Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene *Snf5*

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Summary

Recent data suggest the SWI/SNF chromatin remodeling complex may also act as a tumor suppressor. Utilizing a reversibly inactivating conditional allele, we demonstrate that loss of *Snf5/Ini1/Baf47/SmarcB1*, a core subunit of SWI/SNF, results in highly penetrant cancer predisposition with 100% of mice developing mature CD8$^+$ T cell lymphoma or rare rhabdoid tumors with a median onset of only 11 weeks. Notably, while loss of *Snf5* predisposes to aggressive cancers, it is also required for survival of virtually all nonmalignant cells in vivo. Reversible gene targeting demonstrates a critical and specific role for *Snf5* in tumor suppression, provides a novel system in which to explore the genetic pathways involved in tumor suppression by Swi/Snf, and should be of wide use in evaluating other essential tumor suppressor genes.

Introduction

Tightly compacted chromatin provides an organizational structure for DNA but constitutes a significant barrier to gene expression. Nucleosomal structures impede active transcription and several chromatin remodeling complexes serve to modulate expression of genes by regulating local chromatin structure. These remodeling complexes can be grouped into two broad classes—those that covalently modify histones with acetyl, methyl, or phosphoryl groups and those that utilize the energy of ATP hydrolysis to mobilize nucleosomes. SWI/SNF, a 2 MDa complex made up of 8 to 11 polypeptides, is the most intensively studied of the latter class. Though originally identified in yeast, the complex is present in all eukaryotes and highly evolutionarily conserved. In mammals several variants of SWI/SNF exist, each containing one of two mutually exclusive ATPase subunits, BRG1 or BRM. The complexes further differ in subunit composition but all contain the same active core that, in addition to SNF5, consists of BAF155, BAF170, and either BRG1 or BRM. (Phelan et al., 1999).

While it has been known for a decade that SWI/SNF regulates transcription through chromatin remodeling, it is only recently that several observations have indicated a potential link between SWI/SNF and tumor suppression. BRG1 and BRM bind to the tumor suppressor Rb and are required for Rb-mediated cell cycle arrest (Dunaief et al., 1994; Strobeck et al., 2000; Strober et al., 1996; Trouche et al., 1997; Zhang et al., 2000). Subsequently, association of SWI/SNF with BRCA1 in a biochemical complex was reported (Bochar et al., 2000). Specific, inactivating mutations in *SNF5* were identified in the majority of cases of malignant rhabdoid tumor, a rare but highly aggressive cancer of early childhood, and constituted the first genetic evidence linking SWI/SNF to tumor suppression (Biegel et al., 1999; Sevenet et al., 1999; Versteege et al., 1998). Inactivating mutations in *BRG1* have also been identified in several human tumor cell lines (Wong et al., 2000). Attempts to confirm the significance of these findings in murine models have met with variable success. Mice deficient in either *Brg1* or *Snf5* die early in embryonic development and thus only haploinsufficient mice can be assessed for cancer occurrence (Bultman et al., 2000; Guidi et al., 2001; Klochendler-Yeivin et al., 2000; Roberts et al., 2000). *Brg1* haploinsufficient mice display a mild tumor-prone phenotype with 3/20 mice developing glandular tumors, none of which exhibited loss of heterozygosity for *Brg1*. *Snf5* haploinsufficient mice are prone to developing tumors characterized by rhabdoid cells and bear remarkable resemblance to human malignant rhabdoid tumors. Like Brg1, Brm is present in only a subset of SWI/SNF complexes. *Brm*-deficient mice are viable and heavier than normal, but do not appear tumor prone (Reyes et al., 1998). Of Swi/Snf genes inactivated in mice to date, only Snf5 is a core member present in all Swi/Snf complexes. However, early embryonic lethality in *Snf5*-deficient animals precludes evalua-

SIGNIFICANCE

Chromatin remodeling complexes are essential for appropriate transcriptional regulation of large numbers of genes. Snf5 is a core subunit of the SWI/SNF chromatin remodeling complex and is one of only a few subunits present in all variants of the complex. Recent data suggest that SWI/SNF may serve an important tumor suppressor role for a variety of cancers. However, the data have often been indirect in nature and mouse knockout experiments have been hampered by lethality. While haploinsufficient models have provided support to this association, long latency and low penetrance have hampered further analysis. Utilizing a novel conditional targeting approach that should be useful in the characterization of other tumor suppressor genes, we demonstrate that controlled inactivation of *Snf5* in vivo results in extremely rapid development of aggressive cancers with high penetrance.

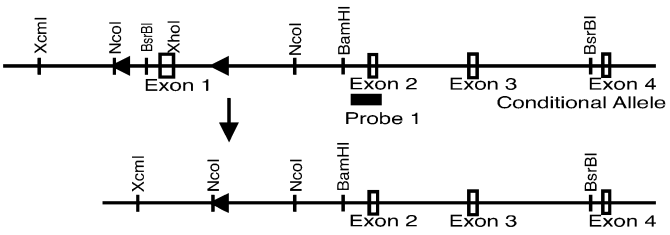

Figure 1. *Snf5* conditional targeting construct

The conditionally targeted allele with the neomycin resistance cassette removed is shown on top and the allele that results following Cre expression is shown below.

tion of tumor development in its absence. Conditional targeting utilizing the loxP-Cre system provides an alternate strategy as a target gene flanked by loxP sites may be inactivated in vivo in liveborn mice by expression of the bacteriophage Cre recombinase (Lewandoski, 2001; Sauer and Henderson, 1989).

In order to circumvent embryonic lethality and evaluate the effect of biallelic loss of *Snf5*, we generated strains of mice with two different conditional mutations at the *Snf5* locus—one a conditionally targeted *Snf5* allele and the other a reversibly inactivating allele. These mice were bred to transgenic strains that express Cre recombinase either in the very early embryo or following induction with interferon. Using this approach, we report a powerful system for evaluating conditional oncogenesis mediated by *Snf5* loss. This approach should also prove useful for evaluating other essential tumor suppressor genes.

Results

Inactivation of *Snf5* in conditional *Snf5*$^{floxed/-}$ mice leads to rapid demise

Previously we generated a targeted *Snf5* null allele (*Snf5*$^{-}$) (Roberts et al., 2000). *Snf5*$^{-/-}$ mice are early embryonic lethal. Haploinsufficient *Snf5*$^{+/-}$ mice are predisposed to rhabdoid tumors that bear a striking histologic resemblance to human malignant rhabdoid tumors. Further follow-up of a cohort of *Snf5*$^{+/-}$ mice has revealed an overall tumor incidence of 12%. In total, 28 mice have developed tumors with a median onset of just over one year. All but one of the tumors was of the rhabdoid type. While this establishes a firm link between *Snf5* and tumor suppression, the low penetrance and high median age have precluded the use of *Snf5*$^{+/-}$ mice as a practical tumor model with which to study the involvement of *Snf5* in critical cellular pathways.

To recapitulate the *Snf5* null mutation utilizing a conditional allele, we generated a targeting construct in which exon 1 is flanked by loxP sites (floxed) (Figure 1). Thus, following exposure to Cre recombinase, exon 1,which contains the initiator ATG, is deleted. Following appropriate targeting in ES cells, conditionally targeted *Snf5*^{floxed} mice were generated.

To confirm that inactivation of the *Snf5*^{floxed} allele recreates a *Snf5*$^{-}$ allele, we bred mice carrying the conditional allele to mice carrying both the *Snf5*$^{-}$ allele (*Snf5*$^{+/-}$) and the *Gata1-Cre* transgene (*Gata1-Cre*$^{+}$). *Gata1-Cre* is expressed very early in embryonic development and results in ubiquitous deletion of floxed target sequences (Mao et al., 1999). As anticipated, Cre-mediated deletion of the floxed allele by the *Gata1-Cre* transgene resulted in lethality of *Snf5*$^{floxed/-}$ embryos (data not shown).

We next considered the use of various lines of transgenic Cre-expressing mice that would be useful in the evaluation of *Snf5* function. We ultimately chose *Mx-Cre* mice for two reasons. First, expression of Cre from the Mx promoter is inducible and could therefore circumvent embryonic lethality caused by loss of *Snf5*. The Mx promoter is activated by injection of interferon or, alternatively, by administration of the interferon-inducer synthetic double-stranded RNA (polyI/polyC), (Kuhn et al., 1995). Second, following induction of *Mx-Cre* mice, Cre-mediated excision occurs to some degree within all tissues with the exception of brain. Widespread excision was essential because the cell of origin of malignant rhabdoid tumor is unknown. We therefore bred *Mx-Cre* mice onto the *Snf5*^{floxed} background.

Following polyI/polyC administration, high levels of Cre-mediated excision were achieved in bone marrow, liver, thymus, and spleen and moderate levels in all other tissues tested including muscle, heart, lung, GI tract, kidney, uterus, ovaries, and testes. The only tissue that lacked excision was brain. Whereas polyI/polyC treatment of 10 *Snf5*$^{floxed/+}$/*Mx-Cre* mice had no effect on survival, treatment of *Snf5*$^{floxed/-}$/*Mx-Cre* mice resulted in dramatic findings. Between 1 and 3 weeks after injection, 90% of *Snf5*$^{floxed/-}$/*Mx-Cre* mice died; the remainder died over the ensuing 4 weeks. This occurred whether mice were injected as neonates or adults. By 1–2 weeks following polyI/polyC administration, mice appeared ill and were profoundly pancytopenic. Mice often displayed extensive bruising of the skin and intestinal hemorrhage (Figures 2A and 2B). Histological analysis of bone marrow revealed an extreme deficiency of hematopoietic cells (Figure 2C). Presumably bone marrow aplasia led to low platelet counts, bleeding, anemia, oxygen deprivation, and death. Several mice developed only transient mild/moderate pancytopenia. Despite daily monitoring and a healthy appearance, these latter mice succumbed to sudden death, the etiology of which is unclear. Of note, a single mouse developed a lymphoma 12 days after injection. These findings demonstrate that continued Snf5 is required for survival of both neonatal and mature mice and is necessary for maintenance of hematopoietic cells within the bone marrow. Rapid demise due to widespread inactivation of *Snf5* in *Snf5*$^{floxed/-}$ mice, however, precludes their use as a tumor model.

Generation of a reversible, inverting conditional *Snf5* allele (*Snf5*^{inv})

We reasoned that decreasing the penetrance of Snf5 loss in bone marrow and other tissues might circumvent lethality because unaffected progenitor cells would proliferate and compensate for the death of affected cells. Moreover, even partial penetrance would still be expected to generate many *Snf5* null cells, which might be highly tumor predisposed. To establish such an animal model, we modified the *Snf5* locus so that it could be reversibly inactivated via Cre-mediated gene inversion, an approach first used by Lam and Rajewsky in the context of B-lymphoid cell function (Lam and Rajewsky, 1998)—here, we apply this approach to cancer modeling.

In construction of traditional conditional alleles with the loxP-Cre recombination system, 34 base pair loxP sites are placed in the same directional orientation flanking a target DNA region. Upon expression of Cre recombinase, crossing-over results in the excision of DNA between the loxP sites (Figure 3A). In con-

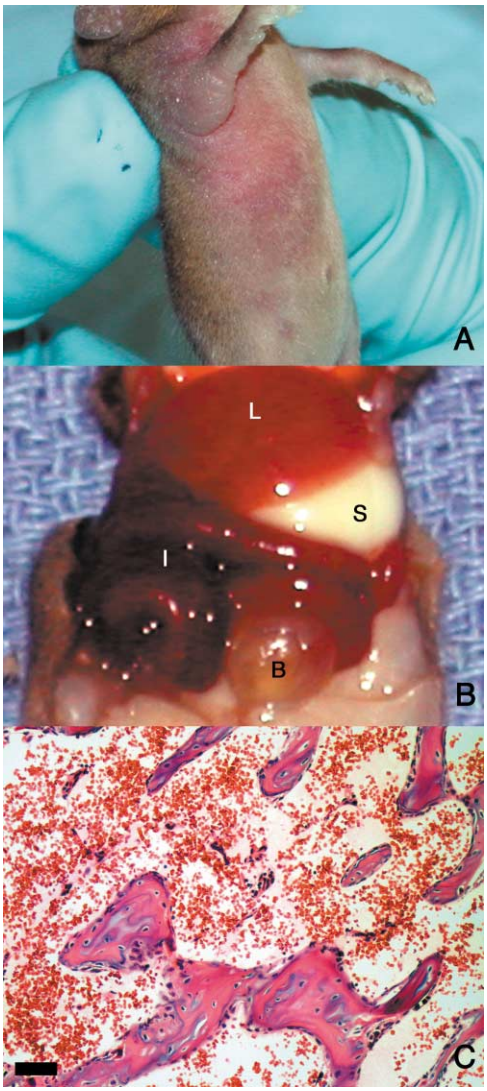

Figure 2. Inactivation of *Snf5* with *Mx-Cre* leads to hemorrhage and pancytopenia

A: Subcutaneous hemorrhage is present over the right thorax and abdomen of a Mx^+ $Snf5^{Cond/-}$ pup that was injected with polyI/polyC one week earlier.
B: Abdominal organs of a Mx^+ $Snf5^{Cond/-}$ mouse injected with polyI/polyC eight days earlier demonstrating extensive intestinal hemorrhage. The color of the intestine (I) is normally close to that of the stomach (S) or bladder (B) but in this case is dark red due to bleeding. Liver (L).
C: A section through the femur of an adult Mx^+ $Snf5^{Cond/-}$ mouse that was injected with polyI/polyC 16 days earlier. The bone marrow contains only mature red blood cells and there is virtually no hematopoietic activity. Reference bar = 50 μm.

trast, Cre-mediated inversion takes place when the flanking loxP sites are placed in opposing orientation (Figure 3B). This is a reversible event since both loxP sites are recreated on the same strand of DNA following inversion. Previously, Lam and Rajewsky (Lam and Rajewsky, 1998) demonstrated that Cre-mediated inversion occurs with high efficiency. We reasoned that following transient expression of the Cre protein within cells a steady state would be rapidly reached between alleles that were in the germline orientation and those in the inverted orientation. Thus, in any given tissue, even if 100% of cells expressed Cre following administration of polyI/polyC, only 50% of the alleles would end up in the inverted orientation if no biological selection were imposed.

We generated the reversible inverting conditional allele of *Snf5*, designated $Snf5^{inv}$ with the targeting vector shown (Figure 3C). We predicted that inversion of exon 1 would inactivate the gene given that mutations within exon 1 predispose to cancer in humans and because no alternative initiation sites are present downstream of the initiator ATG. $Snf5^{inv/inv}$ mice appear normal in the absence of Cre expression (data not shown).

To validate the properties of the $Snf5^{inv}$ allele, we first examined the consequences of expressing Cre recombinase within the germline (or very early embryo) of $Snf5^{inv/-}$ mice through use of the *Gata1-Cre* transgene. As was the case with $Snf5^{floxed/-}$/*Gata1-Cre* mice, $Snf5^{inv/-}$/*Gata1-Cre* mice were embryonic lethal suggesting that loss of *Snf5* in a substantial proportion (or 50%) of cells during embryonic development is incompatible with survival. To test the $Snf5^{inv}$ allele further, we then analyzed polyI/polyC-treated $Snf5^{inv/+}$ mice carrying the *Mx-Cre* transgene. We predicted that there would be no selective disadvantage to inversion of Snf5 in this genetic background. Administration of polyI/polyC was tolerated without any adverse effects. DNA analysis validated the properties of the $Snf5^{inv}$ allele. In organs where induction of the Mx promoter is highly efficient such as bone marrow, ~50% of DNA derived from the $Snf5^{inv}$ allele was in the germline (active) orientation and ~50% in the inverted (inactive) orientation (Figure 3D). In those tissues in which the *Mx-Cre* transgene is less well expressed, a smaller fraction of the $Snf5^{inv}$ derived DNA was in the inverted orientation. Recombination was detected within all tissues with the exception of brain where the *Mx-Cre* transgene is not expressed.

Highly penetrant, rapid tumor formation following Cre-mediated inversion in $Snf5^{inv/-}$ mice

The effect of polyI/polyC-activated Cre expression on the short-term survival of $Snf5^{inv/-}$/Mx-Cre mice was strikingly different from that observed with $Snf5^{floxed/-}$/*Mx-Cre* mice. Rather than succumbing within the three weeks following polyI/polyC administration, $Snf5^{inv/-}$/*Mx-Cre* mice initially appeared normal. However, beginning at 4 weeks following injection, $Snf5^{inv/-}$/*Mx-Cre* mice developed cancer. The susceptibility to tumor formation was dramatic: 100% of the mice developed tumors with a median onset of only 11 weeks post-polyI/polyC (Figure 4). Mice displayed an enlarged lymph node or dramatically enlarged spleens and livers (Figures 5A–5C). This was often accompanied by a mild progressive decrease in blood counts, an increase in circulating eosinophils, and occasionally by circulating tumor cells (Figure 5D). Histologic analysis revealed effacement of normal architecture in involved lymph nodes and spleen (Figure 5E) and peri-portal involvement frequently in liver (Figure 5F). Bone marrow was typically devoid of malignant cells although it often contained moderately increased numbers of eosinophils. At high-magnification the tumors revealed a pleomorphic mixture of small to intermediate sized cells including a predominant population of intermediate sized cells with irregular to cleaved nuclei, vesicular chromatin, and variably distinct nucleoli (Figures 5G and 5H). Mitotic figures were frequent. The histologic appearance of the tumor cells was highly suggestive of malignant lymphocytes.

Tumors were characterized further by immunohistochemistry. Findings were consistent in eight tumors that were analyzed

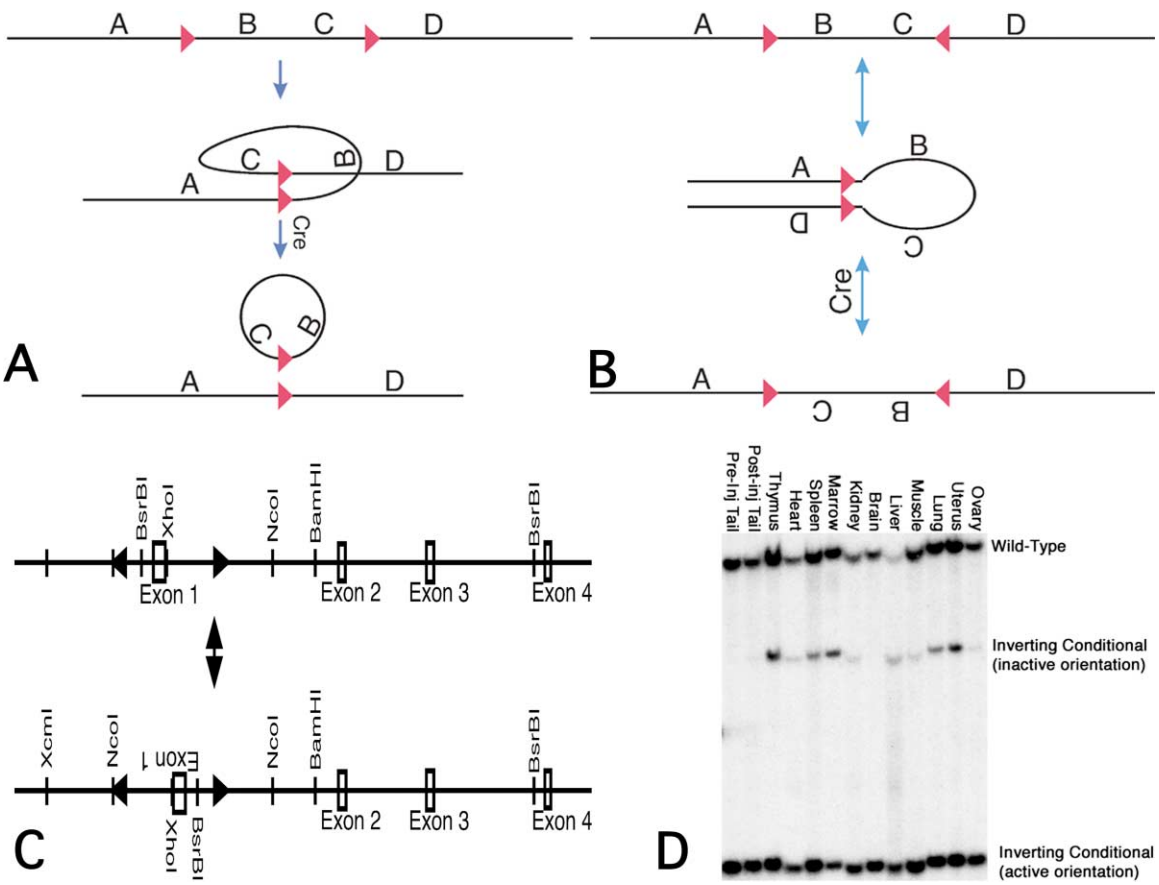

Figure 3. An inverting conditional allele allows for reversible inactivation and results in partial penetrance of *Snf5* loss

A: Diagram of a conditionally targeted allele containing loxP sites in identical orientations. Following expression of Cre, DNA between the LoxP sites is removed and the loxP sites are recreated on separate DNA molecules making the reaction essentially irreversible.

B: Diagram demonstrating the effect of placing loxP sites in opposing orientations. Following expression of Cre, DNA between the loxP sites is inverted. Since both loxP sites remain on the same DNA strand, the reaction is entirely reversible.

C: Inverting conditional *Snf5* construct. The construct was made by reversing the orientation of the floxed neomycin resistance cassette within intron 1 and then transfecting ES clones to remove the neomycin resistance cassette.

D: Southern blot of DNA from the organs of a Mx^{+} $Snf5^{Inv/+}$ mouse one month after injection with polyI/polyC. Lane 1 contains tail DNA obtained from the mouse prior to injection with polyI/polyC and demonstrates presence of the wild-type and inverting conditional allele in the active orientation only. Lane 2 contains DNA from the tail of the mouse obtained following injection with polyI/polyC. The Mx promoter is weakly expressed in tissues of the tail but there is now a faint band corresponding to the inverting allele in the inactive orientation while the majority of the inverting allele remains in the active orientation. In contrast, *Mx-Cre* is highly expressed in thymus, bone marrow, spleen, and liver, and close to 50% of the inverting allele is located in the inactive orientation within these organs—demonstrating that despite close to 100% expression of Cre, only 50% of the inverting allele is in the inactive orientation.

(Figure 6). All tumors were positive for the T cell markers Thy1.2, CD3, and CD8 and negative for CD4, immature T cell (TdT), B cell (B220), and myeloid (Mac-1 and Gr-1) markers. Tumors were highly vascular as assessed by CD31 (PECAM) staining. These findings define the tumors as mature $CD8^{+}$ T cell lymphomas (Morse et al., 2002), a tumor type not seen in *Snf5* haploinsufficient mice.

To examine potential clonality of these mature T cell lymphomas, we performed Southern analysis of the β T cell receptor locus. As shown in Figure 7A, we observed monoclonal rearrangements in each tumor with one tumor containing two discrete rearranged bands. In total, 28 $Snf5^{inv/-}$ mice carrying *Mx-Cre* have been treated with polyI/polyC. Twenty-three developed cancer within 24 weeks following injection and another five were found dead with advanced autolysis precluding evaluation of the cause of death. Of the 23 evaluable mice, 20 developed lymphomas and 3 developed rhabdoid tumors.

Inactivation of *Snf5* in T cell lymphomas of polyI/polyC-treated $Snf5^{inv/-}$ mice

The orientation of the $Snf5^{inv}$ allele was determined in various tissues and tumors in multiple $Snf5^{inv/-}$/*Mx-Cre* mice by Southern blot analysis (Figure 7B). In all tissues other than tumor, virtually all $Snf5^{inv}$-derived DNA was in the normal (active) orientation. In contrast, in lymphoma DNA the majority of $Snf5^{inv}$-derived DNA was in the inverted (inactive) orientation. Since the inverting event is detectable in most tissues only when the $Snf5^{inv}$ allele is paired with a wild-type allele but not when it is paired with a knockout allele (compare Figure 3D to Figure 7B), we infer that loss of *Snf5* is lethal to virtually all cells and yet also predisposes to malignant transformation. The small amount of $Snf5^{inv}$ allele DNA in the functional orientation within the tumor very likely reflects the presence of nonmalignant cells, such as endothelial and inflammatory cells, which are abundant in these tumors.

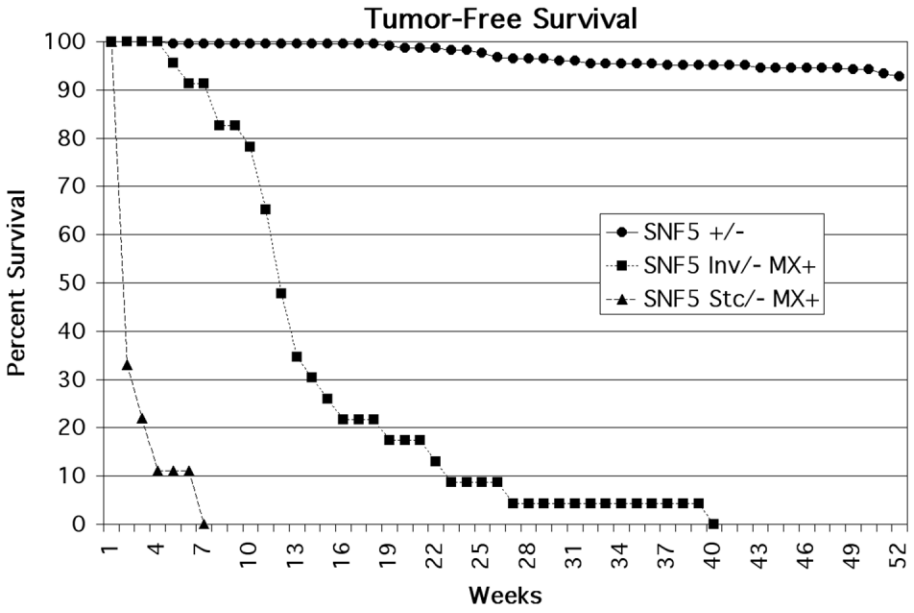

Figure 4. Survival of *Snf5* targeted mice

Snf5$^{+/-}$ mice are predisposed to develop rhabdoid tumors. Overall, there is a 12%–15% penetrance with a median onset of 12 months. Mx^{+} *Snf5*$^{Cond/-}$ mice (designated Stc/−) die rapidly of bone marrow and organ failure following injection with polyI/polyC. 90% of the mice die within 3 weeks and the remainder over the next four weeks. Mx^{+} *Snf5*$^{Inv/-}$ mice succumb to cancer with a penetrance of 100% and median latency of 11 weeks after injection with polyI/polyC.

Effects of *Snf5* loss upon lymphoid subpopulations

The finding of exclusively CD8^{+} mature lymphomas in *Snf5*$^{inv/-}$/*Mx-Cre* mice is particularly interesting since mice normally have a 4:1 predominance of CD4^{+}:CD8^{+} mature T cells. It was recently reported that two other subunits of Swi/Snf, *Brg1* and *Baf57*, are involved in suppression of CD4 expression and activation of CD8 expression during thymocyte development (Chi et al., 2002). We thus sought to evaluate whether *Snf5* loss had differential effects upon lymphocyte subpopulations in spleen and thymus. Splenocytes and thymocytes were evaluated at 12, 18, 24, 36, 48, 96, and 216 hr following induction with polyI/polyC. There was a progressive loss of hematopoietic cells within both tissues as well as in bone marrow. Lymphocyte subsets within the spleen were lost at equal rates following inactivation of *Snf5*. There was no difference in the CD4^{+}:CD8^{+} ratio or in the T cell:B cell ratio in spleen following induction (Figures 8A and 8B). Further, we examined V_{β} rearrangements in splenic T cells and found no change in the relative prevalence of individual V_{β} segments following induction (not shown). Similarly, inactivation of *Snf5* led to an equal loss of thymocyte subsets. No significant differences were detected in CD4^{+}CD8^{-}, CD4^{-}CD8^{+}, or CD4^{+}CD8^{+} thymocyte subsets either in total thymus or in the TCR lo$^{/-}$ fraction (not shown). These data suggest that the development of exclusively CD8^{+} lymphomas is not due to differential lethality within lymphocyte subsets following *Snf5* inactivation.

Cell death following loss of *Snf5*

Since inactivation of *Snf5* led to the loss of large numbers of cells in addition to oncogenic transformation, we sought to determine whether apoptosis occurred following induction. *Snf5*$^{floxed/-}$/*Mx-Cre* mice were induced with polyI/polyC and tissues harvested at 12, 18, 24, 36, 48, 96, and 216 hr post-induction. We focused our analysis upon liver and spleen because these organs are the most common sites of lymphoma occurrence and have high levels of Cre activity. No increase in apoptosis was evident at 12 hr post-induction. However, at 18 to 24 hr following a single dose of polyI/polyC there was increased apoptosis in *Snf5*$^{floxed/-}$/*Mx-Cre* mice when compared either to uninduced mice of the same genotype or to induced *Snf5*$^{floxed/+}$/*Mx-Cre* control mice at the same time points. The increased apoptosis was readily detected by flow cytometric analysis of anexin-V staining of splenocytes (Figures 8C and 8D), by TUNEL staining in liver and spleen (Figures 8E–8H) and by histologcial analysis (Figures 8I and 8J).

Discussion

Highly penetrant, short latency tumor formation upon inactivation of *Snf5*

The data presented here demonstrate that Snf5, a core member of the SWI/SNF chromatin remodeling complex, is vital for tumor suppression in vivo and is also required for the survival of most normal cells. The rapidity with which tumors develop following inducible inactivation of *Snf5* is unprecedented. 100% percent of mice develop tumors with a median latency of 11 weeks. In comparison, the time to tumor development with other tumor suppressors is significantly longer. p53-deficient mice develop tumors at a median age of 20 weeks (not including the 3 weeks of embryonic development without p53), p19/Arf knockout mice at 38 weeks, p16/Ink4a^{-} mice at 60 weeks, and loss of p21/Cip1/Waf1 does not lead to a detectable increase in tumor formation in mice (Brugarolas et al., 1995; Donehower et al., 1992; Serrano et al., 1996; Sharpless et al., 2001). Inactivation of the tumor suppressors *Rb*, *Apc*, *Pten*, *Vhl*, or *Nf1* each leads to embryonic lethality (Bollag et al., 1996; Di Cristofano et al., 2001; Gnarra et al., 1997; Largaespada et al., 1996; Lee et al., 1992; Su et al., 1992). Haploinsufficiency for *Rb* leads to tumor formation with a median latency of 45 weeks while combined *p53* deficiency and *Rb* haploinsufficiency yields tumors at 17 weeks (Williams et al., 1994). Thus, the aggressive cancer-prone phenotype that occurs following reversible inactivation of the *Snf5* gene is striking when compared to other tumor suppressor

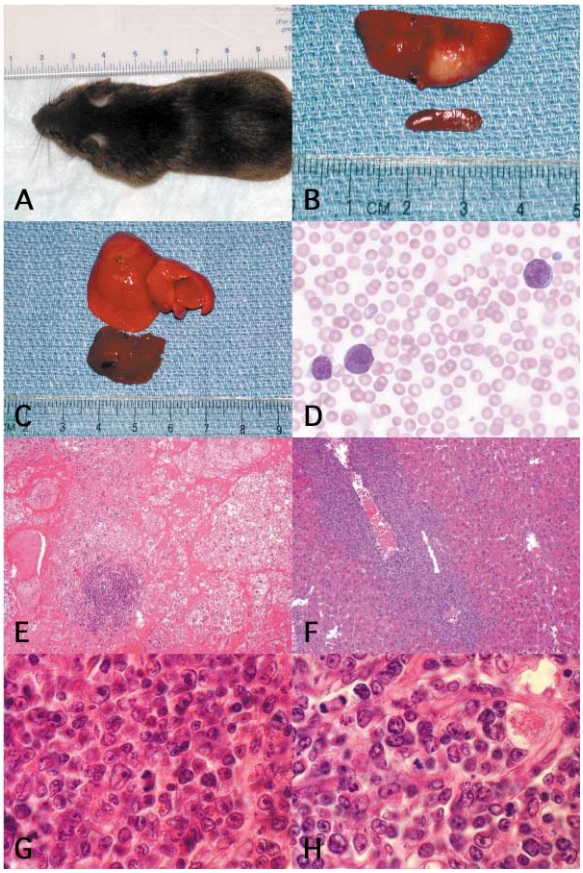

Figure 5. Lymphomas in Mx^{+} $Snf5^{Inv/-}$ mice

A: Mouse demonstrating markedly enlarged lymph node secondary to lymphoma.
B: Spleen from mouse with lymphoma (top) compared to spleen from normal littermate (bottom).
C: Liver from mouse with lymphoma (top) compared to litter from normal littermate (bottom).
D: Peripheral blood smear demonstrating the presence of three malignant white blood cells. Reference bar = 10 μm.
E: Section from involved spleen demonstrating widespread necrosis and an island of malignant lymphocytes. Reference bars = 100 μm.
F: Section through involved liver demonstrating peri-portal infiltration of lymphoma. Reference bars = 100 μm.
G and H: Sections through two different lymphomas demonstrating uniform histologic appearance of the lymphomas. The lymphomas consist of a predominant population of intermediate sized cells with irregular nuclei, vesicular chromatin, and variably distinct nucleoli. Mitotic figures are frequent. Reference bar = 10 μm.

knockouts and indicates a critical role for *Snf5* in preventing cancer.

The limited spectrum of tumors arising after *Snf5* inactivation is particularly interesting. While disruption of tumor suppressors such as *p53*, *p19/Arf*, and *Pten* predisposes to a wide variety of cancers, inactivation of others leads to specific phenotypes. For example, haploinsufficiency for *Rb*, a gene known to be critical in tumor suppression in humans, yields exclusively pituitary adenocarcinomas in mice, an otherwise rare tumor (Hu et al., 1994). Similarly, inactivation of *Snf5* in our system gives rise to two specific types of tumors. The basis for this specificity remains unclear but several possibilities exist. First, since the lymphomas occur early, have a high mitotic rate, and are rapidly progressive, animals may die of lymphoma prior to the appearance of more slowly growing solid tumors. For instance, this may explain why the overall incidence of rhabdoid tumors in the inverting system is the same as in the haploinsufficient mice (10%–15%) despite a significantly earlier onset of 4.6 months in the inverting mice instead of just over 12 months in the haploinsufficient mice. Second, the high proliferative rate of hematopoietic cells combined with the high activity of the *Mx-Cre* transgene in this tissue may select for the occurrence of lymphomas although this would not explain the specific predilection for mature $CD8^{+}$ T cell lymphomas per se. Third, recent data demonstrate roles for the Swi/Snf subunits *Baf57* and *Brg1* in activation of CD4 expression and repression of CD8 expression (Chi et al., 2002). If *Snf5* also plays a role in controlling the development of T cells then loss of *Snf5* may impair T cell development and predispose to malignant transformation of maturing $CD8^{+}$ T cells. However, using flow cytometric analysis we were unable to detect differential effects upon T cell subsets following inactivation of *Snf5*. It must be noted, though, that induction of *Mx-Cre* relies upon increased levels of interferon, and it is thus possible that the presence of interferon obscurred subtle thymic developmental effects following *Snf5* inactivation.

Tumor suppression by other Swi/Snf subunits

It is not yet clear whether tumor suppression is specific to Snf5 or is a more general property of the Swi/Snf complex. Several lines of evidence suggest that the tumor suppressor activity of Snf5 is related to the role of the Swi/Snf complex in chromatin remodeling. First, other chromatin remodeling complexes, particularly histone acetylase (HAT) and deacetylase (HDAC) factors, are involved in recurrent chromosomal translocations and have been implicated in oncogenesis. Second, Brg1 and Brm, mutually exclusive catalytic ATPase-containing subunits of Swi/Snf, each bind to Rb and are required for Rb-mediated cell cycle arrest. (Dunaief et al., 1994; Trouche et al., 1997). Third, the Swi/Snf complex is also part of the Brca1 biochemical complex and Brg1 directly binds to Brca1 (Bochar et al., 2000). Fourth, mutations in *BRG1* have been detected in several human tumor cell lines and, while inactivation of *Brg1* results in embryonic lethality in mice, 3 of 30 *Brg1* haploinsufficient mice developed glandular tumors (Bultman et al., 2000). Most recently, *HLTF*, a member of the Swi/Snf family, was demonstrated to be specifically silenced in human colon cancers (Moinova et al., 2002) and *BAF180* was found to have specific nonsense mutations in two of 40 primary breast cancers (Ramon Parsons, personal communication). Thus, there is accumulating evidence that the entire Swi/Snf complex is involved in growth regulation and may have a widespread role in tumor suppression. Since Snf5 is an invariant subunit in all Swi/Snf complexes, further elucidation of its function and interacting pathways is likely to generate insight into this newly appreciated mechanism of tumor suppression.

While the above data support a generalized role for the Swi/Snf complex in tumor suppression, the fact that distinct tumor-prone phenotypes result following inactivation of different Swi/Snf subunits suggests that the complex does not solely act in a single pathway. Rather, we speculate Swi/Snf may be involved in diverse growth control pathways and inactivation of individual subunits leads to altered expression of only subsets of Swi/Snf-regulated genes. Thus, loss of *Brg1* promotes glandular tumors while inactivation of *Snf5* leads to rhabdoid tumors and

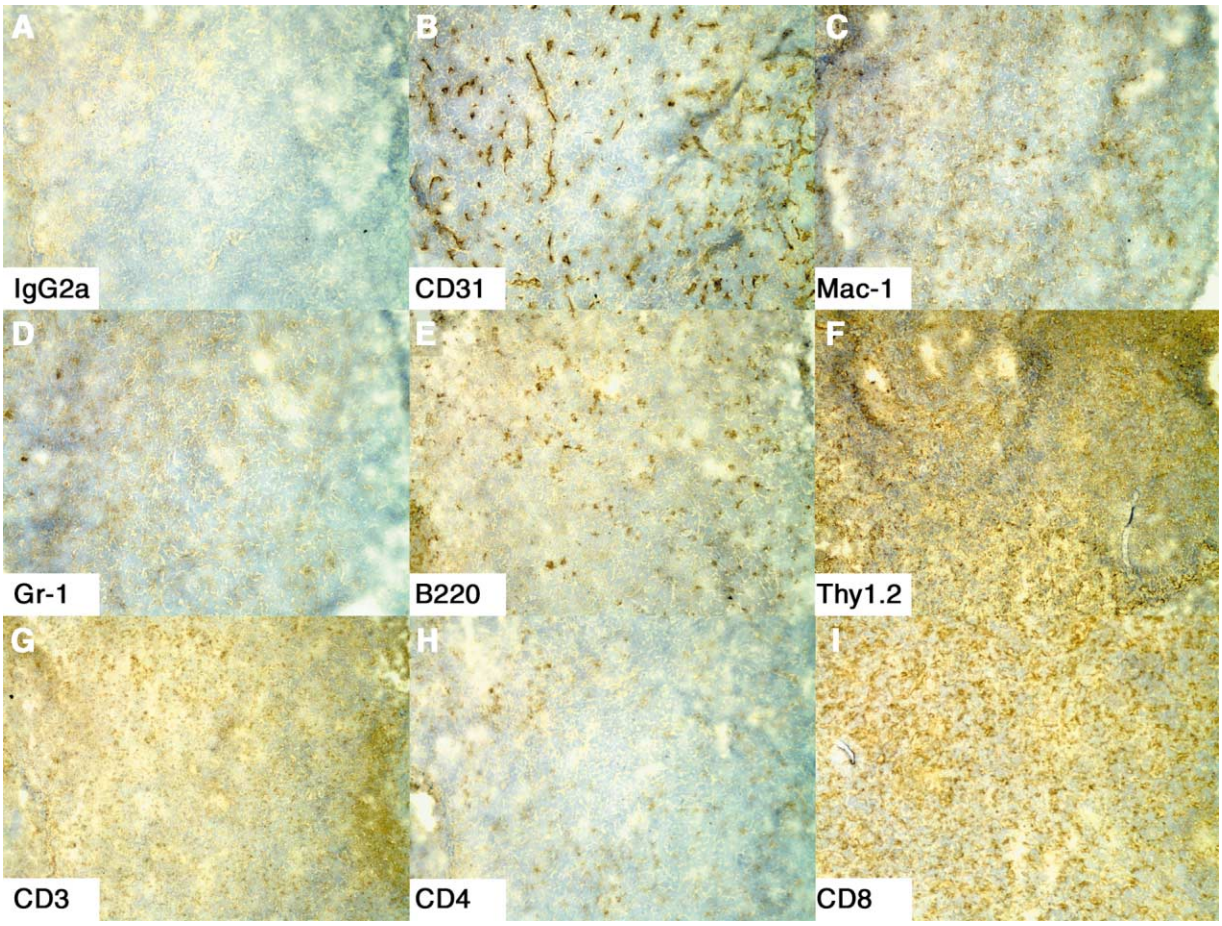

Figure 6. Loss of *Snf5* gives rise to mature CD8$^+$ T cell lymphomas

Immunohistochemical staining was performed on eight lymphomas from Mx$^+$ *Snf5*$^{inv/-}$ mice that had been injected with polyI/polyC. Representative sections are shown.
A: IgG2a negative control.
B: CD31 (PECAM) reveals a high degree of vascularity.
C: Mac-1 stains only scattered macrophage/monocyte cells within the tumor mass.
D: Gr-1 stains only occasional neutrophils within the tumor.
E: B220 identifies a few B cells within the tumor.
F: The vast majority of cells are positive for this T cell marker.
G: The vast majority of cells are also positive for this T cell marker, although it is slightly less intense than thy1.2.
H: All tumors were negative for CD4 with the exception of a very few cells.
I: All tumors were positive in the vast majority of cells for this marker.

lymphomas. Given the high penetrance and rapid lethality caused by lymphomas following conditional inactivation of *Snf5*, it will be interesting to determine whether other, slower growing, tumors occur in the absence of *Snf5* but never become manifest due to early death of lymphoma-laden mice. Ultimately, identification of interacting pathways and downstream targets of *Snf5* as well as evaluation of cancer-prone phenotypes in mice deficient for other Swi/Snf subunits will be required in order to identify specific roles for Snf5 and Swi/Snf in cancer.

Snf5 is required in nonmalignant cells

It is interesting that loss of *Snf5* expression ultimately results in the death of cells and yet also predisposes to malignant transformation. This apparent paradox is not without precedent in oncogenesis as illustrated by findings with c-myc. Overexpression of c-myc causes most primary cells to undergo apoptotic death. However, rare cells that harbor an additional predisposing mutation such as in *ras*, *bcl2*, or *bax* undergo increased proliferation/survival and are predisposed to malignancy (Hoffman et al., 2002). Thus, loss of *Snf5* may result in establishment of an epigenetic state that leads to cell death except in those rare cells that contain additional mutations and progress to cancer.

Snf5 and human cancer

The types of tumors that develop in the *Snf5*$^{inv/-}$ mice carrying the *Mx-Cre* transgene closely resemble specific human tumors. The histologic appearance of the rhabdoid tumors is essentially indistinguishable from human malignant rhabdoid tumors that carry biallelic inactivating mutations in *hSNF5*. The clinical presentation and histologic appearance of mice with mature T cell lymphoma/leukemia is highly reminiscent of human mature T

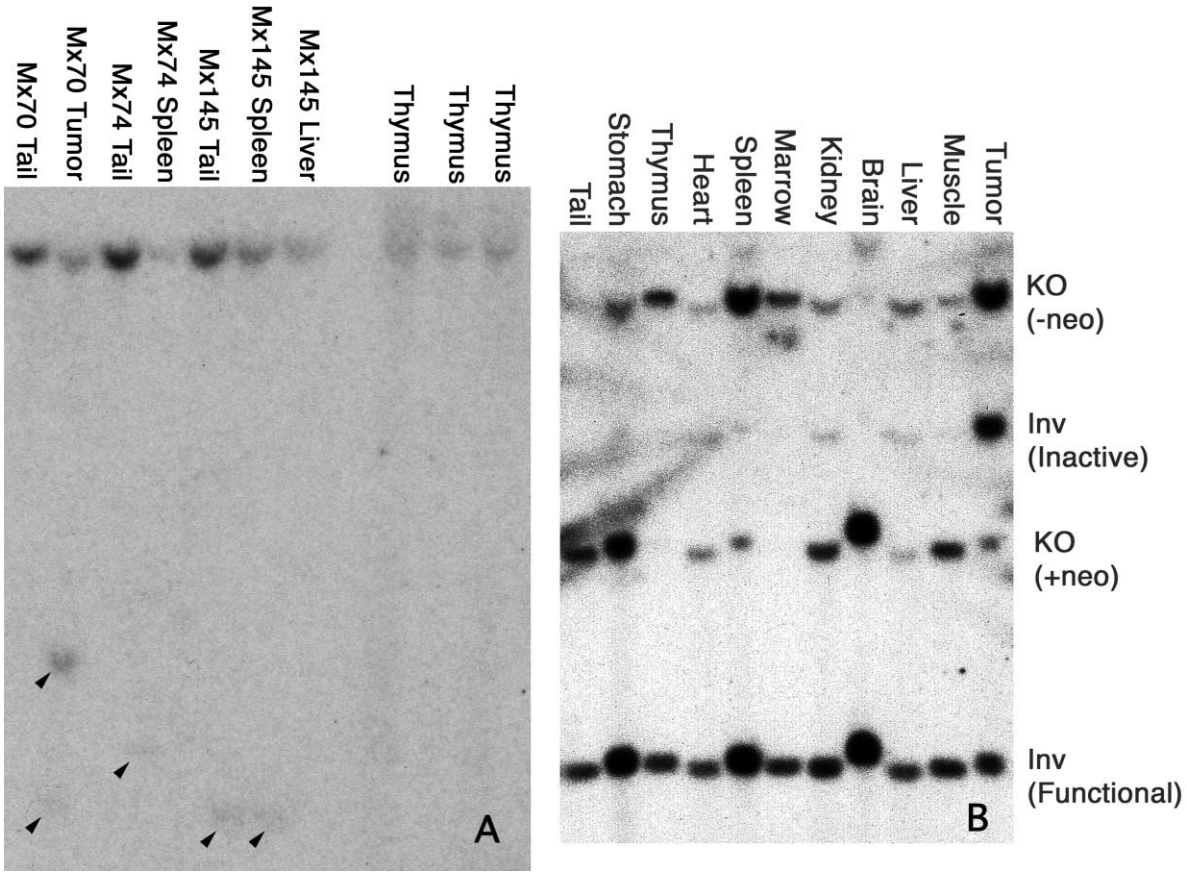

Figure 7. Loss of *Snf5* leads to monoclonal lymphomas and loss of nonmalignant cells

A: Southern blot of DNA obtained from tail and tumor samples of mice with lymphoma. The blot was hybridized with a probe to the V_β region of the T cell receptor. The three thymus lanes demonstrate the germline band at the top and faint polyclonal smears throughout the lane. The tail samples contain only the germline band and each has a distinct clonal band within the tumor sample. In the case of mice Mx74 and Mx145, tumor samples consisted of pieces of involved spleen and liver. **B:** Southern blot of DNA isolated from the organs and lymphoma of a Mx^+ $Snf5^{Inv/-}$ mouse that had been injected with polyI/polyC. The tail DNA was harvested prior to injection and demonstrates the unrearranged bands. Of note, in the knockout allele (−), exon 1 has been replaced with a neomycin resistance cassette surrounded by loxP sites. Thus, in the presence of Cre, the neomycin cassette is removed leading to a change in size on the Southern blot while the allele remains inactive due to continued absence of exon 1. Comparison of the KO(−neo) band to the KO(+neo) band demonstrates that there has been virtually 100% excision within thymus and bone marrow, over 90% excision in spleen, liver, and tumor samples, and 20%–40% excision in the remaining organs with the exception of brain where there is no excision. In contrast, despite high levels of Cre expression, the vast majority of the inverting allele remains in the active orientation with the exception of the tumor sample where the majority is in the inactive orientation.

cell leukemia/lymphoma in which patients typically present with isolated adenopathy or with markedly enlarged spleen and liver. Elevated eosinophil counts and infiltration of tumor masses with nonmalignant inflammatory cells are common in such patients. Mature T cell lymphoma/leukemia is among the most aggressive of human lymphoid neoplasms and is typically refractory to treatment with chemotherapy. Notably, the high frequency of mature T cell leukemia/lymphoma in the $Snf5^{Inv/-}$ mice suggests a potential link to human cancer where 45% of cases of mature T cell prolymphocytic leukemia have loss of genetic material at chromosome 22q11, the location of *SNF5* (Soulier et al., 2001). We are currently investigating the status of *Snf5* within these human tumors.

Advantages of reversible inverting conditional alleles for tumor modeling in mice

Given that many tumor suppressor genes are essential for survival of individual cells or the whole organism, conditional targeting strategies are required in order to evaluate the normal function of these genes and generate useful cancer-prone animal models. Conditional targeting typically makes use of site-specific recombinases, either the Cre-loxP system derived from bacteriophage P1 or the Flp-Frt system derived from *Sacchararomyces cerevisiae* (Lewandoski, 2001). In combination with transgenes that drive tissue-specific Cre/Flp expression, inactivation of a gene of interest can be directed to a specific tissue. However, many tumor suppressor genes are essential for survival, and deletion of the gene within specific tissues/organs may lead to death of the mouse via loss of organ function. The strategy we have used serves to counter this problem and provides several advantages. First, we have utilized an inducible system so that embryonic lethality due to loss of essential gene functions is minimized. Second, the approach leads to reproducible mosaic partial penetrance within multiple tissues. Consequently, significant numbers of cells lose expression of the

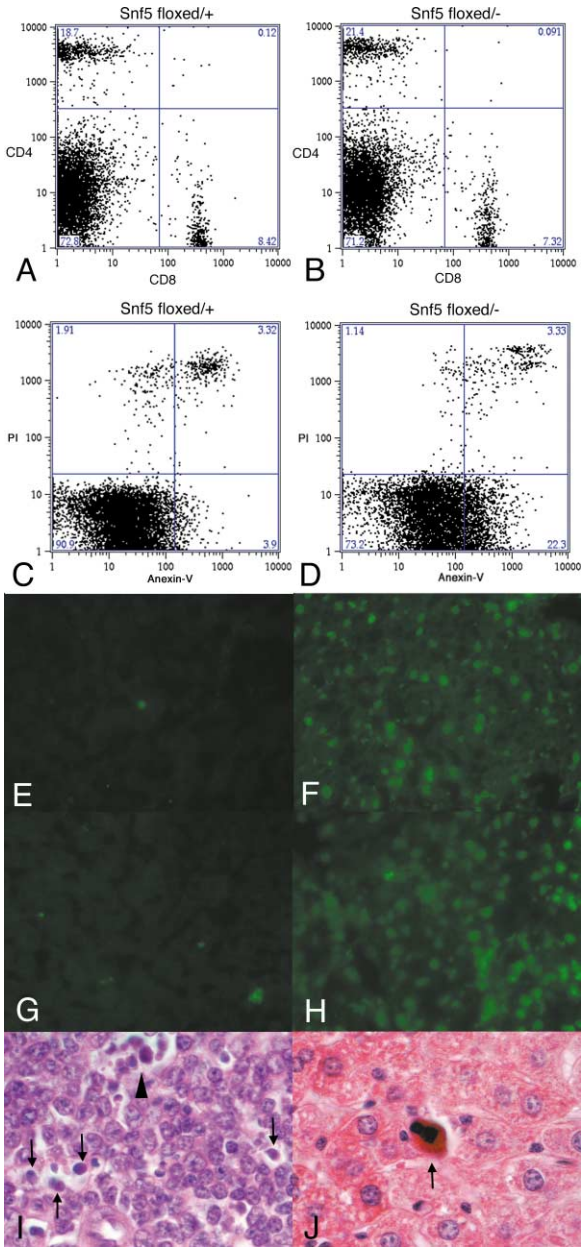

Figure 8. Lymphocyte development and apoptosis following inactivation of *Snf5*

A and B: Flow cytometry of CD4 and CD8 staining of splenocytes from *Snf5*$^{floxed/+}$/*Mx-Cre* control mice (**A**) and *Snf5*$^{floxed/-}$/*Mx-Cre* mice (**B**). The samples shown are from 216 hr post-induction. No differences were apparent at any of the times examined.

C and D: Two-color staining of nonpermeabilized splenocytes with Anexin-V and propidium iodide (PI) from *Snf5*$^{floxed/+}$/*Mx-Cre* control mice (**C**) and *Snf5*$^{floxed/-}$/*Mx-Cre* mice (**D**) are shown at 24 hr post-induction. Viable cells are Anexin-V negative and PI negative (lower left quadrant), necrotic cells are positive for both markers (upper right quadrant) and apoptotic cells are positive for Anexin-V but negative for PI (lower right quadrant). Note the increase in apoptotic cells in the *Snf5*$^{floxed/-}$/*Mx-Cre* mice compared to the control mice.

E: Negative control TUNEL reaction performed without TdT enzyme on liver from a *Snf5*$^{floxed/-}$/*Mx-Cre* mouse at 18 hr after induction.

F: Positive control TUNEL reaction from the same mouse. The liver section was treated with DnaseI prior to performing the TUNEL reaction in order to generate DNA strand breaks.

G: TUNEL reaction performed on a liver section from a *Snf5*$^{floxed/+}$/*Mx-Cre* control mouse 18 hr after induction with polyI/polyC.

targeted gene and become predisposed to malignant transformation, while at least half of cells retain expression and prevent death of the animal due to organ failure.

The system we have developed should also prove ideal for identifying interacting genetic pathways participating in tumor suppression. The tumors that result from inactivation of *Snf5* are highly penetrant and develop over a narrow time course. Thus, examining the effects of other genetic mutations on tumor formation in *Snf5*^{inv} mice provides a robust system in which to pursue how *Snf5* loss contributes to oncogenesis, either through established tumor suppressor pathways or through novel mechanisms.

Experimental Procedures

Generation of *Snf5* conditionally targeted mice

An 8.8 kb region of homology beginning 4.1 kb 5′ of exon 1 and continuing to the BamHI site in intron 1 was used to generate the targeting constructs. A neomycin resistance cassette flanked by loxP sites was blunt-end ligated into the XcmI site 1.4 kb 3′ of exon 1 and this was transformed into *E. coli*. A clone with the loxP sites pointed in the 3′ direction was selected. Polylinker and loxP sites were inserted at the EcoRv site 0.8 kb upstream of exon 1 such that the loxP site pointed 3′. An HSV-TK cassette was inserted at the BamHI site at the 3′ end of the homology region. This construct was electroporated into CJ7 ES cells (strain 129) and clones were selected in G418 and gancyclovir. Six of 114 clones had undergone homologous recombination. Three of these six clones had incorporated the 5′ loxP site and two of these had good karyotypes. In order to remove the neomycin resistance cassette and leave only the loxP sites behind, one of these clones (#108) was electroporated with the pMC-Cre expression plasmid. Fourteen of 96 clones were now G418 sensitive and 4 of these 14 clones retained exon 1. Two of these clones (#7 and #8) had good karyotypes and were injected into blastocysts. Both gave rise to high percentage chimeric mice and both led to germline transmission.

Generation of *Snf5* inverting conditional mice

The inverting construct was generated identically to the conditional construct described above with the exception that a bacterial clone containing the loxP sites of the neomycin cassette pointing in the 5′ direction was used. This construct was electroporated into CJ7 ES cells and clones were selected in G418 and gancyclovir. Twenty of 103 clones underwent homologous recombination and ten of these retained the 5′ loxP site. In order to prevent transcriptional interference from the neomycin resistance gene, one of these clones (#31) was transduced with the pMC-Cre expression plasmid and subclones that had deleted the neomycin cassette but retained exon 1 of *Snf5* were identified. Thirty of 115 clones were now G418 sensitive and 5 of these retained exon 1. Two of these clones (#26 and #28) had good karyotypes and were injected into blastocysts. Both gave rise to high-percentage chimeric mice and both led to germline transmission.

Mx-Cre induction

Induction of *Mx-Cre* in vivo was accomplished via one of two regimens. Neonatal mice (day of life 3) were injected subcutaneously with 200 μg of polyI/polyC (# P-0913, Sigma Chemicals, St. Louis, MO) daily for 5 days. Mature mice (four to eight weeks of age) were injected intraperitoneally with

H: TUNEL reaction performed on a liver section from the same *Snf5*$^{floxed/-}$/*Mx-Cre* mouse in the first two panels. Note the increased apoptosis in comparison to the control mouse.

I: H&E stained spleen section from a *Snf5*$^{floxed/-}$/*Mx-Cre* mouse at 96 hr post-induction. Arrows identify cells with the morphologic appearance of late stage apoptosis while the arrowhead points to a tingible body macrophage that has engulfed several apoptotic cells.

J: Liver section from a *Snf5*$^{floxed/-}$/*Mx-Cre* mouse. The arrow indicates a late stage apoptotic cell.

300 μg of polyI/polyC every other day for five doses. There was no apparent toxicity associated with either of these regimens.

Immunohistochemistry

Immunohistochemistry was carried out utilizing commercially available staining kit (ABC kit, #SC-2019, Santa Cruz Biotechnology, Santa Cruz, CA) and antibodies (PharMingen, San Diego, CA) reactive to the following epitopes: B220 (#01121A), Gr-1 (#557445), Mac-1 (557394), IgG2a (553927), IgG1 (553969), Thy-1.2 (553000), CD31 (557355), IgG2b (553986), CD3 (557306), CD4 (553043), CD8 (558733).

Southern Analysis

Genotyping of mice was done by hybridizing a labeled 0.6 kb XcmI fragment located 3 kb upstream of exon 1 to BamHI digested DNA. Deletion and inversion of the conditional alleles was performed using a labeled 0.38 kb XcmI fragment located 3 kb upstream of exon 1 to SpeI digested DNA. Rearrangement at the T cell receptor β locus was detected using a labeled 1.6 kb BamHI/KpnI fragment located just 5′ of Cβ2 and hybridizing to BglII digested DNA.

TUNEL staining

Detection of DNA strand breaks within apoptotic cells was carried out using the *In Situ* Cell Death Detection Kit (Catalog # 1 684 809, Roche Diagnostics, Mannheim, Germany). Staining of 5μM cryopreserved sections was performed according to the manufacturer's directions with the exception that permeabilization was carried out for 10 min in 0.1% Triton X-100, 0.1% sodium citrate.

Flow Cytometric Analysis

Four-color immunostaining of primary thymocytes and splenocytes in single cell suspension was carried out using the following antibodies: anti-CD4-PE, anti-CD8-FITC, anti-TCRβ-APC, anti-B220-PerCP, anti-CD44-FITC, anti-CD25-APC, anti-CD3-APC, anti-CD8-PerCP, anti-IgM-PE, and anti-IgD-FITC (all antibodies obtained from BD PharMingen, San Diego, CA). Analysis of V_β T cell receptor was carried out with 15 individual FITC-conjugated monoclonal antibodies that recognize V_β 2, 3, 4, 5.1 and 5.2, 6, 7, 8.1 and 8.2, 8.3, 9, 10b, 11, 12, 13, 14 and 17a T cell receptors (V_β TCR Screening Panel, catalog #0143KK, BD PharMingen, San Diego, CA).

Detection of apoptotic cells by flow cytometry was carried out with the Anexin-V-FLUOS Staining Kit according to the manufacturer's instructions (Catalog # 1 858 777, Roche Diagnostics, Mannheim, Germany).

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