACAAAT-3'
UBE2J2 #3	Seq 5'-GGAGGAGGTGGGTTG-3'
	For 5'-GGGGTTAGAGAGAGGTGGA-3'
	Rev 5'-ACACCCTCCCCTACAAATAC-3'
UBE2J2 #4	Seq 5'-AGAGAGAGGTGGAGA-3'
	For 5'-ATTTGTAGGGGAGGGTGTT-3'
	Rev 5'-CTTCCCTCCCCATATACCA-3'
UBE2J2 #5	Seq 5'-GTGGGTTGTAAAGAGAT-3'

	For 5'-AAGGAAGTTAGGGAGTGAGAGA-3'
	Rev 5'-AACACCACTACTCCTACTATCC-3'
ZNF451	Seq 5'-GGGAGTGAGAGAAAG-3'

The *MLH1* pyrosequencing assay was designed by Biotage (REF 40-0055, Qiagen).

Bisulfite sequencing of individual clones

DNA was bisulfite converted as described above, and cloning and sequencing performed using standard methods (Dewdney et al. 2011). The forward and reverse primers used to amplify the CR1L promoter were F 5'-GTGTTTGTTTGGGATAGAGA-3' and R 5'-CCAATAAACCCTCCCCTTACTA-3'. Bisulfite sequencing was performed on the *MIRLET7D* amplicon used for COBRA. PCR products were cloned using the PCR-2.1TOPO TA vector (Invitrogen) and a minimum of 5 clones for each cloning experiment sequenced using ABI Prism BigDye Terminator chemistry v1.1 (Applied Biosystems).

Results and Discussion

Paragraph to be prepared by Dr. Wang's group on description of the MeDIP and MRE combination and Next-Generation Sequencing Results. Table on number of reads (unique, repeats, etc).

Genic regions

Overall the combined MRE/MeDIP ("M&M") data showed that KLE shLuc and shDcrA cells had very similar methylation patterns (Figure S1). The KLE cell line on

average had higher methylation than DNA from normal endometrial tissue, which was to be expected. However several regions of the genome, such as the *OAT* gene, showed differences in methylation (Figure 8). Figure 8 shows the combined methylation data along with additional genomic features at Exons 1-4 and 5 kb upstream of the transcription start site of the *OAT* gene. This gene exhibited higher MeDIP in the shDcrA cells and higher MRE in the shLuc cells, which meant that it gained methylation in the DICER1 knockdown. OAT was one of 21 promoter regions that showed a highly significant difference between the shLuc and shDcrA cells (Table 3). By comparing this data to methylome data from unperturbed KLE cells (data not shown) and transcriptome data from the shLuc and shDcrA cells (described in (Chiappinelli et al. 2012)), we chose six promoter regions to validate.

The methylation gains at *OAT*, *UBE2J2*, and *ZNF451* did not validate by pyrosequencing (Figure 9). Methylation differences were minimal and often showed a small decrease in the shDcr cells instead of the observed increase by M&M analysis. Each pyrosequencing assay covers only a portion of the promoter region, so we designed two further 3' assays at the *UBE2J2* promoter region (Assays 4 and 5). These also did not show a significant difference between shLuc and shDcrA cells (Figure S2). We were not able to design pyrosequencing assays for *CR1L*, *NPHS2*, and *UBE2S* because of the difficulty of amplifying such CG-rich regions. However, bisulfite cloning and sequencing showed the lack of a consistent difference between shLuc and shDcrA at the *CR1L* promoter region (Figure 10). MRE cut sites are marked in Figure 10 to show where the restriction enzymes used to generate DNA fragments for MRE are. The biggest methylation difference should be at these cut sites. We conclude that any differences in

methylation at these promoter regions are too subtle for our methods to validate and can perhaps only be detected by genomic methods. Interestingly, the finding that a *DICER1* hypomorph cell line loses methylation at specific CpG islands (Ting et al. 2008) was not confirmed by a genome-wide approach using MBP-Sequencing (Serre, Lee and Ting 2010). These methods analyze populations of cells; single-cell analysis would enable more precise measurements.

Repetitive Elements

DICER1 depletion did, however, cause a loss of methylation genome-wide at the Alu family of repetitive elements, but not at other repetitive elements such as the L1 family (Figure 11). The Alus are type of transposable element (TE) that were first described by Barbara McClintock in maize (McClintock 1950) and make up at least 10% of the human genome (Lander et al. 2001). These pieces of DNA are characterized by their ability to transpose themselves into different places in the host genome ("jumping genes"). Alu elements are a type of SINE (short interspersed element); these elements are typically 75-100 nt in length and are transcribed by RNA Polymerase III (Deininger et al. 2003). After transcription, they use reverse transcriptase (encoded in the L1 transposable elements) to create a DNA copy that inserts into a different part of the genome. As abundant Alu insertion would create significant genome instability, there is strong selection to stably silence these elements. They are silenced by chromatin modifications and DNA methylation in normal human tissues, but may lose silencing and be expressed in cancer or other disease states (Belancio, Hedges and Deininger 2008). Our finding that reduced DICER1 levels cause a loss of methylation at Alu elements was consistent with a

recent report showing that low DICER1 levels in macular degeneration cause an accumulation of Alu transcripts (Kaneko et al. 2011). The authors imply that DICER1 may process Alu transcripts. The Alu transcripts being processed by DICER1 may be necessary to initiate stable transcriptional silencing of the repetitive elements, which have a closed chromatin structure and DNA methylation. DICER1 may thus be involved in transcriptional silencing or RNA processing of Alu transcripts. As DICER1 is generally reduced and Alu transcription and mobility is generally increased in tumorigenesis (Batzer and Deininger 2002), this may be an important part of the genomic instability associated with cancers. However, as Alu elements make up at least 10% of the human genome, there is a possibility that we observed this subtle change in methylation simply because of the abundance of this element and therefore its large genomic "signal".

The fact that we observed an effect on methylation only at repetitive elements is intriguing. The interaction of the RNAi system with heterochromatin formation was first described in the fission yeast *S. pombe* at heterochromatic repeats flanking the centromere of each chromosome. These repetitive regions have bidirectional transcription that creates double-stranded RNA. A Dicer homolog and an RNA-dependent RNA polymerase are necessary for formation of the resulting siRNA, which then associates with the Argonaute protein and brings a chromatin modifier, in this case an H3K9 methyltransferase, to put silencing marks on the chromatin (Buhler, Verdel and Moazed 2006; Verdel and Moazed 2005). *S. pombe* lacks the DNA methylation mark, but the plant *Arabidopsis thaliana* has heavily methylated repetitive DNA and utilizes the RNAi system to silence these repeats. In this case the process is similar to that in *S. pombe*, but the RITS complex is able to recruit DNA methyltransferases (Teixeira and Colot 2010).

Both plants and fission yeast have an RNA-dependent RNA polymerase that is necessary for generation of long dsRNA; the strongest argument against RNAi-directed chromatin silencing in higher organisms is that flies, mice, and humans lack an RNA-dependent RNA polymerase (Allis et al. 2007). However, a different class of small RNAs, the Dicer-independent piRNAs, have been shown to repress repetitive regions in flies, mice, and humans; and, in the case of mammalian cells, an imprinted region, by DNA methylation (Esteller 2011). Unfortunately, deleting DICER1 would have no effect on piRNAs; future experiments knocking down piRNA processing enzymes and determining the effects on DNA methylation will help to elucidate the roles of these proteins. Evidence for DICER1 involvement in heterochromatin comes from the chicken globin locus, which requires DICER1 to recruit the Argonaute protein Ago2 and initiate silencing histone modifications (Giles, Ghirlando and Felsenfeld 2010). Constitutive heterochromatin, including the domain in the globin locus and centromeres in fission yeast, thus may require RNAi for appropriate chromatin packaging. Our data on loss of methylation at Alu repetitive regions in shDcr cells implies a conserved role for DICER and small RNA maintenance of heterochromatin at repetitive regions.

In conclusion, DICER1's effects on DNA methylation in human cells are minimal. DICER1 however does appear to be involved in maintenance of methylation at the Alu elements. As these make up at least 10% of the human genome, this function may be very relevant to genome stability. On the other hand, we may have not achieved a good enough knockdown of the DICER1 protein to see effects on methylation, but we observed other physiological effects when DICER1 was reduced to ~10% of it's endogenous levels (Chiappinelli et al. 2012). Further directions include characterization

of histone modifications in DICER1 knockdown cells; these are an alternate way to effect gene expression and may be influenced by RNAi.

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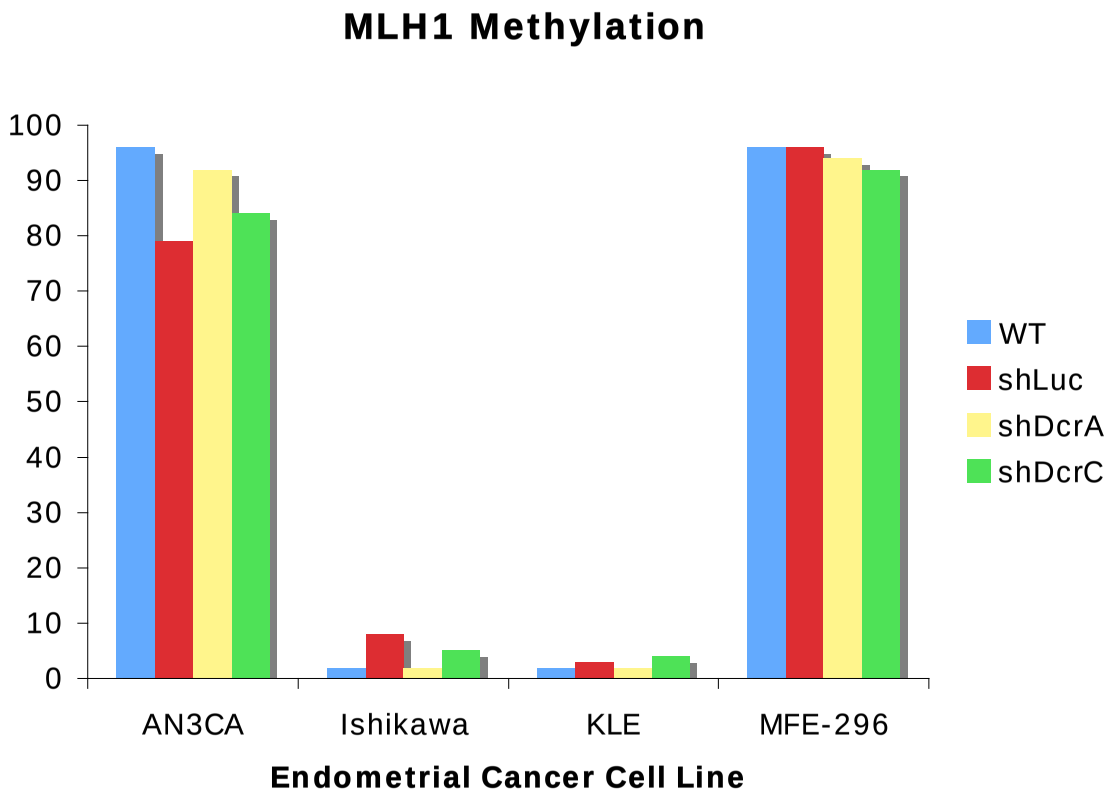


Figure 1. *MLH1* methylation in shDcr cells. *MLH1* methylation was assessed by Pyrosequencing in the endometrial cancer cell lines AN3CA, Ishikawa, KLE, and MFE-296 in wt (unperturbed) cells, shLuc, and shDcr cells.

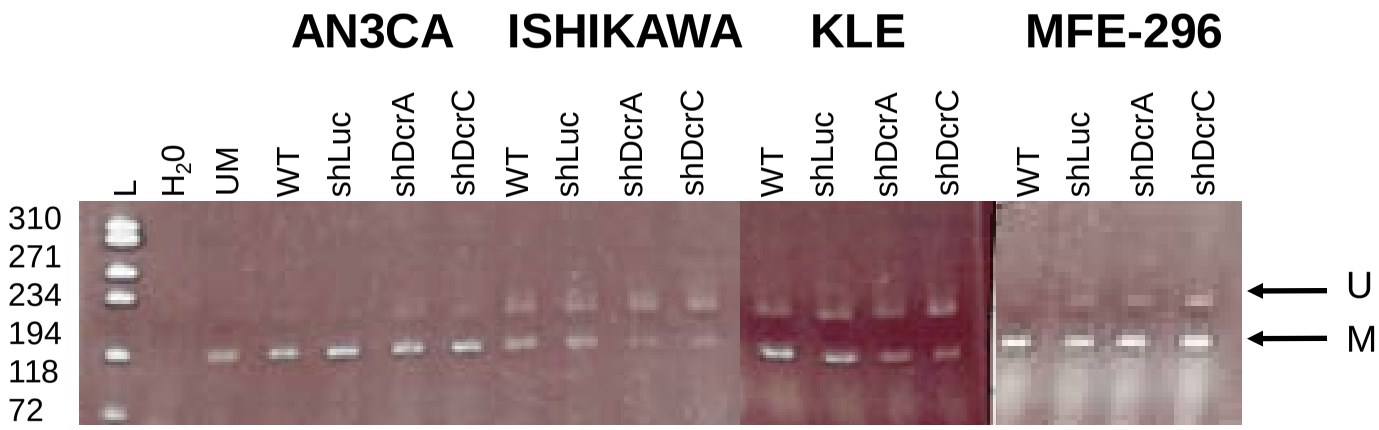
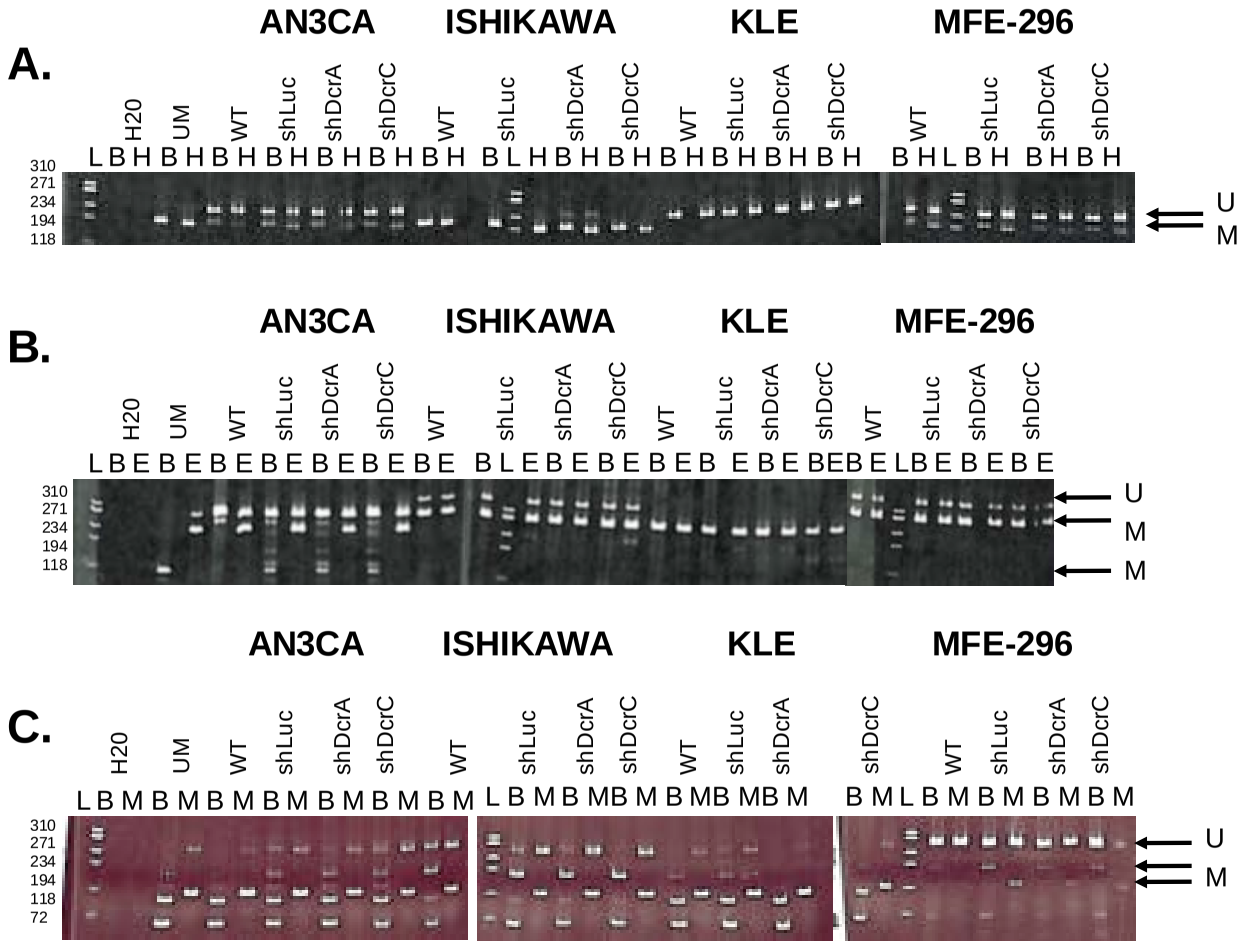


Figure 2. *RSK4* methylation in shDcr cells. COBRA was performed at the *RSK4* locus. The resulting 156 bp amplicon was digested with ZraI) and if methylated produced 123 and 33 bp fragments. H_2O = water, UM = universally methylated control. B = BstUI, E = EcoRV. U = unmethylated, M = methylated. L = ΦX ladder.


A.
AN3CA
ISHIKAWA
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MFE-296
H20
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234
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118
U
M
B.
AN3CA
ISHIKAWA
KLE
MFE-296
H20
UM
WT
shLuc
shDcrA
shDcrC
L B E B E B E B E B E B E B E B L E B E B E B E B E B E B E B E B E L B E B E B E
310
271
234
194
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M
C.
AN3CA
ISHIKAWA
KLE
MFE-296
H20
UM
WT
shLuc
shDcrA
shDcrC
L B M B M B M B M B M B M B M B L B M B MB M B MB MB M B M L B M B M B M B M B M
310
271
234
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118
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U
M

Figure 3. *PY2B4, SFRP1,* and *TITF1* methylation in shDcr cells.
A. COBRA for the *PY2B4 (*Sestrin-3) gene was performed. The resulting 218 bp amplicon was digested with BstUI and HinfI to produce 178 and 40 and 172 and 46 bp bands, respectively.

B. COBRA for the *SFRP1* gene was performed . The resulting 265 bp amplicon was digested with BstUI and EcoRV to produce 214 and 51 and 110, 56, 35, 31, 29, and 2 bp bands, respectively.

C. COBRA for the *TITF1* gene was performed. The resulting 225 bp amplicon was digested with BstUI and MboI to produce 100, 63, and 62, and 115 and 110 bp bands, respectively. H2O = water, UM = universally methylated control. B = BstUI, E = EcoRV. U = unmethylated, M = methylated. L = ΦX ladder.

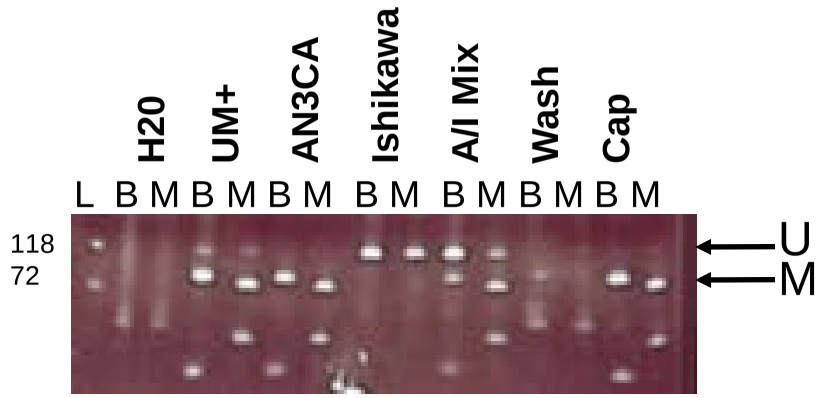
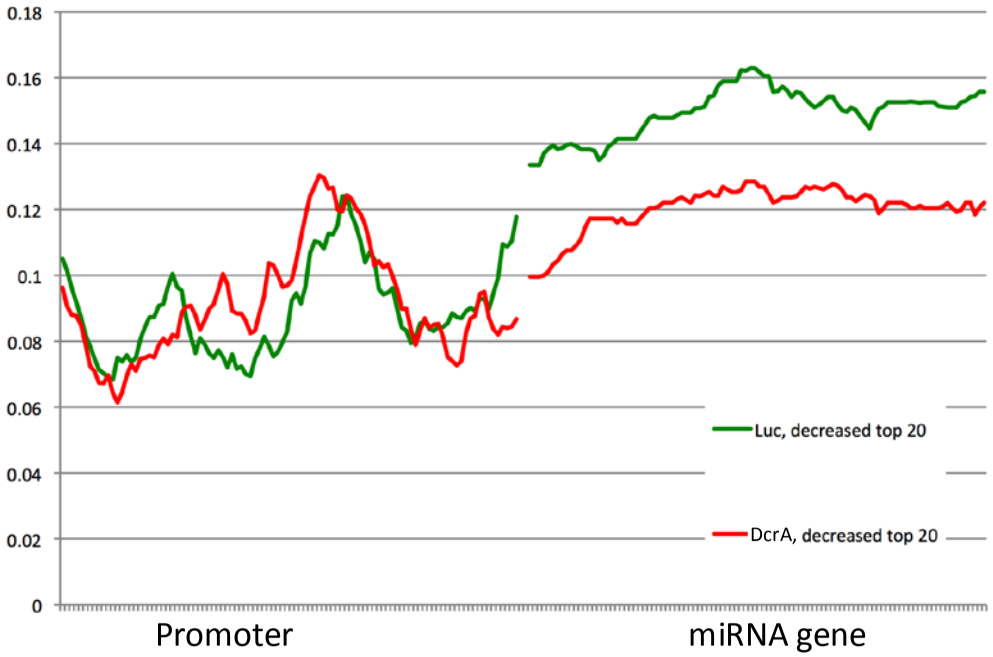


Figure 4. Demonstration of Methylminer™ capture of methylated component of DNA using the *MLH1/EMP2AIP1* promoter region as an example. DNA from AN3CA and Ishikawa cell lines was subjected to Methylminer™ preparation . DNA isolated before, during, and after the prep was assessed using *MLH1* COBRA. The resulting 115 bp amplicon was digested with BstUI and MboI to produce 83 and 32 and 76 and 39 bp bands, respectively. H2O = water, UM+ = universally methylated control. B = BstUI, E = EcoRV. U = unmethylated, M = methylated. L = ΦX ladder. A/I Mix = 50% AN3CA DNA, 50% Ishikawa DNA. Wash = excess from the prep, Cap = captured DNA (should be 100% methylated).



Ting Wang

Figure 5. Methylation at miRNA genes in shDcr cells. MeDIP was performed on KLE shLuc and shDcrA DNA as previously described .This graph represents the methylation at the 20 most decreased miRNAs.

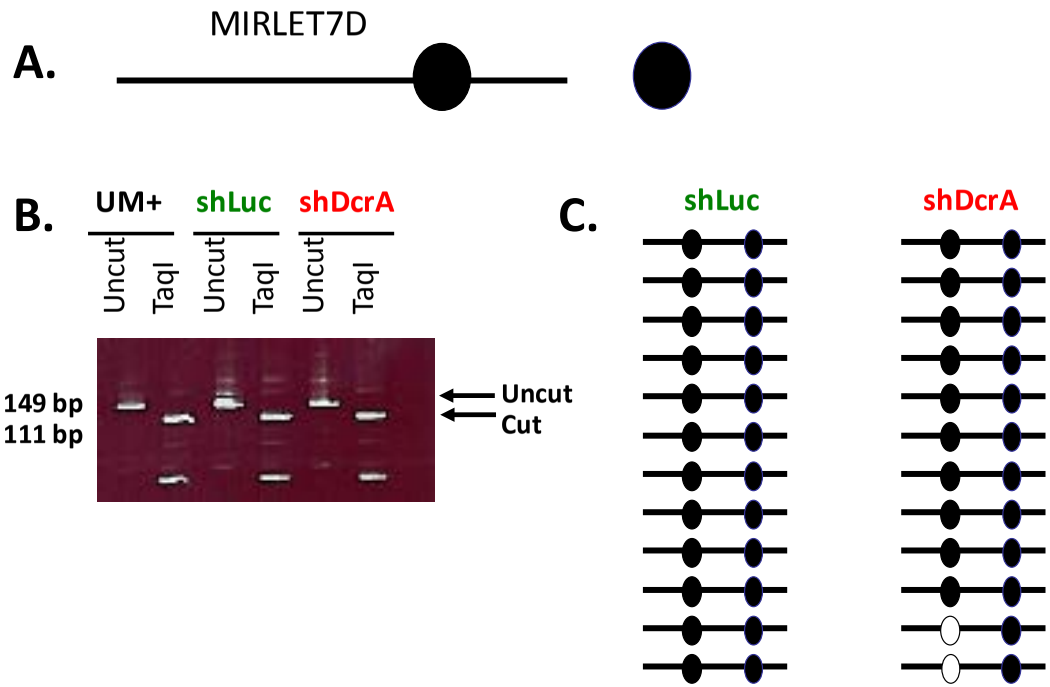


Figure 6. Validating the change in methylation at the *MIRLET7D* gene. A) Schematic of the *MIRLET7D* gene body: one CpG site is within the gene and the second, providing a cut site for TaqI in B), is downstream of the gene. B) COBRA was performed . The resulting 149 bp amplicon was digested with TaqI to produce 111 and 38 bp bands. UM+ = universally methylated control. C) Bisulfite sequencing was performed on the *MIRLET7D* amplicon used for COBRA . Black circles indicate methylated CpG and white circles indicate unmethylated CpG.

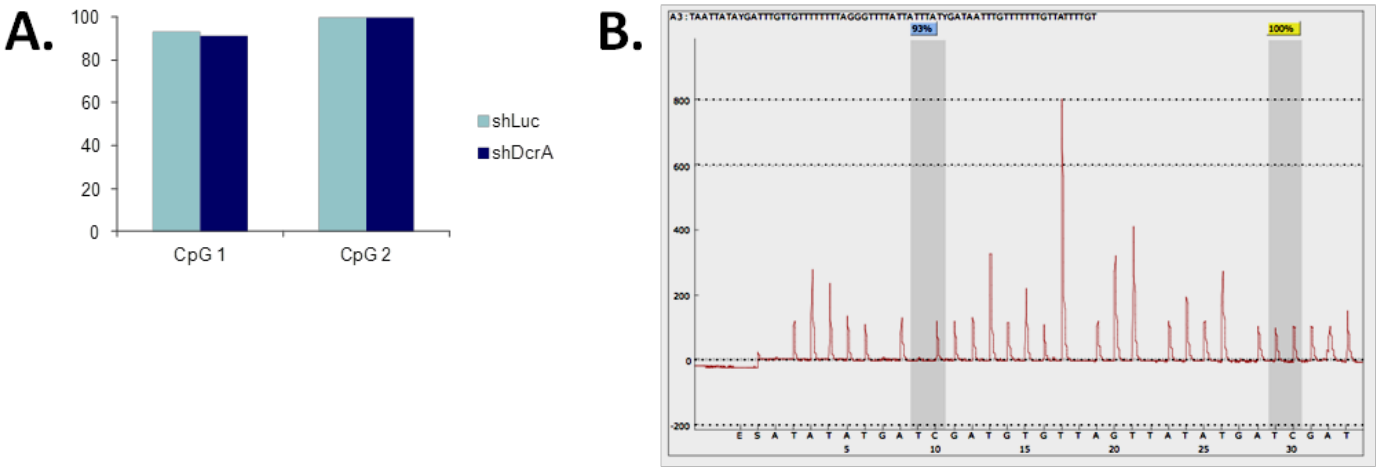


Figure 7. *MIRLET7D* methylation. The *MIRLET7D* gene was assessed by pyrosequencing . A) Pyrosequencing results for the two CpGs of *MIRLET7D.* B) Sample pyrogram of *MIRLET7D* in KLE shLuc cells.

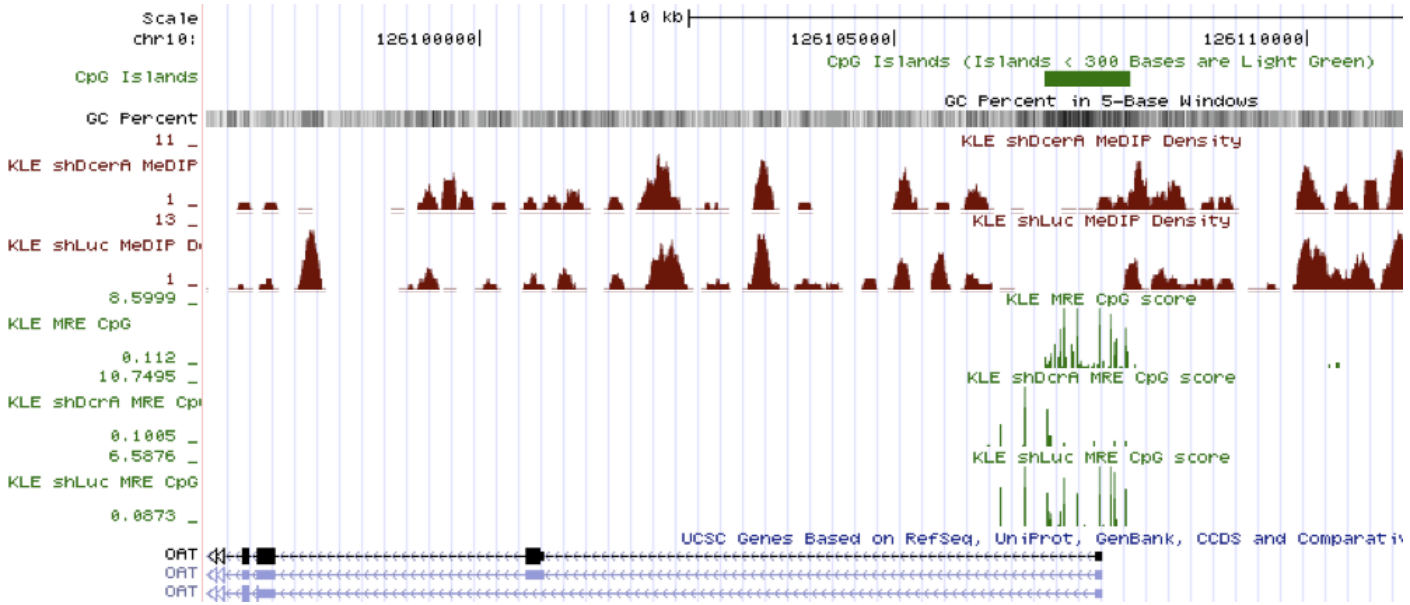


Figure 8. Browser shot at the promoter and genic region of the OAT gene. Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

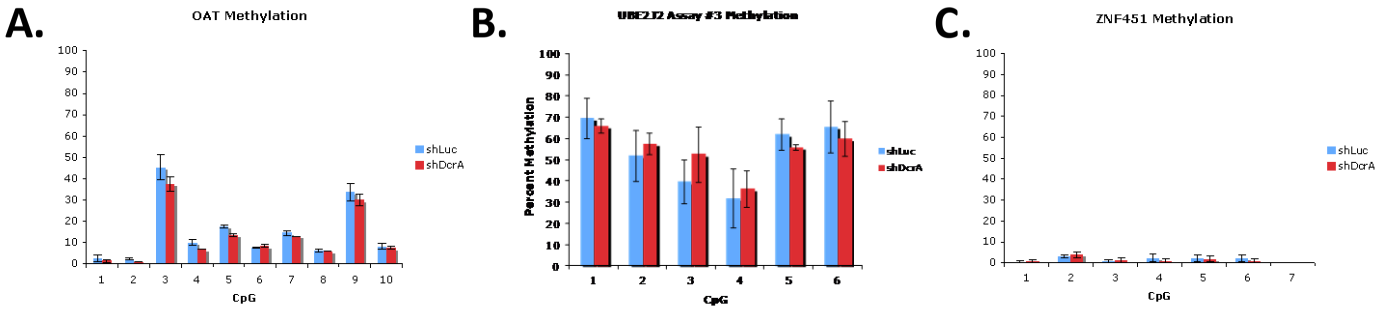


Figure 9. Methylation at genic regions is unchanged by DICER1 depletion. Pyrosequencing assays determine the amount of methylation at the promoter regions of A) *OAT*, B) *UBE2J2*, and C) *ZNF451*. Average of three independent experiments is shown. Error bars are SEM.

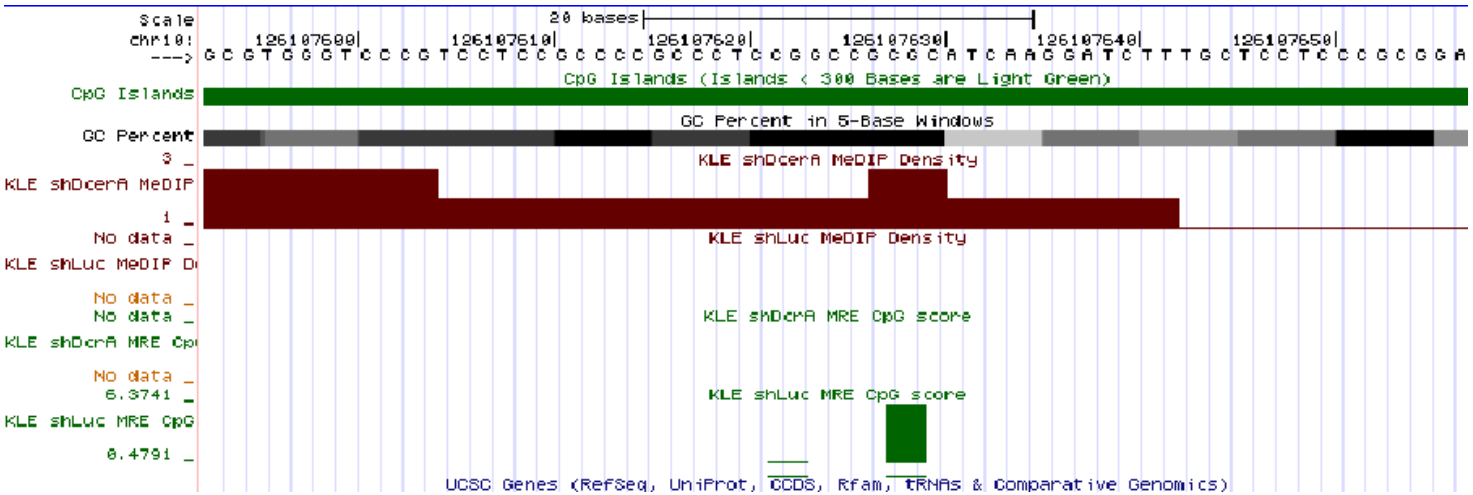


Figure 9D. Browser shot at the area assessed in Figure 9A (OAT promoter). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

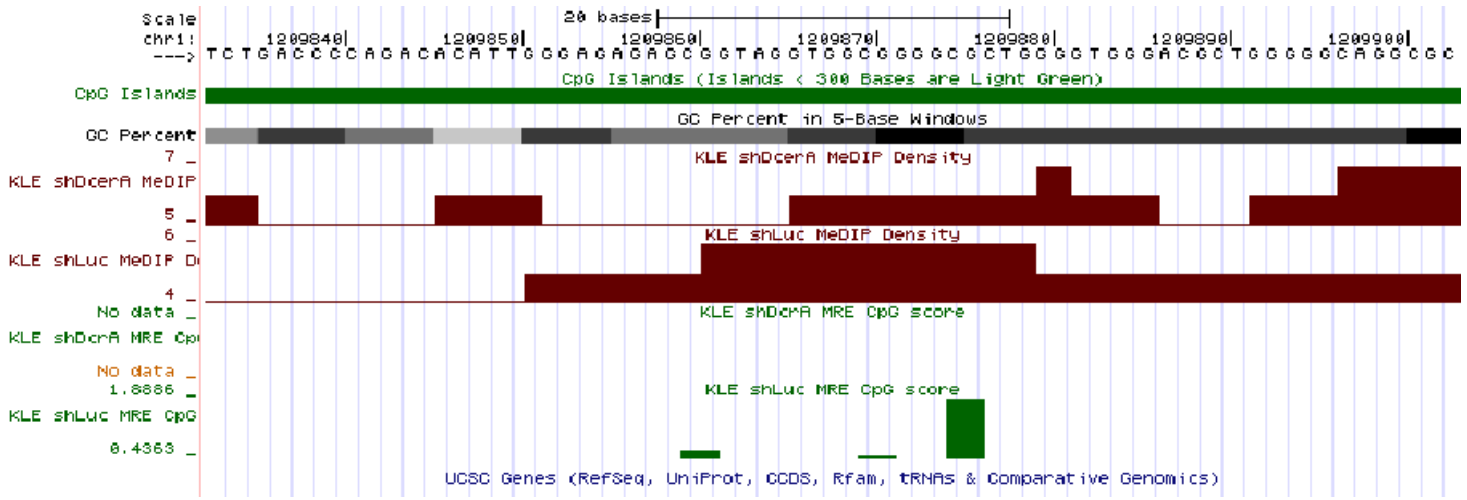


Figure 9E. Browser shot at the area assessed in Figure 9B (UBE2J2 promoter). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

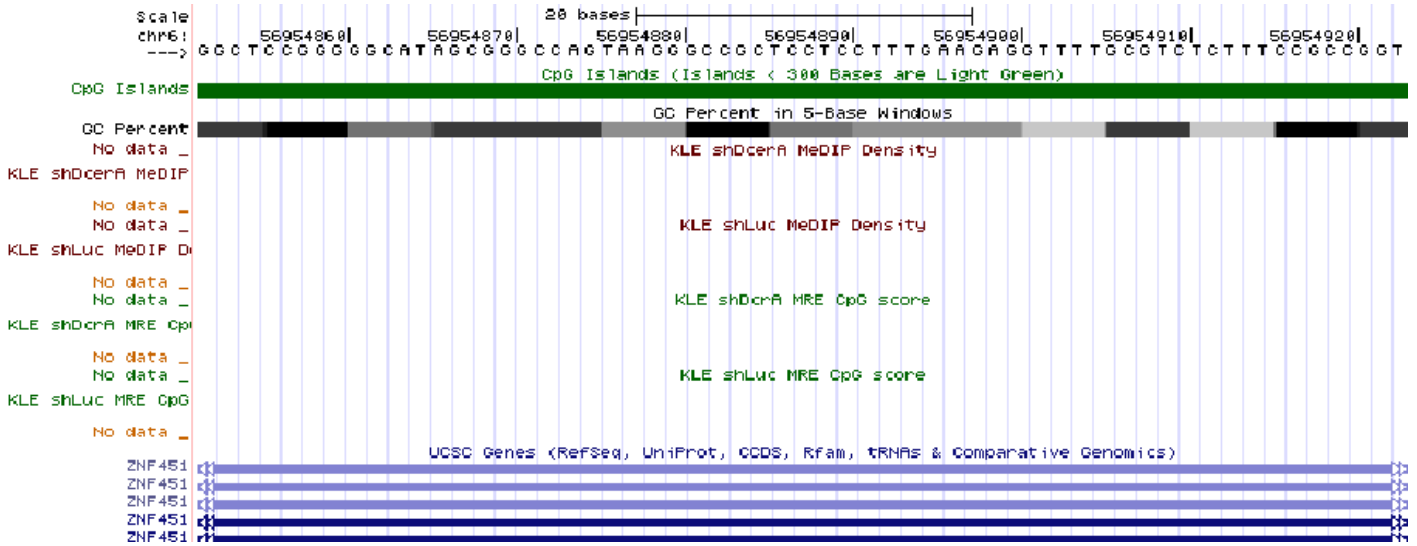


Figure 9F. Browser shot at the area assessed in Figure 9C (UBE2J2 gene). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

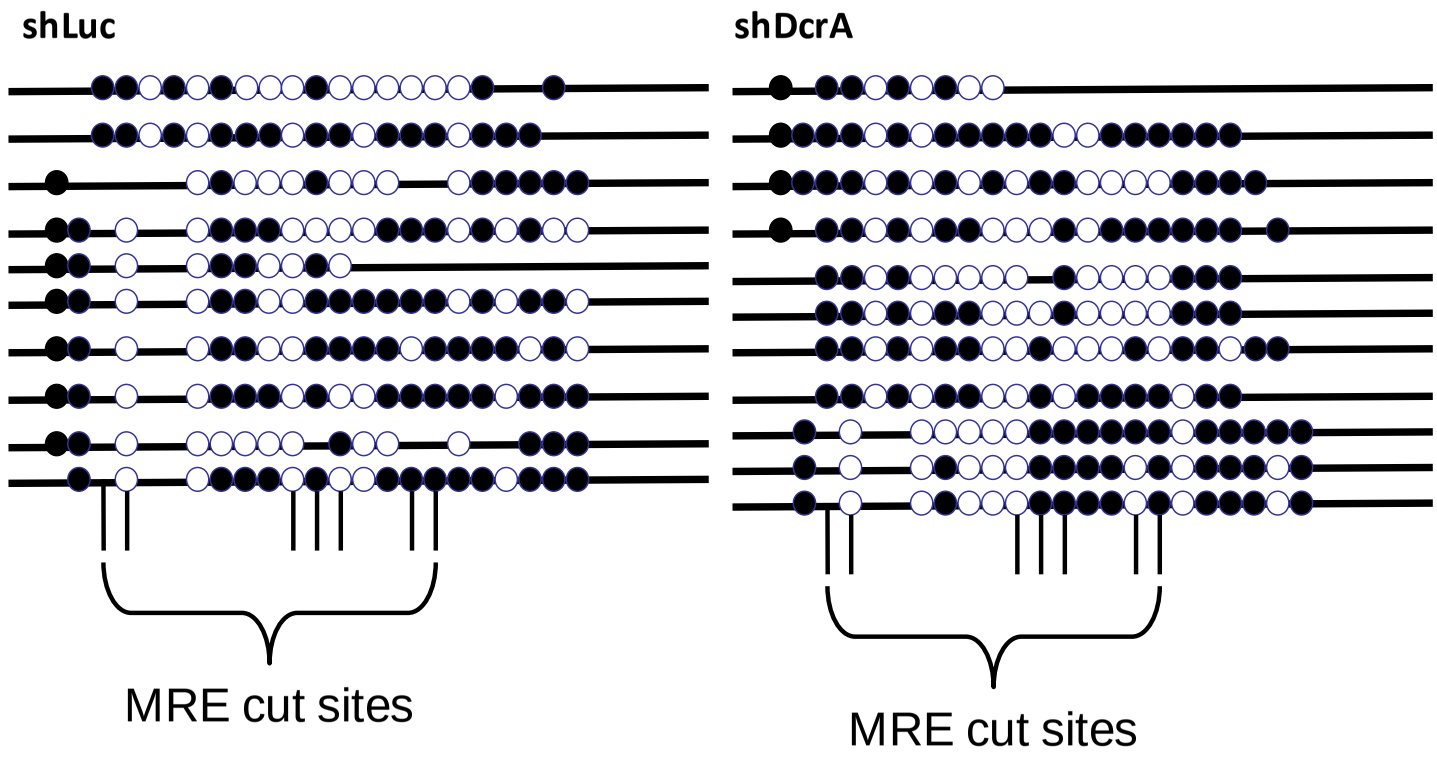


Figure 10A. Cloning and Sanger sequencing results for the *CR1L* gene. DNA from KLE shLuc and KLE shDcrA cell lines. Black circles indicate methylated CpGs and white circles indicate unmethylated CpGs.

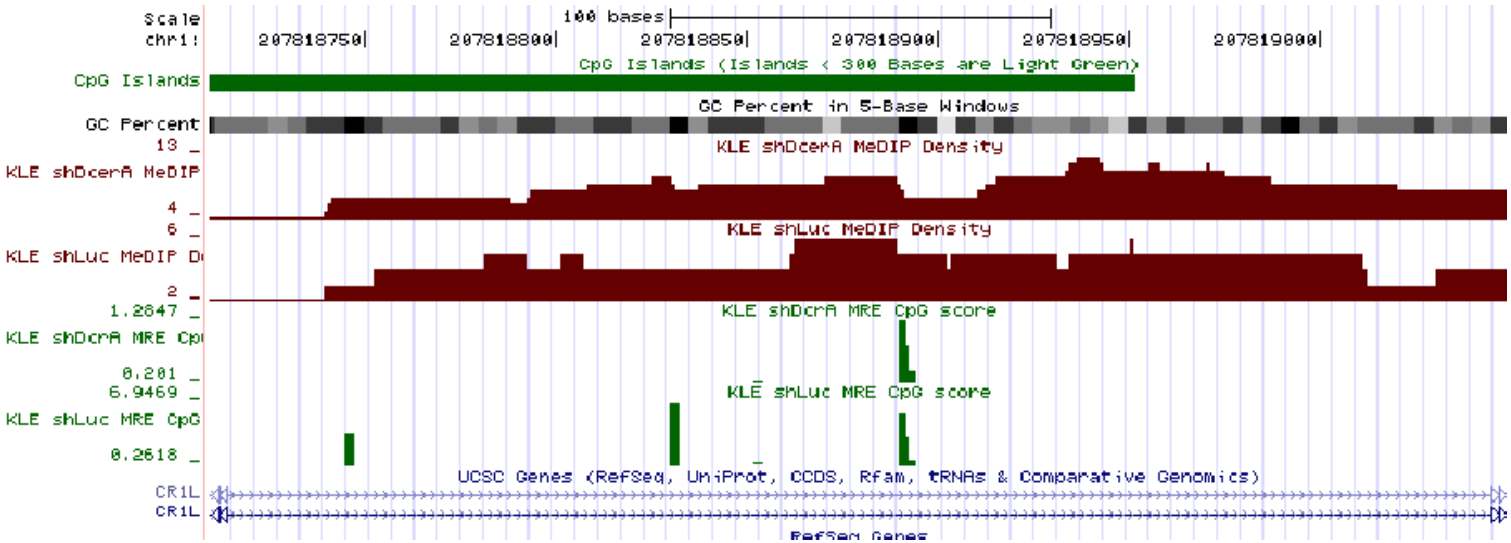


Figure 10B. Browser shot at the area assessed in Figure 10A (CR1L gene). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

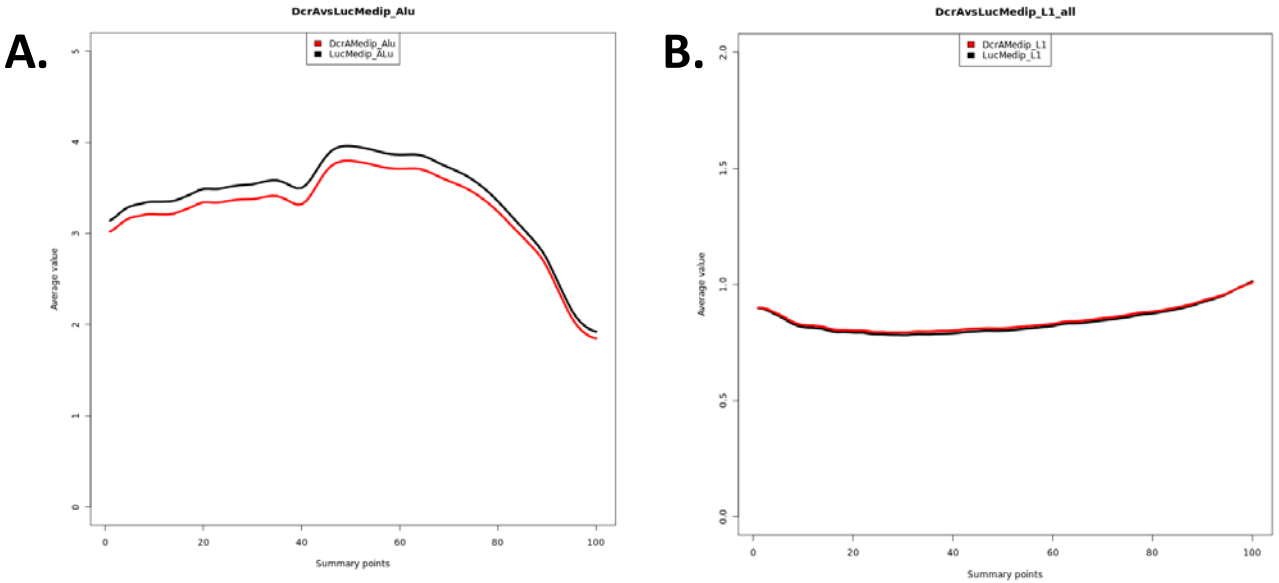


Figure 11. DICER1 depletion causes the Alu elements (A) but not the L1 repeats (B) to lose methylation. Black line indicates KLE shLuc DNA and red line indicates KLE shDcrA DNA.

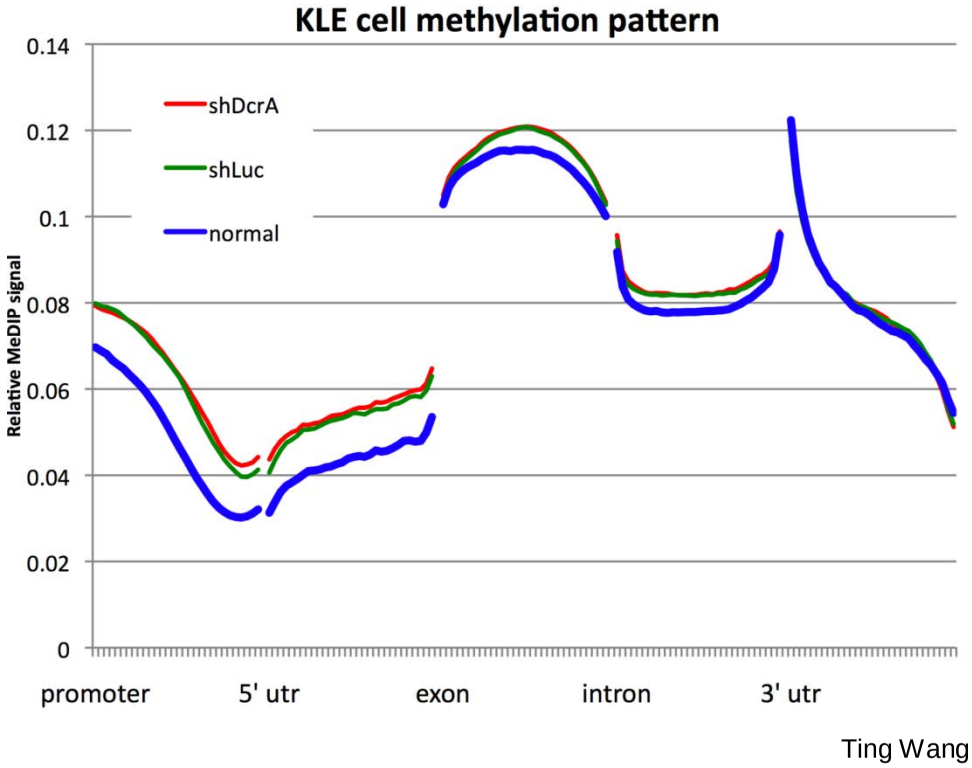


Figure S1. MeDIP data show very little difference in DNA methylation across the genome for normal endometrium compared to KLE shLuc and KLE shDcrA cell lines.

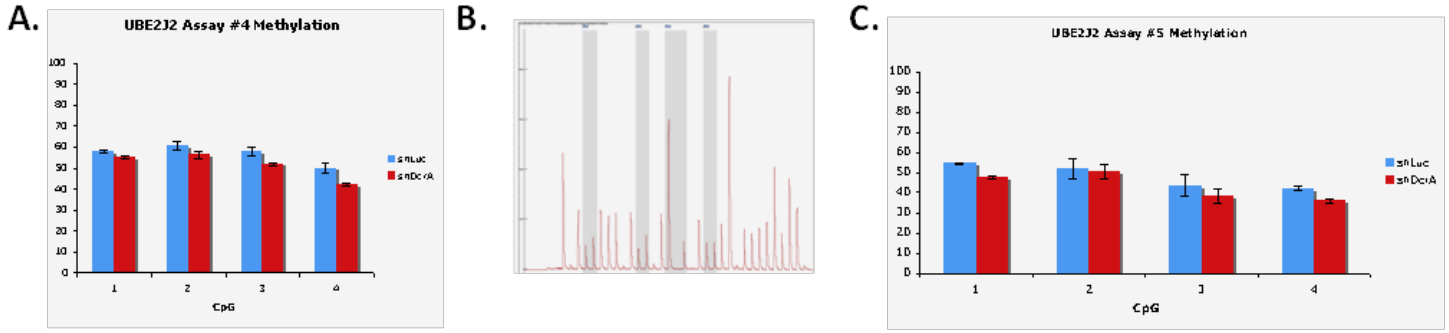


Figure S2. Pyrosequencing assays for the 3' region of the *UBE2J2* promoter. Assay #4 is shown in A) (summary of data) and B) (sample pyrosequencing trace). Assay #5 is shown in C). Average of three independent experiments is shown. Error bars are SEM

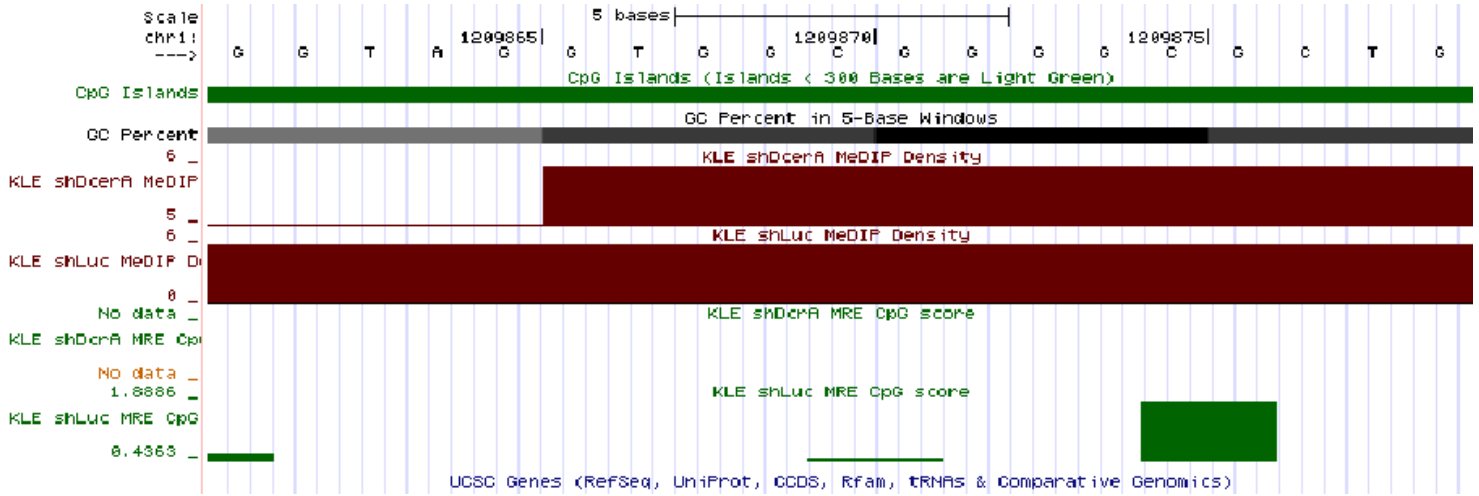


Figure S2D. Browser shot at the region assessed in Figure S2A (UBE2J2 promoter). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

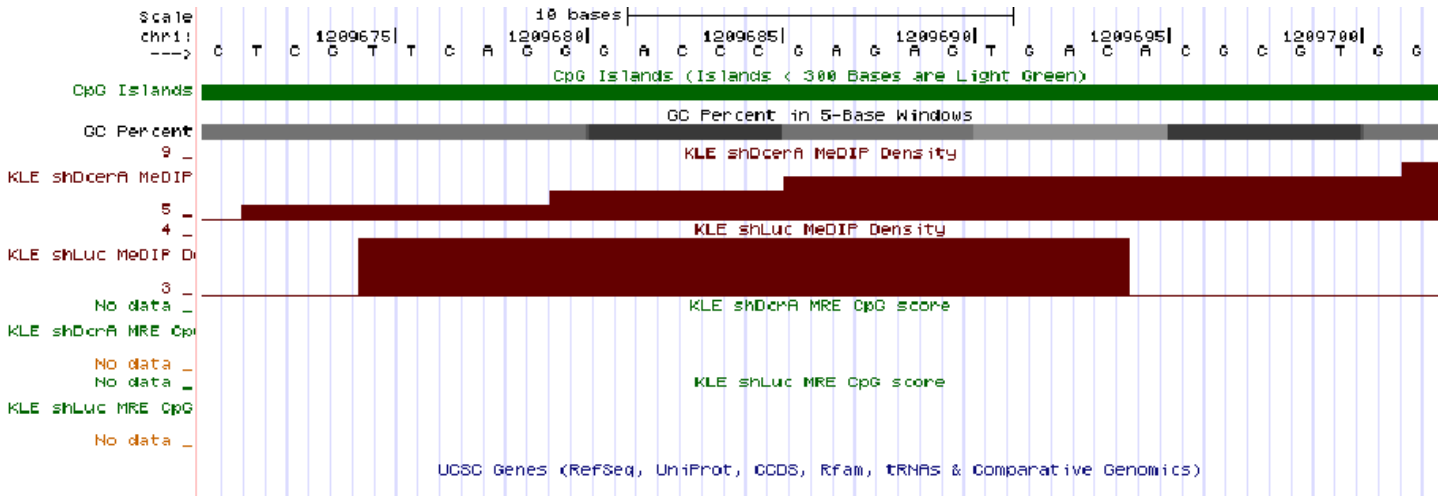


Figure S2E. Browser shot at the region assessed in Figure S2C (UBE2J2 promoter). Red tracks are MeDIP and green tracks are MRE. KLE unperturbed, shLuc, and shDcrA cell lines are shown.

Gene	RNA-Seq Fold Upregulation (shDcrA/controls)	CpG Island	qRT-PCR Validation	COBRA
AC016995.3	30	Yes	N.D.	N.D.
AL512802.1	3	No	N.D.	N.D.
BAD	4	Yes	No	N.D.
IFI44	6	No	Yes	N.D.
KB-1839H6.1	4	No	N.D.	N.D.
NDUFA6	5	Yes	Yes	Unmethylated
RNF123	10	Yes	No	N.D.
RP11-96L14.1	5	No	N.D.	N.D.
SLC2A4RG	4	Yes	No	N.D.
SVIL	3	Yes	Yes	Unmethylated

Table 1. Summary of validation attempts at regions that showed higher expression in the mRNA-sequencing (described in Chapter Three). Regions that showed upregulation in shDcrA cells and had canonical CpG islands were selected. Expression of BAD, IFI44, NDUFA6, RNF123, SLC2A4RG, and SVIL mRNAs was assessed by quantitative RT-PCR. If the transcript upregulation was validated by qRT-PCR, COBRA was performed to determine if the promoter regions exhibited DNA methylation. N.D. = not determined.
*Capitalized letters indicate unmethylated C converted to T by bisulfite treatment.

Table 2. Changes in microRNA gene body methylation by MeDIP fail to validate by other methods.

	Expression	Methylation in DICER1 Knockdown vs. Control	
MicroRNA	**Nanostring**	**MeDIP**	**COBRA/Cloning/ Pyrosequencing**
MIRLET7B	Decreased	Decreased	Unchanged
MIRLET7D	Decreased	Decreased	Unchanged
MIR107	Decreased	Decreased	Unchanged
MIR183	Decreased	Decreased	Unchanged
MIR450	Decreased	Decreased	Unchanged
MIR542	Decreased	Decreased	Unchanged
MIR146A	Decreased	Same	Unchanged

Table 1. Promoter regions with significant differences in methylation from M&M analyses.

Gene Name	Promoter	delta-MeDIP (Luc-Dcr)	delta-MRE (Luc-Dcr)	p-value	q-value	RNA-Seq DcrA/Controls
ACOXL	chr2:111487149-111492149	-7	76	2.01E-10	8.85E-06	Not Expressed
AGTR1	chr3:148412657-148417657	-20	67	1.73E-13	4.02E-08	Not Expressed
ANKMY1	chr2:241495405-241500405	-22	32	1.24E-07	0.000947064	Not Expressed
B3GALT6	chr1:1164628-1169628	-26	34	4.75E-07	0.002414903	0.9
CR1L	chr1:207815457-207820457	-11	160	9.32E-11	5.11E-06	Not Expressed
ECHDC3	chr10:11781355-11786355	-9	90	4.37E-10	1.64E-05	Not Expressed
LHX2	chr9:126770888-126775888	-12	58	3.12E-08	0.00036818	Not Expressed
LYN	chr8:56789385-56794385	-11	58	2.13E-09	5.45E-05	Not Expressed
NPHS2	chr1:179543084-179548084	-11	89	3.00E-09	7.05E-05	Not Expressed
OAT	chr10:126105545-126110545	-7	80	4.48E-08	0.000468518	0.24
PCP4L1	chr1:161225516-161230516	-18	89	2.86E-12	3.41E-07	Not Expressed
PDZRN4	chr12:41579249-41584249	-2	150	2.62E-07	0.001578603	Not Expressed
PYCARD	chr16:31212251-31217251	-9	85	1.24E-07	0.000945047	Not Expressed
SLC35F3	chr1:234037678-234042678	-6	79	1.22E-06	0.004471015	Not Expressed
SLITRK2	chrX:144896346-144901346	-12	81	5.94E-08	0.000558685	Not Expressed
STAG3	chr7:99772537-99777537	-2	99	3.43E-07	0.001925883	Not Expressed
SYCP2	chr20:58505209-58510209	-7	108	5.17E-10	1.88E-05	0.95
UBE2J2	chr1:1207234-1212234	-14	142	1.20E-11	9.75E-07	-0.47
UBE2S	chr19:55917325-55922325	-15	146	2.02E-15	8.96E-10	Not Expressed
ZC3HAV1L	chr7:138718775-138723775	-11	56	1.08E-07	0.000855823	Not Expressed
ZNF451	chr6:56951827-56956827	-9	84	1.39E-09	4.11E-05	0.04

CHAPTER FIVE: Dissertation Conclusions and Future Directions

I began my Ph. D. program in 2007, four years after the completion of the human genome sequence and 65 years after the term “epigenetic” was coined by the developmental biologist Conrad Waddington (Waddington 2012). In my first year as a Developmental Biology graduate student, I took the Chromatin Structure and Gene Expression (Biology 5282) course, outlined by the landmark “Epigenetics” textbook first published in 2007 (Allis et al. 2007). The Chromatin Structure and Gene Expression course set the path for my graduate work on mechanisms of epigenetic control. Epigenetics research grew and changed remarkably during my graduate school experience. In 2007, epigenetics was thought of more as a series of phenomena than a field of science. Today, epigenetics is its own discipline. Undergraduate molecular biology students are taught that two cells with the same genome can have different phenotypes, attributed to epigenetic differences. A major international effort was recently mounted to map chromatin modifications throughout the genome (Rosenbloom et al. 2012). During graduate school, I was fortunate to TA two courses, one that focused on developmental biology and another that focused on genome organization and chromatin modifications, such that my knowledge of epigenetics evolved with the field and concordantly with my research.

Conclusions

My graduate work focused on epigenetic modifications in human malignancies. It began with an in-depth analysis of one well-known tumor suppressor gene that is silenced in endometrial and other cancers, then evolved to a whole-genome study of effects of perturbation of a key factor in epigenetic regulation. By characterizing transcription at the

MLH1 locus in Chapter Two, I showed that this locus had high levels of noncoding RNA. This corroborated the genome-wide data on the abundance of noncoding RNA in the mammalian transcriptome. I also noted that nucleosome positioning at the active *MLH1* locus was not as previously reported, at least in endometrial cancer. These results show that chromatin modifications and transcription at specific loci likely differ between cell types and as such, the massive amounts of genomic data being generated on chromatin modifications (Rosenbloom et al. 2012) should not be assumed to apply to all cell types. While there is one human genome, there are many human epigenomes.

Although the 2003 "completed" human genome was heralded as the eventual key to treating human disease via targeted therapies and personalized medicine (Wade 2003), we have since learned from efforts to sequence cancer genomes that the process from finding a mutation to characterizing the biological and potential tumorigenic effects of this mutation is quite involved (Cancer Genome Atlas Research Network 2008; Cancer Genome Atlas Research Network 2011). It is also difficult to distinguish important mutations from the plethora of mutations in a cancer cell. So-called "driver" mutations are selected for during clonal expansion of tumors and confer growth advantages, while "passenger" mutations are not selected for but are casualties of highly proliferating cells with genomic instability. Finding and characterizing "driver" mutations is and will continue to be a focus of cancer genomics research (Stratton, Campbell and Futreal 2009). The transcriptome and methylome sequencing efforts described in Chapters Three and Four detail my attempts to technically and biologically validate genome-wide observations.

I observed an interferon response upon DICER1 knockdown, normally a reaction to viruses or other foreign particles in a human cell, and postulated that this might be due to endogenous retrovirus reactivation. Endogenous retroviruses are retroviruses that integrated into the human genome very long ago, and like other mobile elements, are stably silenced to protect genome stability. While investigating the role of DICER1 in transcriptional silencing (detailed in Chapter Four), I thought that perhaps small RNAs might be involved in silencing endogenous retroviruses and that loss of DICER1 could reactivate these elements. Figure 1 shows the levels of the HERV-K subfamily of endogenous retroviruses in control and DICER1 knockdown cells. The HERV-K family is expressed, but by RT-PCR (Figure 1A) and deep sequencing (Figure 1B) does not differ between shDcr and control cells. Thus reactivation of endogenous retroviruses does not appear to be responsible for activating the interferon response.

Future Directions

My work on the transcriptome sequencing in shDcr cells showed that these cells exhibited a canonical interferon response. I observed that shDcr cells were characterized by tumorigenic phenotypes including enhanced migration and increased growth in soft agar. This work did not, however, establish a link between the interferon response and tumor phenotypes. Transfecting the cells with PolyI:C, a positive control for interferon signaling, caused cell death and was not informative for either assay. PolyI:C is almost too intense a stimulation of the IFN response and is likely to trigger more apoptosis than the milder phenotype of the DICER1 knockdown. It is more likely that the low-level, chronic interferon signature I observed might contribute to tumor phenotypes. Cancers

are known to arise from conditions such as inflammatory bowel disease and prostatitis (Mantovani et al. 2008; Slattery et al. 2009). Chronic inflammation has been shown to be mutagenic (Franco et al. 2008) and to cause epigenetic changes affecting gene expression (Hahn et al. 2008). Ras/Raf signaling works together with the inflammatory state of pancreatitis to initiate pancreatic cancer (Guerra et al. 2007), and STAT3 has been well established as a tumor-promoting molecule (Iliopoulos, Hirsch and Struhl 2009). As phospho-STAT3 is known to be an oncogene, I assessed the levels of pSTAT3 in shDcr and control cells. pSTAT3 was not significantly increased in shDcr cells (Figure 2) and so it does not seem that STAT3 is contributing to tumorigenesis in this model.

While I established a novel role for DICER1 in the control of the interferon response, there are still many unanswered questions about how the interferon response affects cancer cells. In the future we will follow up on interferon response components that were increased in shDcr cells and may be involved in metastasis, such as CXCL10. This protein is secreted by melanoma metastases (Amatschek et al. 2011) and promotes invasiveness of breast and colon cancer cells (Shin et al. 2010; Zipin-Roitman et al. 2007). It is also upregulated at the mRNA level in human ovarian cancers and a mouse ovarian cancer model driven by deletions of Dicer1 and Pten in the fallopian tube (Kim et al. 2012). The upregulation of this protein by IFN and NFκB signaling could be a cause of the increased migration and growth in soft agar observed in the DICER1 knockdown cells.

Another way that low DICER1 might contribute to tumorigenesis is through its effects on chromatin modifications. While I did not see changes in methylation at coding regions, I did observe a loss of methylation at Alu elements (Chapter Four). I am

currently working with Ting Wang's laboratory (Center for Genome Sciences & Systems Biology, Washington University in St. Louis) to validate this result using a PCR amplicon that amplifies most Alu subfamilies, then subjecting this amplicon to reduced representation bisulfite sequencing (RRBS) (Meissner et al. 2005). If we are able to validate this result, it will point to a role for DICER1 in heterochromatin regulation at repetitive regions. The interaction of RNAi with chromatin was first described at pericentromeric repetitive heterochromatin in *S. pombe* (Hall et al. 2002; Volpe et al. 2002), and a recent publication implied that DICER1 was necessary for heterochromatin maintenance at the globin locus in chickens (Giles, Ghirlando and Felsenfeld 2010). DICER1 could be necessary to process Alu RNA, as suggested by Kaneko *et al.* (Kaneko et al. 2011), and the small RNAs generated could be involved in heterochromatin maintenance at the Alu elements. Recent work (Tarallo et al. 2012) shows that loss of DICER1 and subsequent accumulation of Alu RNA activates the NLRP3 inflammasome and triggers a cell autonomous immune response, very similar to the one I observed in endometrial cancer cells. Several components of the inflammasome complex are upregulated at the mRNA level in the shDcr cells, but further work will need to be done to determine whether pre-miRNAs or Alu RNA are activating the immune response in the endometrial cancer cells.

Given Alu repetitive elements make up 10% or more of the genome, the greater part of global hypomethylation in cancer has often been attributed to methylation loss at Alus. Lower DICER1 levels in solid tumors go along with this. Interestingly, about 20% of human miRNAs are driven by Pol III promoters that have Alus in them (Borchert, Lanier and Davidson 2006). Thus loss of methylation globally at Alu elements in shDcr

cells could be explained by increased miRNA transcription in response to DICER1 knockdown and the resulting loss of mature miRNAs. Increased transcription at miRNA promoters containing Alus could lead to loss of methylation at these elements. The RRBS experiments under way will allow Dr. Wang's laboratory to map back the Alus sequenced to the genome and to determine which elements specifically lose methylation.

The experiments performed in this thesis address only the *in vitro* consequences of DICER1 depletion in tumor cells. To gain insights into tumor biology *in vivo,* I utilized an orthotopic mouse model of endometrial cancer (first described in (Kamat et al. 2007)) in which we injected endometrial cancer cells (AN3CA) into the uterine horn of mice and imaged the cancers over time. We did not see a significant difference in tumor burden between control (shRFP) and shDcrA cells. Interestingly, the shRFP (control) lost DICER1 expression *in vivo,* according to RNA analysis after the tumors were resected. This speaks to the selection pressure for reduced DICER1 in tumors *in vivo,* but it also a very small sample size. This result shows the need for reliable methods to downregulate genes in human cells growing orthotopically or in xenografts on mouse models.

Another way to determine the effect of reduced DICER1 *in vivo* is to assess primary tumors with known DICER1 levels. Future directions include determining the levels of interferon response genes in primary tumors with high and low levels of DICER1. However, because we acquired RNA from primary tumors mixed with infiltrating cells from the immune system, it would be necessary to use microdissected endometrial cancer tissue to determine the effect on the cell-autonomous immune response I observed. Microdissection has been successfully performed to separate epithelium from stroma in breast cancer (Kurose et al. 2001) and study the two

components in isolation. There is no reason this could not be done for endometrial cancer samples to allow us to see the effect of low DICER1 on the cell autonomous immune response *in vivo.*

While the RNA-Sequencing method used to characterize the transcriptome of shDcr cells was well-established, methylome sequencing developed as I proceeded through graduate school. Thus I was able to learn and try out new methods to map genome-wide methylation in DICER1 knockdown cells. From this experience I have learned that technical and biologic validation of genome-wide differences is crucial. In addition, genome-wide techniques such as "M + M" have the ability to characterize changes in nongenic regions such as the Alu elements that chip- or array-based methods do not.

The Next Generation Sequencing methods I used to assess the effects of reduced DICER1 in endometrial cancer cells made possible the identification of molecular phenotypes that I would have missed by taking a traditional gene-by-gene approach, such as the characterization of the *MLH1/EPMA2IP1* promoter. My thesis research taught me about the power of genomic approaches as well as the care and time necessary to validate results and meaningfully connect them with tumorigenesis. The human genome sequence and novel genomic methods have accelerated the pace of cancer research. Genomic discoveries hold promises for new cancer therapies and potentially approaches to the prevention of cancers. Putting genomic discoveries into biologic context is a daunting challenge. New paradigms focused on contextualizing genomics will be needed to capitalize on genomic discovery efforts, and further mechanistic studies are necessary.

Epigenomics, an even newer field, may hold particular relevance for cancer treatments. Epigenetic therapies for cancer have been considered since the DNA methyltransferase inhibitor 5-azacytidine and its analog 5-aza-2'-deoxycytidine were found to be effective in myelodysplastic syndrome (MDS), the precursor to acute myeloid leukemia (Issa et al. 2004; Lubbert 2000; Silverman et al. 2002; Wijermans et al. 2000). These drugs are nucleoside analogs that become incorporated into DNA upon replication and covalently bind up the DNA methyltransferases, preventing methylation of cytosines. They have recently been shown to be effective against both solid and hematologic tumors. A low initial dose triggers a persistent response in cancer cells, specifically inhibiting the clonogenic properties of putative "cancer stem cells" (Shen and Laird 2012; Tsai et al. 2012). A recent study showed that a small molecule enhancer of TRBP miRNA processing specifically inhibits cancer cells (Melo et al. 2011). Histone Deacetylases (HDACs) remove acetyl groups from histone to silence genes and are often overexpressed in cancer. HDAC inhibitors are used to reactivate silenced genes and have been very successful in treating many types of cancer, especially as combination therapies (Spiegel, Milstien and Grant 2012). A recent study in non-small cell lung cancer showed that DNA methyltransferase inhibitors are also successful as combination therapy (Juergens et al. 2011). These molecules are appealing cancer drugs because they change modifications to the DNA, not the DNA itself, and because they appear to preferentially target cancer cells with minimal side effects. At the 2012 American Association for Cancer Research Conference (Chicago, IL) there were dozens of talks and posters describing molecular mechanisms and clinical trials of molecules inhibiting DNA methyltransferases and HDACs. Current concerns are related to the nonspecific

nature of changing global chromatin modifications. Clearly basic science research is required to address these concerns.

I hope to make a contribution to this work during my postdoctoral research with Dr. Stephen Baylin (Johns Hopkins University Medical School) determining the molecular mechanisms by which DNA methyltransferase inhibitors target cancer cells as well as their effects on genome organization within the nucleus.

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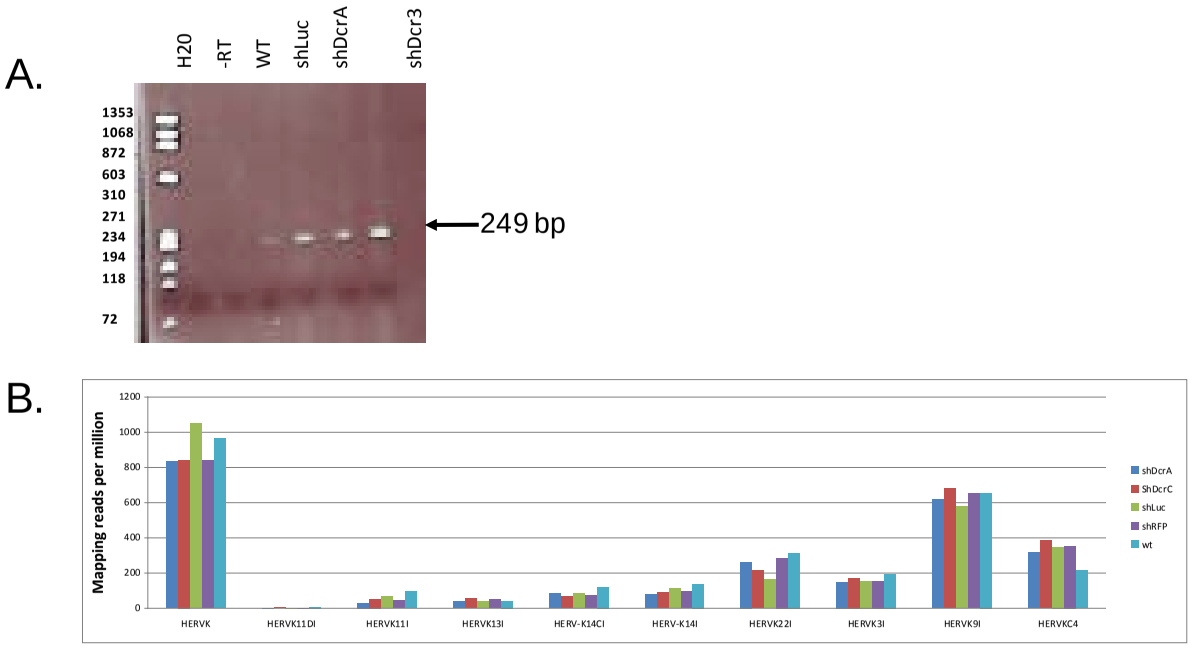

Figure 1. Endogenous retroviruses are not activated by DICER1 knockdown. A) Endogenous retroviruses were measured by conventional RT-PCR in KLE cells. Primers used were 5'-TCCCCTTGGAATACTCCTGTTTTY-3' and 5'-CATTCCTTGTGGTAAAACTTTCCAYTG-3' (Galindo et al., 2006). B) Relative levels of endogenous retrovirus families from RNA-Sequencing in KLE cells (Chiappinelli et al 2012).

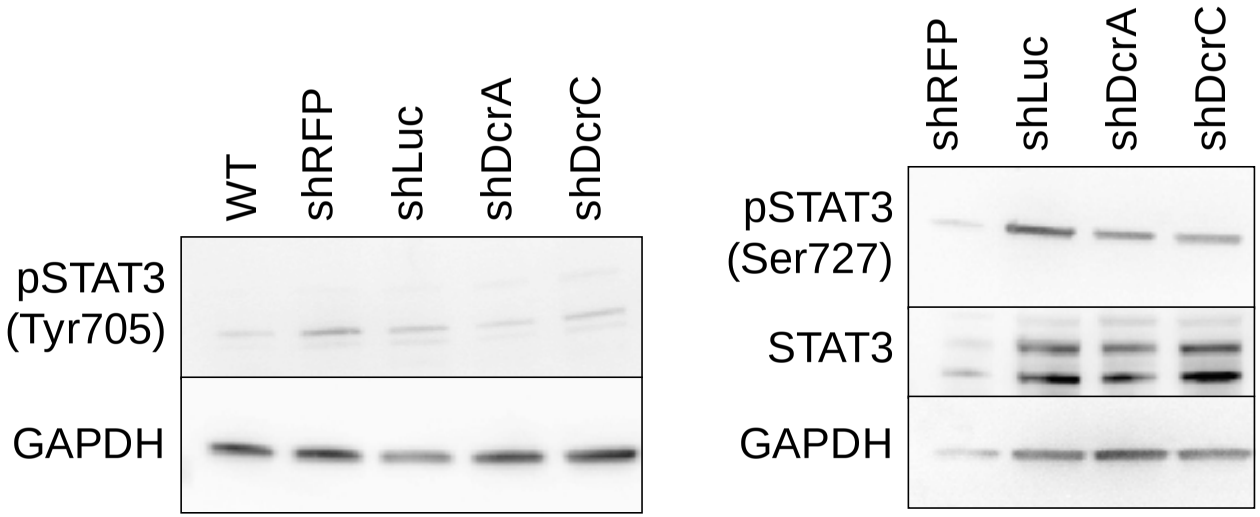

Figure 2. pSTAT3 is not activated by DICER1 knockdown. KLE cell lysates were probed for pSTAT3 Tyr705 and Ser727, using STAT3 and GAPDH to normalize. Western blots were performed as previously described (Chiappinelli et al., 2012).

APPENDIX A: Infrequent methylation of the DUSP6 phosphatase in endometrial cancer

The following is a manuscript published in Gynecologic Oncology (August 2010).

Infrequent methylation of the DUSP6 phosphatase in endometrial cancer

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Abstract

Objective: Dual-specificity phosphatase six (DUSP6, MKP3, or PYST1) dephosphorylates phosphotyrosine and phosphothreonine residues on ERK-2 (MAPK1) to inactivate the ERK-2 kinase. DUSP6 is a critical regulator of the ERK signaling cascade and has been implicated as a tumor suppressor. DNA methylation in the first intron of DUSP6 abrogates expression in a subset of pancreatic cancers. We sought to determine whether DUSP6 was similarly silenced by methylation in endometrial cancer, a tumor type in which there is frequent activation of the ERK pathway.

Methods: 109 endometrial cancers were analyzed for DUSP6 methylation using combined bisulfite restriction analysis (COBRA). The cohort included 70 primary endometrioid endometrial cancers, 21 primary endometrial tumors of adverse histological types, and 18 endometrial cancer cell lines. Primary tumors, cell lines, and normal endometrial tissues were analyzed for DUSP6 mRNA levels using quantitative RT-PCR and pERK levels by Western blots and/ or immunohistochemistry.

Results: Methylation of the first intron of the DUSP6 gene was seen in 1/91 primary endometrial cancers investigated. The methylated tumor was also methylated at the more 5' regulatory region of DUSP6. Q-RT-PCR revealed that DUSP6 transcript levels varied widely in primary endometrial tumors. DUSP6 mRNA levels did not correlate with pERK status in primary tumors, consistent with the existence of negative feedback loops activated by pERK that result in transcription of DUSP6.

Conclusion: DUSP6 methylation is a rare event in endometrial cancer. Silencing of the DUSP6 phosphatase is unlikely to contribute to constitutive activation of the ERK kinase cascade in endometrial cancer.

Introduction

Endometrial cancer is the most common gynecological malignancy in the United States, with 42,160 new cases and 7,780 deaths predicted in 2009 (Jemal et al. 2009). Although most women present with early stage disease and are cured with a hysterectomy, approximately 15% of patients suffer from recurrent or persistent disease that is often fatal (Creutzberg et al. 2000). Discovery of the molecular lesions that contribute to endometrial tumorigenesis will provide opportunities for targeted therapies for endometrial cancer.

Endometrioid endometrial carcinomas comprise about 80% of uterine cancers. Several key genetic events associated with the development of endometrioid endometrial cancer have been described. Inactivating mutations in the PTEN tumor suppressor and gain-of-function CTNNB1 mutations are seen in 26-80% and 25-38% of tumors respectively (Hecht and Mutter 2006). Gain-of-function mutations in the ERK kinase cascade (FGFR2 or KRAS2), leading to ERK activation, are seen in 20-30% of tumors (Byron et al. 2008). However, FGFR2 and KRAS2 mutations do not explain ERK-2 activation in all cases. ERK activation (pERK) is seen in over 60% of endometrial cancers ((Mizumoto et al. 2007), and our unpublished data). The ERK kinase cascade is normally initiated by the binding of growth factors (ligands such as EGF and FGF) to cell-surface receptor tyrosine kinases, resulting in autophosphorylation of the tyrosine kinase domains of the intracellular protein of the receptor. This in turn triggers G-protein-mediated activation of the RAS kinase, which phosphorylates the RAF effector, which phosphorylates ERK-2 (MAPK1). ERK-2 has many phospho-targets involved in transcriptional regulation, translational regulation, and control of the cell cycle.

Mutations in genes in the ERK kinase pathway contribute to the development of a variety of cancers. In endometrioid endometrial cancer, activating FGFR2 mutations are identified in 10-16% of endometrioid tumors and activating KRAS2 mutations in 10-30% of endometrioid tumors (Byron et al. 2008; Dutt et al. 2008). These mutations occur exclusively of one another (Byron et al. 2008). In addition to mutational activation of the ERK cascade, increased ERK activation can result from silencing of the DUSP6 phosphatase that normally serves to inactivate ERK-2 (Xu et al. 2005).

A number of dual-specificity phosphatases regulate specific kinases in normal mammalian cells. DUSP1, DUSP2, and DUSP4 localize to the nucleus and target JNK, p38, and ERK; DUSP5, DUSP6, DUSP7, and DUSP9 localize to the cytoplasm and target ERK. All of the phosphatases are expressed in normal human uterine tissue (Expressed Sequence Tag Database 2010). The mouse knockout of DUSP6 shows no gross abnormalities, but has significantly increased phospho-ERK (Owens and Keyse 2007). RNAi-mediated knockdowns of DUSP6 result in increased phospho-ERK, showing a direct relationship between the level of this phosphatase and pERK (Chan et al. 2008; Zeliadt, Mauro and Wattenberg 2008).

DUSP6 has been identified as a tumor suppressor gene and is inactivated in several different types of cancer. A recent study showed that ~18% of primary lung cancers exhibit loss of heterozygosity at the DUSP6 locus. DUSP6 expression shows an inverse correlation with grade in lung cancer (Okudela et al. 2009) and DUSP6 has been implicated as a tumor suppressor gene in non-small-cell lung cancer (Zhang et al. 2010). The accumulation of reactive oxygen species in ovarian cancer causes ubiquitination and proteasomal degradation of DUSP6, leading to increased ERK-2 activity and cell

proliferation (Chan et al. 2008). A third mechanism of inactivation, DNA methylation, has been observed in pancreatic cancer cell lines and primary tumors (Furukawa et al. 1998; Zeliadt, Mauro and Wattenberg 2008). Pancreatic cancers, like endometrial cancers, show frequent mutational activation of KRAS2 (Almoguera et al. 1988), which leads to increased pERK levels. Methylation of intron 1 of DUSP6 is associated with reduced expression of DUSP6 (Xu et al. 2005). The region of intron 1 methylated in pancreatic cancer has promoter activity and includes a binding site for the ETS2 transcription factor. ETS2 is a target of ERK-2 and ERK-2 and DUSP6 are involved in a negative feedback loop. As phosphorylated (activated) ERK-2 accumulates in the cell, it phosphorylates ETS2, which in turn transcriptionally activates DUSP6, which functions by removing phosphate groups to inactivate ERK-2 (Ekerot et al. 2008; Furukawa et al. 2008). DUSP6 has also been shown to be upregulated through negative feedback by high levels of fibroblast growth factor (FGF) and KRAS2 (Owens and Keyse 2007). DUSP6 expression is higher in Stage I than Stage II endometrial cancers (Wu et al. 2005). Given the high frequency with which mutational activation of the ERK signaling pathway is seen in endometrial cancers, we hypothesized that methylation of the DUSP6 gene leading to low expression of DUSP6 might also contribute to constitutive activation of the ERK kinase cascade. We evaluated DUSP6 methylation in a large cohort of endometrial cancers representative of all grades, stages and histologic types.

Materials and Methods:

Preparation of Nucleic Acids:

All primary endometrial tumors and normal endometrium specimens analyzed were collected as part of IRB-approved studies (Washington University Medical Center Human Research Protection Office protocols HRPO-91-507, -93-0828 and -92-242). Histologic grading and typing were performed by gynecologic pathologists. All primary tumors evaluated had ≥70% neoplastic cellularity. Staging was determined using 1988 criteria from the International Federation of Gynecology and Obstetrics. Tissue specimens and blood were obtained at the time of surgery and stored at -70° C until nucleic acids were extracted. Genomic DNA from tumor tissues, normal endometrium, and cell lines was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA). Total cellular RNA was extracted from tumors and cell lines using the Trizol reagent (Invitrogen).

DNA from eighteen endometrial cancer cell lines and one pancreatic cell line was also investigated. The cell lines were AN3CA, HEC1A, HEC59, HHUA, HOVA, Ishikawa, KLE, MDA H2774, MFE280, MFE296, MFE319, RL952, Sawano, TEN, UAC1053, UACC210, UACC297, and MiaPaCa-2.

Bisulfite Conversion:

DNA bisulfite conversion was performed using a commercially available kit (EZ DNA Methylation Gold™ Kit, Zymo Research, Orange, CA).

DUSP6 COBRA Assays:

COBRA (Combined Bisulfite Restriction Analysis) was performed as previously described (Xiong and Laird 1997). We used two rounds of amplification (nested PCR). Three assays were designed at the DUSP6 5' upstream region, 5' UTR, and intron 1. The primers used in nested PCR, amplicon sizes, and restriction digestions used are presented in Table 1.

Restriction fragments were resolved on 10% polyacrylamide gels, stained with ethidium bromide, and photoimaged with a UV camera (ImageSTore 500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA). Band intensities were captured and quantified using the program ImageJ (National Institutes of Health, Bethesda, MD).

cDNA preparation and quantitative RT-PCR:

Total RNA preparation was used as a template to generate first-strand cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative gene expression was performed using SYBR® Green (BioRad) methods (Whitehead et al. 2005) and relative expression was calculated using the ΔΔCT method. Quantitative RT-PCR primers were: Forward 5' CCCCTTCCAACCAGAATGTA 3', Reverse TGCCAAGAGAAACTGCTGAA 3'. GAPDH was used as the reference gene, PCR primers were: Forward 5' TGCACCACCAACTGCTTAGC 3', Reverse 5' GGCATGGACTGTGGTCATGAG 3'.

Immunohistochemistry:

Immunohistochemistry was performed for a subset of primary tumors investigated by COBRA. Five micrometer sections of paraffin-embedded, formalin-fixed tissues were

obtained of eight endometrioid tumors. MKP-3 staining was performed with anti-MKP3 antibody (sc-8598, goat anti-human, polyclonal Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 1:100 dilution followed by a biotinylated secondary antibody at 1:500 dilution (Donkey anti-goat, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, CA). Signals were developed with the 3,3'-diaminobenzidine (DAB) Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, CA). ERK and phospho-ERK staining was performed using anti-ERK1/2 (#9012, rabbit anti-human, Cell Signaling Technology, Inc., Danvers, MA) and anti-phospho-ERK (Thr 202/Tyr 204, #9101S, rabbit anti-human, Cell Signaling Technology, Inc., Danvers, MA) at 1:100 dilution. Signals were developed with the 3,3'-diaminobenzidine (DAB) Substrate Kit for Peroxidase (BioCare Medical, Concord, CA).

Western Blots:

Protein was extracted using lysis buffer containing a mixture of protease and phosphatase inhibitors. ERK and phospho-ERK were detected using the same antibodies used for IHC (1:1000 dilutions). Goat anti-Rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a secondary antibody at a concentration of 1:1250.

Results

Three COBRA assays were used to evaluate DUSP6 methylation (Figure 1A). Assay 3, located in intron 1, includes the region previously shown to be methylated in pancreatic cancers. Methylation of this region prevents transcription factor binding and is associated with loss of DUSP6 expression (Dutt et al. 2008; Expressed Sequence Tag Database 2010; Xu et al. 2005). The 5' upstream region, as well as exon 1 and intron 1, are CpG rich. Because the CpG methylation that is associated with gene silencing most often involves promoter regions upstream of the transcription start site (Cedar and Bergman 2009), we further evaluated more 5' regions of DUSP6 for methylation, using additional COBRA Assays 1 and 2.

BstUI (CGCG) and *MboI* (GATC) restriction digests were used to evaluate methylation in intron 1 (Assay 3) in 91 primary uterine tumors, representing a diverse group of grades, stages, and histologies. A single endometrioid tumor (2070, stage IC, grade 2) showed methylation at both the *MboI* and *BstUI* sites (Table 2, Figure 1B). None of the 18 endometrial cancer cell lines showed methylation in intron 1 of DUSP6. MiaPaCa-2, the pancreatic cancer cell line previously shown to have DUSP6 methylation and very low expression of DUSP6 (Xu et al. 2005), had approximately 40% methylation at the *BstUI* and *MboI* sites by COBRA (Figure 1B).

We evaluated more 5’ sequences for methylation using COBRA assays to determine whether the methylation seen in tumor 2070 and the MiaPaCa-2 cell line was restricted to intron 1 (Assay 3 in our studies, Figure 1A). Assays 1 and 2, in the 5' upstream region and 5' UTR respectively, were evaluated in tumor 2070 and MiaPaCa-2. In addition, seven endometrial cancer cell lines (AN3CA, HEC1A, Ishikawa, KLE,

MFE296, RL952, and SKUT1B), 3 normal endometrial tissues, and 33 primary tumors from the cohort evaluated with Assay 3 were evaluated for methylation with Assays 1 and 2. None of the samples evaluated showed methylation at Region 1 (data not shown). Tumor 2070 and the MiaPaCa-2 cell line, however, showed methylation in Region 2 using *BstUI* and *HpyCH4IV* COBRA (data not shown).

Quantitative RT-PCR showed DUSP6 mRNA was reduced in the MiaPaCa-2 cell line compared to all cell lines, tumors, and normal tissues assessed (Figure 2A). DUSP6 expression varied widely in the normal endometrial tissues, endometrial cancer cell lines, and tumors investigated (Figure 2A). DUSP6 transcript levels in normal endometrial tissues varied approximately two-fold (range 272 to 601 arbitrary expression units relative to the MiaPaCa-2 cell line). Expression in endometrial cancer cell lines ranged from 56 to 861 units and in primary endometrioid endometrial cancers from 55 to 889 units (Figure 2A). DUSP6 transcript levels were not correlated with the pERK levels as assessed by Western blots and IHC (Figure 2B,C). Tumor 2070, which has *DUSP6* methylation, did not show a substantial reduction in DUSP6 expression at the mRNA level. Samples with low DUSP6 expression at the mRNA level (2027T, 1570T, 1474T, 1655T, etc.) did not show methylation at any region of the DUSP6 gene. Immunohistochemistry revealed DUSP6 expression in all tumors evaluated, including the specimen 2070 with 5' UTR and intron 1 methylation (data not shown).

Discussion

To the best of our knowledge this study is the first to assess DUSP6 methylation in a large cohort of endometrial cancers. We conclude that methylation of DUSP6 is an

infrequent event in endometrial cancers based on our observation of a single methylated case among 91 tumors investigated. None of the eighteen endometrial cancer cell lines evaluated showed DUSP6 methylation, further supporting our conclusion that DUSP6 methylation is uncommon in endometrial cancers. In contrast to previous findings in pancreatic cancers (Xu et al. 2005), DUSP6 intron 1 methylation did not appear to affect mRNA or protein expression as assessed by quantitative RT-PCR and IHC. However, we had a single example of a primary tumor with DUSP6 methylation and it is difficult to speculate as to why the observed methylation was not associated with reduced expression. One possible explanation for the difference in DUSP6 expression in the endometrial cancer we observed and what has been described for pancreatic adenocarcinomas could be the extent of the methylation. Tumor 2070 had an estimated 20% methylation of DUSP6 at intron 1. The pancreatic adenocarcinomas with lowered DUSP6 expression were shown to have ≥40% methylation at intron 1 of DUSP6 (Xu et al. 2005) and the 20% methylation observed in sample 2070 may not be sufficient to affect DUSP6 expression.

We observed methylation at the putative 5' regulatory region of DUSP6 in sample 2070 as well as MiaPaCa-2, a pancreatic cell line with low expression of DUSP6. Methylation at the more 5' region of the DUSP6 sequence has not been previously reported. The significance of this methylation is unknown; however, the importance of methylation at the 5' regions of genes has been well characterized (Cedar and Bergman 2009; Herman and Baylin 2003). Methylation in 5' regulatory regions can contribute to recruitment of repressive proteins, a closed chromatin structure, and gene silencing. The closed chromatin state may spread downstream from the 5' region (Jones et al. 1998;

Kass, Goddard and Adams 1993). Methylation could thus be initially targeted to either the 5' region or intron 1 of DUSP6, then spread to other regions, effectively silencing expression of the gene. It is presently unclear which region is methylated first *in vivo*.

The low rate of *DUSP6* methylation in endometrial cancers was somewhat unexpected given the fact many endometrial cancers have a CpG island methylator phenotype and, like pancreatic cancers, have frequent mutations in the ERK signaling pathway (Arafa et al. 2008; Joensuu et al. 2008; Whitcomb et al. 2003). Hypermethylation of promoter regions and the resultant CpG island methylator phenotype (CIMP) as seen in endometrial cancers is a form of epigenetic deregulation (Herman and Baylin 2003; Lujambio and Esteller 2009). The absence of methylation at the DUSP6 promoter in cancers that often show abnormal methylation of promoter regions could reflect strong selection for DUSP6 expression and regulation of ERK-2 phosphorylation in endometrial cancers and/or selection against tumor cells in which the DUSP6 promoter is methylated. Alternatively, the sequence or genomic context of DUSP6 could make it relatively resistant to methylation. Our methylation studies did not address the possibility of post-transcriptional or translational repression of DUSP6 expression; the variation in DUSP6 transcript levels could be explained by microRNA regulation or other post-transcriptional events.

Quantitative RT-PCR showed low DUSP6 mRNA levels in the MiaPaCa-2 cell line with DUSP6 methylation compared to the endometrial normal tissues, cancer cell lines, and primary cancers, consistent with transcriptional silencing by methylation. Endometrial cancer cell lines with low phospho-ERK (AN3CA, SKUT1B) exhibited high levels of DUSP6 mRNA. HEC1A, with high levels of pERK, had low DUSP6 mRNA

expression, and Ishikawa and MFE296 had medium levels of both pERK and DUSP6, consistent with DUSP6 regulation of ERK phosphorylation. The level of DUSP6 expression we saw in the Ishikawa cell line is similar to what has been previously reported (Cui et al. 2006). However, KLE and RL952 did not fit this expression pattern (Figure 2). While seventeen primary endometrial cancers assessed showed a large variation in DUSP6 transcript expression, there appears to be no relationship between DUSP6 mRNA and phospho-ERK status. Three normal endometrium tissues were also assessed and exhibited medium-high levels of DUSP6 mRNA (Figure 2A). An explanation for the lack of correlation between DUSP6 mRNA and phospho-ERK could be that other phosphatases are at work, such as DUSP5, DUSP7, or DUSP9 (Owens and Keyse 2007). Feedback loops in place in response to activated ERK-2, FGF, and KRAS could also affect levels of DUSP6 when pERK levels are high.

Phosphorylated ERK-2 is seen in >60% of endometrioid endometrial cancer cases, including some that lack activating mutations upstream in the pathway ((Mizumoto et al. 2007), and our unpublished data). KRAS2 and FGFR2 mutations are common in endometrioid endometrial cancers (Byron et al. 2008) but do not account for all of the cases with activated ERK. We hypothesized that aberrant hypermethylation of the DUSP6 gene and silencing of the DUSP6 ERK-2 phosphatase could be an additional mechanism of constitutive activation of the ERK kinase pathway in endometrial cancers. Given current interest in MEK inhibitors (MEK phosphorylates ERK) as biologic therapies for cancer, understanding how ERK activity is regulated is of increasing importance (Adjei et al. 2008; Haura et al. 2010).

This study shows that DUSP6 methylation is uncommon in endometrial cancer. Further studies are required to determine whether the high rate of activated ERK seen in endometrial cancers is attributable to as yet unknown upstream activation events and whether DUSP6 activity is deregulated by other mechanisms in pERK-positive endometrial cancers.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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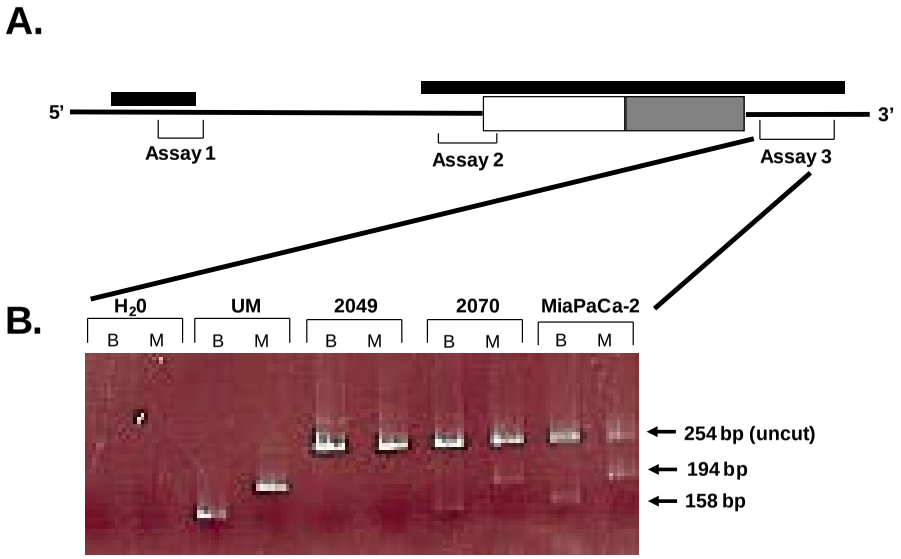

Figure 1. COBRA Assays for the DUSP6 gene. (A) Schematic representation of the DUSP6 gene**.** Black line on the left represents the 5' regulatory region (1200 bp), white rectangle represents the 440 bp 5' UTR of Exon 1, grey rectangle represents the coding sequence of Exon 1 (400 bp). Black line on the right represents Intron 1 (400 bp shown). Black rectangles represent CpG islands. Brackets indicate COBRA Assays 1, 2, and 3. **(B)** Representative COBRA; Assay 3 in intron 1**.** The universally methylated (UM) control shows the expected 158 and 194 bp bands for *BstUI* and *MboI* digests, respectively. Tumor 2049 is unmethylated whereas tumor 2070 and Mia-PaCa-2 show methylation. B: BstUI digestion, M: MboI digestion.

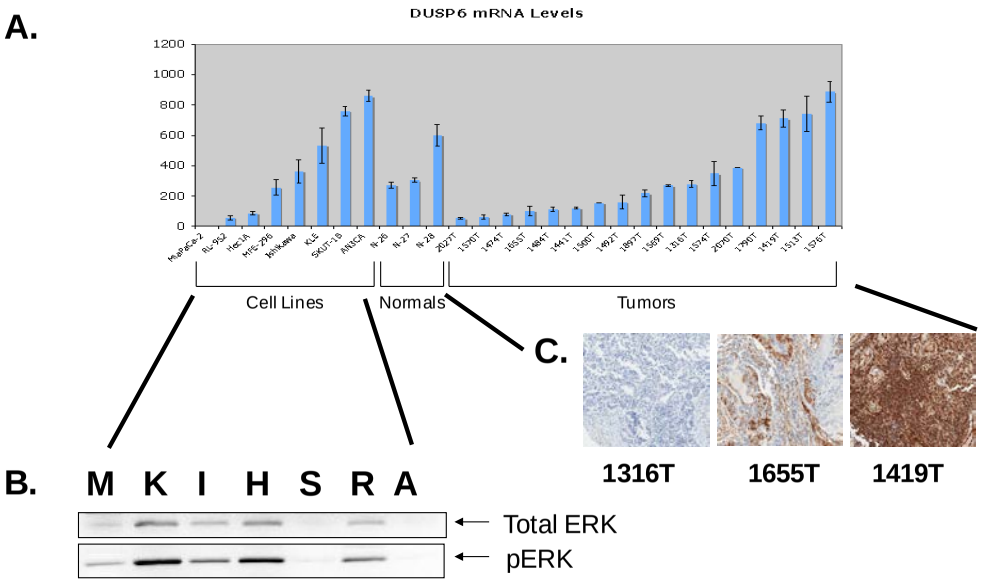

Figure 2. A. Level of DUSP6 mRNA in cancer cell lines assessed by Q-RT-PCR, shown as fold change relative to the MiaPa-Ca-2 cell line. All experiments were performed in triplicate and replicated at least once. Error bars indicate SEM. B. Western blot to assess total ERK and pERK levels in endometrial cancer cell lines.
C. Representative examples of very low (1316T), medium (1419T), and high (1655T) pERK expression in primary tumors, by immunohistochemistry.

Table 1. Primers and restriction digests used for COBRA assays.

Assay	Primers*	Amplicon Size	Restriction Digest Products
Region 1	Rd1 For 5'gaTatgTTtTTTtgTTaaTtgtaa 3'	147 bp	*BstUI* (*CGCG*) 117 & 30 bp
(5' putative regulatory region)	Rd1 Rev 5'aacaaactcttaAAtcaAtcc 3'		*AciI* (*CCGC*) 62, 59 & 26 bp
	Rd2 For 5'agTtTTttgTTTagaaatTattaa 3'		
	Rd2 Rev 5'caAtccaAtActtttactAtattc 3'		
Region 2	Rd1 For 5'aagtgTTTtggtttatgtgTTTtg 3'	196 bp	*BstUI* 157 and 39 bp
(5' regulatory region, 5' UTR)	Rd1 Rev 5'tctaatccctccctccaaAA 3'		*HpyCH4IV* (*ACGT*) 85, 74 & 37 bp
	Rd2 For 5'ttgtgaatgaTaaaTtTattaaTa 3'		
	Rd2 Rev 5'tttActatctcttAAactcaAcct 3'		
Region 3	Rd1 For 5'tgTtgTtTaagaagTtTaagg 3'	254 bp	*BstUI* 158, 45, 42, 7 & 2 bp
(intron 1)	Rd1 Rev 5'tttAcatccccaacaatct 3'		*MboI* (*GATC*) 194 & 60 bp
	Rd2 For 5'ggattgaaaataTTtTtgTtT 3'		
	Rd2 Rev 5'tcctAcaaatcttaattcaaa 3'		

*Capitalized letters indicate unmethylated C converted to T by bisulfite treatment.

Table 2: Clinical and molecular features of tumors analyzed for DUSP6 expression.

Breakdown of tumors analyzed for DUSP6 methylation	N (% methylated)
Primary uterine cancers (N)	91 (1.1 %)
Stage	
I	49 (2.0%)
II	7 (0%)
III	26 (0%)
IV	9 (0%)
Histology	
Endometrioid	70 (1.4%)
Grade 1 (33)	
Grade 2 (21)	
Grade 3 (16)	
Papillary serous	7 (0%)
Clear cell	7 0%)
Carcinosarcoma	7 (0%)
ERK Kinase Cascade Mutation Status	
Wild type	55 (1.8%)
FGFR2 mutation	4 (0%)
KRAS2 mutation	8 (0%)
Unknown	24 (0%)

Grading and staging was performed according to FIGO staging.

APPENDIX B: Evaluating the frequency of *TARBP2* mutations in endometrial cancer

A subset of colon and endometrial cancer cell lines with methylation of *MLH1* and the resulting loss of mismatch repair and MSI phenotype have loss of function *TARBP2* mutations (Melo et al. 2009). Insertions or deletions in one of the two poly-C tracts in Exon 5 of the *TARBP2* gene are presumed uncorrected strand-slippage mutations (fairly common in MSI cancers) and abrogate expression of the TRBP protein, which normally forms a complex with DICER1 and assists in microRNA processing (Melo et al. 2009). A similar mutation was characterized in a poly-A tract of the *ATR* gene in MSI-positive endometrial cancer and was associated with poor clinical outcomes (Lewis et al. 2005; Zighelboim et al. 2009). *TARBP2* mutations cause destabilization of the DICER1 protein and subsequent microRNA processing defects. Melo *et al.* (Melo et al. 2009). screened four endometrial cancer cell lines and found that SKUT1B had an insertion in the *TARBP2* gene. They did not evaluate primary endometrial tumors but identified *TARBP2* mutations in 25.4% of MSI colon cancer and 14% of MSI gastric cancer primary tumors. In order to determine how frequent this specific *TARBP2* mutation is in endometrial cancer, I sequenced the two C tracts in Exon 5 of *TARBP2* in endometrial cancer cell lines and primary tumors. I confirmed the C insertion in the SKUT1B cell line, but did not observe any *TARBP2* mutations the AN3CA cell line (Figure 1). In Figure 1, SKUT1B is a C_7/C_8 heterozygote, compared to AN3CA, a wild type C_7/C_7. The sequencing was performed with the reverse primer, thus one allele of SKUT1B is shifted to the left upon the C insertion. This result is concordant with that of Melo *et al* (Melo et al. 2009). However, no mutations were seen in four other cell lines and 64 MSI primary tumors. I conclude that this mutation is, in fact, not common in endometrial cancer.

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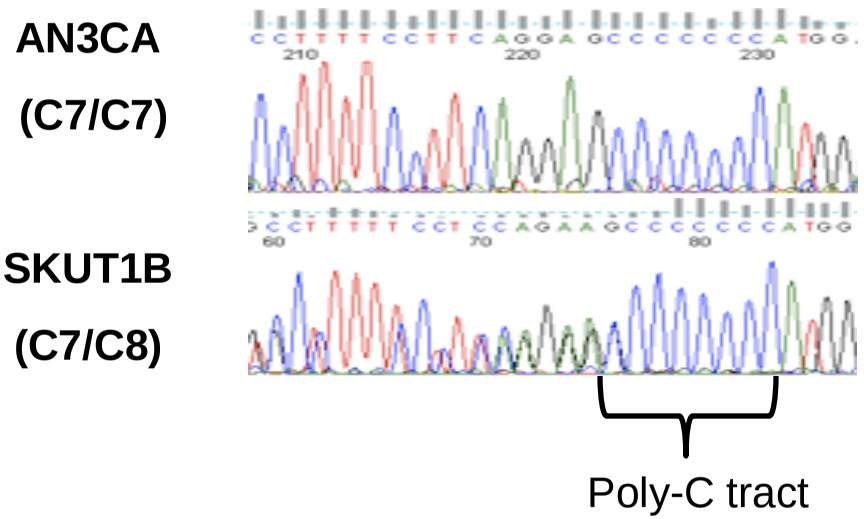

Figure 1. TARBP2 mutation in the SKUT1B cell line. Cancer cell lines were grown as previously described (Byron, Gartside et al. 2008) and DNA isolated as described in (Chiappinelli, Rimel et al. 2010). A portion of Exon 5 of the TARBP2 gene was amplified by PCR using the primers from (Melo, Ropero et al. 2009) and was subjected to cloning and Sanger sequencing as described in (Zighelboim, Schmidt et al. 2009). The Poly-C tract is indicated by the brackets.

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Education

B.S. in Biology and Music, Haverford College, May 2007.
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Employment

1999	Intern, Friendship Animal Hospital, Washington, DC
2002	Intern, The George Washington University School of Medicine, Washington, DC
2006	Intern, Center for Cancer Research, National Naval Medical Center, Bethesda, MD

Awards and Fellowships

National Merit Scholarship Finalist, 2003

Presidential Scholar Nominee, 2003

Cancer Biology Pathway Fellow, Siteman Cancer Center, Washington University in St. Louis (September 2008-September 2010)

Student Representative, Developmental Biology Program, Washington University in St. Louis (January 2009-December 2011)

Student Poster Award, the Molecular Genetics and Genomics Program Retreat, 2010

Student Representative (Washington University), The 9th International Student Seminar, Kyoto, Japan, 2011

The Teaching Citation, Washington University in St. Louis (December 2011)

AACR-Bristol-Myers Squibb Oncology Scholar-in-Training Award (AACR Annual Meeting, Chicago, Illinois, April 2012)

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Publications:

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Rimel BJ, Arur S, Khatri A, Thompson D, **Chiappinelli KB**, Schedl T, et al. ERK phosphorylation of DICER1 results in nuclear localization in mammalian cells. *Manuscript in preparation.*

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Science Education:

Chiappinelli KB, Moss BL, Lenz DS, Holt GE, Holt LE, Woolsey TA. Refining a K-12 Science Outreach Program. *Manuscript in preparation.*

Chiappinelli KB. The Young Scientist Program: Fostering diversity in science and public science literacy. *ASBMB Today.* October 2011.

Invited talks:
A genomics approach to understanding DICER1's role in tumorigenesis. **Chiappinelli KB**, Haynes BC, Schillebeeckx M, Mitra RD, Brent MR, Wang T, Goodfellow PJ. *The 9th International Student Seminar, Kyoto, Japan.* March 2011.

Posters:
Chiappinelli KB, Haynes BC, Brent MR, Goodfellow PJ. A genomics approach to understanding DICER1's role in tumorigenesis: Interferon responses in the cancer cell. *The American Association for Cancer Research Annual Meeting, Chicago, Illinois.* April 2012.

Kizer N, Yin Y, Trinkhaus K, **Chiappinelli KB**, Thompson DM, Ma L, Goodfellow PJ, Thaker P. Glycogen synthase kinase 3β inhibition as a therapeutic approach in the treatment of endometrial cancer. *The Society for Gynecologic Oncology Annual Meeting, Austin, Texas.* March 2012.

Lynch J, Moss B, **Chiappinelli KB**, Mosher J, Woolsey TA. The Young Scientist Program: successful use of a volunteer based outreach program created by graduate and medical students to improve science education in the St Louis Public School system. *The American Association of Immunologists Annual Meeting, San Francisco, California.* May 2011.

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Ramsingh G, Kobolt DC, Trissal M, **Chiappinelli KB**, Wylie T, Koul S, Chang LW, Nagarajan R, Fehniger TA, Goodfellow P, Magrini V, Wilson RK, Ding L, Ley TJ, Mardis ER, Link DC. Complete characterization of the microRNAome in a patient with acute myeloid leukemia. *AACR Translational Cancer Medicine, San Francisco, California.* July 2010.

Saharia A, Teasley DC, Dao B, **Chiappinelli KB**, Stewart SA. FEN1 facilitates replication fork reinitiation and ensures telomere stability. *AACR The Role of Telomeres and Telomerase in Cancer Research, Fort Worth, Texas*. March 2010.

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Teacher, Writing Course, Summer Focus (May 2009-August 2010)
Student Director (August 2010- December 2011)
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Assistant Director, Levine School of Music Summer Music and Arts Day Camp, Bethesda, MD (2004, 2005, 2007)

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