

Epigenetic inactivation of the tumor suppressor BIN1 drives proliferation of SNF5-deficient tumors

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Abbreviations: RT, rhabdoid tumor; SNP, single nucleotide polymorphism

Emerging evidence demonstrates that subunits of the SWI/SNF chromatin remodeling complex are specifically mutated at high frequency in a variety of human cancer types. SNF5 (SMARCB1/INI1/BAF47), a core subunit of the SWI/SNF complex, is inactivated in the vast majority of rhabdoid tumors (RT), an aggressive type of pediatric cancer. SNF5-deficient cancers are diploid and genomically stable, suggesting that epigenetically based changes in transcription are key drivers of tumor formation caused by SNF5 loss. However, there is limited understanding of the target genes that drive cancer formation following SNF5 loss. Here we performed comparative expression analyses upon three independent SNF5-deficient cancer data sets from both human and mouse and identify downregulation of the *BIN1* tumor suppressor gene as a conserved event in primary SNF5-deficient cancers. We show that SNF5 recruits the SWI/SNF complex to the *BIN1* promoter, and that the marked reduction of *BIN1* expression in RT correlates with decreased SWI/SNF occupancy. Functionally, we demonstrate that re-expression of *BIN1* specifically compromises the proliferation of SNF5-deficient RT cell lines. Identification of *BIN1* as a SNF5 target gene reveals a novel tumor suppressive regulatory mechanism whose disruption can drive cancer formation.

Introduction

Subunits of SWI/SNF chromatin remodeling complexes are specifically mutated in a variety of cancers. Specific biallelic inactivation of the core SWI/SNF subunit SNF5 (also known as SMARCB1, INI1 and BAF47) is present in 98% of malignant rhabdoid tumors (RT).^{1,2} RT are aggressive cancers that are poorly differentiated, locally invasive, frequently metastatic and highly lethal. SNF5 mutation has also been implicated in a familial cancer predisposition syndrome as well as in the genesis of familial schwannomatosis, multiple meningiomas, epithelioid sarcomas and extraskeletal myxoid chondrosarcomas.³⁻⁷ Recently, additional SWI/SNF subunits, including ARID1A (BAF250A), PBRM1 (BAF180) and BRG1, have been found to be specifically mutated at high frequency in subsets of ovarian, kidney and lung cancers, further demonstrating a broad role for SWI/SNF complexes in tumor suppression.⁸⁻¹² Consequently, elucidation of the mechanisms that drive formation of these cancers is of great interest.

SWI/SNF complexes occupy a key position at the intersection between epigenetic regulation and tumor suppression.

These evolutionarily conserved complexes utilize the energy of ATP hydrolysis to mobilize nucleosomes, remodel chromatin and regulate transcription of numerous target genes. Transcriptional regulation by SWI/SNF complexes has been implicated in the balanced control of proliferation and differentiation in multiple tissues.^{13,14} Mammalian SWI/SNF complexes are comprised of a SWI2/SNF2 family ATPase (either BRG1 or BRM), common core subunits (SNF5, BAF155 and BAF170) and 4–8 additional subunits that vary by cellular lineage, including ARID1A/BAF250A and PBRM1/BAF180.^{13,14} Mouse models have revealed a potent and specific role for SNF5 as a tumor suppressor. Heterozygous mice are prone to developing RT, and conditional, biallelic inactivation leads to a mixture of lymphomas and RT in 100% of mice with a median onset of only 11 weeks.¹⁵⁻¹⁸ *Snf5*-deficient murine tumors develop with remarkable rapidity in comparison to mice with mutations of other tumor suppressors such as p53 (20 weeks), p19^{ARF} (38 weeks) or p16^{Ink4a} (60 weeks), thus indicating an essential role for SNF5 in suppressing cancer.¹⁹⁻²¹

Recent evidence suggests that epigenetically driven changes in gene expression may drive tumorigenesis following SNF5

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inactivation. Despite their aggressive nature, RT are diploid, and, aside from loss of SNF5 itself, the large majority are indistinguishable from normal cells on high-density SNP arrays.²² The dysfunctional epigenetic state caused by SNF5 loss occurs in part due to disrupted epigenetic antagonism between SWI/SNF and Polycomb complexes.²³ The altered epigenetic landscape that arises following SNF5 loss promotes the transcriptional activation of several oncogenes (*c-MYC*, *CCND1*, *GLI1*, *AURKA*), which have been identified as SNF5 targets and are upregulated in SNF5-deficient tumors.^{22,24-27} The tumor suppressor *p16^{INK4A}* is also regulated by SNF5 in RT cell lines and mouse embryonic fibroblasts, although it remains to be shown whether *p16^{INK4A}* silencing is common in primary RT.^{23,28,29} Thus, tumor suppressors epigenetically inactivated in cancers following SNF5 loss have remained elusive.

The *BIN1* (bridging integrator 1/box-dependent Myc interactor 1/amphiphysin II) gene encodes a collection of approximately 10 alternatively spliced SH3 adaptor proteins, which have varied expression patterns and subcellular localization.³⁰ Several lines of evidence indicate that BIN1 exhibits tumor suppressor activity. BIN1 isoforms can inhibit cellular transformation by the K-RAS, C-MYC and E1A oncogenes or by dominant-negative p53.³¹ This inhibition may be mediated by physical associations between BIN1 and MYC or the RAS-specific guanine nucleotide exchange factors SOS-1 and SOS-2, which may function to inhibit MYC-induced gene activation and limit invasiveness of RAS-dependent cancers, respectively.³²⁻³⁵ Furthermore, *Bin1* mutant mice are cancer prone, and *Bin1* deficiency has also been shown to play an important role in the ability of cancer cells to avoid detection and destruction by the immune system.^{36,37}

Interestingly, loss of *BIN1* expression without genetic mutation has been observed in several human cancers, suggesting the mechanism of inactivation may primarily be epigenetic.^{31,38,39} Promoter hypermethylation or aberrant splicing have been suggested as possible causes for *BIN1* downregulation, but a mechanism for cancer-specific *BIN1* suppression is largely unclear.^{38,40} In this study, we provide evidence that *BIN1* is a novel SNF5 target whose epigenetic deregulation drives proliferation of SNF5-deficient tumor cells. Identification of aberrant epigenetic silencing of *BIN1* as a conserved event in SNF5-deficient cancers illustrates how changes in target gene expression due to aberrant chromatin remodeling complex activity can drive rapid tumorigenesis.

Results

Loss of *BIN1* expression in human and murine SNF5-deficient tumors. The lack of detectable alteration in DNA repair caused by SNF5 loss together with the resulting genomically stable cancers suggest a central role for epigenetically driven changes in gene expression in the etiology of RT.^{2,22} To search for targets essential for the tumor suppressor activity of SNF5, we compared genome-wide expression data that we had previously generated from primary RT of the brain (also known as atypical teratoid/rhabdoid tumor or AT/RT) to that from primary medulloblastomas that we had generated. We also included other data sets

from the Gene Expression Omnibus representing cancer types not known to be driven by SNF5 inactivation, including Wilms tumor, neuroblastoma, Ewing sarcoma, clear cell sarcoma of the kidney, prostate cancer, ovarian cancer and lung cancer. As an initial test of this approach, we utilized comparative marker selection, an algorithm that identifies the genes whose differential regulation is most closely correlated with a particular class of samples, to query expression of *SNF5* (<http://genepattern.broadinstitute.org/>).⁴¹ As expected, low levels of *SNF5* expression were strongly associated with RT, and *SNF5* ranked as one of the 10 (out of 4498) most downregulated genes in RT compared with the other pediatric and adult cancers used in the analysis (Table 1).

In order to identify potential targets of SNF5, we next used the GeneNeighbor algorithm (GenePattern) to identify genes whose expression levels were most similar to those of SNF5 across both SNF5-deficient and SNF5-expressing human cancers.⁴¹ We reasoned that such genes would represent candidates for novel tumor suppressor targets that are aberrantly silenced following SNF5 inactivation. The nearest gene neighbor of *SNF5* was *BIN1*, a gene with known tumor suppressor properties (cosine distance score = 0.008, Fig. 1A). Comparative marker selection also identified *BIN1* as the ninth most downregulated gene in RT, whereas downregulation of *BIN1* was not strongly correlated with any other tumor type analyzed (Table 1). These analyses indicate that, like *SNF5*, *BIN1* is specifically downregulated in RT (Table 1 and Fig. 1B). Furthermore, the expression data suggest downregulation of all *BIN1* isoforms, as the *BIN1* probes on the array platforms recognize all isoforms (NetAffx Analysis Center).

We next sought to validate these findings in an independent data set by evaluating *BIN1* expression in SNF5-deficient tumors originating in a different organ. We utilized comparative marker selection to analyze recently published gene expression data from pediatric kidney tumors that included RT of the kidney, cellular mesoblastic nephroma and samples of Wilms tumor and clear cell sarcoma independent from those analyzed in Figures 1A and B.⁴² *BIN1* was in the top 1% of genes downregulated in RT of the kidney compared with clear cell sarcoma, Wilms tumor and cellular mesoblastic nephroma (Table 1). Consequently, downregulation of *BIN1* in RT is independent of tissue of origin.

To evaluate the relationship between SNF5 and BIN1 in a third cancer system, we examined the expression of *Bin1* in murine CD8⁺ lymphomas driven by deletion of *Snf5*.¹⁸ *Bin1* expression was strongly decreased in the lymphomas compared with wild-type CD8⁺ cells (Fig. 1D), indicating that loss of *BIN1* expression is a conserved event in SNF5-deficient tumors.

SNF5 recruits the SWI/SNF complex to the *BIN1* promoter to activate expression. We next investigated the mechanism underlying altered expression of *BIN1* to determine whether the downregulation was directly due to SNF5 loss. We retrovirally transduced the SNF5-deficient RT cell line BT16 with either FLAG-tagged SNF5 or vector control. *BIN1* was reproducibly upregulated upon SNF5 reintroduction (Fig. 2A). As a positive control, we analyzed expression of *p16^{INK4A}*, a gene that has been previously shown to be upregulated upon SNF5 reintroduction

into RT cell lines.²⁸ To further evaluate the relationship between SNF5 and BIN1, we next tested whether expression of *Bin1* is dependent upon SNF5 in normal mouse cells. Deletion of *Snf5* via a retrovirus expressing Cre recombinase in *Snf5^{flax/flax}* primary murine embryonic fibroblasts resulted in a 1.4-fold decrease ($p = 0.0134$) in *Bin1* mRNA levels (Fig. 2B), indicating that *Bin1* expression is also dependent on SNF5 in primary cells.

To more specifically evaluate the mechanism of *BIN1* regulation, we evaluated whether loss of SNF5 resulted in aberrant silencing of *BIN1* due to altered recruitment of SWI/SNF to the *BIN1* promoter. We reintroduced SNF5 into BT16 cells as above and performed chromatin immunoprecipitation analysis of the SWI/SNF ATPase BRG1 at the *BIN1* promoter. We observed specific SNF5-dependent enrichment of BRG1 (Fig. 3), suggesting that SNF5 facilitates recruitment of the SWI/SNF complex to the *BIN1* promoter to drive *BIN1* expression.

***BIN1* silencing promotes proliferation of SNF5-deficient cancer cells.** Having found that loss of SNF5 directly leads to *BIN1* downregulation due to decreased recruitment of the SWI/SNF complex, we sought to determine whether silencing of *BIN1* contributes to the proliferation of tumor cells driven by SNF5 loss. We introduced the two ubiquitous *BIN1* isoforms, isoform 9 and isoform 10, into BT16 cells (Fig. 4A and B). Given the potential for negative selection against *BIN1* expression in RT cells, we expressed the isoforms as fusions with AcGFP and DsRed to both label infected cells and ensure that fluorescence levels correlated with *BIN1* expression. Transduction of the *BIN1* isoforms into U2OS, an osteosarcoma cell line that expresses endogenous *BIN1* (Fig. 4C), had no effect on proliferation (Fig. 4D). In contrast, transduction of *BIN1* into BT16 cells blocked cell proliferation (Fig. 4E).

Discussion

SNF5-deficient RT are almost exclusively diploid and genomically stable and lack recurrent genetic abnormalities on SNP arrays other than at the SNF5 locus. Rather, deregulation of expression of known SNF5 targets, such as *CCND1*, *GLII*, *RHOA*, *AURKA* and *c-MYC*, without evidence of genetic amplification suggests that epigenetically based alteration of gene expression is a key mechanism of SNF5-mediated tumor suppression.^{22,24-27,43,44} SWI/SNF complexes are specifically enriched at promoters, where they contribute to chromatin remodeling that facilitates epigenetic control of gene expression.⁴⁵ Thus, defective remodeling, perhaps akin to “epigenetic instability,” caused by SNF5 loss could conceivably deregulate many target genes and activate pathways that cooperatively drive cancer growth, thus amplifying the effects of inactivation of a single gene and providing an explanation for the rapid tumorigenesis caused by SNF5 loss.^{22,46} In this study, we show that aberrant silencing of *BIN1* is a direct effect of SNF5 loss in primary human RT of brain and kidney, murine *Snf5*-deficient lymphoma as well as cell culture models of RT, and that loss of *BIN1* expression is required for the proliferation of SNF5-deficient tumor cells. Our data supports a model in which SNF5 is required for normal expression of *BIN1* via recruitment of SWI/SNF chromatin remodeling

Table 1. Downregulation of *SNF5* and *BIN1* are specific events in RT

Phenotype test	Gene	Rank	Fold change
Rhabdoid Tumor (CNS) vs. All	<i>BIN1</i>	9/4498 down	-1.75
	<i>SNF5</i>	10/4498 down	-1.68
Medulloblastoma vs. All	<i>BIN1</i>	2883/4607 down	-1.10
	<i>SNF5</i>	3055/4222 up	+1.06
Ewing's Sarcoma (GSE1825) vs. All	<i>BIN1</i>	2018/4152 down	-1.05
	<i>SNF5</i>	1460/4677 up	+1.09
Wilms Tumor (GSE2712) vs. All	<i>BIN1</i>	1825/4690 up	+1.12
	<i>SNF5</i>	1036/4690 up	+1.17
Neuroblastoma (GSE1825) vs. All	<i>BIN1</i>	167/4568 up	+1.23
	<i>SNF5</i>	2179/4568 up	+1.09
Clear Cell Sarcoma of Kidney (GSE2712) vs. All	<i>BIN1</i>	2836/4561 up	+1.12
	<i>SNF5</i>	3876/4561 up	+1.03
Primary Prostate Cancer (GSE6919) vs. All	<i>BIN1</i>	2601/4340 up	+1.19
	<i>SNF5</i>	2050/4340 up	+1.21
Lung Adenocarcinoma (GSE7670) vs. All	<i>BIN1</i>	2755/4410 up	+1.07
	<i>SNF5</i>	3812/4419 down	-1.03
Primary Invasive Breast Tumor (GSE1456) vs. All	<i>BIN1</i>	2418/4377 down	-1.11
	<i>SNF5</i>	3347/4377 down	-1.05
Ovarian Carcinoma (GSE7463) vs. All	<i>BIN1</i>	1470/4383 up	+1.20
	<i>SNF5</i>	3858/4383 up	+1.04
Gadd et al., 2010 (GSE11482)			
Phenotype test	Gene	Rank	Fold change
Rhabdoid Tumor (Kidney) vs. All	<i>BIN1</i>	90/7490 down	-2.35
	<i>SNF5</i>	4/7490 down	-2.72
Cellular Mesoblastic Nephroma vs. All	<i>BIN1</i>	844/6270 up	+1.49
	<i>SNF5</i>	5531/6270 up	+1.04
Wilms Tumor vs. All	<i>BIN1</i>	5544/6574 up	+1.05
	<i>SNF5</i>	149/6574 up	+1.76
Clear Cell Sarcoma of Kidney vs. All	<i>BIN1</i>	3907/5323 up	+1.06
	<i>SNF5</i>	5524/7998 down	-1.12

Merged, Rank Ordered Data set. The Comparative Marker Selection algorithm on the GenePattern website (<http://genepattern.broadinstitute.org/>) was used to identify genes whose up- or downregulation is most closely correlated with a particular class of tumor samples (i.e., RT) compared with all others in the data set combined (All). Genes were either upregulated (up) or downregulated (down) in a tumor type when compared with all others and were ranked based on their association with the tumor phenotype in question. Fold change represents the degree of up- or downregulation of a gene in the tumor type being tested compared with the other tumors.

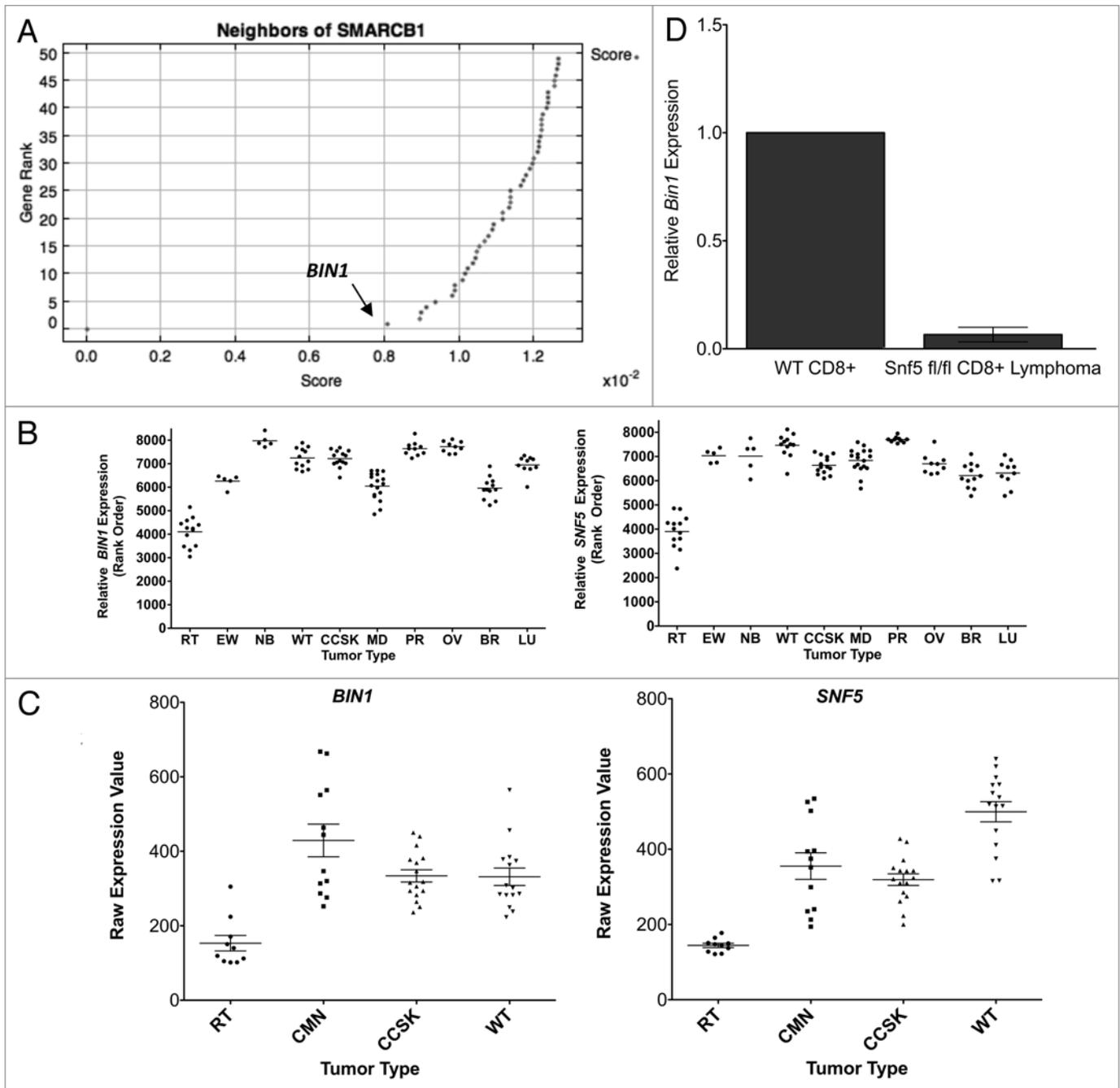


Figure 1. For figure legend, see page 1960.

activity to the *BIN1* promoter. This is consistent with data from a recent genome-wide study of SWI/SNF localization in human cells, which showed SNF5 occupancy throughout the *BIN1* promoter.⁴⁵ Consequently, in the absence of SNF5, *BIN1* expression is silenced.

Several lines of evidence indicate that negative regulation of MYC represents a major aspect of BIN1-mediated tumor suppression. BIN1 inhibits MYC-induced gene activation in vitro and is required for apoptosis caused by MYC overexpression.^{33,47} BIN1 and MYC physically interact, and their levels are inversely

correlated, suggesting that BIN1 may affect MYC expression or protein stability.^{33,34} Further, forced expression of BIN1 selectively reduces colony formation in neuroblastoma cell lines with multiple copies of *N-MYC*, suggesting selective pressure for BIN1 inactivation in tumors driven by *MYC*.⁴⁸ This is consistent with our observations of specific *BIN1* downregulation and *MYC* overexpression in SNF5-deficient tumors compared with other tumor types.²² Furthermore, like BIN1, SNF5 has also been shown to regulate MYC target activation, and *SNF5* and *BIN1* were identified as two of only 17 genes whose knockdown overrides

Figure 1 (See previous page). *BIN1* downregulation is a conserved event in SNF5-deficient tumors. (A) Expression data from 9 RT, 4 RT cell lines and 17 medulloblastomas merged with samples from data sets downloaded from the Gene Expression Omnibus [Ewings Sarcoma and Neuroblastoma (GSE1825), Wilms Tumor and clear cell sarcoma of the kidney (GSE2712), prostate cancer (GSE6919), ovarian cancer (GSE7463), lung cancer (GSE7670) and breast cancer (GSE1456)] and normalized by replacing the raw expression value for each gene with its rank in each individual microarray sample, from lowest to highest. The nearest neighbors of SNF5 (genes whose expression values followed similar patterns across the microarray samples) were identified in the merged, normalized data set using the GeneNeighbor algorithm on the GenePattern website. *BIN1* is the top gene neighbor of *SNF5*, with a cosine distance score of 0.008 relative to *SNF5*. (B) Both *BIN1* and *SNF5* are specifically downregulated in RT compared with other tumors. A non-parametric (Mann-Whitney) test was performed based on the ranks of *SNF5* and *BIN1* gene expression in RT and non-RT ($p < 0.0001$). A scatterplot of this data was generated using GraphPad Prism. EW, Ewings sarcoma; NB, neuroblastoma; WT, Wilms tumor; CCSK, clear cell sarcoma of the kidney; MD, medulloblastoma; PR, prostate cancer; OV, ovarian cancer; BR, breast cancer; LU, lung cancer. (C) Independent verification of specific *BIN1* downregulation in RT of the kidney. Expression data from a recently published study of pediatric kidney tumors (38) was downloaded from GEO (GSE11482) and an unpaired, two-tailed t-test was performed based on the raw expression value of *SNF5* and *BIN1* in RT and non-RT ($p < 0.0001$). CMN, cellular mesoblastic nephroma; CCSK, clear cell sarcoma of the kidney; WT, Wilms tumor. Note that the WT and CCSK samples presented here are independent from the samples analyzed in (A and B). (D) *Bin1* expression is downregulated in CD8⁺ lymphomas caused by conditional inactivation of Snf5. Quantitative real-time PCR was performed using cDNA prepared from wild-type and Snf5-deficient lymphoma CD8⁺ T cells purified from three mice of each genotype. *Rps8* was used as a reference gene to calculate the dCt. An unpaired T-test was used to calculate p-values from the dCts. Relative expression was determined using the ddCt method. Error bars represent standard error (SE) of at least three independent samples.

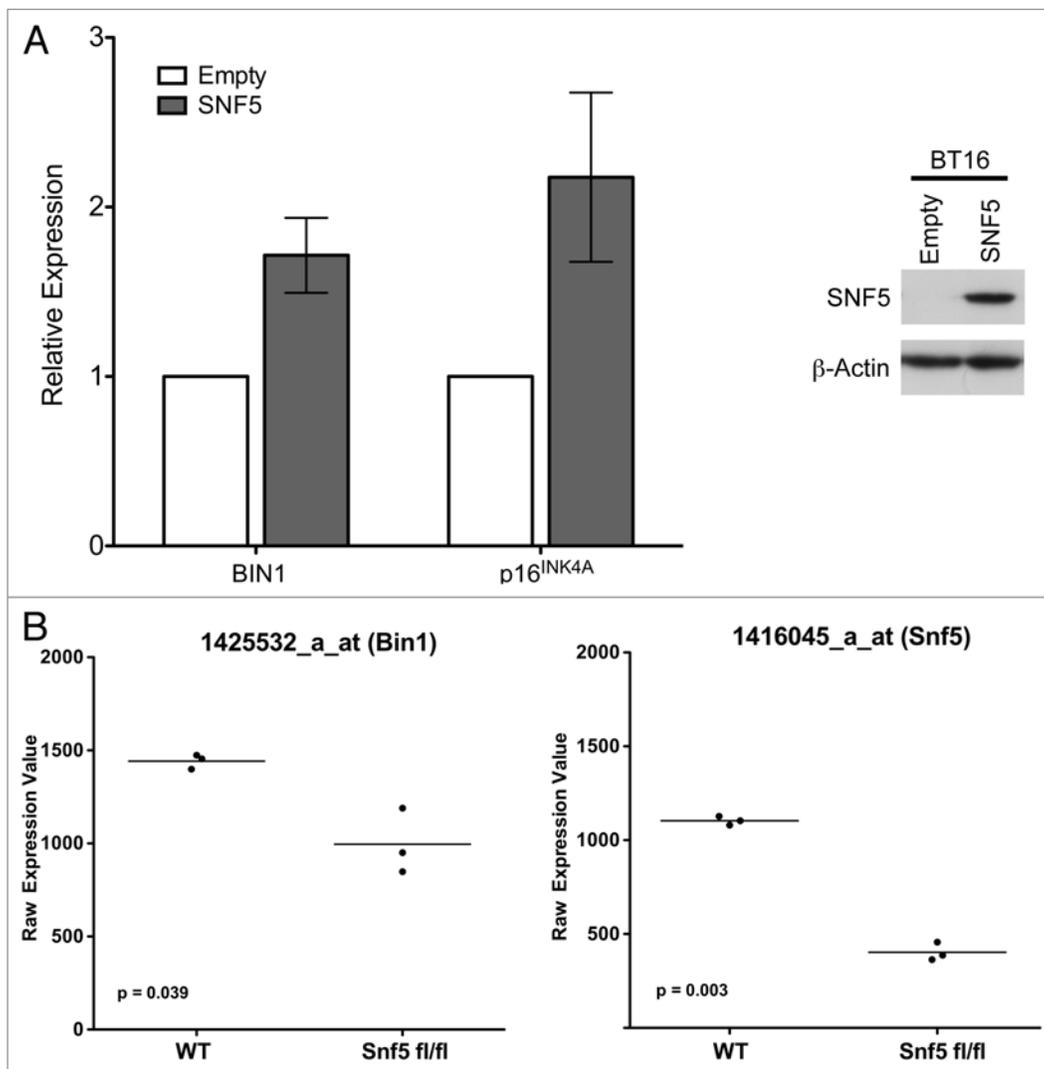


Figure 2. SNF5 regulates *BIN1* expression. (A) Reintroduction of SNF5 into two SNF5-deficient RT cell lines derived from either brain or kidney tumors leads to increased *BIN1* expression. Quantitative real-time PCR was performed using cDNA prepared from BT16 cells retrovirally transduced with either empty vector or FLAG-SNF5. Expression analysis of *p16^{INK4A}* was included as a positive control. Error bars represent SE of three independent experiments. (B) Excision of *Snf5* in primary MEFs leads to decreased *Bin1* expression. RNA from three independent WT and *Snf5* fl/fl MEFs was applied to an Affymetrix 430A 2.0 array. Hybridization values for *Bin1* in WT and *Snf5* fl/fl MEFs were compared using an unpaired, two-tailed t-test ($p = 0.039$). A scatterplot of this data was generated using GraphPad Prism.

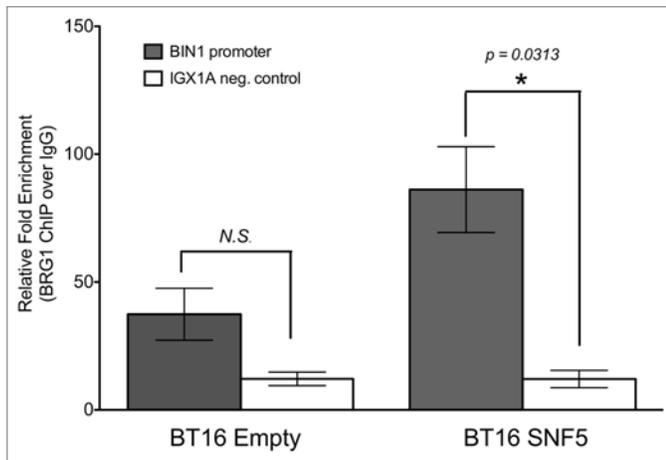


Figure 3. SNF5 recruits the SWI/SNF complex to the *BIN1* promoter. Chromatin from BT16 cells retrovirally transduced with either empty vector or FLAG-SNF5 was immunoprecipitated with an anti-BRG1 antibody. Data are presented as relative fold enrichment of BRG1 ChIP over IgG ChIP. Error bars represent standard error (SE) of three independent samples. A paired t-test was performed comparing the relative fold enrichment of BRG1 ChIP over IgG ChIP at the *BIN1* promoter and at the IGX1A negative control region.

MYC-dependent oncogene-induced senescence driven by constitutive *BRAF* activation.⁴⁹⁻⁵¹ Collectively, these data suggest that SNF5 and BIN1 may act in a common pathway in which SNF5 controls the expression of the effector BIN1. This is supported by gene expression studies in a murine model of muscle differentiation, in which induction of *Bin1* by the myogenic transactivator MyoD is blocked by a dominant-negative SWI/SNF ATPase.⁵²

The fact that expression of *BIN1* is attenuated in human cancer without frequent genetic mutation underscores both the importance of BIN1 activity in tumor suppression and the potential impact of epigenetic deregulation of gene expression in cancer.³⁹ Given our previously published work demonstrating that SNF5-deficient RTs are genomically stable, and that transcriptional deregulation of target genes is associated with the RT phenotype, identifying SNF5 targets provides insight into the mechanisms driving the proliferation of these cancers and suggests potential novel therapeutic targets. It is interesting to note that BIN1 has been implicated in the regulation of other targets reported to be regulated by SNF5. For example, BIN1 is a known negative regulator of c-MYC, a proliferation-promoting gene upregulated in RT. Additionally, BIN1 has been shown to genetically interact with Rho GTPases and could conceivably contribute to the increased Rho activity observed in RT cells.^{44,52,53} Finally, while the cellular function of BIN1 is not completely understood, its ability to interact with many binding partners through its SH3 domain and its ability to translocate to the nucleus suggests that BIN1 may be an example of a bridge between epigenetic gene regulation and transduction of multiple types of extracellular signals.^{30,54,55} Disruption of such coordinated signaling activity following SNF5 loss would be analogous to our finding that upstream control of Hedgehog signaling becomes uncoupled from Hedgehog target gene transcription in the absence of SNF5,

collectively raising the possibility of a role for SNF5 in coordinated transduction of lineage-specific growth and differentiation signaling, an area for future investigations.^{24,56}

Identification of *BIN1* as a SNF5 target gene whose down-regulation promotes the genesis of SNF5-deficient tumors may also have implications for the treatment of these aggressive cancers. For example, the gene indoleamine-2,3-dioxygenase (*IDO*) is an immunomodulatory enzyme constitutively overexpressed in many human tumors, whose elevation can promote evasion of immune surveillance by tumors. *Ido* is regulated by BIN1 in mice and is required for immune escape by MYC/HRAS-transformed *Bin1*-deficient primary cells. An orally bioavailable IDO inhibitor was found to potentiate the effects of several chemotherapeutic agents in a BIN1-deficient breast cancer mouse model.³⁷ Additionally, BIN1 has recently been implicated in the negative regulation of the DNA repair enzyme PARP1.⁵⁷ Unrestrained PARP1 activity in the absence of BIN1 leads to increased DNA repair capacity and resistance to chemotherapeutic agents, which would be consistent with the lack of DNA damage sensitivity in *Snf5*-deficient cells.^{22,57} Given that *BIN1* is silenced following SNF5 loss, existing IDO and PARP inhibitors already in clinical trials are worthy of evaluation in SNF5-deficient cancer models.

Identification of *BIN1* as a SNF5 target gene silenced in RT constitutes a novel mechanism of tumor suppression and demonstrates the potential of mining cancer transcriptomes to establish novel pathways that would not otherwise be identified by sequencing of cancer genomes. Integration of genome-wide expression data with epigenomic analyses will provide crucial insight into the contributions of alterations of the chromatin landscape to transcriptional programs that promote oncogenesis. SNF5-deficient tumors represent a useful system in which to initiate such studies, given that they are diploid, appear genomically stable and are initiated by mutation of a chromatin regulator. As mutations in other SWI/SNF subunits have recently been found at high frequency in several other types of human cancer, it will be important to determine whether these cancers are driven by deregulation of similar pathways.

Materials and Methods

Tumor gene expression array and gene neighbor analysis. The RT and medulloblastoma microarray data sets were previously described in reference 22. Briefly, snap-frozen primary tumor samples were collected from brain tumor resections collected at Children's Hospital Boston, Children's Hospital of Philadelphia and St. Jude's Research Hospital for Children under approval from their respective Institutional Review Boards. To identify the genes with expression patterns closest to SNF5 across a panel of human tumors, we compared expression data from 9 RT, 4 RT cell lines and 19 medulloblastomas with samples from the following data sets downloaded from the Gene Expression Omnibus: 5 Ewing sarcomas and 5 neuroblastomas (GSE1825), 12 Wilms Tumors and 14 clear cell sarcomas of the kidney (GSE2712), 10 prostate cancers (GSE6919), 9 ovarian cancers (GSE7463), 10 lung cancers (GSE7670) and 12 breast cancers (GSE1456). For each individual data set, the CollapseData set

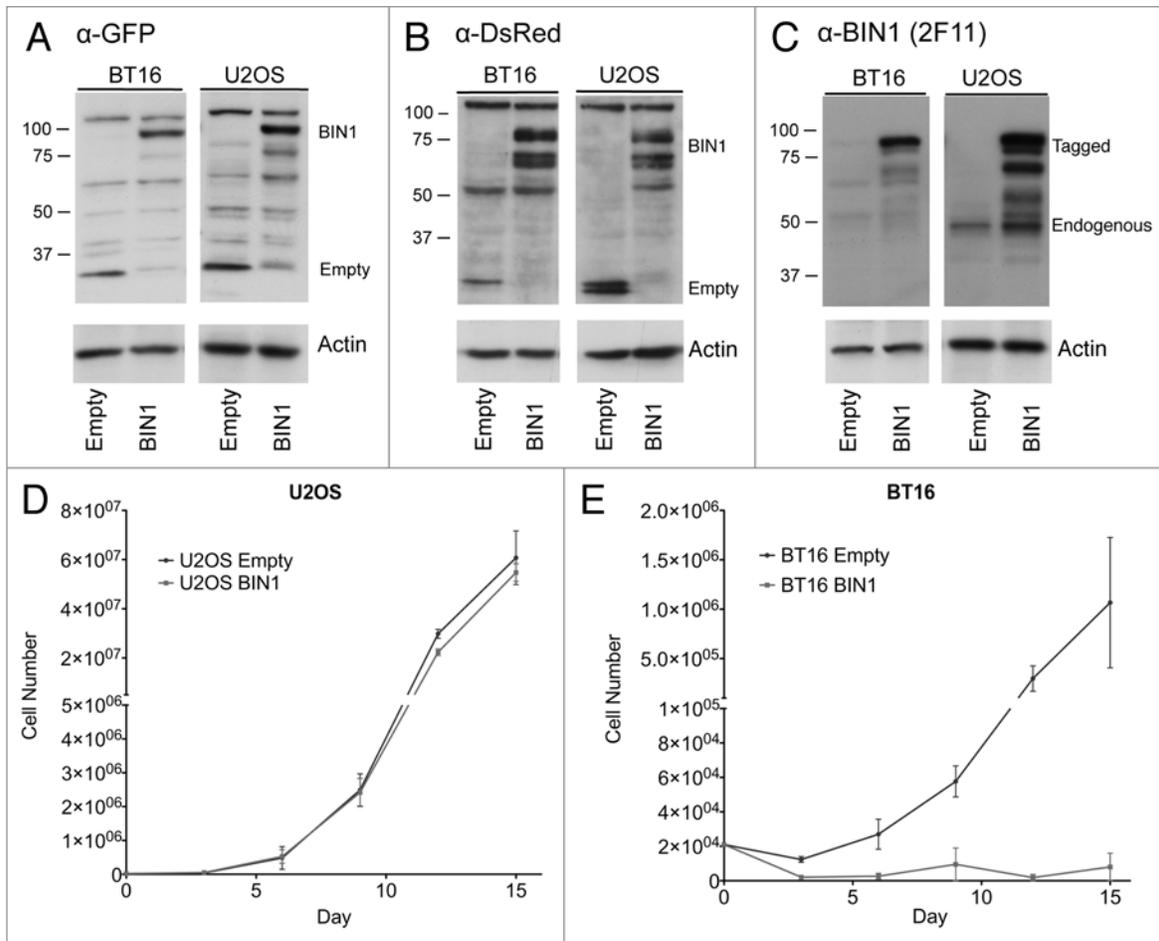


Figure 4. Aberrant epigenetic silencing of BIN1 is required for the proliferation of SNF5-deficient tumor cell lines. (A and B) Western blots showing expression of the AcGFP (A) and DsRed monomer (B) BIN1 fusion proteins in unsorted cell lines. (C) Western blot showing that the fusion proteins are translated in frame and are recognized by a BIN1 antibody. Note endogenous expression of BIN1 in the U2OS osteosarcoma cell line. (D and E) Forced expression of the ubiquitous BIN1 isoforms has no effect on the proliferation of a BIN1-expressing cell line (D), but specifically inhibits proliferation of the RT cell line BT16 (E), indicating that these growth of these cells is dependent on loss of BIN1 expression. Error bars represent standard error (SE) of two independent replicates.

tool from the Gene Set Enrichment Analysis desktop application (Broad Institute) was used to create a new data set using single gene symbols as the identifier for the maximum probe value. The data sets were then merged using an R programming script that only retained genes that were common among the different Affymetrix platforms (U133A 2.0, U133A and U95Av2) and normalized the samples by replacing the raw expression value for each gene with its rank in each individual microarray sample, from lowest to highest. Comparative marker selection was performed using a two-sided t-test to assess differential expression with zero permutations (because several tumor classes had fewer than 10 samples) of the phenotype test one vs. all. The nearest neighbors of SNF5 (genes whose expression values followed similar patterns across the microarray samples) were identified in the merged, normalized data set using the GeneNeighbor algorithm on the GenePattern website (genepattern.broadinstitute.org). The raw expression data from Gadd et al.⁴² was downloaded from GEO (GSE11482), normalized using robust multichip average on the GenePattern website and processed

using comparative marker selection (two-sided t-test with 1,000 permutations).⁴¹

Immunoblots. Whole-cell lysates were prepared by incubating cells on ice in RIPA lysis buffer for 20 min followed by centrifugation at 15,000x g for 10 min. Supernatant was collected and protein concentration determined using Bradford reagent (Bio-Rad). Proteins were separated using SDS-PAGE and transferred to PVDF membranes (Millipore). The following antibodies were used: SNF5 (Bethyl; A301-087A), BIN1/Amphiphysin II (Santa Cruz Biotechnology; 2F11 clone, sc-23918), actin-HRP (Abcam; 20272-200), DsRed-monomer (Clontech; 632496), AcGFP (Clontech, 632375) and HRP conjugated secondary antibodies (Jackson ImmunoResearch).

CD8⁺ T-cell isolation and quantitative real-time PCR. Murine CD8⁺ T cells were isolated from *Snf5* wild-type spleens (n = 3) or from *Snf5*-deficient CD8⁺ lymphomas (n = 3) from the same line of 129-BL6 mice using the MACS CD8⁺ T-cell isolation kit (Miltenyi Biotec). Total RNA was extracted using Trizol reagent (Invitrogen) and reverse-transcribed by the

Reverse Transcription System (Promega). Quantitative real-time PCR was performed using the iQ SYBR Green Supermix and iCycler (Bio-Rad). For analysis, gene expression was normalized to *Rps8*. *mBin1* Fwd: TTC GGA CCT ATC TGG CTT CTG, Rev: CCT CCT GAA GAC ACT CAC TCA; *mSnf5* Fwd: ACA GTG GAG ATT GCC ATC CG, Rev: ACG CCT CAT TCG CCT TGT GT; *mRps8* Fwd: GCG AAA ACC CTA CCA CAA GA, Rev: GAA AAG TTC CCC ACA TCC AA.

Mouse embryonic fibroblast expression array. Primary WT and *Snf5*-conditional MEFs were cultured and harvested as described previously and infected two times with pBabe-puro^r-Cre retroviral supernatant at 4 h intervals (with 4 µg hexadimethrine bromide per mL retrovirus).^{22,29} Cells were stably selected in medium containing puromycin (2.5 µg/mL) 48 h after infection. Total RNA was isolated using Trizol reagent (Invitrogen) and applied to Affymetrix 430A 2.0 arrays. CEL files were pre-processed using ExpressionFileCreator and the robust multichip average algorithm on GenePattern and scatterplots generated using GraphPad Prism software.

SNF5 reintroduction: expression analysis and chromatin immunoprecipitation. BT16 cells were cultured in DMEM supplemented with 10% Fetalplex (Gemini Bio-Products) and 1% penicillin/streptomycin. RT cell lines were infected two times with either pBabe-puro^r or pBabe-puro^r-FLAG-hSNF5 (a kind gift of R. Kingston) retroviral supernatant at 3 h intervals with 4 µg hexadimethrine bromide per mL retrovirus. Cells were stably selected in medium containing puromycin (1.0–1.5 µg/mL) 48 h after infection. Western blots and quantitative real-time PCR were performed as described above. For analysis, gene expression was normalized to *GAPDH* (Fwd: AAT CCC ATC ACC ATC TTC, Rev: ATG AGT CCT TCC ACG ATA CC). The *BINI* primers were designed to recognize all *BINI* isoforms. *BINI* Fwd: GCA GTG CGT CCA GAA TTT CAA, *BINI* Rev: CCA ATC GGG CTC ATA CAC CT. *p16^{INK4A}* Fwd: GAA GGT CCC TCA GAC ATC CCC, *p16^{INK4A}* Rev: CCC TGT AGG ACC TTC GGT GAC.

Chromatin immunoprecipitation was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling) supplemented with Protein G Sepharose Fast Flow (GE Healthcare). Approximately 1.5 x 10⁷ cells were used per IP. Chromatin was

digested using 3.5 µL MNase per 4.5 x 10⁷ cells and sonicated 3x for 10 sec pulses with the Fisher Sonic Dismembrator Model 100 at power setting 3. BRG1 was immunoprecipitated with 10 µL α-SNF2β/BRG1 (Upstate/Millipore, 07–478, lot numbers DAM1647877, DAM1718075, R0706B0022). Quantitative real-time PCR was performed as described above. IGX1A negative control primers (SABiosciences) were used to demonstrate specific target binding enrichment. *BIN1* ChIP primers: Fwd-CTT CGC CGC ACT TTC TCT TTG AT, Rev-AGT AAT CTC CTG CAA CCC AGG AA.

***BIN1* reintroduction.** *BIN1* isoform 9 and 10 cDNA was cut from the EcoRI site of pcDNA3.1 (provided by G. Prendergast) and, respectively, cloned into the EcoRI site of pRetroQ-AcGFP-C1 and pRetroQ-DsRed-monomer-C1 (Clontech), which both contain a hybrid CMV/MSV promoter to drive gene expression. Retroviral supernatant was harvested after co-transfection of 293T cells with a pRetroQ-*BIN1* construct and the pCL-10A1 retroviral packaging vector using FuGeneHD transfection reagent (Roche). To reintroduce both *BIN1* isoforms, cells were first infected as above with pRetroQ-DsRed-*BIN1* isoform10, stably selected in medium containing puromycin (1.0–1.5 µg/mL) then infected with pRetroQ-AcGFP-*BIN1* isoform9. BT16 and U2OS cells were cultured as above. The top 50% of cells expressing both DsRed and AcGFP were sorted using the FACSaria II cell sorting system (BD Biosciences) and plated 2–5 x 10⁴ per well. Cells were counted every 3 d after sorting for 15 d total with the Countess Automated Cell Counter (Invitrogen).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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