Epigenetic Antagonism between Polycomb and SWI/SNF Complexes during Oncogenic Transformation

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DOI 10.1016/j.ccr.2010.09.006

SUMMARY

Epigenetic alterations have been increasingly implicated in oncogenesis. Analysis of Drosophila mutants suggests that Polycomb and SWI/SNF complexes can serve antagonistic developmental roles. However, the relevance of this relationship to human disease is unclear. Here, we have investigated functional relationships between these epigenetic regulators in oncogenic transformation. Mechanistically, we show that loss of the SNF5 tumor suppressor leads to elevated expression of the Polycomb gene EZH2 and that Polycomb targets are broadly H3K27-trimethylated and repressed in SNF5-deficient fibroblasts and cancers. Further, we show antagonism between SNF5 and EZH2 in the regulation of stem cell-associated programs and that Snf5 loss activates those programs. Finally, using conditional mouse models, we show that inactivation of Ezh2 blocks tumor formation driven by Snf5 loss.

INTRODUCTION

Epigenetic modifications, somatically heritable changes in gene expression derived from changes in chromatin structure not from changes in DNA sequence, serve crucial roles in cell fate decisions and are increasingly appreciated as having roles in oncogenic transformation (Jones and Baylin, 2007; McKenna and Roberts, 2009; Sparmann and van Lohuizen, 2006). Global alterations in covalent histone-tail modifications, such as acetylation, methylation, and phosphorylation, are frequently observed in cancer, as are aberrant expression of enzymes mediating these reactions (Wang et al., 2007a). Alterations in nucleosome positioning are also contributors to oncogenesis, and specific mutations in chromatin remodeling complexes that utilize ATP to reposition nucleosomes contribute to the formation of a growing list of tumors (Lin et al., 2007; Wang et al., 2007b). While genomic instability is present in most cancers, a subset of aggressive cancers are diploid and largely devoid of genomic alterations suggesting that genomic instability is dispensable for cancer formation and further highlighting important contributions of epigenetic alterations in oncogenesis (McKenna et al., 2008; McKenna and Roberts 2009). Gaining insight into the mechanisms by which epigenetic alterations drive cancer formation is an area of intense interest in cancer research because unlike DNA mutations epigenetic modifications are reversible, raising the possibility that therapeutics that specifically target epigenetic modifiers may be more effective in the treatment of cancer. While epigenetically based changes are increasingly recognized, the underlying mechanisms and contributions of individual chromatin modifying enzymes are not well understood.

Significance

While epigenetic alterations are widely present in cancers, in the context of genomic instability it has been difficult to determine the degree to which epigenetic alterations serve as primary drivers of tumorigenesis. Cancers initiated by mutation of the SNF5 chromatin remodeling subunit, despite being highly aggressive and lethal, are diploid and genomically stable, making them an ideal model for this purpose. Here, we demonstrate that the rapid onset of tumor formation following SNF5 loss arises due to imbalanced epigenetic antagonism between the SWI/SNF complex and the Polycomb complex PRC2. Collectively, our work reveals essential roles for epigenetic modifications during tumor formation and demonstrates that inactivation of EZH2 can have therapeutic efficacy against cancer in vivo.
Proteins from the Polycomb group (PcG) contribute to epigenetically based gene silencing during development and evidence is emerging to suggest that they may serve important roles during oncogenic transformation (Bracken and Helin, 2009; Simon and Lange, 2008). The PcG protein EZH2 is highly expressed in a range of cancer types, including breast, prostate, and lymphomas, and is often correlated with advanced stages of tumor progression and poor prognosis, although the mechanisms underlying the upregulation of EZH2 are poorly understood. EZH2 serves as the catalytic subunit in the PRC2 polycomb repressor complex and mediates gene silencing by catalyzing the trimethylation of histone 3 lysine 27 (H3K27) at the promoters of target genes. A number of studies, using tumor cell lines, have concluded that EZH2 may contribute to oncogenic transformation. Similarly, reducing the levels of EZH2 leads to growth inhibition and reduced tumor formation in tumor cell line transplantation models (Varambally et al., 2002; Bracken et al., 2003; Simon and Lange 2008). Collectively, these findings have led to the hypothesis that overexpression of EZH2 is an important driver of oncogenesis and that targeted inactivation of EZH2 may have therapeutic efficacy against these cancers in vivo.

Accumulating evidence has suggested that SWI/SNF complexes oppose epigenetic silencing by PcG proteins. Antagonistic relationships between SWI/SNF components and PcG proteins were first uncovered via genetic studies in Drosophila where mutations in core components of the SWI/SNF complex were found to suppress defects in body segment identity conferred by mutations in PcG proteins (Kennison and Tamkun, 1988). Insight into the underlying mechanism came when it was discovered that PcG proteins maintain repression of Hox genes during embryogenesis, while the SWI/SNF complex promotes Hox gene activation (Kennison, 1995; Tamkun et al., 1992). Subsequent in vitro work with mammalian proteins showed that SWI/SNF complexes mediate changes in gene expression by utilizing the energy of ATP to reposition nucleosomes and remodel chromatin and that this enzymatic activity can be counteracted by PcG proteins (Shao et al., 1999; Francis et al., 2001). Last, re-expression of SNF5 into a SNF5-deficient tumor cell line led to increased activation of the tumor suppressor protein p16INK4a and removal of PcG proteins at the p16INK4a locus (Kia et al., 2008; Oruetxebarria et al., 2004). Intriguingly, while PcG proteins are frequently overexpressed in cancer, core components of the SWI/SNF complex are frequently inactivated in cancer.

The SWI/SNF complex represents a novel link between epigenetic regulation and tumor suppression. Inactivation of the core SNF5 subunit leads to aggressive cancer formation and a familial cancer predisposition syndrome. Malignant rhabdoid tumors, which arise in the brain, kidney, and other soft tissues following biallelic inactivation of SNF5, are aggressive and highly lethal cancers that strike young children. The list of tumors with frequent loss of SNF5 is expanding and includes several aggressive cancers (Reisman et al., 2009; Roberts and Biegel, 2009). Mouse models have validated the tumor suppressor function of Snf5 as heterozygous Snf5 mice are tumor prone and biallelic conditional inactivation of Snf5 leads to fully penetrant cancer formation with a median onset of 11 weeks (Guidi et al., 2001; Kloochendler-Yeivin et al., 2000; Roberts et al., 2000, 2002). Furthermore, accumulating evidence raises the possibility that SWI/SNF complexes have a more widespread role in preventing tumorigenesis as specific mutations of this complex have been identified in cancers of the lung, breast, prostate, and pancreas (Roberts and Orkin, 2004; Medina et al., 2008; Reisman et al., 2009). While perturbations in SWI/SNF complexes can lead to aggressive widespread tumor formation, the mechanism underlying the formation of these tumors has remained elusive. Accumulating evidence suggests that epigenetic changes play a critical role in the genesis of cancer. The presence of genome instability and widespread genetic mutations in the majority of cancers makes it difficult to evaluate the role of epigenetic alterations as it is unclear which, if any, of these epigenetic alterations are primary drivers of cancer and which are secondary passengers. In contrast, SNF5-deficient cancers, despite being highly aggressive, are diploid and genomically stable, suggesting a critical epigenetic influence and making them an ideal model with which to elucidate epigenetic drivers of oncogenesis (McKenna et al., 2008; McKenna and Roberts 2009).

Here, we investigate a functional relationship between SNF5 and EZH2 in oncogenic transformation by examining the role of EZH2 in driving SNF5-deficient tumors.

RESULTS

Ezh2 Is Upregulated in Snf5-Deficient Tumors

To investigate whether activation of PcG function contributes to the oncogenic drive caused by SNF5 loss, we evaluated PcG gene expression in SNF5-deficient tumors. We first examined PcG gene expression signatures from primary CNS rhabdoid tumors and cell lines compared with expression signatures from normal cerebellum. MRTs expressed higher levels of EZH2 (Figure 1A; p < 0.0001), whereas other PcG transcripts were upregulated in some samples but were not consistently affected (Figures S1A–S1D; EED, p = 0.54; SUZ12, p = 0.03; BMI1, p = 0.97). In order to evaluate the relative magnitude of this effect, we compared the levels of PcG transcripts in SNF5-deficient samples with prostate cancers because these tumors have been shown to express EZH2 at high levels in metastatic cancers or low levels in primary tumors (Varambally et al., 2002). We utilized previously published microarray data sets downloaded from the Gene Expression Omnibus database repository (GEO) (Edgar et al., 2002) and found that EZH2 was most highly expressed in MRTs exceeding even the levels in metastatic prostate cells (Figure 1B, p = 0.01; Actin control, Figure S1E). Taken together, these results reveal elevated levels of EZH2 in SNF5-deficient tumors.

We next sought to determine whether PcG genes were also upregulated in primary tumors that arise following conditional inactivation of Snf5 in the mouse T cell lineage. In this mouse model of cancer, inactivation of Snf5 in peripheral T cells rapidly gives rise to fully penetrant CD8+ T cell lymphomas, thus providing a tumor model with which we could investigate functional relationships between PcG proteins and the SWI/SNF complex during oncogenic transformation (Isakoff et al., 2005; Roberts et al., 2002; Wang et al., 2009b). Comparison of gene expression signatures from purified CD8+ Snf5-deficient lymphoma T cells to wild-type control CD8+ T cells revealed markedly increased levels of the Ezh2 transcript in all lymphoma
samples (Figure 1C; Figure S1F; p = 0.0002). These analyses also showed a trend toward upregulation of other PcG proteins although this was not as consistent (Figure 1C; Figure S1G–S1J; Eed, p = 0.19; Suz12, p = 0.08; Bmi1, p = 0.09). Ezh2 upregulation was confirmed using immunoblotting, which revealed a robust increase of Ezh2 protein in Snf5-deficient lymphomas (Figure 1D).

**Snf5 Directly Represses Ezh2 Transcription in Mouse Embryonic Fibroblasts**

To determine whether the elevated levels of Ezh2 detected in Snf5-deficient tumors was a primary effect of Snf5 inactivation, we next evaluated PcG expression in mouse embryonic fibroblasts (MEFs) conditional for Snf5. We first utilized whole-genome expression data sets that we generated from Snf5-deficient MEFs compared with control MEFs to evaluate changes in PcG gene expression caused by Snf5 loss (Isakoff et al., 2005). The PcG gene Ezh2 was reproducibly upregulated in all samples following Snf5 depletion (p = 0.004), whereas the expression of other PcG transcripts, Eed (p = 0.50), Suz12 (p = 0.76), and Bmi1 (p = 0.15), did not show consistent patterns of upregulation (Figure 1E; Figure S1K–S1O). Ezh2 protein levels also increased following Snf5 inactivation (Figure 1F). To gain insight into whether the SWI/SNF complex directly regulates Ezh2 expression, we tested for binding of Snf5 at the genomic locus of Ezh2 in MEFs. Using chromatin immunoprecipitation (ChIP), we examined Snf5 and RNA Polymerase II occupancy at Ezh2 compared with three negative control locations and observed enrichment of Snf5 at the Ezh2 gene, but not at control genes (Figures S1P and S1Q).

**Ezh2 Drives Epigenetic Silencing of p16INK4a Following SNF5 Loss**

Downregulation of the p16INK4a tumor suppressor occurs following inactivation of SNF5 and may contribute to oncogenesis (Isakoff et al., 2005; Oruetxebarria et al., 2004). p16INK4a has also been shown to be a target of EZH2 and to be repressed by PcG-mediated silencing (Kia et al., 2008; Kotake et al., 2007; Shen et al., 2008). Further, re-expression of SNF5 in human MRT cell lines leads to accumulation of SWI/SNF at the p16INK4a promoter and is associated with loss of PcG proteins (Kia et al., 2008). Therefore, we sought to utilize p16INK4a as a model target to investigate the functional relationship between EZH2 and SNF5 in primary cells. We crossed Snf5-conditional mice to Ezh2-conditional mice and isolated MEFs of three genotypes: Snf5-conditional, Ezh2-conditional, and Snf5-Ezh2 doubly conditional. We evaluated p16INK4a expression after inactivation of Ezh2 or Snf5 or both. Consistent with previous reports, Snf5 inactivation alone resulted in silencing of p16INK4a (Isakoff et al., 2005). In contrast, inactivation of Ezh2 abrogated the downregulation effect of Snf5 loss upon p16INK4a expression (Figure 2A). Finally, inactivation of Ezh2 abrogated the downregulation effect of Snf5 loss upon p16INK4a expression (Figure 2B).
and resulted in wild-type p16INK4a levels in double deficient MEFs (Figure 2A). To test whether this antagonism was reversible, 3 days after inactivation of Snf5 and Ezh2, we reintroduced Snf5 and found that p16INK4a expression was restored to a high level, demonstrating reversibility (Figure S2). Since Ezh2 is thought to mediate epigenetic gene silencing through the addition of tri-methyl groups to lysine 27 of histone H3, we evaluated whether this histone modification was elevated at the p16INK4a locus after Snf5 inactivation. Using chromatin immunoprecipitation (ChIP), we quantified the relative levels of H3K27 trimethylation at the p16INK4a locus after Snf5 inactivation in MEFs. Snf5 inactivation led to elevated levels of H3K27 trimethylation at the p16INK4a gene, consistent with a role for Ezh2 in driving epigenetic gene silencing after Snf5 inactivation (Figure 2B).

To evaluate the relevance to human cancers, we next investigated whether EZH2 was essential for maintaining the epigenetic silencing of p16INK4a in a human SNF5-deficient MRT cell line. Using a shRNA against EZH2, we were able to achieve greater than 90% knockdown of EZH2, as determined at the protein level. Expression of the EZH2 shRNA, but not the non-silencing shRNA, led to increased expression of p16INK4a (Figure 2C), showing that EZH2 is required for epigenetic silencing of p16INK4a.

Repression of Polycomb Signatures in Snf5-Deficient Tumors and Following Snf5 Inactivation in MEFs

The findings at the p16INK4a locus were supportive of epigenetic antagonism between SNF5 and EZH2. However, downregulation of p16INK4a is unlikely to be the full mechanism by which SNF5 loss drives cancer formation as tumor onset occurs much more quickly, with higher penetrance, and in a different spectrum following Snf5 inactivation than p16INK4a inactivation (Krimpenfort et al., 2001; Sharpless et al., 2001). Also, humans with biallelic p16INK4a mutations are not predisposed to develop MRT (Kim and Sharpless, 2006; Roberts and Biegel, 2009). Last, both SWI/SNF and PcG bind the genome at many loci rather than just at p16INK4a. Consequently, we sought to determine whether the epigenetic antagonism between SNF5 and EZH2 was more broad. We began with an analysis of the expression levels of PcG targets using Gene Set Enrichment Analysis (GSEA). PcG targets vary substantially by lineage, and as the cell of origin of MRTs is unknown but the tumors are poorly differentiated, we chose to utilize a gene set of PcG targets that had been defined in embryonic stem cells (Ben-Porath et al., 2008). We found that these PRC2 targets were downregulated in MRTs compared with normal brain (Figure 3A; p < 0.001). Consistent with this, a gene set consisting of H3K27me3 modified targets defined from embryonic stem cells (Ben-Porath et al., 2008; Lee et al., 2006) was similarly negatively enriched in rhabdoid tumors (Figure S3A; p < 0.001). We next used a gene set that we generated by performing ChIP-chip of H3K27me3 from primary murine cerebellum to identify H3K27me3 bound targets that were expressed to some degree to determine whether this gene set was downregulated in SNF5-deficient rhabdoid tumors. These genes were even more strongly downregulated in MRTs (Figure 3B; p < 0.001).

We next turned to our conditional mice to determine whether the cancers caused by ablation of Snf5 in this model system displayed similar alterations in PcG target genes. We thus examined the expression of a gene set consisting of genes regulated by Ezh2 within the T cell lineage (Su et al., 2005) and found that they are also repressed in Snf5-deficient CD8+ T cell lymphomas compared with normal CD8+ T cells (Figure 3C; p < 0.001).
Figure 3. SNF5 Loss Leads to Broad Repression of PcG Targets and Activation of Stem Cell-Associated Programs

GSEA is a method that determines whether a set of genes shows differences between two biological states. The normalized enrichment score (NES) reflects the degree to which a gene set is upregulated (positive NES) or downregulated (negative NES). Corresponding p values are indicated.

(A and B) (A) GSEA of PRC2 targets from stem cells (Ben-Porath et al., 2008) and (B) H3K27me3 enriched genes from cerebellum in expression data from human MRT samples compared with normal cerebellum.

(C) GSEA of T cell-specific Ezh2-regulated genes (Su et al., 2005) in expression data from purified CD8+ Snf5-deficient lymphomas compared with CD8+ T cells purified from a wild-type mouse.

(D and E) (D) GSEA of human fibroblast-specific EZH2-regulated genes (Bracken et al., 2006) and (E) MEF-specific EZH2-regulated genes in expression data from Snf5-deficient MEFs compared with control MEFs.

(F) GSEA of MEF-specific EZH2-regulated genes in expression data from Snf5 Ezh2-deficient MEFs compared with control MEFs.

(G–L) (G) GSEA of stem cell-associated program in expression data from human MRT samples compared with normal cerebellum, (H) purified CD8+ Snf5-deficient lymphomas compared with CD8+ T cells purified from a wild-type mouse, (I) Snf5-deficient MEFs compared with control MEFs, (J) human fibroblasts where EZH2 levels have been knocked down compared with control fibroblasts, (K) Ezh2-deficient MEFs compared with control MEFs, and (L) Snf5 Ezh2-deficient MEFs compared with control MEFs. The stem cell-associated expression signatures (Ben-Porath et al., 2008; Wong et al., 2008) and EZH2 knockdown expression data (Bracken et al., 2006) were previously published.

See also Figure S3. Gene sets are listed in Table S1.
A key question is whether repression of PcG-regulated genes is simply a consequence of oncogenic transformation or whether it is a primary consequence of Snf5 inactivation. We therefore utilized Snf5-deficient embryonic fibroblasts to evaluate the effect of Snf5 inactivation upon PcG-regulated gene expression in primary nontransformed cells. We found that a gene set consisting of genes regulated by Ezh2 in human fibroblasts (Bracken et al., 2006) was repressed following Snf5 inactivation in MEFs (Figure 3D; p < 0.001). We next utilized our Ezh2 conditional MEFS to identify an independently derived set of Ezh2-regulated genes (Table S1) and found that this gene set was also downregulated in Snf5-deficient MEFS (Figure 3E; p < 0.001). To evaluate specificity and to rule out a general repressive effect caused by Snf5 inactivation, we examined expression of Ezh2-regulated genes from an unrelated differentiated lineage, T cells, and found them not to be repressed following Snf5 inactivation in MEFS (Figure S3B) indicating lineage specificity. Last, we analyzed MEFS derived from Snf5fl/fl-Ezh2fl/fl double conditional embryos and found that the absence of Ezh2 indeed blocked the downregulation of these targets otherwise caused by Snf5 inactivation (Figure 3F). Taken together, these results show that Snf5 inactivation leads to broad repression of lineage-specific PcG-regulated genes and that this repression is dependent upon the presence of Ezh2.

**Snf5 Inactivation Causes Elevated Levels of H3K27me3 at Lineage-Specific PcG Targets**

The antagonism between Snf5 and Ezh2 in the control of gene expression programs prompted us next to investigate the underlying mechanism by using ChIP to test whether increased levels of histone H3K27 trimethylation were present. We thus performed ChIP-PCR for H3K27me3 from both Snf5-deficient CD8+ T cell lymphomas and from wild-type CD8+ T cells to ask whether increased levels of H3K27me3 were present at genes downregulated by Snf5 loss. We tested 25 downregulated and 5 nondownregulated control genes. Eighteen of the 25 experimental genes demonstrated statistically significant increases in H3K27me3 in the Snf5-deficient lymphomas; an additional 4 of the 25 genes had increased H3K27me3 levels but were not statistically significant; 2 of the 25 genes were unchanged and only 1 of the 25 displayed reduced levels of H3K27me3 in the lymphoma (Figure 4A). In contrast, H3K27me3 levels were not significantly altered in any of the five control genes. Consequently, increased levels of H3K27me3 are present at the promoters of genes downregulated in Snf5-deficient lymphomas.

We next investigated whether increased H3K27 trimethylation and elevated levels of Ezh2 were directly contributing to changes in gene expression programs following Snf5 inactivation in the MEF model system. We utilized ChIP-chip to define a set of 508 high-confidence genes bound by H3K27me3 modified histones in wild-type MEFS and then evaluated how this gene set was affected by inactivation of Ezh2 and Snf5. Examination of the list revealed several gene families including Hox, Pax, and Fox genes that have previously been shown to be regulated by PcG proteins (Bracken et al., 2006; Table S1). As expected, given that Ezh2 and the PRC2 complex trimethylate H3K27, these targets were upregulated in Ezh2-deficient MEFS (Figure 4B). In contrast, Snf5 inactivation led to further downregulation of the targets (Figure 4C). Coinactivation of Ezh2 blocked the downregulation of these genes otherwise caused by Snf5 loss, demonstrating an essential role for Ezh2 in this process (Figure 4D). Of note, the effects of Snf5 inactivation were specific as genes not bound by H3K27me3 modified histones in wild-type MEFS were not downregulated (Figure 4E). We next evaluated the effect of Snf5 inactivation upon the prevalence of genes modified by H3K27me3 and found that it led to a 1.9-fold increase (Figure S4).

To gain insight into levels of H3K27me3 at specific genes, we evaluated its density at promoters. Interestingly, while a 2-fold elevation of H3K27me3 was present at p16Ink4a, the effect was even more pronounced at numerous other targets (Figure 4F). At a few polycomb targets, such as Hoxb1, there appeared to be minimal change (Figure 4F). To evaluate the effect of Snf5 loss upon Ezh2 targets overall, we examined the set of genes that are upregulated following Ezh2 inactivation. Inactivation of Snf5 led to increased signal intensity of both H3K27me3 and Ezh2 binding overlapping the TSS and upstream portion of these genes (Figure 4G), an effect not seen at control genes such as actin and Rps8 (Figure 4F) or at a randomly chosen set of repressed genes not expressed in wild-type MEFS (Figure 4H).

**Balanced Expression of Stem Cell-Associated Programs by Ezh2 and Snf5**

We next sought to investigate the mechanistic basis by which disruption of the SNF5-PcG balance drives oncogenic transformation. PcG genes have been implicated in the control of stem cell identity via the repression of lineage-specific targets (Boyer et al., 2006; Lee et al., 2006). Similarly, the SWI/SNF complex has recently been implicated in the control of stem cell self-renewal and pluripotency (Ho et al., 2009; Kidder et al., 2009). It has been proposed that oncogenic transformation may depend upon aberrant ectopic activation of stem cell-related self-renewal programs (Ben-Porath et al., 2008; Wong et al., 2008). Therefore, we evaluated the expression gene signatures associated with identity of embryonic stem cells in Snf5-deficient tumors. We utilized previously published embryonic stem cell-associated gene sets and found that these genes are significantly upregulated in both human MRTs (p < 0.001) and in murine Snf5-deficient lymphomas (p < 0.001) (Figures 3G and 3H; Figures S3C and S3D; Wong et al., 2008; Ben-Porath et al., 2008). As a higher fraction of tumor cells than control cells are proliferating, we sought to determine whether proliferation-associated genes were driving this enrichment. Therefore, we used a previously published “proliferation-corrected” gene set from which the proliferation-associated genes had been removed (Somerville et al., 2009). Significant enrichment remained intact using this gene set as well (MRT, p < 0.001; Snf5-deficient lymphomas, p < 0.001, Figures S3E and S3F). Again, we sought to determine whether activation of the stem cell-associated signature was simply a secondary consequence of oncogenic transformation or whether it was a primary effect due to Snf5 loss. Therefore, we examined the expression of these gene sets in Snf5-deficient MEFS and found enrichment of both the stem cell-associated signatures (p < 0.001) and proliferation deficient stem cell-associated signatures (p < 0.001) in Snf5-deficient MEFS (Figure 3F; Figures S3G and S3H). Consequently,
the upregulation of stem cell-like programs occurs in primary cells following Snf5 depletion and is not simply a consequence of transformation. We next evaluated whether the gene expression signatures associated with stem cells were oppositely regulated by EZH2. To test this, we examined the expression of these gene...
signatures in previously published microarray data from human embryonic fibroblasts in which EZH2 had been knocked down (Bracken et al., 2006). We found that the stem cell-associated signatures and a proliferation-deficient stem cell-associated signature were downregulated following EZH2 knockdown in primary cells, an effect opposite to that caused by Snf5 loss (Figure 3 J; Figures S3 I and S3J). We next utilized our Ezh2-conditional MEFs and similarly found that these stem cell-associated signatures were downregulated following Ezh2 inactivation (Figure 3K; Figures S3K and S3L). Finally, we utilized MEFs from Snf5fl/fl-Ezh2fl/fl double conditional embryos and found that inactivation of Ezh2 entirely blocked enrichment of the stem cell-associated signature otherwise caused by Snf5 loss (Figure 3L; Figures S3M and S3N).

**Ezh2 Is Required for Growth of SNF5-Deficient Tumor Cells**

The antagonism between SNF5 and EZH2 in the control of gene expression prompted us to ask whether PcG activity is a key driver of oncogenic transformation following Snf5 loss. We began by evaluating the effect of EZH2 knockdown in human SNF5-deficient MRT lines and found that shRNA-mediated knockdown of EZH2 caused a decrease in cell proliferation (Figure 5A; Figure S5). To confirm that the slowed proliferation was not due to off-target effects conferred by expressing the shRNA, we monitored proliferation after knocking down EZH2 using a different system. We transiently transfected a smartpool of siRNAs that target EZH2 and then monitored growth by counting cells after 72 hr. Using this siRNA-mediated technology, we were also able to achieve greater than 90% knockdown of EZH2 and similarly observed a reproducible reduction in proliferation compared with cells that were treated with control siRNAs (Figure 5B). Thus, EZH2 is required for proliferation of SNF5-deficient cancer cells.

To gain further insight into how EZH2 is driving tumor formation, we next evaluated whether the decreased proliferation following EZH2 knockdown was a result of either apoptosis or cellular senescence. While EZH2 knockdown revealed no detectable apoptotic phenotype as measured by cleavage of PARP1 (Figure 5C), a significant increase in senescence-associated β-galactosidase expression was detected (Figure 5D). Further, these cells were larger and flatter, consistent with senescent morphology. These results suggested that EZH2 contributes to tumorigenesis, at least in part, by preventing the activation of cellular senescence.

**Ezh2 Is Essential for Tumor Formation In Vivo**

Given our results thus far, we hypothesized that imbalanced function between EZH2 and SNF5 might serve crucial roles in driving tumor formation. Therefore, we sought to test in vivo, using a formal genetic approach, whether inactivating Ezh2 could rescue the tumor phenotype conferred by Snf5 inactivation in the T cell lineage. Importantly, inactivation of Snf5 in peripheral T cells confers rapid onset of mature CD8+ T cell lymphomas in 100% of the mice, thus allowing us to quickly investigate contributions of Ezh2 to oncogenesis in vivo (Roberts...
et al., 2002; Wang et al., 2009b). Therefore, we interbred Snf5 and Ezh2 conditional mice in the presence of the CD4-Cre transgene and evaluated whether inactivation of Ezh2 would ameliorate the phenotypes caused by Snf5 loss in this lineage. While inactivation of Snf5 leads to a block in development and a reduction in peripheral T cell numbers to 30% of wild-type (Figures 6A and 6B; Roberts et al., 2002), Ezh2 inactivation had no effect upon the number or distribution of peripheral T cells (Figures 6A and 6B), consistent with previous studies (Su et al., 2005).

We aged cohorts of CD4-Cre expressing Snf5fl/fl, Ezh2fl/fl, and Snf5fl/fl-Ezh2fl/fl double conditional mice to monitor for tumor onset. Strikingly, while Snf5 inactivation led to fully penetrant and rapid tumor formation (Figure 6C), inactivation of Ezh2 completely blocked tumor onset driven by Snf5 loss (Figure 7).

**DISCUSSION**

**Epigenetics in Tumor Development**

Epigenetic alterations likely contribute to the formation of most, if not all cancers. However, in the setting of genomic instability it is difficult to evaluate the contributions of epigenetic changes as it is unclear which are primary drivers and which arise as
secondary passengers due to instability (McKenna and Roberts, 2009). We recently showed that SNF5-deficient cancers, both in humans and mice, are diploid and genomically stable, demonstrating that disruption of this chromatin remodeling complex can substitute for chromosomal instability in the genesis of aggressive, lethal cancers (McKenna et al., 2008). Further, this suggests that epigenetic alterations play a central role in driving the formation of these malignancies. Given that they are initiated by perturbation of a chromatin remodeling complex, their rapid onset, aggressive nature, and genomic stability, SNF5-deficient tumors constitute an ideal system with which to evaluate the epigenetic mechanisms that drive cancer formation.

While previous studies have demonstrated antagonism between PcG proteins and the SWI/SNF complex in regulating Hox gene expression in Drosophila, the extent of this relationship in mammalian development and disease has remained uncertain (Tamkun et al., 1992; Kennison 1995). Our studies show this epigenetic relationship is broad based in mammalian cells and demonstrate that balanced function between SWI/SNF and PcG serves to prevent tumor formation (Figure 8). As SWI/SNF is capable of displacing PcG proteins from the INK4a/ARF locus, our findings are consistent with a model where SWI/SNF and PcG complexes bind mutually exclusive of one another, a model supported by nonoverlapping binding profiles on polytene chromosomes and from genome-wide localization studies in mammalian embryonic stem cells (Armstrong et al., 2002; Kia et al., 2008; Ho et al., 2009; Kidder et al., 2009). Consequently, the antagonistic relationship between PcG proteins and the SWI/SNF complex also serves important roles in preventing tumor formation in mammals and is a critical component of tumor formation following Snf5 loss.

**EZH2 and Oncogenesis**

Accumulating evidence has suggested that EZH2 contributes to cancer formation. This evidence comes from studies examining the expression of EZH2 in different tumors, where the highest levels correlate with the most aggressive tumors and the worst prognosis (Sparmann and van Lohuizen 2006; Simon and Lange 2008). Additional studies in a variety of tumor cell lines have shown that reduced levels of EZH2 leads to slowed proliferation and a decreased capacity to form tumors when transplanted into mice, leading to the proposal of Ezh2 as a possible therapeutic target (Bracken et al., 2003; Crönquist and Van Ness, 2005; Kleer et al., 2003; Simon and Lange, 2008; Varambally et al., 2002). However, it has remained unclear whether hyperactivation of EZH2 has roles in the initiation and maintenance of primary cancers in vivo and whether targeted inactivation of EZH2 or its downstream function would be therapeutically useful. Using an in vivo cancer model, we show that inactivation of Ezh2 is sufficient to block tumor formation that occurs after Snf5 loss. A formal possibility is that Ezh2 could be an essential gene whose absence prevents cell growth. Several lines of evidence argue against this and for a specific relationship between PcG and SWI/SNF. First, as noted above, PcG and SWI/SNF serve antagonistic roles in control of Hox gene expression (Tamkun et al., 1992; Kennison 1995). Second, PcG blocks chromatin remodeling mediated by SWI/SNF in vitro assays (Francis et al., 2001; Shao et al., 1999). Third, re-expression of Snf5 leads to PcG eviction from the INK4a/ARF locus (Kia et al., 2008). Fourth, Ezh2 loss does not have a negative effect upon T cell number in vivo (Su et al., 2005). Last, we have found that the effects of Ezh2 inactivation in vivo to be quite specific. For instance, in a model of osteosarcoma, Ezh2 is largely dispensable for bone development and Ezh2 inactivation does not ablate cancer formation (J. Perry and S. Orkin, personal communication). Consequently, EZH2 does not serve an essential role in all cancers but rather likely drives cancer formation via specific activation.

Our results also provide mechanistic insight into the regulation of EZH2 expression. The SWI/SNF complex is thought to regulate activation or repression of genes via alterations in nucleosome positioning. Our work shows that Snf5 binds directly to the Ezh2 promoter and serves to prevent high-level expression. That Snf5 is an important regulator of Ezh2 expression is also supported by genome-wide ChIP- Chip analyses of Brg1 binding in embryonic stem cells where it was found that Ezh2 is a target of the SWI/SNF complex (Ho et al., 2009). Thus, our work establishes that Ezh2 is bound and regulated by Snf5, and required for tumor formation after Snf5 loss. In addition to Ezh2, several other PcG genes including Suz12, Eed, and Bmi1 are bound by Brg1 in embryonic stem cells, and these genes are also upregulated in some primary human cancers lacking Snf5, although to a lesser degree than EZH2. Therefore, Snf5 inactivation may lead to overexpression of other PcG genes which also contribute to oncogenesis. Indeed, while clear evidence linking SUZ12 and EED to oncogenic transformation is still lacking, Bmi1 has been shown to possess clear oncogenic properties (Sparmann and van Lohuizen 2006; Simon and Lange 2008). Collectively, our work establishes that the regulatory relationship between SWI/SNF and PcG complexes extends across multiple cell types and that perturbation of this relationship can contribute to disease.
Hyperactivated Stem Cell-Associated Programs: A Potential Mechanism Underlying Tumors that Arise Following Snf5 Inactivation or Overexpression of PcG Proteins

Stem cell-associated signatures have recently been found to be enriched in tumors with more aggressive behavior and poorer prognosis (Ben-Porath et al., 2008; Wong et al., 2008). While these signatures are upregulated in many cancers and are an important contributing factor during oncogenic transformation, the mechanisms underlying specific activation of these programs are not well understood. PcG proteins have a dynamic role in safeguarding stem cell identity by maintaining the repression of lineage-specific genes, and hyperactive PcG function may drive tumorigenesis through the repression of lineage-specific genes and reversion to a more stem cell-like fate associated with an increased capacity for self-renewal and proliferation. The SWI/SNF complex has roles in lineage-specific differentiation and perturbations in SWI/SNF activity block differentiation, and thus may force cells to retain properties of a stem cell. Furthermore, while PcG and SWI/SNF complexes have opposing functions in the regulation of Hox gene expression in Drosophila, it is not clear whether this antagonistic relationship exists broadly during development or in the regulation of genetic programs in stem cells. Our results show stem cell-associated signatures are enriched in Snf5-deficient cancers and most importantly that Snf5 inactivation leads to upregulation of stem cell-associated programs in primary, untransformed cells. This thus identifies Snf5 as a key regulator of these genetic signatures. Conversely, PcG proteins regulate genetic programs in stem cells and PcG overexpression has been shown to promote increased proliferation and contribute to oncogenic transformation. We show that reduction of Ezh2 levels in primary cells leads to downregulation of stem cell-associated signatures, demonstrating antagonistic effects of Snf5 and Ezh2 upon stem cell-associated programs. Last, we show that activation of the stem cell-associated signature caused by Snf5 loss is dependent upon the presence of Ezh2. Collectively, our studies establish Snf5 as a regulator of these genetic programs. Our results may be therapeutically relevant to tumors caused by inactivation of Snf5 by providing an in vivo model cancer system with which to test novel Ezh2 inhibitors, and may additionally be applicable to the wide variety of cancers in which Ezh2 is overexpressed. Most cancers caused by Snf5 loss are aggressive and respond poorly to current therapies. For example, despite intensive therapy most children affected with atypical teratoid rhabdoid tumor of the CNS, a highly malignant neoplasm driven by inactivation of Snf5, die within a year of diagnosis (Chi et al., 2009). Thus, more effective treatments are needed. Unlike genetic mutations, which in the context of cancer are essentially irreversible, epigenetic modifications are reversible and thus represent attractive targets for intervention. Our results suggest that targeted inactivation of Ezh2, or alternatively blockade of the epigenetic changes driven by Ezh2, may be effective in the treatment of patients bearing tumors driven by Snf5 inactivation.

**Targeted Therapy of Ezh2**

Our results may be therapeutically relevant to tumors caused by inactivation of Snf5 by providing an in vivo model cancer system with which to test novel Ezh2 inhibitors, and may additionally be applicable to the wide variety of cancers in which Ezh2 is overexpressed. Most cancers caused by Snf5 loss are aggressive and respond poorly to current therapies. For example, despite intensive therapy most children affected with atypical teratoid rhabdoid tumor of the CNS, a highly malignant neoplasm driven by inactivation of Snf5, die within a year of diagnosis (Chi et al., 2009). Thus, more effective treatments are needed. Unlike genetic mutations, which in the context of cancer are essentially irreversible, epigenetic modifications are reversible and thus represent attractive targets for intervention. Our results suggest that targeted inactivation of Ezh2, or alternatively blockade of the epigenetic changes driven by Ezh2, may be effective in the treatment of patients bearing tumors driven by Snf5 inactivation.

**EXPERIMENTAL PROCEDURES**

**Snf5 and Ezh2 Inactivation and Ezh2 Knockdown**

Excision of Snf5 and Ezh2 in MEFs was carried out as previously described (Isakoff et al., 2005; McKenna et al., 2008). Ezh2 knockdown was achieved using siRNAs and shRNAs targeting Ezh2. For details regarding Ezh2 knockdown and cell culture experiments, see Supplemental Experimental Procedures.
Cancer Cell
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Immunoblots
The following antibodies were used: SNF5 (Bethyl, A301-087A), EZH2 (Cell Signaling; 3147), Actin-HRP (Abcam; 20272-200), p16INK4a (Santa Cruz sc-1207 and sc-468), p19ARF (Bethyl; A300-340A and Novus; NB200-106), PARP1 (Cell signaling; 9542), and HRP-conjugated secondary antibodies (Jackson Immunoresearch). For details, see Supplemental Experimental Procedures.

Murine Models
All mouse experiments adhere to guidelines at the Animal Resources Children’s Hospital (ARCH) at the Children’s Hospital in Boston and have been approved by the Institutional Animal Care and Use Committee (IACUC).

Chromatin Immunoprecipitation and ChIP-chip
A two-step crosslinking method was done for the Snf5 ChIP according to Nowak et al. (2005) with some slight modifications. The Bethyl, A301-087A Snf5 antibody, was used. p values were determined using a paired t test. The H3K27me3 and Ezh2 ChIP experiments were performed similarly to the above method using the H3K27me3 antibody (Upstate; 07-449) or the Ezh2 antibody (Millipore; 17-662) and a one-step crosslinking method where formaldehyde (1%) was added directly to the culture media and incubated for 10 min at 37°C. p values were determined using an unpaired t test for the H3K27me3 enrichment in Snf5-deficient and WT MEFS were normally distributed (mean = 0.09). p values were calculated as P(X > x) where X is the difference in MAT scores and x is the difference for the indicated gene. For details, see Supplemental Experimental Procedures.

Gene Expression Data
Whole-genome expression analyses from Snf5-deficient lymphomas, Ezh2-deficient MEFS, and Snf5 Ezh2-deficient MEFS were generated as previously described (Isakoff et al., 2005). Expression data sets were analyzed using the Gene Pattern data analysis software (Reich et al., 2006). GSEA was performed as described previously (Subramanian et al., 2005). For details regarding the generation and analysis of whole-genome expression data, see Supplemental Experimental Procedures.

ACCESSION NUMBERS
Whole-genome expression and ChIP-chip data are deposited in the GEO data repository (accession number GSE23659).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Experimental Procedures, five figures, and one table and can be found online at doi:10.1016/j.ccr.2010.09.006.

ACKNOWLEDGMENTS
Grant support was from the American Cancer Society New England Division-SpinOdyssey Postdoctoral Fellowship #PF-07-261-01-DDC from the American Cancer Society and the Ruth L. Kirstechn National Research Service Award Fellowship 1 F32 CA130312-01A1 from the National Cancer Institute (B.G.W.). This work was supported in part by a U01 NCI Mouse Models of Cancer Consortium Award (S.H.O. and C.W.M.R.) and a PHS award R01CA113794 (C.W.M.R.). C.W.M.R. is a recipient of an Innovative Research Grant from Stand Up 2 Cancer. The Garrett B. Smith Foundation, the Claudia Adams Barr Foundation, the Sarcoma Foundation of America (C.W.M.R.), and a R01 CA109467 research project grant (S.L.P.) provided additional support. S.H.O. is an Investigator of the HHMI. We thank Jen Perry for helpful discussions and permission to cite unpublished data. We also thank Pablo Tamayo for help with cross-platform analysis of microarray data, Pengcheng Zhou for isolation of murine cerebellum cells, and David James for the BT16 cell line. Received: February 1, 2010 Revised: June 3, 2010 Accepted: August 17, 2010 Published: October 18, 2010

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Cancer Cell 18, 316–328, October 19, 2010 ©2010 Elsevier Inc. 327


