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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Primary inflammatory breast cancer (IBC) accounts for approximately 3% of new breast cancers in the US. This form of locally advanced breast cancer is rapidly metastatic and, because of this disease's rapid progression, the effectiveness of aggressive multimodality treatment is limited; the 5-year disease-free, mean survival rate is less than 45%, making IBC the most lethal form of breast cancer. Here, we report that RhoC GTPase expression is regulated by the NfκB pathway. Specifically, p65 binds to and activates the RhoC promoter leading to increased RhoC mRNA expression and RhoC-mediated motility and invasion in IBC cell lines, but not control metastatic breast cancer cell lines. Additionally, we report that IBC has an additional copy of chromosome 1, possibly leading to an additional mechanism of increased gene expression. Finally, although we did not find any recurrent gene fusions in IBC by high throughput transcriptome sequencing, by microRNA array, we found that miR-31 and miR-31* are specifically downregulated in IBC cell lines. Taken together, we have identified several molecular alterations which drive the aggressive phenotype of IBC cell lines and propose that these may represent important targets for future studies of IBC.					
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## Introduction:

Primary inflammatory breast cancer (IBC) accounts for approximately 3% of new breast cancers in the US. This form of locally advanced breast cancer is characterized clinically by erythema, warmth, and dimpling of the skin that arise rapidly, typically within six months. IBC is generally not associated with precursor lesions and is rapidly invasive from the outset, especially to the skin and lymphatics, and is highly angiogenic and metastatic. Because of this disease's rapid progression, the effectiveness of aggressive multimodality treatment is limited; the 5-year disease-free, mean survival rate is less than 45%, making IBC the most lethal form of breast cancer (Beahrs et al., 1957). This rapid progression is due to the development of distant metastases, indicating that the tumors quickly acquire the ability to invade and metastasize during tumor development. This suggests that the unique aggressive inflammatory phenotype of IBC is the result of a limited number of concordant genetic alterations. As such, IBC constitutes an excellent paradigm to understand aggressive phenotypes in breast cancer. Previously, our laboratory has found concordant and consistent overexpression and of RhoC GTPase in tissue samples from patients with IBC as compared to stage-matched non-IBC (Kleer et al., 2004; van Golen et al., 1999). We have also demonstrated that RhoC GTPase occupies an integral role in the aggressive phenotype of IBC (Hakem et al., 2005; Wu et al., 2004). With the increasing evidence that RhoC and other ras-homology family proteins play a significant role in other cancers (Sahai and Marshall, 2002; van Golen et al., 2000), the therapeutic importance of inhibiting RhoC activity is clear, highlighting the crucial need to uncover the the molecular mechanisms leading to RhoC-driven metastatic phenotype of IBC. In spite of this need, however, a model explaining the mechanisms of RhoC overexpression in breast cancer does not exist. The goal of this award is to establish such a model. ***Our central hypothesis was that overexpression of RhoC GTPase in metastatic breast cancer is due to gene amplification, epigenetic deregulation, transcription factor deregulation, and/or enhanced or differential mRNA stability. Because of these cellular and molecular alterations, early stage IBC is subject to rapid metastatic spread through downstream effectors signaling for invasion and angiogenesis.***

## Body:

From my total work in breast cancer research and in graduate school, I have been able to publish 15 papers including a first author *Cancer Cell* paper on inhibition of transcription factor-driven cancers and a first author *Cancer Cell* review on high throughput sequencing as well as additional co-author manuscripts in *Science*, *PNAS*, *Nature Biotechnology* and *Cancer Cell* (2). I currently have first author papers in submission at both *Nature* and *Cancer Research* and expect to be able to submit an additional breast cancer manuscript related this proposal before my thesis defense in March.

As I matriculated through graduate school, I originally thought that I was going to work full time in Dr. Merajver's lab (first graduate school rotation) studying IBC. However, as I was granted this award I was also choosing to transfer into Dr. Arul Chinnaiyan's lab. Fortunately, I was granted permission by the DOD to transfer the award to follow me to continue working on this project from Dr. Chinnaiyan's lab. Because of this, we have been able to establish an effective and highly collaborative meeting with Dr. Merajver where I attend her bi-weekly lab meetings

and work with a technician in her lab to help complete this project. This has given me a lot of unique experiences. For example, learning how to create well defined experimental protocols and making sure that the technician has the appropriate materials and controls to execute each experiment. As such, continuing this DOD pre-doctoral grant has given me the opportunity to continue existing collaborations and to continue improving my leadership skills through working with a technician on a daily basis.

In addition to working with a technician, I have also had the opportunity to train five undergraduate students through the University of Michigan Undergraduate Research Opportunities Program and one Master's degree student. The students have learned several different protocols including PCR, restriction digests, Gateway cloning, DNA miniprep, DNA maxiprep, RNA isolation, cDNA synthesis, qRT-PCR, PCR, Western blotting, transfections of both large DNA vectors and siRNA into mammalian cells, cell culture, production of lentivirus and lentiviral transduction, cell invasion assays, cell growth assays and propidium iodide staining. Additionally, I have led a bi-weekly cancer biology journal club meeting with all of the students in our lab (26 undergraduates). At the end of each semester, I help the students compile their results to present at a lab meeting and at an Undergraduate research forum by both poster presentation and lecture. Importantly, three of the students that I have trained have been awarded NIH summer fellowships that funded their work in the lab for the entire summer.

In addition to directly working with undergraduate students in the lab, as part of the statement of work the training plan I am was a graduate assistant teaching Cancer Biology for incoming graduate students. For this course, I co-ordinated different lectures and also prepare and give several lectures throughout the semester. For example, this year I will be teaching lectures on GTPase oncogenes like RhoC, DNA damage and translocations as well as the use of high throughput sequencing for modern cancer biology. Additionally, I led review courses for the other professors' lectures and had weekly open office hours for the students. Finally, as part of the training program I have been able to host several speakers for a University of Michigan speaker series and co-ordinate student discussions with the lectures by providing background reading and a background lecture for incoming speakers.

While I have found reward in the successes that students have experienced after working with them in the various teaching formats, I have also been able to learn several new experimental techniques that I would not otherwise have had the opportunity to learn without this training grant including Solexa high throughput Transcriptome sequencing, Fluorescence in situ hybridization as well as running aCGH and microRNA arrays. Perhaps more interesting is the analysis algorithms that I am helping to develop, including those used to identify novel gene fusions from paired end sequencing data (8), for the analysis of my global profiling data from these IBC cell line samples. While little is known about the molecular origins of inflammatory breast cancer, we have made significant advances not only in the acquisition of large profiling data sets of DNA copy number, microRNA expression and transcriptome sequencing, but also in software development to analyze this data. Currently, we are in the process of completing an integrated analysis from all three profiling platforms. Additionally, we have unexpectedly found that the two IBC cell lines SUM149 and SUM190 have an extra copy of chromosome 1. Because several other stage matched breast cancer cell lines do not have this extra copy of chromosome 1, we are exploring the occurrence of chromosome 1 amplification in IBC clinical samples. The

significance of this finding is still unclear, but will be explored in more detail if a clinical correlation is observed.

The opportunity to work on developing novel techniques and protocols for this project has led directly to opportunities to improve my communication and professional skills. Within the last two years, I have presented some of the work at the American Association for Cancer Research Meeting in Denver, Colorado (April 2009 and April 2010). At those meetings, I was a co-author or first author on six posters on both the role of RhoC GTPases in IBC and other breast cancers as well as co-author on an abstract that I presented by podium presentation. Additionally, this research led to a scholarship to attend a keystone conference in Victoria, British Columbia. For this meeting, I wrote a meeting summary that was published as part of the conference proceedings. Following the research for this project, I received an independent nomination to become an American Association of Cancer Research Associate council member. For my work on molecular profiling of IBC and my overall thesis project, I received the Lindau-Nobel Prize graduate student award as well as received the Rackham “Excellence in Research” award for best thesis project by a graduate student at the University of Michigan. Finally, this research has led directly to the generation of preliminary data that was used to produce a grant, which I co-authored and is funded through the Susan G. Komen foundation N012788-00 (11-PAF00190).

### **Key Research Accomplishments:**

**Specific Aim 1: *To delineate if and how gene amplification in RhoC GTPase occurs in breast cancer and to identify novel gene fusions in inflammatory breast cancer.***

- Completed RhoC FISH and discovered that IBC cell lines do not have amplification of the RhoC locus, but carry an extra copy of chromosome 1. (**Figure 1**)
- Acquired 244k Agilent aCGH data for several cell lines including the two IBC cell lines, SUM149 and SUM190. Recurrent aberrations between the two IBC cell lines were not observed.
- Completed the Illumina bead station microRNA profiling chip V2 of cell line panel including HME, MCF10A, SUM149, SUM190, HCC1937 and BT20. This led to the identification of has-miR-31 and the anti-sense hsa-miR-31\* as downregulated in the IBC cell lines, but not the control cell lines (**Table 1**). This observation was confirmed using Taqman qPCR probes to analyze mature miR-31 and miR-31\* expression across the panel of cell lines. Because miR-31 has recently been shown to suppress metastatic breast cancer (Valastyan et al., 2009), we are currently exploring the specific role of miR-31 in IBC.
- Sequenced the RNA transcriptome of both SUM149 and SUM190 using massively parallel, high throughput paired-end sequencing on a SOLEXA GA2 from Illumina. While we found and pursued several gene fusions, we were unable to identify any recurrent gene fusions using our integrated techniques. As such we have decided to pursue the SOLEXA data in more detail by analyzing the role of non-coding RNAs (ncRNAs) in both IBC and highly metastatic breast cancer. To do this, we are attempting to identify and validate ncRNAs that are specifically expressed in either IBC, triple negative or metastatic breast cancer. Additionally, we have generated ChIP-Sequencing (ChIP-SEQ) libraries of 17- $\beta$ -estradiol treated MCF7 and BT474 cells in order to assess which of these ncRNAs may be estrogen responsive. To demonstrate the success of these experiments, **Figure 2** shows ChIP-Seq coverage maps of the estrogen-regulated gene

*GREB1* from MCF7 and BT474 cell lines starved for 48 hours and then treated with either 1nM 17- $\beta$ -estradiol or vehicle for 48 hours. CHIP-assays were performed with antibodies against ER $\alpha$  or a histone mark of activated transcription, H3K4-Me3.

**Specific Aim2:** *To determine how DNA methylation status and histone modifications regulate the RhoC GTPase promoter, and to assess the ability of the small molecule drugs 5-azacytidine and Trichostatin A to alter the metastatic phenotype depicted by an IBC cell line model.*

- Completed Illumina bead station microRNA profiling chip V2 of cell line panel including HME, MCF10A, SUM149, SUM190, MDA-MB-231, HCC1937 and BT20 treated with 5-azacytidine or Trichostatin A.
- Prepared RNA transcriptome libraries of both SUM149 and SUM190 treated 5-azacytidine or Trichostatin A for sequencing on an Illumina SOLEXA GA2.
- Treatment of MCF10A and HME cells with either 5-azacytidine or Trichostatin A revealed no significant increase in RhoC mRNA expression suggesting that the molecular mechanism leading to RhoC overexpression does not involve the activation of genes repressed by either methylation or deacetylation.

**Specific Aim3:** *To characterize the consequences of down regulating the expression of the transcription factors FoxP3, HoxA3, HoxB7, HoxB8, HoxD9, HoxD10, CREB and NF $\kappa$ B1, all of which contain highly conserved binding sites in the putative RhoC GTPase promoter, on molecular pathways regulating cell proliferation, survival and the metastatic phenotype, using an RNAi model system of human IBC cell lines.*

- Established stable shRNA knockdown cell lines for FoxP3, HoxA3, HoxB7, HoxB8, HoxD9, HoxD10, CREB and NF $\kappa$ B1 in SUM149 cells.
- Identified NF $\kappa$ B1 as a key regulator of RhoC mRNA and protein expression in SUM149 and SUM190 cells. (Figure 3)
- Completed chromatin immunoprecipitation assays that demonstrated enhanced p65 binding at 2/3 putative NF $\kappa$ B1 binding sites in the RhoC promoter. This binding pattern was unique to SUM149 cells. (Figure 4A and B)
- Established a 4.0kbp RhoC promoter reporter system. Importantly, transient transfections assays with this promoter reporter system demonstrated increased activity in the SUM149 cells, but not in MCF10A cells. This suggests that the RhoC promoter activity is deregulated in IBC leading to RhoC overexpression. (Figure 4C)
- Developed site mutants of RhoC promoter reporter system.
- Demonstrated that downregulation of p65 in IBC cells leads to loss of cell motility and invasion. (Figure 5)

**Specific Aim4:** *To determine the distribution and stability of RhoC GTPase transcription variants in altering the half-life of the different mRNAs, thereby, regulating the total RhoC GTPase protein expression.*

- Established RhoC and GAPDH probes for northern blot analysis. This experiment demonstrated that RhoC mRNA decay is not differential between IBC and non-IBC control cell lines.

## Reportable outcomes:

- Published a manuscript detailing the methodology for identification of gene fusions in epithelial cancers, “Chimeric transcript discovery by paired-end transcriptome sequencing.” (Maher et al., 2009)
- Published a review titled, “Translocations in epithelial cancers.” (Brenner and Chinnaiyan, 2009)
- A manuscript was accepted for publication at *Mol. Cancer Res*, “RhoC Expression and Head and Neck Cancer Metastasis” (Islam et al., 2009)
- Completed a book chapter that was accepted for publication, “The Rho GTPases in Cancer” (*In Press*)
- Published co-author manuscript disseminating from this proposal on the role of polycomb group proteins in aggressive breast cancer (attached).
- Manuscript is about to be submitted, “p65 drives RhoC GTPase expression and the metastatic phenotype in Inflammatory Breast Cancer”
- Research has led to an additional breast cancer grant that I co-authored through the Susan G. Komen foundation.

## Conclusions:

Since the submission of the original application and initiation of the DOD breast cancer training program, I have completed the core courses in Genetics, Biochemistry, Cell Biology and Ethics required by the University’s CMB program as well as comprehensive courses in Cancer Biology, Pharmacology, Proteomics, Bioinformatics of Sequence Alignment and Mathematical Models in Biology. I have completed a comprehensive preliminary exam on a subject unrelated to this DOD award (my thesis project) as required by the CMB program. On work directly disseminating from the hypotheses presented in the original DOD Breast cancer award, I have been first author or co-author on three manuscripts and one book chapter accepted for publication. From my total work in breast cancer research and in graduate school, I have been able to publish 15 papers including a first author *Cancer Cell* paper on inhibition of transcription factor-driven cancers and a first author *Cancer Cell* review on high throughput sequencing as well as additional co-author manuscripts in *Science*, *PNAS*, *Nature Biotechnology* and *Cancer Cell* (2). I currently have first author papers in submission at both *Nature* and *Cancer Research* and expect to be able to submit an additional breast cancer manuscript related this proposal before my thesis defense in March. Based on this work, I have received national and international awards for the breadth of my thesis work. Additionally, I was awarded two follow-up research grants emanating from this research including one that was funded by the Susan G. Komen breast cancer research foundation. Finally, I was also awarded a young investigator grant to support my transition to an independent faculty position, for which I am currently interviewing.

## Figures

**Figure 1. Fluorescence in situ hybridization (FISH) using a *RHOC* locus probe.** Normal breast tissue is shown on the left as well as the IBC cell lines SUM149 and SUM190. An interphase spread of SUM190 is shown. In both SUM149 and SUM190 cells, three copies of chromosome 1 are present as confirmed by additional cytogenetic analysis using a centromeric

probe for chromosome 1 leading to the additional copy of *RHOC*. Representative images are shown.

**Figure 2. ChIP-SEQ positive control analysis.** Chromatin immunoprecipitation-sequencing (ChIP-SEQ) using anti-ER $\alpha$  or anti-H3K4-tri-methylation antibodies on MCF7 or BT474 cells treated with or without 1nM 17- $\beta$ -Estradiol as indicated. Plots show read accumulations in reads per kilobase million were aligned to the genome using HPEAK software as previously described (Yu et al.). Analysis of the *GREB1* locus reveals increased binding of ER $\alpha$  and H3K4-tri-methylation in both cell lines following stimulation with 17- $\beta$ -Estradiol.

**Figure 3. p65 regulates RhoC mRNA expression in SUM149 cells.** Following the targeted shRNA screen, p65 was identified as a potential regulator of RhoC mRNA expression. QPCR analysis of SUM149 cells treated with p65 siRNA demonstrates that RhoC mRNA expression decreases with p65 knockdown. p65 knockdown was confirmed and IL6, a known target of p65, was used to demonstrate functional p65 knockdown. Importantly, the p65 siRNA did not alter p105 mRNA expression. Reactions were run in quadruplicate three times. Standard deviation is shown in the error bars.

**Figure 4. p65 binds to the *RHOC* promoter.** **A)** schematic shows putative p65 binding sites in the *RHOC* proximal promoter. **B)** ChIP analysis of p65 binding in HME, MCF10A, SUM149 and MDA-MB-231 cells demonstrates that p65 is enriched in the SUM149 cell line at p65 binding sites 1 and 3, but not in the control cell lines. **C)** *RHOC* promoter reporter activity demonstrates that the *RHOC* promoter, but not an empty vector control is highly active in the SUM149 cell line, but not in MCF10A cells. Data is shown relative to a renilla control used to normalize for transfections efficiency. All experiments were run in triplicate and standard deviation is shown on the bar plots.

**Figure 5. p65 expression is required for SUM149 cell motility and invasion.** **A)** Representative photomicrographs of cell motility assays in SUM149 cells treated with shRNA as indicated. **B)** Quantification of cell motility assays. **C)** As in **A**, except boyden chamber transwell migration assays. Chambers were coated with 100 $\mu$ L matrigel 4 hours prior to seeding cells in serum free media. Forty eight hours later, representative images were taken to assess invasion through 8.0 $\mu$ M pores. Cells were stained with crystal violet. **D)** Quantification of cell invasion. Cells were released from the membrane with acetic acid and quantified by colorimetric analysis at 560nm. Percent maximum invasion is shown. All experiments were run in triplicate.

**Table 1. Analysis of microRNA array data.** MicroRNAs that were greater than two-fold down- or up-regulated were compared across cell lines to identify microRNAs that were recurrently differential among IBC cell lines, but not several other control cell lines.

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Figure 1

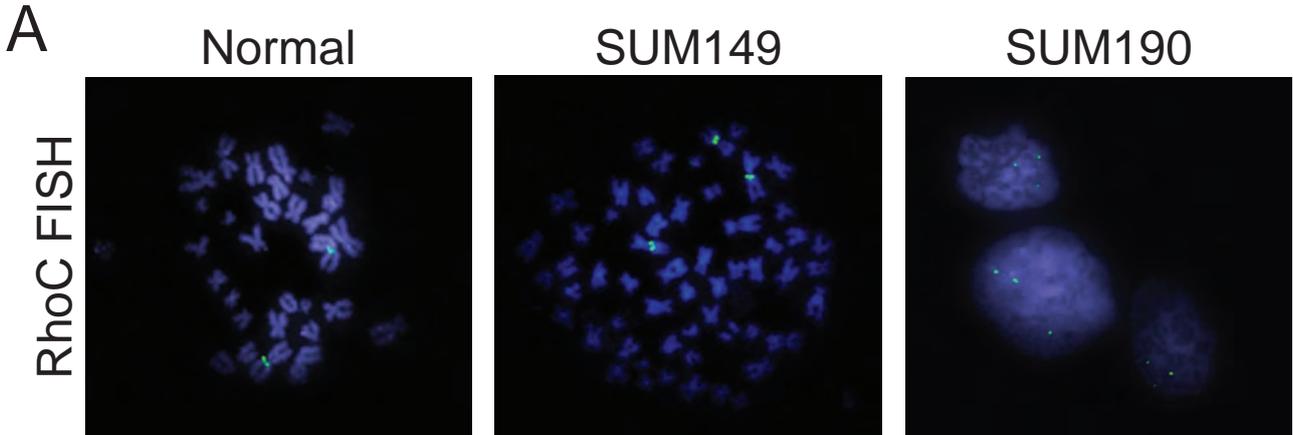
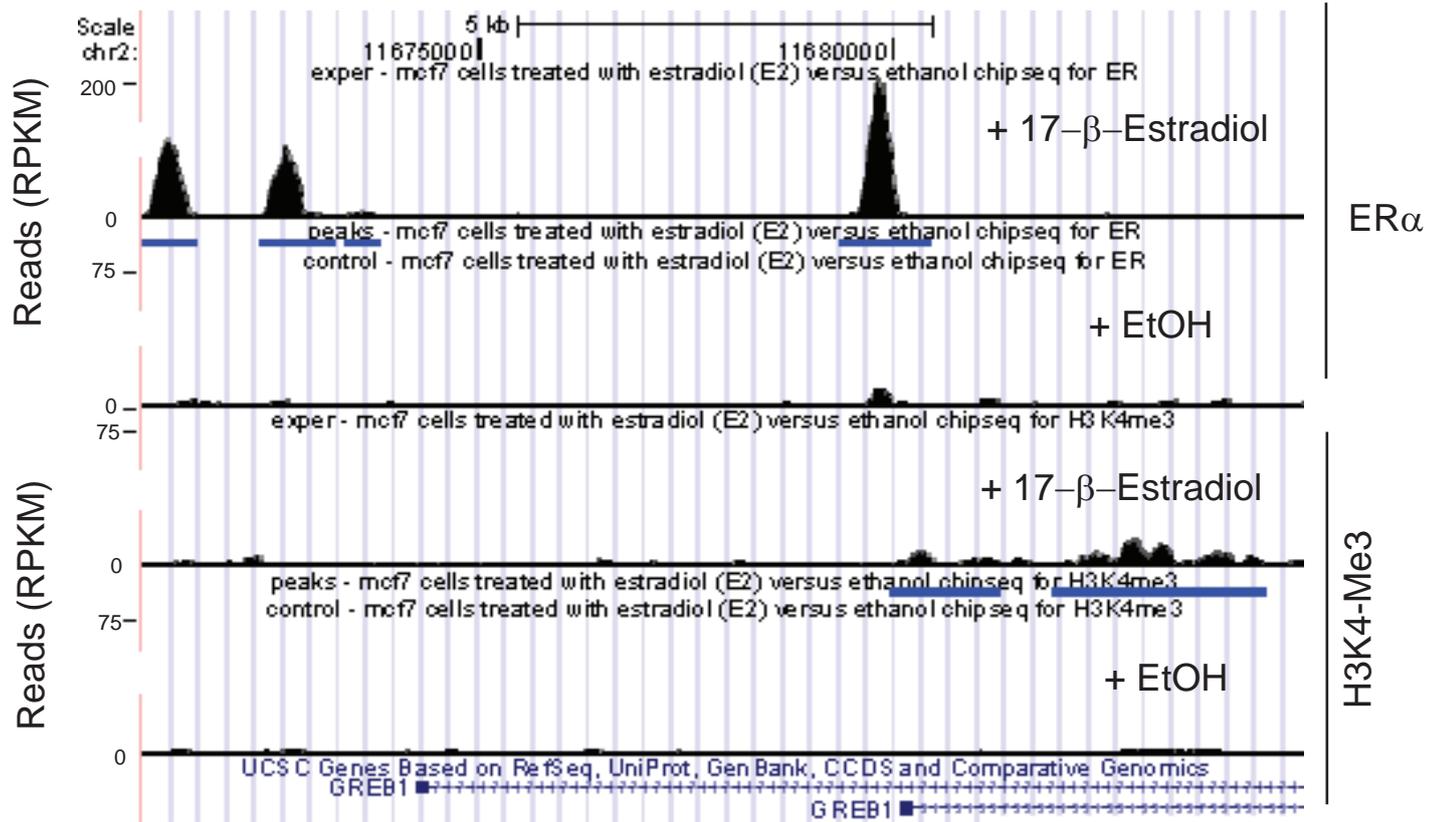
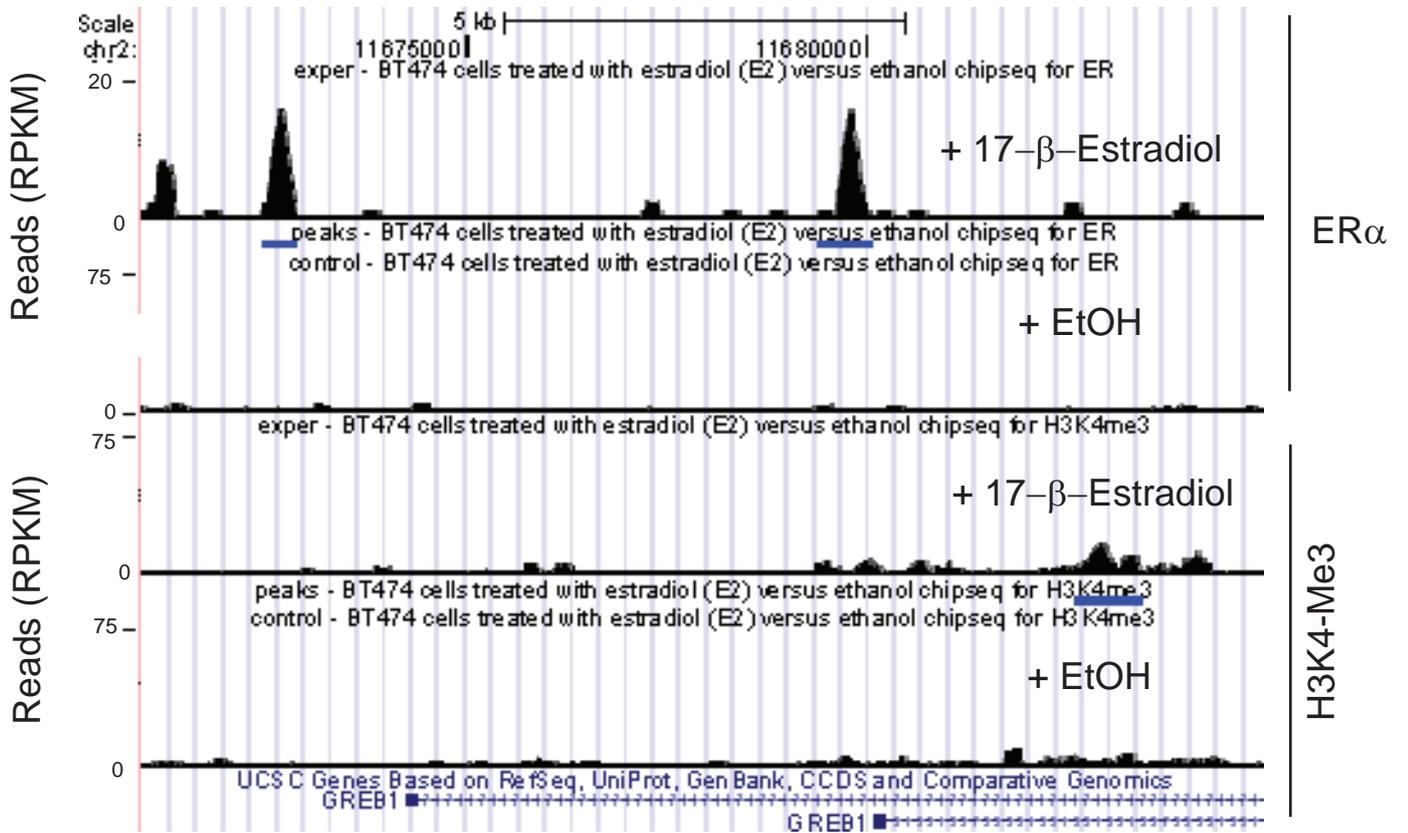


Figure 2  
MCF7

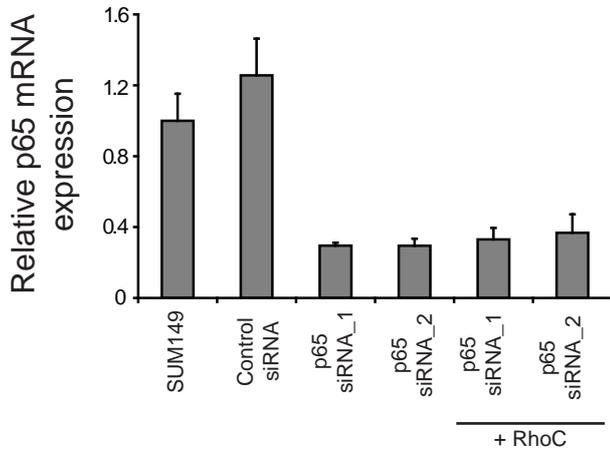


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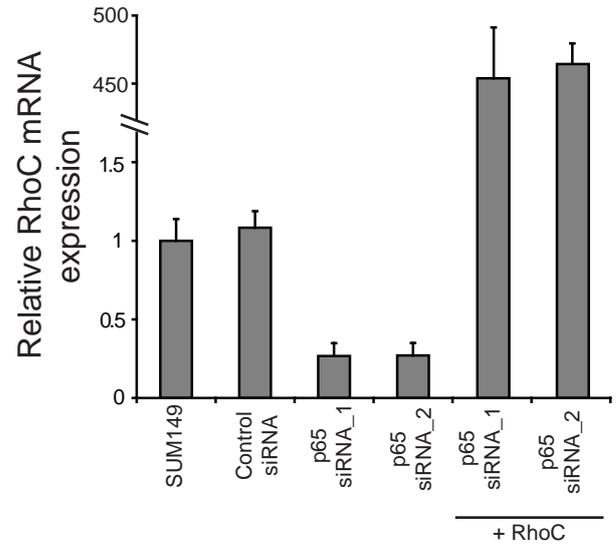


# Figure 3

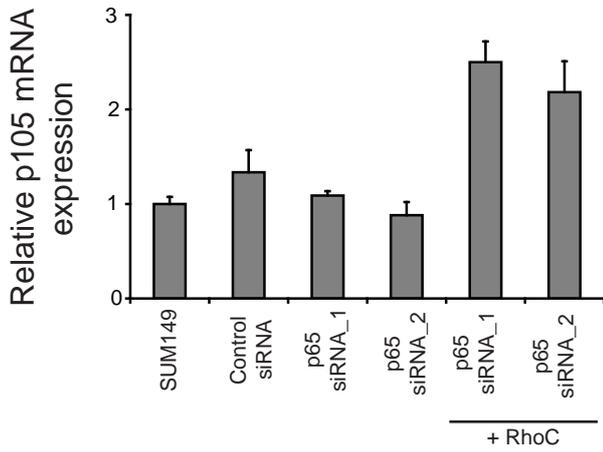
A



B



C



D

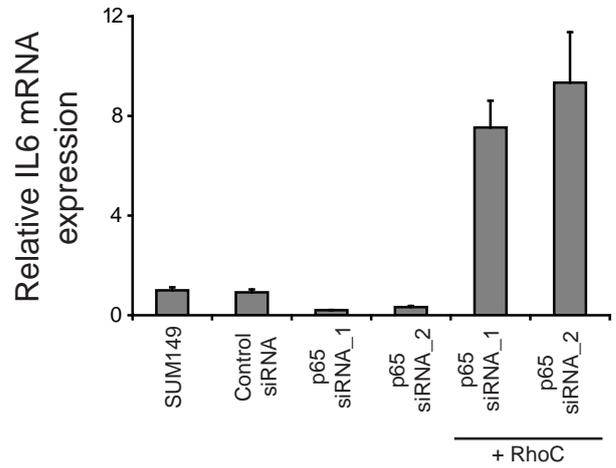
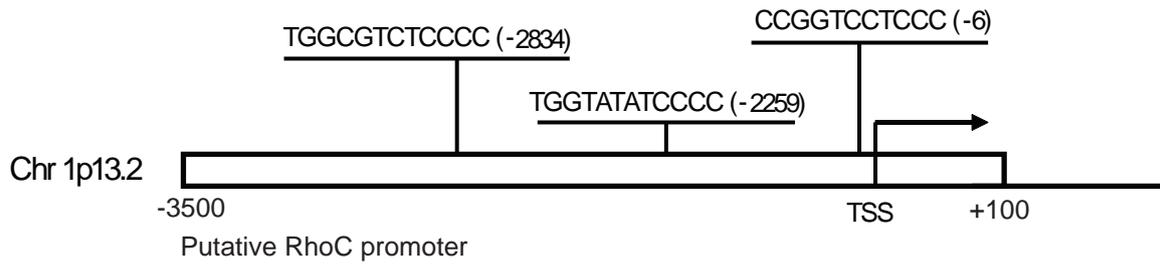
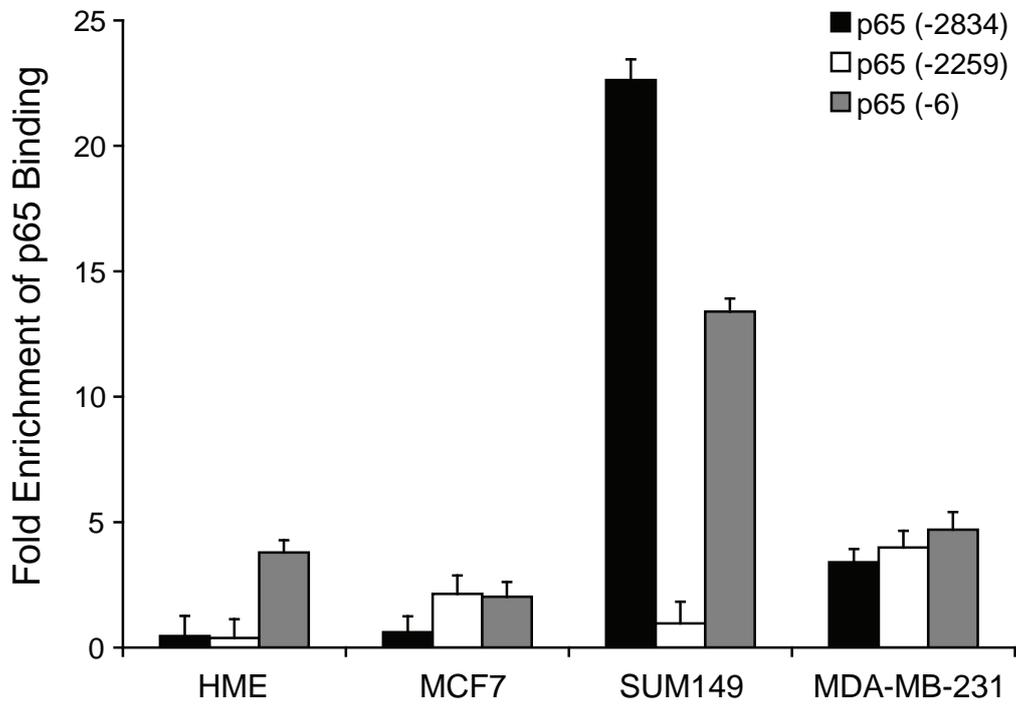


Figure 4

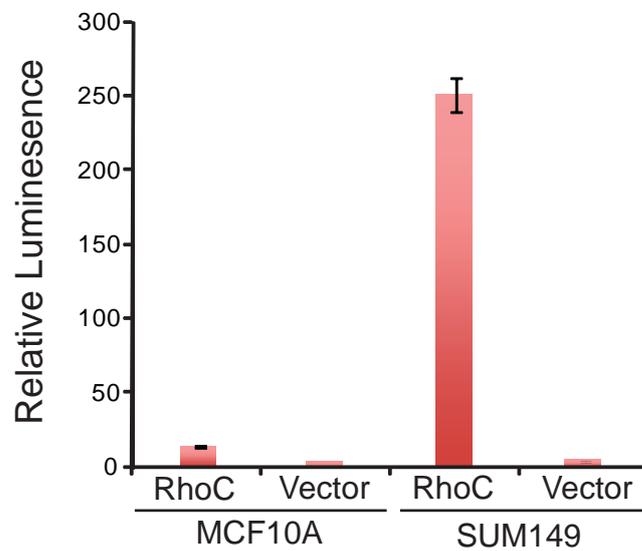
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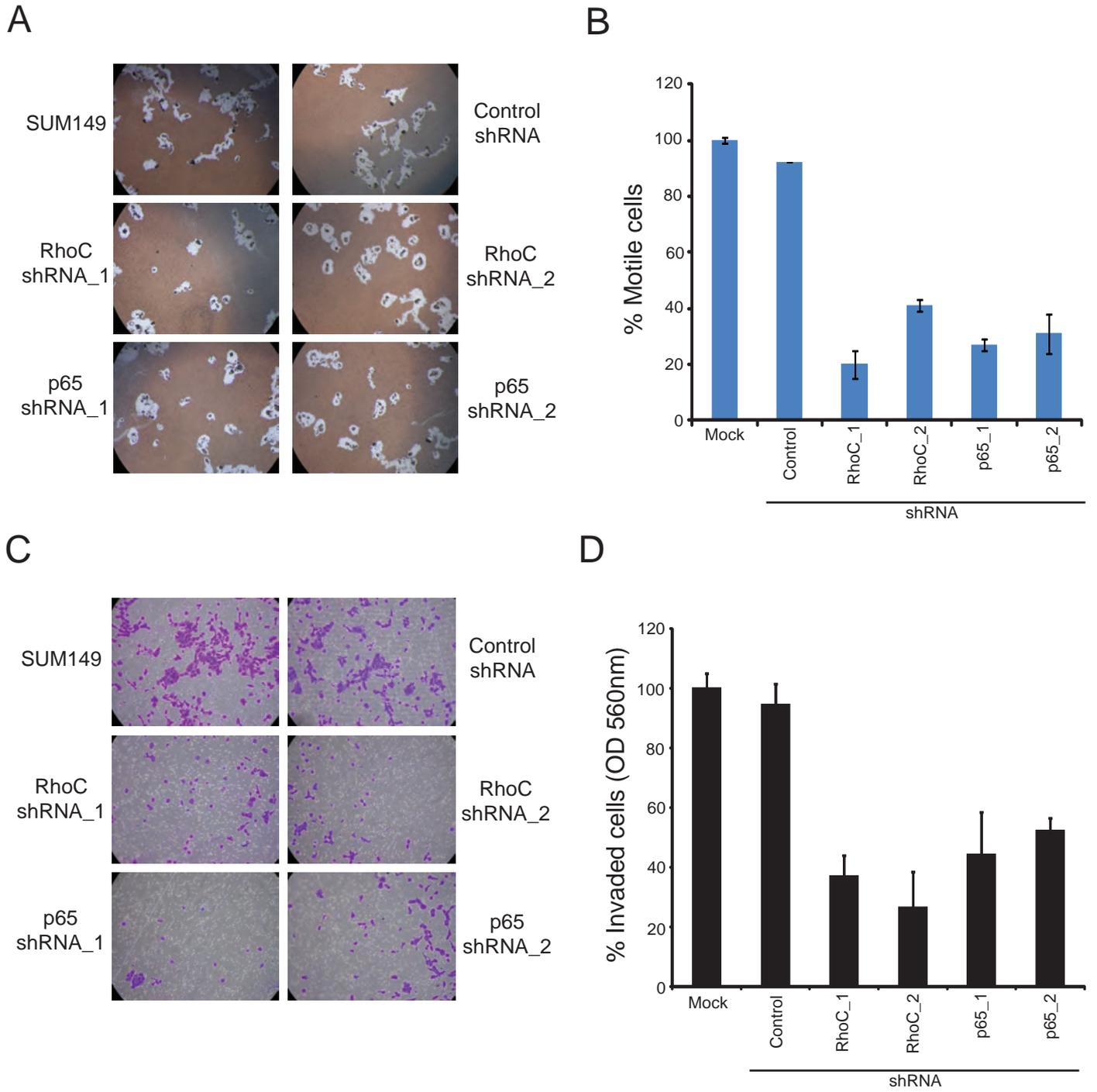
B



C



# Figure 5



# Table1

TargetID NORM: HME	Prostate				Breast				
	PrEC	RWPE	PC3	DU145	MCF10A	SUM149	SUM190	BT20	HCC1937
hsa-miR-296-5p	0.832594	4.446693	0.282801	2.938353	0.954421	0.228308	0.168524	0.192569	0.192236
hsa-miR-708	0.597511	1.740731	0.02235	0.023809	0.786263	0.027347	0.024853	0.023295	0.025006
hsa-miR-663	1.742116	0.405908	0.15265	0.387818	0.66306	0.175342	0.078522	0.475149	0.106728
hsa-miR-31*	1.43628	1.84149	0.659765	0.909713	0.627522	0.028181	0.021377	0.595517	0.986864
hsa-miR-31	1.105523	1.126393	0.910725	0.981528	0.614428	0.031018	0.023398	0.758384	0.972423

Table 1: Summarized Illumina microRNA array bead station data. microRNAs that were greater than 2-fold down or upregulated in both IBC cell lines SUM149 and SUM190, but not other breast (MCF10A, BT20 and HCC1937) or prostate (PrEC, RWPE, PC3 and DU145) cells as compared to HME cells were identified. Validations were performed by qPCR using Taqman probes specific for both miR-31 and miR-31\*.

# Coordinated Regulation of Polycomb Group Complexes through microRNAs in Cancer

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## SUMMARY

Polycomb Repressive Complexes (PRC1 and PRC2)-mediated epigenetic regulation is critical for maintaining cellular homeostasis. Members of Polycomb Group (PcG) proteins including EZH2, a PRC2 component, are upregulated in various cancer types, implicating their role in tumorigenesis. Here, we have identified several microRNAs (miRNAs) that are repressed by EZH2. These miRNAs, in turn, regulate the expression of PRC1 proteins BMI1 and RING2. We found that ectopic overexpression of EZH2-regulated miRNAs attenuated cancer cell growth and invasiveness, and abrogated cancer stem cell properties. Importantly, expression analysis revealed an inverse correlation between miRNA and PRC protein levels in cell culture and prostate cancer tissues. Taken together, our data have uncovered a coordinate regulation of PRC1 and PRC2 activities that is mediated by miRNAs.

## INTRODUCTION

Polycomb group (PcG) proteins are evolutionarily conserved regulators of gene silencing important in metazoan development (Surface et al., 2010), stem cell pluripotency (Pereira et al., 2010), and X chromosome inactivation (Cao et al., 2002; Margueron and Reinberg, 2011). PcG proteins form multiprotein repressive complexes called PRCs. Both PRC1 and PRC2 play a critical role in the maintenance of normal and cancer stem cell populations (Ezhkova et al., 2009; Lukacs et al., 2010; Pietersen et al., 2008). Dysregulation of PcG proteins can contribute to

a number of human diseases, most notably, cancer (Bracken and Helin, 2009; Margueron and Reinberg, 2011).

Key components of the human PRC2 include the histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2), and its binding partners, Embryonic Ectoderm Development (EED) and Suppressor of Zeste 12 (SUZ12), which function as a multi-subunit complex that trimethylates histone H3K27. PRC2 is thought to be recruited to target genomic loci by long noncoding RNAs (ncRNAs) such as HOTAIR (Gupta et al., 2010; Kaneko et al., 2010; Rinn et al., 2007). EZH2, which is the enzymatic component of PRC2, is elevated in aggressive forms of prostate

### Significance

Polycomb group (PcG) proteins are chromatin-modifying complexes that regulate epigenetic silencing and play an important role in determining cell fate. PcG proteins form two major complexes, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). PRC2 methylates histone H3 on lysine27 (H3K27), a chromatin mark that stimulates PRC1 to enact gene silencing at target genes. Employing in vitro and in vivo cancer models and human tumor studies, we demonstrate that PRC2 and PRC1 coordinate their functions through regulation of specific microRNAs. Increased PRC2 activity in cancer leads to repression of these microRNAs, and subsequent increase of PRC1 components. Thus, we propose that key microRNAs link PRC2 to PRC1 forming an integral regulatory axis of the epigenetic silencing machinery.

and breast cancer (Kleer et al., 2003; Varambally et al., 2002), as well as multiple other solid tumors (Matsukawa et al., 2006; Sudo et al., 2005). Loss of microRNA (miRNA)-101, has been shown to be one mechanism that leads to elevated EZH2 and PRC2 activity in tumors (Cao et al., 2010; Chiang et al., 2010; Friedman et al., 2009; Varambally et al., 2008; Wang et al., 2010). Also, miR-26a was reported to target EZH2 in cancer and myogenesis (Lu et al., 2011; Wong and Tellam, 2008). Accumulating evidence suggests that increased activity of PRC2 is oncogenic as measured by cell proliferation (Bracken et al., 2003; Varambally et al., 2002), cell invasion (Cao et al., 2008; Kleer et al., 2003), anchorage-independent growth (Bracken et al., 2003; Kleer et al., 2003), maintenance of tumor-initiating cells, tumor xenograft growth (Yu et al., 2007b), and metastasis *in vivo* (Min et al., 2010).

A key collaborator of PRC2 in epigenetic silencing is human PRC1, which comprises B lymphoma Mo-MLV insertion region 1 (BMI1), RING1 (also known as RING1A or RNF1) and RING2 (also known as RING1B or RNF2), and functions as a multiprotein complex to ubiquitinate histone H2A at lysine 119 (uH2A) (Cao et al., 2005; Wang et al., 2004). The prevailing hypothesis is that PRC2-mediated trimethylation of H3K27 recruits PRC1 to gene loci, which enacts chromatin condensation and epigenetic silencing of target genes (Bracken and Helin, 2009). Like PRC2 component EZH2, BMI1 and RING2 have been shown to be elevated in a number of tumor types (Gliinsky et al., 2005; Sánchez-Beato et al., 2006) and regulate self-renewal of embryonic stem cells and cancer stem cells (Galmozzi et al., 2006; Valk-Lingbeek et al., 2004). The mechanism of how PRC2 and PRC1 coordinate their functions is still unclear. In this study, we sought to explore the regulatory axis between PRCs and whether miRNAs mediate the synergy between the two complexes.

## RESULTS

### PcG Proteins Are Regulated by miRNAs

Previously, it has been reported that EZH2, the methyltransferase subunit of the PRC2 complex, is repressed by miR-101 (Friedman et al., 2009; Varambally et al., 2008) and miR-26a (Lu et al., 2011; Wong and Tellam, 2008). We hypothesized that PcG proteins (comprising the mammalian PRC complexes) may in general be regulated by miRNAs. To test this hypothesis, we knocked down Dicer, a key protein required for miRNA processing, by employing Dicer-specific siRNA duplexes. By immunoblot analysis, we found that PRC2 proteins EZH2, EED, and SUZ12, and PRC1 proteins BMI1 and RING2 were increased significantly by three different Dicer siRNA duplexes (Figure 1A; see Figure S1A available online). These experiments support the general notion that miRNAs function to repress PcG expression.

### Identification of EZH2-Regulated miRNAs

To explore miRNAs regulated by PRC2 globally, we knocked down EZH2 in DU145 prostate cancer cells with a validated siRNA targeting EZH2 and monitored miRNA expression with Illumina BeadChips. In parallel, we compared these miRNA profiles with DU145 cells relative to four benign epithelial cell lines of either prostate (PrEC and RWPE) or breast (H16N2 and HME) origin. We primarily observed miRNAs that were

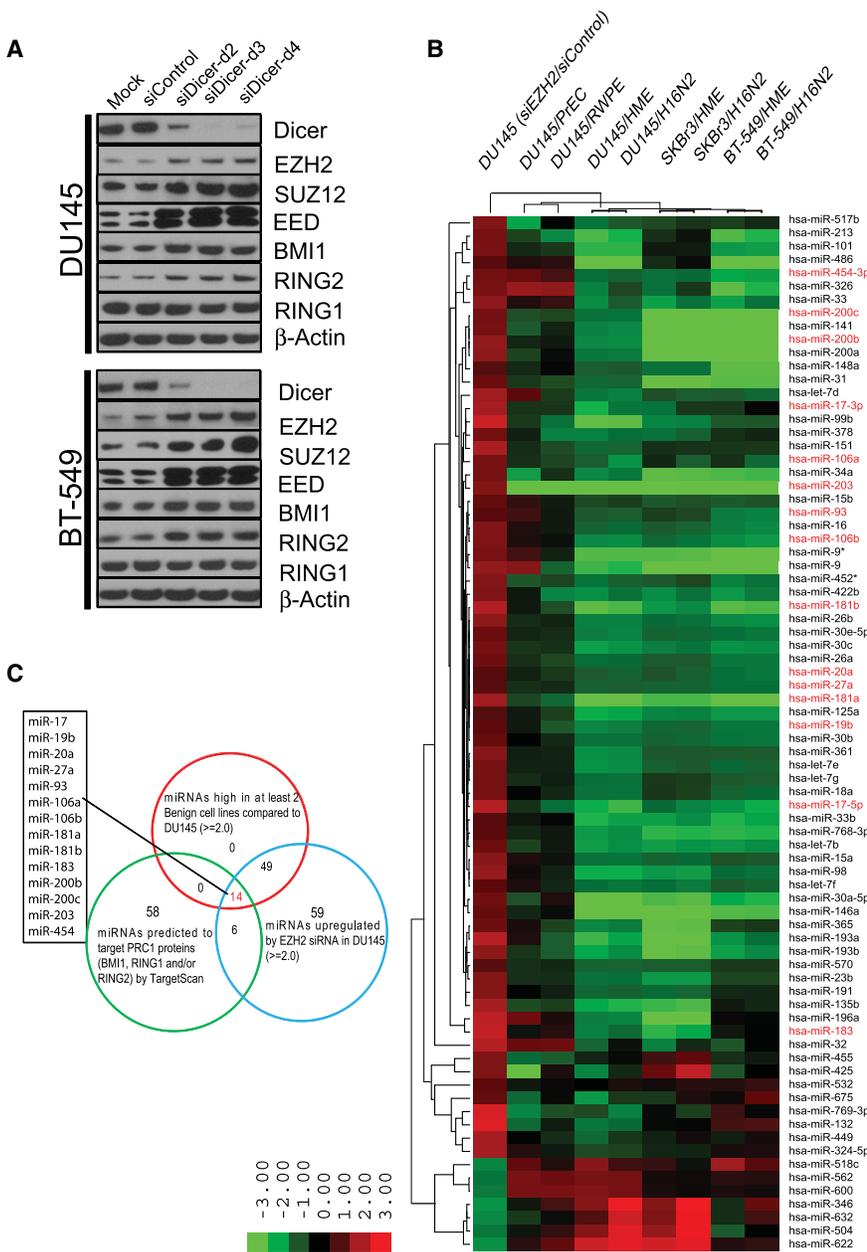
decreased in cancer cells relative to benign that are targets of repression by EZH2, and thus PRC2. We found 63 miRNAs that were downregulated in DU145 cells compared with the normal cell lines, and inhibition of EZH2 by knockdown restored expression of these miRNAs (Figure 1B; Table S1). Similarly, the expression levels of these 63 miRNAs were downregulated in breast cancer cells BT-549 and SKBr3 compared with breast benign epithelial cells H16N2 and HME (Figure 1B; Table S1). Using miRNA target analysis ([www.targetscan.org](http://www.targetscan.org)), we identified 14 miRNAs as top candidates with the following properties: (1) upregulated by EZH2 knockdown in DU145 cancer cells which express high levels of PRC2; (2) higher in benign cell lines compared with DU145 cells, and (3) predicted to bind to the 3' untranslated region (UTR) of target PRC1 components based on TargetScan (Figure 1C). Thirteen of the 14 miRNAs meeting these criterion fell into several known miRNAs clusters and families, including miR-200b and miR-200c in the miR-200 family, which has previously been reported to repress BMI1 (Shimono et al., 2009; Wellner et al., 2009). Of the 14 miRNAs, only miR-203, which is also known to target BMI1 (Wellner et al., 2009), does not belong to any known cluster or family (Figure S1B).

### EZH2-Regulated microRNAs Inhibit Expression of PRC1 Proteins BMI1 and RING2

To pinpoint the specific miRNAs that target PRC1 (out of the 14 that were nominated by computational approaches) (Figure 1C), we overexpressed each of them in BT-549 and DU145 cancer cell lines and monitored EZH2, BMI1, and RING2 protein expression (Figure 2A; Figure S2A). Of these, miR-181a, b decreased RING2 protein levels, miR-203 decreased BMI1 protein levels while miR-200b,c decreased both BMI1 and RING2 (Figure 2A). Attenuation of these PRC1 members resulted in decreased global ubiquityl-H2A, a known PRC1 substrate and mark of gene repression. Furthermore, PRC1 targets including p16INK4A (Jacobs et al., 1999a) and p21 (Waf1/Cip) (Fasano et al., 2007) were derepressed (Figure 2A). Several of the miRNAs computationally predicted to inhibit PRC1 failed to do so by overexpression including miR-17, miR-19b, and others (Figure S2A). Similar to protein levels, real-time qPCR showed miR-181a,b and miR-200b,c decreased RING2 transcript levels and miR-200b,c and miR-203 decreased BMI1 transcript levels in BT-549 cells (Figure 2B). As expected, overexpressing miR-200b or miR-203 decreased BMI1 occupancy on known PRC1 target gene p16, p19 (Jacobs et al., 1999b), p21, and HoxC13 (Cao et al., 2005) regions (Figure S2B).

To further corroborate our miRNA overexpression studies, we also extinguished expression of miRNAs using antagomiRs (Krützfeldt et al., 2005). Consistent with our predictions, antagomiR-200b, antagomiR-200c, and antagomiR-203 increased BMI1 protein levels, while antagomiR-181a, antagomiR-181b, antagomiR-200b, and antagomiR-200c increased RING2 protein levels in H16N2 cells (Figure 2C).

To evaluate whether these miRNAs directly bind to the 3' UTR of BMI1 or RING2, we cloned the predicted binding sites of the wild-type or mutant 3' UTR into a luciferase reporter system and cotransfected them with miRNA expression vectors into BT-549 cells (Figure 2D; Figures S2C–S2F). As expected, inhibition of luciferase activity was observed in cells transfected with constructs containing wild-type binding sites but not the mutant



**Figure 1. PcG Proteins Are Regulated by miRNAs**

(A) Knockdown of Dicer in DU145 and BT-549 cells by three different Dicer-specific duplexes and PcG protein expression was assessed.

(B) miRNA profiling of DU145 prostate cancer cells in which EZH2 was knocked down compared with DU145 cancer cells relative to benign cells HME, PrEC, RWPE, and H16N2. Shades of red represent increased gene expression while shades of green represent decreased expression.

(C) A Venn diagram depicting 14 miRNAs that were upregulated by EZH2 knockdown, had high endogenous levels in normal cells, and were predicted to target PRC1 proteins.

See also Figure S1 and Table S1.

Further, we observed similar expression changes in these microRNAs upon stable overexpression of miR-101 or EZH2 shRNA in DU145 and SKBr3 cells (Figure S3A). Also we observed that miR-101 was increased in DU145 cells in which EZH2 was stably knocked down, suggesting the existence of feedback regulation between EZH2 and miR-101. In contrast, overexpression of EZH2, but not EZH2ΔSET (which is missing its catalytic SET domain), decreased miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 levels in H16N2 cells (Figure S3B).

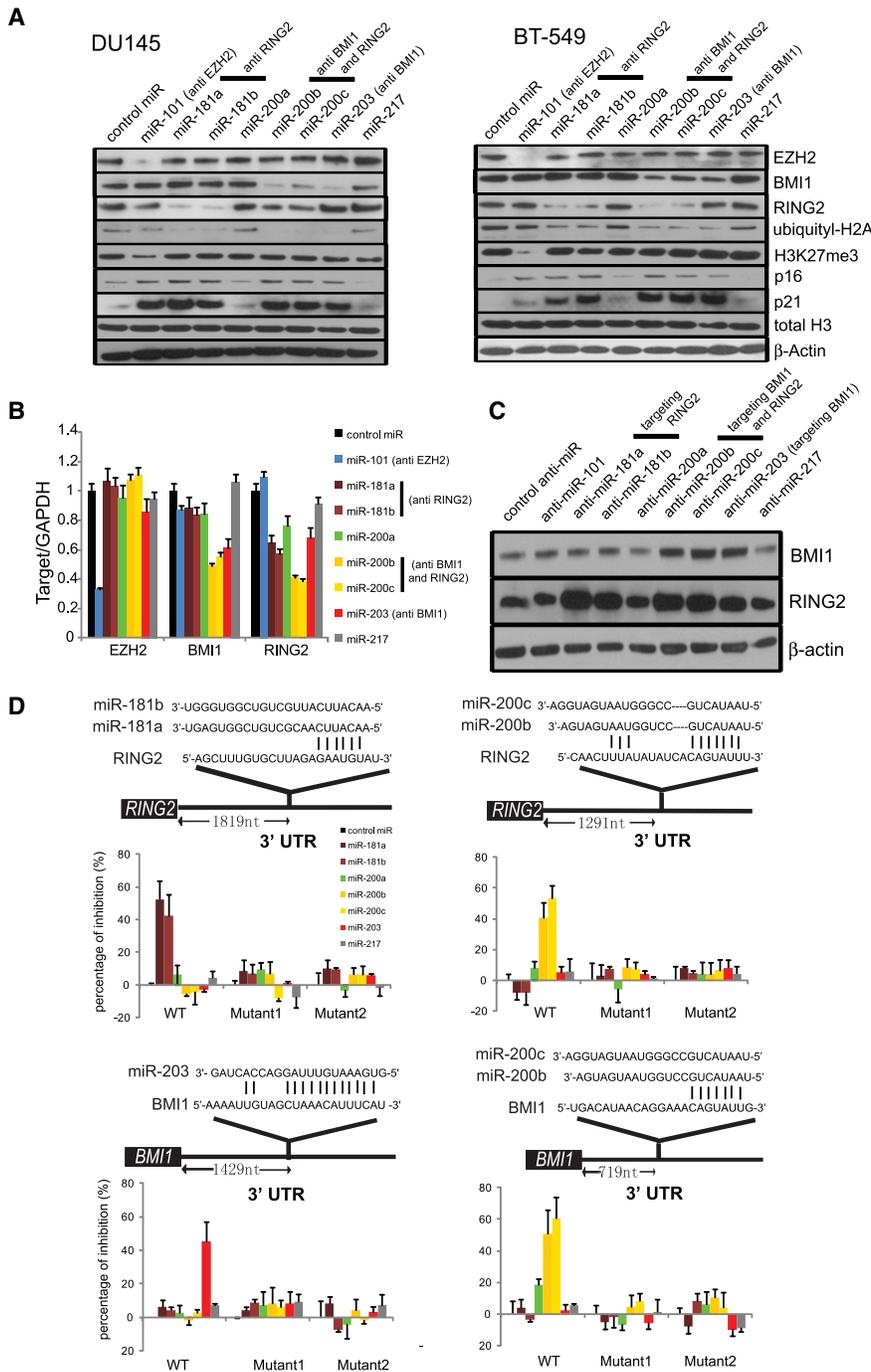
Next, we treated DU145 cells with the global histone methylation inhibitor, deazaneplanocin A (DZNep), that depletes PRC2 and thus attenuates H3K27me3 (Tan et al., 2007). Interestingly, DZNep treatment led to derepression of the putative PRC2-targeted miRNAs including miR-181a,b, miR-200a,b,c, and miR-203 (Figure 3B). This effect was both concentration and incubation time dependent. Control microRNAs, miR-217, miR-219, and miR-21 were not affected by DZNep treatment.

In addition to DZNep, we evaluated other chemical inhibitors of epigenetic pathways. As HDAC activity is essential for EZH2 function (Cao et al., 2008; Kleer et al., 2003), and EZH2 directly or indirectly facilitates DNA methylation (Viré et al., 2006), we predicted that treatment with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and/or the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) would inhibit EZH2-mediated epigenetic modifications, leading to an increase in miRNA expression. Treatment of BT-549 and DU145 cells with 5-aza-dC or SAHA alone or in combination, resulted in a marked increase in miR-181a,b, miR-200a,b,c, and miR-203 expression, suggesting epigenetic regulation of these microRNAs (Figure 3C).

Importantly, when we overexpressed EZH2 by adenovirus in DZNep or SAHA and 5-aza-dC-treated DU145 cells, EZH2 could

constructs. The RING2 3' UTR reporters were downregulated by miR-181a, miR-181b, miR-200b, and miR-200c while the BMI1 3' UTR reporters were downregulated by miR-200b, miR-200c, and miR-203 (Figure 2D).

We next determined whether the miRNAs that regulate PRC1 were directly regulated by PRC2 in BT-549 and DU145 cells. Cells were transfected with either a validated EZH2 siRNA or miR-101 (both of which target and downregulate the PRC2), and expression levels of target miRNAs were measured by real-time PCR. miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 expression levels were increased in EZH2 siRNA or miR-101-transfected cells. Expression of miRNAs miR-217 and miR-219, two control microRNAs not predicted to be regulated by EZH2, were not altered (Figure 3A).



**Figure 2. PRC2-Regulated miRNAs Repress PRC1 Proteins BMI1 and RING2**

(A) Overexpression of indicated miRNAs in DU145 and BT-549 cells and expression of PRC components, PRC2 histone mark H3K27me3, PRC1 target histone mark ubiquityl-H2A and indicated genes by immunoblot analysis.  $\beta$ -actin and total H3 were used as loading controls.

(B) As in (A), except transcript level was assessed in BT-549 by qPCR.

(C) Transfection of indicated antagomiRs (anti-miR) in H16N2 cells and immunoblot analysis for BMI1 and RING2.  $\beta$ -actin was used as a loading control.

(D) TargetScan analysis depicting potential binding sites for EZH2-regulated miRNAs in the 3' UTR of BMI1 and RING2. Luciferase reporter assays with wild-type or mutant 3' UTR constructs of BMI1 or RING2 demonstrate that miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 repress BMI1 and/or RING2 activity.

All bar graphs are shown with  $\pm$  SEM. See also Figure S2.

that a negative feedback system between PRC2-regulated miRNAs and PRC1 may exist. Furthermore, an EZH2-specific siRNA (Figure S3F) or treatment with 5-aza-dC and SAHA, either alone or in combination (Figure 3D), markedly decreased the H3K27me3 occupancy in these regions.

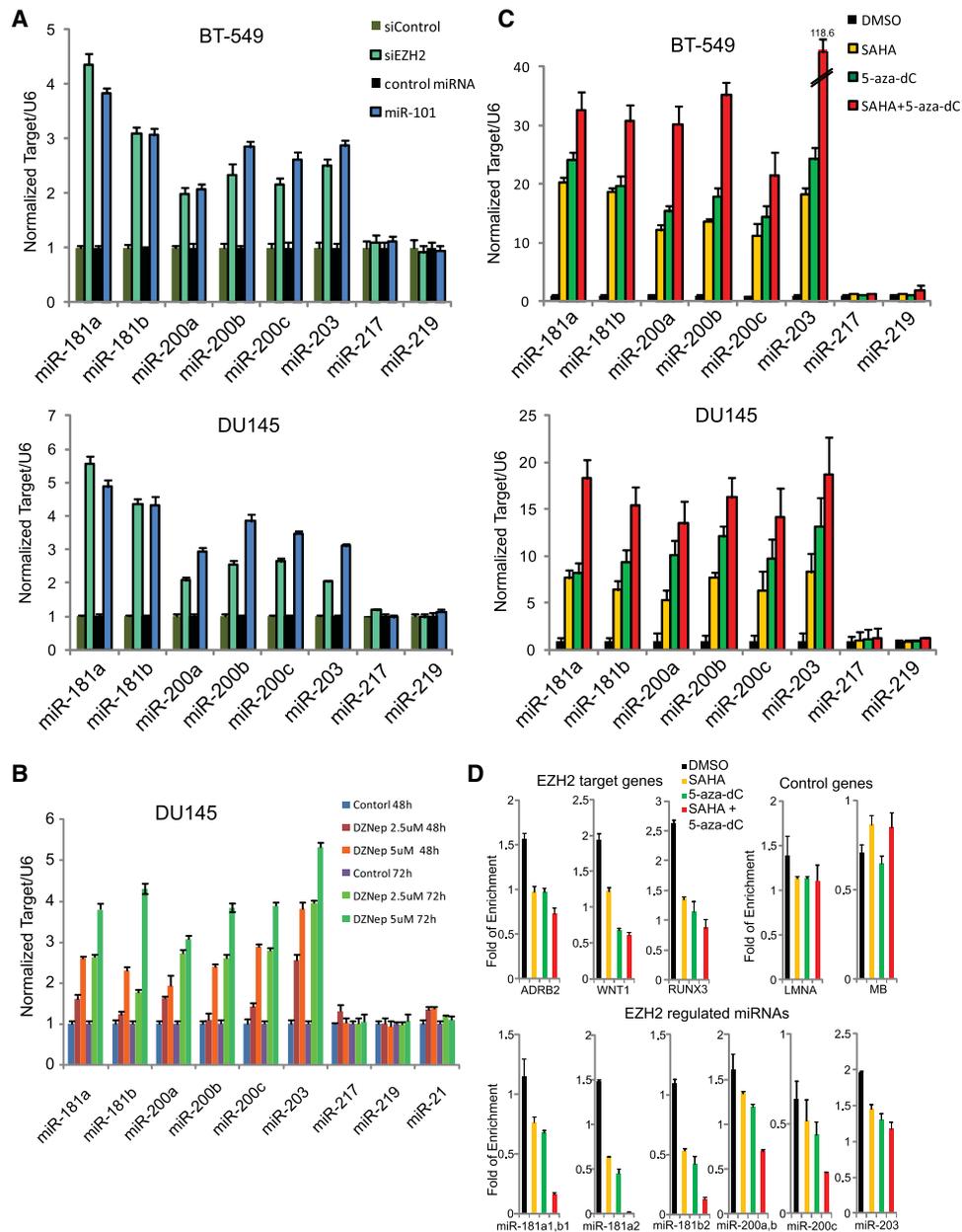
### EZH2-Regulated miRNAs Attenuate Growth, Invasiveness, and Self-Renewal of Cancer Cells

Because EZH2 has been shown to repress several tumor suppressor genes (Cao et al., 2008; Chen et al., 2005; Fujii et al., 2008; Min et al., 2010; Yu et al., 2007b, 2010), we postulated that the EZH2-regulated microRNAs also functioned as tumor suppressors. Consistent with this hypothesis, overexpression of either miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, or miR-203 markedly attenuated BT-549 and DU145 cell proliferation to levels similar to that of cells overexpressing miR-101 (Figure 4A

completely abolish DZNep-mediated miRNA upregulation (Figure S3C), and partially decreased SAHA and 5-aza-dC-mediated miRNA upregulation (Figure S3D) presumably because SAHA and 5-aza-dC also inhibited HDAC and DNMT activities.

To confirm that EZH2 regulates these microRNAs by epigenetic repression, we performed chromatin immunoprecipitation (ChIP) assays with anti-H3K27me3, EZH2 and BMI1 antibodies in BT-549 cells. Interestingly, H3K27me3 and EZH2 occupied the PRC2-regulated miRNAs regions as expected. In addition, BMI1 also occupied these regions (Figure S3E), suggesting

\* $p < 0.001$ , \*\* $p < 0.01$ ; Figure S4A). Likewise, overexpression of either miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, or miR-203 inhibited the in vitro invasive potential of BT-549 and DU145 cells through modified Boyden chambers coated with Matrigel (Figure 4B, \* $p < 0.005$ , \*\* $p < 0.02$ ). However, overexpressing EZH2-repressed miRNAs had no effect on the invasiveness of RWPE-UBE2L3-KRAS and RWPE-SLC45A3-BRAF stable cells, in which fusion proteins UBE2L3-KRAS (Wang et al., 2011) and SLC45A3-BRAF (Bonci et al., 2008; Palanisamy et al., 2010) confer neoplastic properties to RWPE cells



**Figure 3. PRC2 Silences Multiple miRNAs by Epigenetic Mechanisms**

(A) Taqman miRNA qPCR analysis of indicated miRNAs in BT-549 and DU145 cells in which EZH2 was knocked down using siRNA or miR-101 (a microRNA which targets EZH2). Quantitative microRNA levels were normalized against U6.

(B) As in (A), except DZNep at two different doses and time points was incubated with DU145 cells.

(C) As in (A), except SAHA and/or 5-aza-dC was used in BT-549 and DU145 cells.

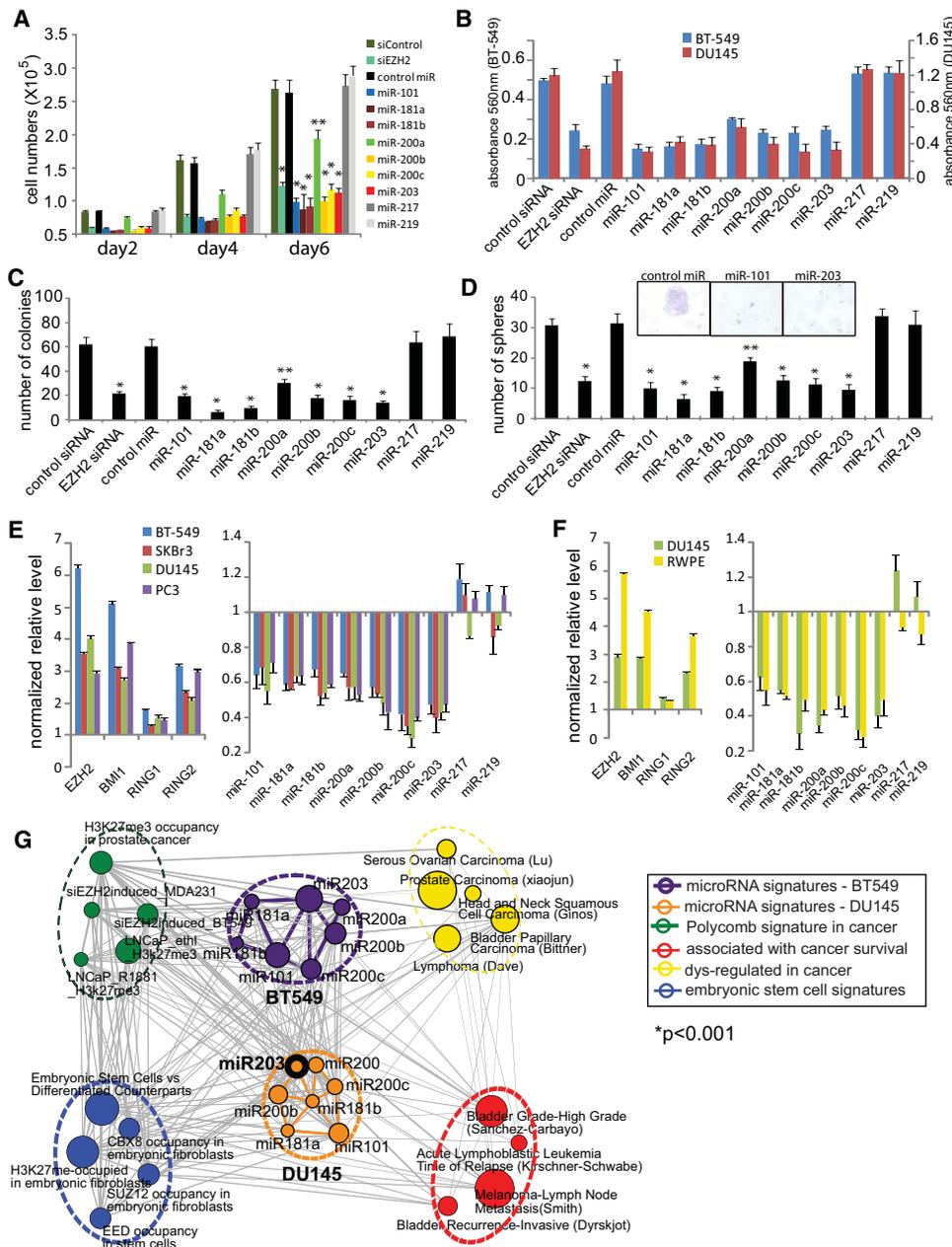
(D) ChIP-qPCR analysis of H3K27me3 at indicated genes and microRNAs in BT-549 cells treated with SAHA and/or 5-aza-dC.

All bar graphs are shown with  $\pm$  SEM. See also Figure S3.

(Figure S4B), suggesting that EZH2-repressed miRNAs miR-181a,b, miR-200b,c, and miR-203 may inhibit cell invasion through acting on PRC1 proteins. However, EZH2-repressed miRNAs still decreased RWPE-UBE2L3-KRAS and RWPE-SLC45A3-BRAF proliferation (Figure S4C), consistent with a critical role of PcG proteins in cell growth.

To investigate whether miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, or miR-203 inhibit anchorage-independent

growth, we performed soft agar colony formation assays. Similar to miR-101 and EZH2 knockdown controls, overexpression of miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 markedly suppressed DU145 colony formation (Figure 4C, \* $p < 0.001$ , \*\* $p < 0.01$ ). Next, we evaluated the ability of DU145 to form prostatospheres in sphere-promoting cell media. This assay serves as a surrogate measure of stem cell-like phenotypes, and cells that are able to form spheres have



**Figure 4. PRC2-Mediated Regulation of microRNAs Potentiates the Cancer Cell Phenotype**

(A) Overexpression of PRC2-regulated miRNAs, but not control miR-217 or miR-219, inhibited BT-549 cell proliferation. EZH2 siRNA and miR-101 overexpression were positive controls and miR-217 and miR-219 overexpression were negative controls. \* $p < 0.001$ , \*\* $p < 0.01$ . (Student's t test).

(B) Overexpression of PRC2-regulated miRNAs decreased BT-549 and DU145 cell invasion in vitro. \* $p < 0.01$ . (Student's t test).

(C) Overexpression of PRC2-regulated miRNAs suppressed DU145 anchorage-independent growth in soft agar. \* $p < 0.01$ . (Student's t test).

(D) Overexpression of EZH2-regulated miRNAs decreased prostatosphere formation by DU145 cells. \* $p < 0.01$ . (Student's t test). Representative images of prostatospheres (scale bar: 100  $\mu\text{m}$ ) were shown in the inset.

(E) qPCR analysis demonstrating EZH2, BMI1 and RING2 transcript levels were higher in spheres compared with monolayer culture, while miR-101, miR-181a,b, miR-200a,b,c, and miR-203, but not miR-217 or miR-219, were lower in spheres compared with monolayers. Expression level of each gene was normalized to GAPDH or U6 and normalized to corresponding monolayer cultured cell line.

(F) qPCR analysis showing EZH2, BMI1 and RING2 levels were higher in sorted CD24-/CD44+ DU145 and RWPE cells compared with the unsorted population, while miR-101, miR-181a,b, miR-200a,b,c, and miR-203, but not miR-217 or miR-219, were lower in CD24-/CD44+ DU145 and RWPE cells compared with an unsorted population.

(G) Genes regulated by EZH2-repressed miRNAs cluster into multiple functional concepts. BT-549 and DU145 cells were transfected with EZH2-repressed miRNAs followed by gene expression profiling and Molecular Concepts analysis. Each node represents a molecular concept or set of biologically related genes. miR-101, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 (miRNA signatures, purple for BT-549, orange for DU145) were enriched

enhanced stem cell characteristics (Lawson et al., 2007). We found that miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 overexpression, as well as miR-101 overexpression and EZH2 siRNA controls, significantly inhibited the ability of DU145 cells to form spheres in this assay (Figure 4D, \* $p < 0.001$ , \*\* $p < 0.01$ ). Intriguingly, several genes implicated in pluripotency and cellular reprogramming by induced pluripotency, such as Klf4, Sox2, and c-Myc, were markedly downregulated by miR-200b, miR-200c, and miR-203, and marginally decreased by miR-101, miR-181a, miR-181b, and miR-200a expression, but not by miR-217 or miR-219 controls (Figure S4D). Relative to the human embryonic stem cell H7, BT-549 and DU145 cancer cells have comparable expression levels of iPSC factors and PcG proteins (Figure S4E).

Next, we measured expression levels of EZH2, BMI1, RING2, and key microRNAs relevant to this study in spheres and monolayers. In BT-549, SKBr3, DU145, and PC3 cells, we observed that EZH2, BMI1, and RING2 levels were higher in spheres than in monolayers; conversely miR-101, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 levels were lower in spheres than in monolayers (Figure 4E). Using DU145 and RWPE parental cell lines, we employed flow cytometry to isolate cells with high expression of the CD44 surface antigen and low expression of the CD24 surface antigen (CD24-/CD44+), a cell population enriched for stem cell-like phenotypes (Hurt et al., 2008). We measured EZH2, BMI1, RING2, and miRNA levels in CD24-/CD44+ cells compared with total, unsorted cells. We observed that EZH2, BMI1, and RING2 levels were increased in CD24-/CD44+ cells, but miR-101, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 expression were decreased in this cell population (Figure 4F). Taken together, the data provide compelling evidence for the coordinated regulation of PRC2, PRC1, and miRNAs in the maintenance of a differentiated cellular state and inhibition of stem cell-like phenotypes.

In order to understand the functional biology of the miRNAs identified in this study, we sought to identify global gene expression patterns and molecular pathways to which they might contribute. We conducted gene expression microarray analyses of DU145 and BT-549 cells transfected with control miRNA, miR-101, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, or miR-203. As shown in Table S2 and Table S3, EZH2-repressed miRNAs targeted many predicted genes. When we analyzed the miRNA-regulated genes using Molecular Concepts Maps (MCM) (Tomlins et al., 2007b), as expected, molecular concepts associated with these miRNAs were highly overlapping, showing a high correlation to gene sets representing multiple cancers, metastatic cancer processes, cancer survival, Polycomb Group targets, and stem cell-related genes (Figure 4G; Table S4).

In order to further examine the molecular link between PRC1 and PRC2 activities, we generated DU145 cells stably overexpressing miR-200b and miR-203 (Figure S5A) and monitored levels of BMI1 and RING2. BMI1 and RING2 were decreased in miR-200b stable cells while only BMI1 was decreased in miR-203 stable cells. In addition, uH2A, the histone modification mediated by PRC1, was similarly decreased in both miR-200b-

and miR-203-expressing cells. Interestingly, BMI1, RING2, and uH2A, as well as EZH2 and H3K27me3, were decreased in miR-101 stable expressing DU145 cells (Figure 5A) suggesting that prolonged knockdown of PRC2 components leads to suppression of PRC1. Using cell count and Boyden chamber invasion assays, we found that similar to miR-101, miR-200b and miR-203 stably expressing cells grew more slowly and were less invasive than vector-transfected cells (Figures 5B and 5C). Intriguingly, coexpression of BMI1 or EZH2 (control) without the 3' UTR both restored the proliferation and invasion properties of DU145 cells despite the presence of miR-101, miR-200b, or miR-203 (Figures 5B and 5C). Importantly, murine xenograft experiments demonstrated that DU145 cells with stable knockdown of PRC1 proteins BMI1 or RING2 (Figure S5B), or expressing miR-181b (Figure S5C), miR-200b, or miR-203 grew more slowly than the vector control in vivo ( $p = 0.0001$ , Figures 5D and 5E).

#### EZH2-Regulated miRNAs Inversely Correlate with PRC Protein Levels in Prostate Cancer

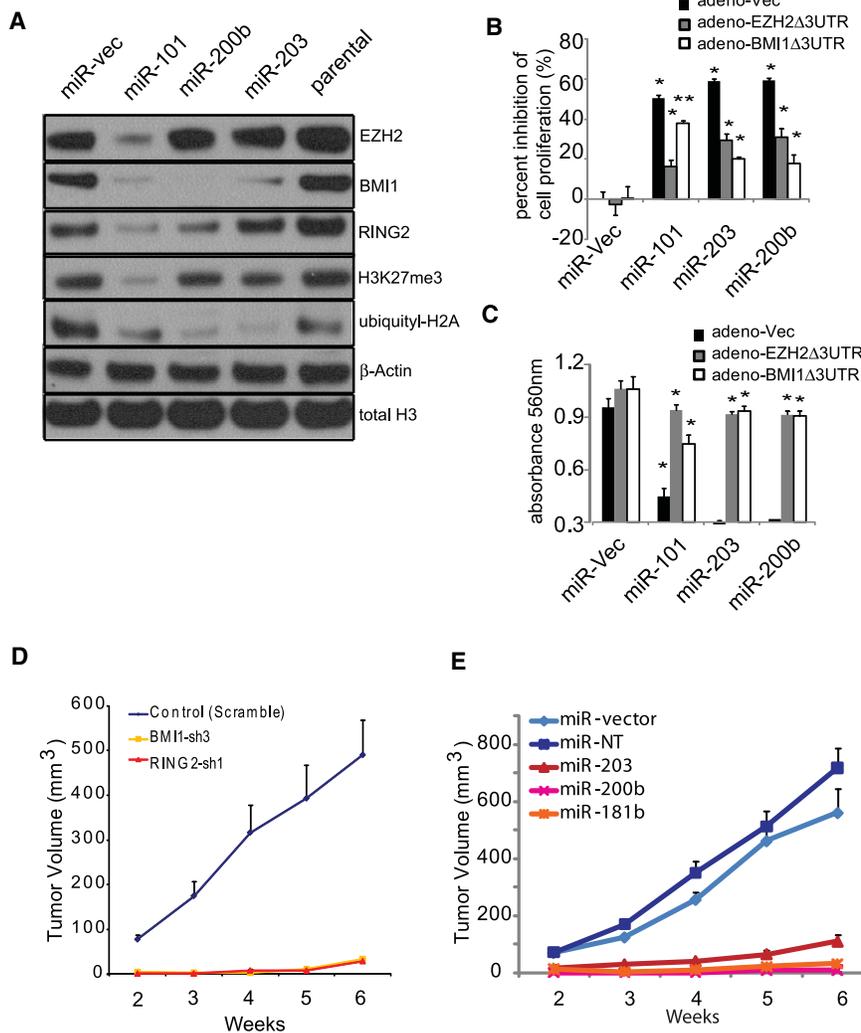
Since miR-101, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 appear to play an important role in cancer progression, we next measured the endogenous expression levels of these miRNAs by qPCR analysis of a cohort of benign prostate, localized, and metastatic prostate cancers in which we had measured miR-101, miR-217, and EZH2 levels previously (Varambally et al., 2008). As expected, miR-181a, miR-181b, miR-200a, miR-200b, miR-200c, and miR-203 levels were lowest in metastatic prostate cancer tissues, and highest in benign prostate tissues (Figure 6A). In addition, immunoblot analyses showed that BMI1, RING2, and uH2A, as well as EZH2, but not RING1, were increased in metastatic prostate cancer compared with benign tissues and localized cancer samples (Figure 6B; Figure S6A). EZH2 levels were highly correlated with BMI1, RING2, and H2A protein levels (Figure S6B), further supporting a molecular link between PRC1 and PRC2 expression and activities during cancer progression. As expected, ChIP assays showed that H3K27me3-marked chromatin occupied the miR-203 upstream region in metastatic prostate cancer, but not in localized prostate cancer (PCA) (Figure S6C). Similarly, DNA methylation of the miR-203 genomic region was observed in localized and metastatic prostate cancer but not benign prostate tissue (Figure 6C). Taken together, these data suggest that EZH2-mediated epigenetic repression of miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 results in an upregulation of PRC1 proteins BMI1 and RING2 and histone code ubiquityl-H2A in advanced prostate cancer.

#### DISCUSSION

This study unravels the intricacies in the regulation of the polycomb protein complexes mediated by various miRNAs, and substantiates the essential role played by PRC in cancer. We demonstrated that increased PRC2 activity results in repression of numerous miRNAs that are known to be important in the

for concepts related to cancer (yellow), cancer survival (red), stem cell likeness (blue), and function of polycomb group (green). All bar graphs are shown with  $\pm$ SEM.

See also Figure S4, and Table S2, Table S3, and Table S4.



**Figure 5. PRC2-Repressed miRNAs Inhibit Tumor Growth**

(A) DU145 cells stably overexpressing miR-101, miR-200b, and miR-203 demonstrated repression of EZH2, BMI1, or RING2, as well as decreased H3K27me3 and ubiquityl-H2A (uH2A) levels. (B and C) Coexpression of EZH2Δ3'UTR or BMI1Δ3'UTR rescued cell proliferation (B), and invasiveness (C) of DU145 cells stably overexpressing miR-101, miR-203, or miR-200b. (D) Stably knocking down BMI1 or RING2 by BMI1-specific shRNA (BMI1-sh3) or RING2-specific shRNA (RING2-sh1) decreased DU145 tumor growth in mice. N = 8 for DU145 control (scramble), BMI1-sh3, and RING2-sh1, respectively, were used for the xenograft. (E) Stable overexpression of miR-181b, miR-200b, or miR-203 decreased DU145 tumor growth in mice. DU145 miR-vector (N = 9), miR-NT (non-targeting) (N = 8), miR-181b (N = 8), miR-200b (N = 8), or miR-203 (N = 7) were used for the xenograft experiment. DU145 stable pools were injected subcutaneously. All bar graphs are shown with ± SEM. See also Figure S5.

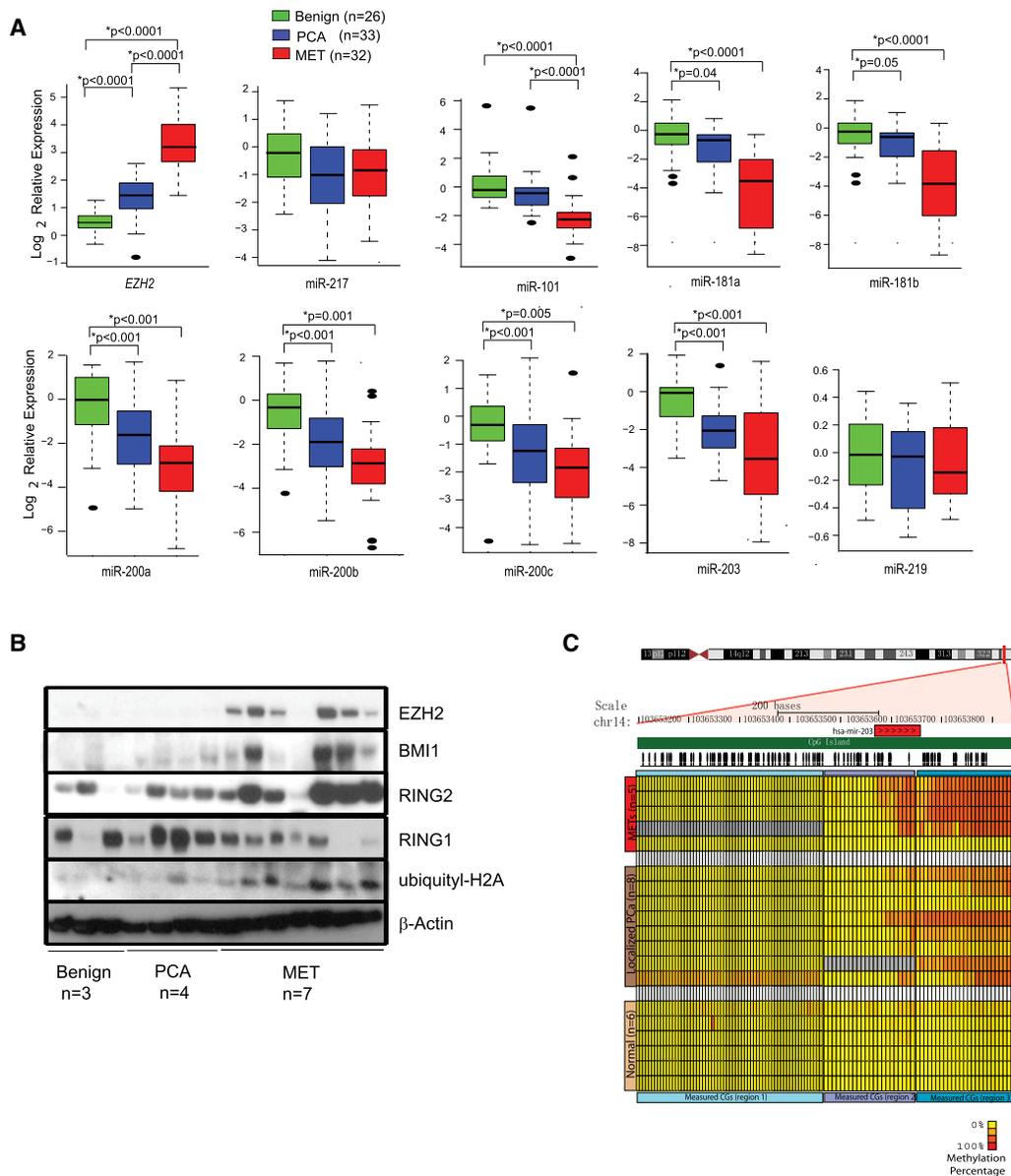
maintenance of stem cell-like phenotypes in cancer cells. We show that PRC2 epigenetically represses miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 expression by facilitating H3K27me3 trimethylation at these loci, and that exogenous overexpression of miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 inhibits a cancer phenotype in vitro. Furthermore, miR-181b, miR-200b, and miR-203 overexpression suppressed prostate tumor formation and growth in mouse xenografts. Recently, several groups have also reported roles for miR-200b, miR-200c, and miR-203 in controlling stem cell differentiation (Yi et al., 2008), epithelial-to-mesenchymal transition (EMT) (Park et al., 2008; Wellner et al., 2009), and cancer progression (Faber et al., 2008; Shimono et al., 2009).

Here, we demonstrated that PRC1 proteins BMI1 and RING2 are direct targets of miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 in breast and prostate cancer. Furthermore, we observed a significant negative correlation between PRC2 expression and miR-181a, miR-181b, miR-200b, miR-200c, and miR-203, as well as a strong positive correlation between EZH2, BMI1, and RING2 protein levels. Intriguingly, earlier studies suggested a discrepancy between BMI1 protein and

miR-203 function as tumor suppressors during prostate cancer progression.

Interestingly, several recent studies have reported similar microRNA-protein regulatory networks that play critical roles in cancer. In one study, the *RAS* proto-oncogene was shown to be coordinately regulated by the *let-7* family of miRs (Johnson et al., 2005). Likewise, the miR-15a-miR-16-1 cluster, located on chr13q14, was proposed to serve as a tumor suppressor in prostate tissue by regulating levels of cancer-related genes such as *BCL2*, *CCND1*, and *WNT3A* (Bonci et al., 2008). Recently, Poliseno et al. (2010) reported a proto-oncogenic miRNA-dependent network in prostate cancer progression in which the miR-106b~25 cluster regulates *PTEN* expression and cooperates with *MCM7* in cellular transformation. These studies, along with our present study, strongly suggest that dysregulation of miRNA and target protein networks may contribute to cancer development.

Here, we propose a model for a coordinated PRC2-PRC1 oncoprotein axis, and epigenetic link between H3K27me3 and ubiquityl-H2A, mediated by PRC2-regulated miRNAs (Figure 7). Recently, Iliopoulos et al. (2010) reported that miR-200b



**Figure 6. Coordinated Expression of PcG Proteins and PRC Regulatory miRNAs in Prostate Cancer Progression**

(A) Expression of indicated miRNAs as assessed by q-PCR in benign prostate, clinically localized prostate cancer and metastatic prostate cancer tissues. Data for EZH2, miR-217, and miR-101 were reported previously (Varambally et al., 2008) and displayed here for comparison (Student's t test).

(B) Immunoblot analysis of EZH2, BMI-1, RING2, RING1, and ubiquityl-H2A in benign prostate, clinically localized prostate cancer, and metastatic prostate cancer.

(C) Bisulfite sequencing analysis of the miR-203 genomic region revealed cancer-specific DNA methylation in a region proximal to miR-203 in prostate cancer tissues.

All bar graphs are shown with  $\pm$  SEM. See also Figure S6.

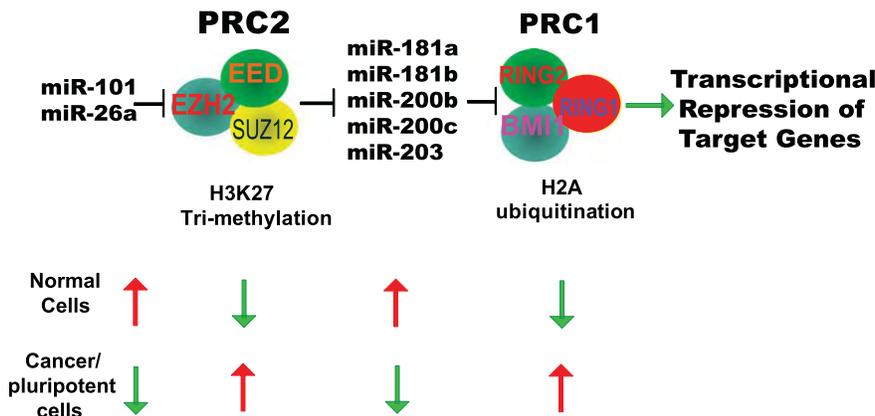
regulates PRC2 protein SUZ12 in a manner similar to that of miR-101, lending further support for microRNA-mediated PRC activity during cancer progression. These findings offer multiple targets for therapeutic interventions in the treatment of aggressive cancers (Garzon et al., 2010).

## EXPERIMENTAL PROCEDURES

### Cell Lines

Breast cancer cell line BT-549 was grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 0.023 IU/ml insulin and 10% FBS (Invitrogen) in 5% CO<sub>2</sub> cell culture

incubator; breast cancer cell line SKBr3 was grown in RPMI 1640 (Invitrogen) with 10% FBS (Invitrogen) in 5% CO<sub>2</sub> cell culture incubator; and prostate cancer cell line DU145 was grown in MEM with 10% FBS in 5% CO<sub>2</sub> cell culture incubator. Immortalized breast cell lines HME and H16N2 were grown in F-12 Nutrient Mixture with 5  $\mu$ g/ml Insulin (Sigma, St. Louis, MO), 1  $\mu$ g/ml Hydrocortisone (Sigma), 10 ng/ml EGF (Invitrogen), 5 mM Ethanolamine (Sigma), 5  $\mu$ g/ml Transferrin (Sigma), 10 nM Triiodo Thyronine (Sigma), 50 nM Sodium Selenite (Sigma), 10 mM HEPES (Invitrogen) and 50 unit/ml Penstrep (Invitrogen), 10% CO<sub>2</sub>. The PrEC (Lonza, Conshohocken, PA) and RWPE (ATCC, Manassas, VA) cells were grown in their respective medium as specified by the suppliers. miR-181b, miR-200b, and miR-203 overexpression constructs were obtained from Openbiosystems (Huntsville, AL).



**Figure 7. A Proposed Model Role for microRNAs in Regulating PRCs**

Specifically, PRC2 is molecularly linked to PRC1 via a set of regulatory miRNAs.

every other day) and/or 1  $\mu$ M suberoylanilide hydroxamic acid (SAHA) for 2 days. DU145 cells were treated with 2.5 or 5  $\mu$ M deazaneplanocin A (DZNep) for 2 or 3 days followed by RNA extraction or chromatin immunoprecipitation.

#### Cell Proliferation Assay and Basement Membrane Matrix Invasion Assays

Invasive breast cancer cell BT-549 and prostate cancer cell DU145 were transfected with miRNAs or controls. The cell proliferation and invasion

assays were performed as described (Cao et al., 2008; Kleer et al., 2003; Varambally et al., 2008; Yu et al., 2007b).

#### Soft Agar Colony Formation Assays

A 50  $\mu$ l base layer of agar (0.6% Agar in DMEM with 10% FBS) was allowed to solidify in a 96-well flat-bottom plate prior to the addition of a 75  $\mu$ l miRNAs or control-transfected or stable DU145 cell suspension containing 4000 cells in 0.4% Agar in DMEM with 10% FBS. The cell containing layer was then solidified at 4C for 15 min prior to the addition of 100  $\mu$ l of MEM with 5% FBS. Colonies were allowed to grow for 21 days followed by counting and imaging under a light microscope.

#### Spheres Culture

Spheres culture was performed as described (Dontu et al., 2003; Yu et al., 2007a). Briefly, cells (1000 cells/ml) were cultured in suspension in serum-free DMEM-F12 (Invitrogen), supplemented with B27 (1:50, Invitrogen), 20 ng/ml EGF (BD Biosciences), 0.4% bovine serum albumin (Sigma), and 4  $\mu$ g/ml insulin (Sigma). To propagate spheres in vitro, spheres were collected by gentle centrifugation, dissociated to single cells as described (Dontu et al., 2003; Yu et al., 2007a), and then cultured to generate prostatospheres of the next generation. Spheres larger than 50  $\mu$ m were counted.

#### Gene Expression Profiling

Expression profiling was performed using the Agilent Whole Human Genome Oligo Microarray (Santa Clara, CA) according to the manufacturer's protocol. BT-549 and DU145 cells were transfected with miRNAs or negative control for precursor microRNA. Over- and underexpressed signatures were generated by filtering to include only features with significant differential expression (Log ratio,  $p < .01$ ) in all hybridizations and 2-fold average over- or under-expression (Log ratio). Gene expression data are deposited into GEO (GSE26996).

#### Gene Set Enrichment Analysis

Molecular Concept Map (MCM) analysis was performed using gene list of putative targets to search for all concepts available in the Oncomine database as previously described (Yu et al., 2007c). Representative concepts with significant enrichment ( $p < 0.001$ ) were displayed as a network (Figure 4G; Table S4).

#### Prostate Tumor Xenograft Model

All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan and conform to their relevant regulatory standards. Five-week-old male nude athymic BALB/c nu/nu mice (Charles River Laboratory, Wilmington, MA) were used for examining tumorigenicity. To evaluate the role of BMI1 and RING2 knockdown, or miR-181b, miR-200b, and miR-203 overexpression in tumor formation, the DU145 stably overexpressing BMI1 shRNA, RING2 shRNA, scramble shRNA, miR-181b, miR-200b, miR-203, nontargeting miR or vector control cells were propagated and  $5 \times 10^6$  cells were inoculated subcutaneously into the dorsal flank of mice ( $n = 7$  for miR-203,  $n = 9$  for vector control, and  $n = 8$  for Scramble, BMI1-sh3, RING2-sh1, miR-181b, miR-200b, and miR-NT,

Lentiviruses were generated by the University of Michigan Vector Core. BMI1, RING2 and control shRNA lentivirus were obtain from Sigma. Prostate cancer cell line DU145 was infected with lentiviruses expressing BMI1 shRNA, RING2 shRNA, miR-181b, miR-200b, and miR-203 or controls only, and stable cell lines were generated by selection with 300  $\mu$ g/ml puromycin (Invitrogen).

#### Benign and Tumor Tissues

In this study, we utilized tissues from clinically localized prostate cancer patients who underwent radical prostatectomy as a primary therapy between 2004 and 2006 at the University of Michigan Hospital. Samples were also used from androgen-independent metastatic prostate cancer patients from a rapid autopsy program described previously (Tomlins et al., 2005, 2007a). The detailed clinical and pathological data are maintained in a secure relational database. This study was approved by the Institutional Review Board at the University of Michigan Medical School. Informed consent was also obtained from all subjects through the Institutional Review Board at the University of Michigan Medical School. Both radical prostatectomy series and the rapid autopsy program are part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence Tissue Core.

#### Illumina microRNA Profiling

Total RNA (500 ng) from each sample was labeled and hybridized on the Human v2 microRNA Expression BeadChips (Illumina, San Diego, CA) according to the manufacturers recommendations. BeadChips were scanned with the Illumina iScan Reader. Data were then average median normalized before generating differential expression values between treated and control samples.

#### microRNA Transfection, AntagomiR Transfection, and Small RNA Interference

Knockdown of EZH2 or Dicer was accomplished by RNA interference using siRNA duplexes (Dharmacon, Lafayette, CO) as previously described (Varambally et al., 2002). Precursors of respective microRNAs, antagomiRs and negative controls were purchased from Ambion (Austin, TX). Transfections were performed with oligofectamine (Invitrogen). EZH2 siRNA duplexes sequences, (duplex 1: GAGGTTCCAGACGAGCTGAT; duplex 2: AGACTCT GAATGCA GTTGC).

#### miR Reporter Luciferase Assays

The 50 bp of wild-type or mutant 3' UTR of BMI1 and RING2 containing the predicted miR-181a,b, miR-200b,c or miR-203 binding sites (as described in Figures S2C–S2F) were cloned into the pMIR-REPORT miRNA Expression Reporter Vector (Ambion). BT-549 cells were transfected with miRNAs or controls and then cotransfected with wild-type 3' UTR-luc or mutant 3' UTR-luc, as well as pRL-TK vector as internal control for luciferase activity. After 48 hours of transfection, the cells were lysed and luciferase assays were conducted using the dual luciferase assay system (Promega, Madison, WI). Each experiment was performed in triplicate. Drug Treatment.

BT-549 and DU145 cells were treated with 5  $\mu$ M 5-aza-2'-deoxycytidine (5-aza-dC) for 6 days (fresh media change containing the drug was performed

respectively). Tumor size was measured every week, and tumor volumes were estimated using the formula  $(\pi/6)(L \times W^2)$ , where  $L$  = length of tumor and  $W$  = width.

#### Bisulfite Modification and Methylation-Specific PCR of miR-203 in Prostate Tissues

Bisulfite conversion was carried out using EZ DNA methylation gold kit (Zymo Research Corporation, Orange, CA) according to manufacturer's instructions. Purified DNA (2  $\mu$ l) was used as template for PCRs with primers (Integrated DNA Technologies Inc., San Diego, CA) and synthesized according to bisulfite converted DNA sequences for the regions of interest using the Meth-primer software (Li and Dahiya, 2002). The PCR product was gel purified and cloned into pCR4 TOPO TA sequencing vector (Invitrogen, Carlsbad, CA). Plasmid DNA isolated from ten colonies from each sample was sequenced by conventional Sanger Sequencing (University of Michigan DNA Sequencing Core). The "BIQ Analyzer" (Bock et al., 2005) online tool was used to calculate the methylation percentage and to generate the bar graphs.

#### ACCESSION NUMBERS

Coordinates have been deposited in Gene Expression Omnibus database with accession code GSE26996.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.06.016.

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