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# The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression

Agnes Klochendler-Yeivin, Laurence Fiette[1], Jaqueline Barra[2], Christian Muchardt, Charles Babinet[2] and Moshe Yaniv[+]

Unité des Virus Oncogènes, CNRS URA 1644, [1]Unité d'Histopathologie and [2]Unité de Biologie du Développement, CNRS URA 1960, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

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**The assembly of eukaryotic DNA into nucleosomes and derived higher order structures constitutes a barrier for transcription, replication and repair. A number of chromatin remodeling complexes, as well as histone acetylation, were shown to facilitate gene activation. To investigate the function of two closely related mammalian SWI/SNF complexes *in vivo*, we inactivated the murine *SNF5/INI1* gene, a common subunit of these two complexes. Mice lacking SNF5 protein stop developing at the peri-implantation stage, showing that the SWI/SNF complex is essential for early development and viability of early embryonic cells. Furthermore, heterozygous mice develop nervous system and soft tissue sarcomas. In these tumors the wild-type allele was lost, providing further evidence that *SNF5* functions as a tumor suppressor gene in certain cell types.**

## INTRODUCTION

A number of chromatin remodeling complexes have been described in recent years. These complexes were shown to loosen the nucleosomal structure and facilitate access of transcription factors to DNA (Kingston and Narlikar, 1999). Biochemically, several of these complexes have a fairly similar activity, suggesting possible overlapping functions. It is obvious that a genetic approach is essential to unravel their specific function *in vivo*.The SWI/SNF multi-protein complex is a prototype of one class of these complexes. The SWI/SNF complexes are evolutionarily conserved and implicated in transcriptional activation of a considerable number of genes through chromatin remodeling (Carlson and Laurent, 1994; Peterson, 1996). However, recent evidence suggests that they may also play a role in transcriptional repression (Holstege *et al.*, 1998). Two related multisubunit SWI/SNF complexes have been characterized in mammalian cells. These complexes share most of their subunits but can be distinguished by their SWI2/SNF2-related ATPase/helicase catalytic subunit, which is either *Brm* (*SNF2*α) or *Brg1* (*SNF2*β) (Wang *et al.*, 1996). Even though both subunits exhibit intrinsic chromatin remodeling activity *in vitro*, full activity is achieved only when supplemented with BAF170/BAF155 and hSNF5 (INI1), which are conserved subunits of all SWI/SNF complexes (Phelan *et al.*, 1999). Transfection studies in mammalian cell lines, and genetic studies in *Drosophila* and mice, implicate the catalytic subunit of the SWI/SNF complexes in the regulation of cell cycle progression, possibly by collaborating with pRb in the control of E2F activity (Dunaief *et al.*, 1994; Strober *et al.*, 1996; Muchardt *et al.*, 1998; Reyes *et al.*, 1998; Staehling-Hampton *et al.*, 1999; Zhang *et al.*, 2000). Moreover, *hSNF5* was shown to be mutated in early childhood malignant rhabdoid tumors (MRT) and in various malignancies of the CNS (Versteege *et al.*, 1998; Sevenet *et al.*, 1999a).

We have recently shown that the gene encoding murine SNF2α helicase can be inactivated without impairing development. In these *SNF2α*$^{-/-}$ mice, increased expression of the SNF2β protein appears to compensate for the loss of SNF2α protein (Reyes *et al.*, 1998). To investigate whether SWI/SNF complexes are indeed essential for development and to explore the role of SNF5 in oncogenesis, we undertook the inactivation of the *SNF5* gene.

[+]Corresponding author. Tel: +33 1 45 68 85 12; Fax: +33 1 40 61 30 33; E-mail: yaniv@pasteur.fr

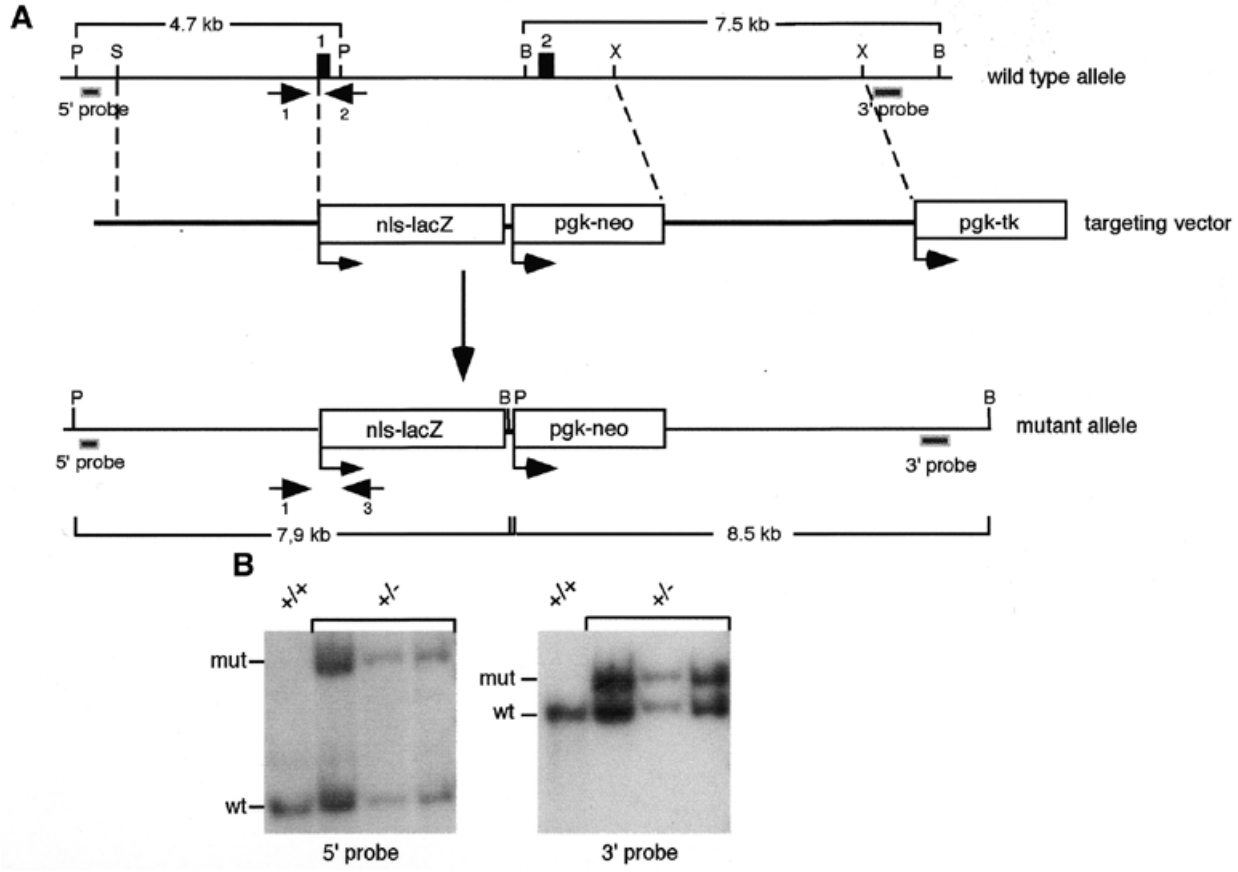


**Fig. 1.** Targeted disruption of the *SNF5* gene. (**A**) Schematic representation of the gene targeting strategy. Exons are indicated by black boxes. The positions of 5′ and 3′ external probes are represented by gray boxes. Primers used for PCR analysis of embryos and mice are indicated by arrows. P, *Pst*I; X, *Xba*I; B, *Bam*HI; S, *Sac*I. (**B**) Southern blot analysis of genomic DNA from G418/gancyclovir-resistant ES clones. *Pst*I- and *Bam*HI-digested DNAs were hybridized with the 5′ and 3′ probes, respectively. One wild-type (+/+) and three heterozygous (+/–) clones are presented. The existence of a single integration site at the targeted locus was verified using *Bam*HI-digested DNA and a neo probe, giving a single 8.5 kb fragment (not shown).

## RESULTS AND DISCUSSION

We generated a loss-of-function mutation of the *SNF5* gene in embryonic stem (ES) cells by replacing exons 1 and 2 with a *lacZ* reporter gene, encoding β-galactosidase with a nuclear localization signal (Figure 1A). Western blot analysis of whole cell extracts using a polyclonal antibody raised against the full-length human SNF5 did not reveal any truncated SNF5 or chimeric LacZ–SNF5 protein in these ES cells, excluding the presence of an interfering protein. We obtained chimeric mice originating from two different heterozygous ES cell clones. These mice transmitted the *SNF5–lacZ* mutation to F1 offspring, which developed normally.

Analysis of β-galactosidase activity in heterozygous adult animals revealed that *SNF5* was expressed ubiquitously in all organs. Similarly, *SNF5* was expressed in all embryonic cells during post-implantation development (data not shown). To investigate whether the *SNF5* gene was activated at earlier time points, we monitored β-galactosidase activity in fertilized oocytes and in early cleavage stage embryos. Crosses of $SNF5^{+/lacZ}$ males with wild-type females showed that the onset of *SNF5* zygotic expression occurs at the four-cell stage and is maintained, at least until the blastocyst stage, both in the inner cell mass (ICM) and the trophectoderm (Figure 2A). In contrast, crosses of wild-type males with heterozygous females revealed the presence of maternal stores of protein in fertilized oocytes and in two-cell embryos (Figure 2B). The widespread expression of *SNF5* in pre-implantation embryos raised the possibility that the SWI/SNF chromatin remodeling activity may be essential for early development.

To explore this issue we bred $SNF5^{+/LacZ}$ animals. No nullizygous animals were born from these heterozygous intercrosses, indicating that the absence of SNF5 causes embryonic lethality (Table IA). To determine the developmental stage at which $SNF5^{-/-}$ mice die, embryos from heterozygous intercrosses were isolated at various gestational times and genotyped by PCR. Analysis of embryos from 6.5 and 7.5 days p.c. showed no nullizygous embryos, but 28% of decidua were empty (only 2% detected in crosses between wild-type and *SNF5* heterozygous mice), which might represent resorption sites of homozygous mutant embryos. To test whether $SNF5^{-/-}$ embryos survive to the blastocyst stage, embryos were isolated at 3.5 days p.c. and genotyped. Nullizygous blastocysts were identified in a Mendelian distribution (Table IB). They did not show any developmental delay and were morphologically indistinguishable from wild-type blastocysts (not shown). Thus, $SNF5^{-/-}$ embryos

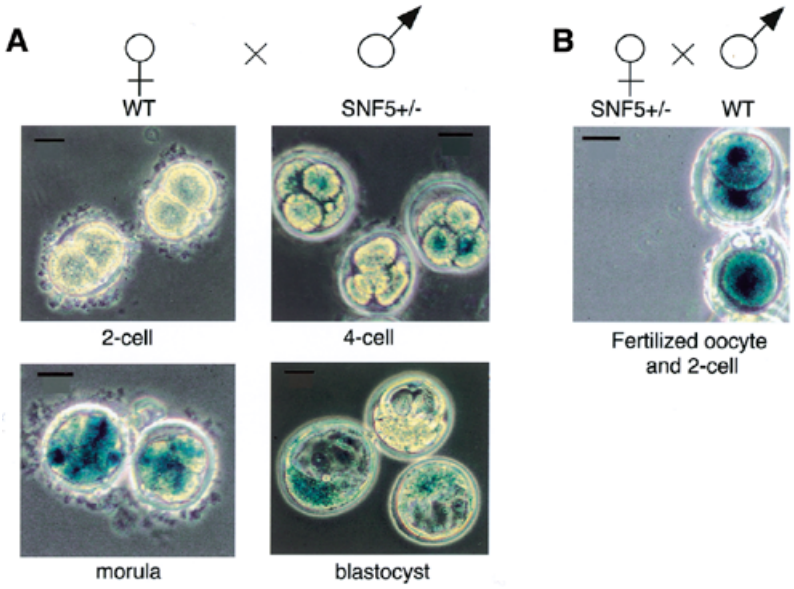


**Fig. 2.** Expression of the *SNF5–lacZ* allele in pre-implantation embryos. All cleavage stages were obtained from *in vitro* culture initiated at the one-cell stage. Fertilized oocytes were collected from mated superovulated females: (**A**) wild-type B6SJL F1 female × $SNF5^{+/lacZ}$ C57Bl6 × 129Sv male; (**B**) $SNF5^{+/lacZ}$ C57Bl6 × 129Sv female × wild-type C57Bl6 × 129Sv males. Recovery of oocytes and culture conditions were performed as described (Hogan, 1994). β-galactosidase activity was detected by whole mount staining with X-gal substrate. Scale bar, 20 μm.

**Table I.** Genotype analysis of offspring [(A) postnatal; (B) embryos] from heterozygous intercrosses

A

| Breeding | Number of litters | Number of each genotype (percent of total) | | |
|---|---|---|---|---|
| | | +/+ | +/– | –/– |
| C57BL/6X129/Sv (mixed) | 20 | 40 (36%) | 71 (64%) | 0 |
| 129/Sv (inbred) | 13 | 17 (30%) | 40 (70%) | 0 |
| Total | 33 | 57 (34%) | 111 (66%) | 0 |

B

| | Number of each genotype (percent of total) | | |
|---|---|---|---|
| | +/+ | +/– | –/– |
| E7.5 | 9 (30%) | 21 (70%) | 0 |
| E6.5 | 8 (29%) | 20 (71%) | 0 |
| E3.5 | 10 (23%) | 25 (57%) | 9 (20%) |

develop successfully to the blastocyst stage, but die shortly after implantation, before day E6.5. To determine whether the developmental defect in $SNF5^{-/-}$ mice was due to a proliferation or survival defect following the blastocyst stage, 3.5-day embryos derived from heterozygous intercrosses were placed in culture and monitored for outgrowth potential. Roughly one-third of *SNF5*-null embryos failed to emerge from the zona pellucida, while the others emerged but the trophectoderm did not spread and the ICM stopped growing after 1 day in culture (Figure 3A). Enzymatic removal of the zona pellucida prior to plating gave identical results. As shown in Figure 3A, in wild-type and heterozygous embryos the trophoblasts migrated out of the compact blastocysts, attached to the substrate and formed polyploid giant cells. The ICM increased in size and by day 4 formed a discernible node. These features were not observed in any of the mutant blastocysts. The failure of *SNF5*-null embryos to hatch or attach *in vitro* indicates that the trophectoderm is defective. By day 4 in culture, both the ICM and trophectoderm of mutant-derived cells invariably died. We explored further the cause of $SNF5^{-/-}$ cell death by TUNEL staining, which detects extensive DNA fragmentation resulting from apoptotic cell death. $SNF5^{-/-}$ blastocysts cultured for 96 h displayed widespread TUNEL signal, while apoptotic cells could not be detected in wild-type and heterozygous blastocysts after 4 days in culture (Figure 3A). These results indicate that *SNF5* is essential for the viability of early embryonic cells in culture and its

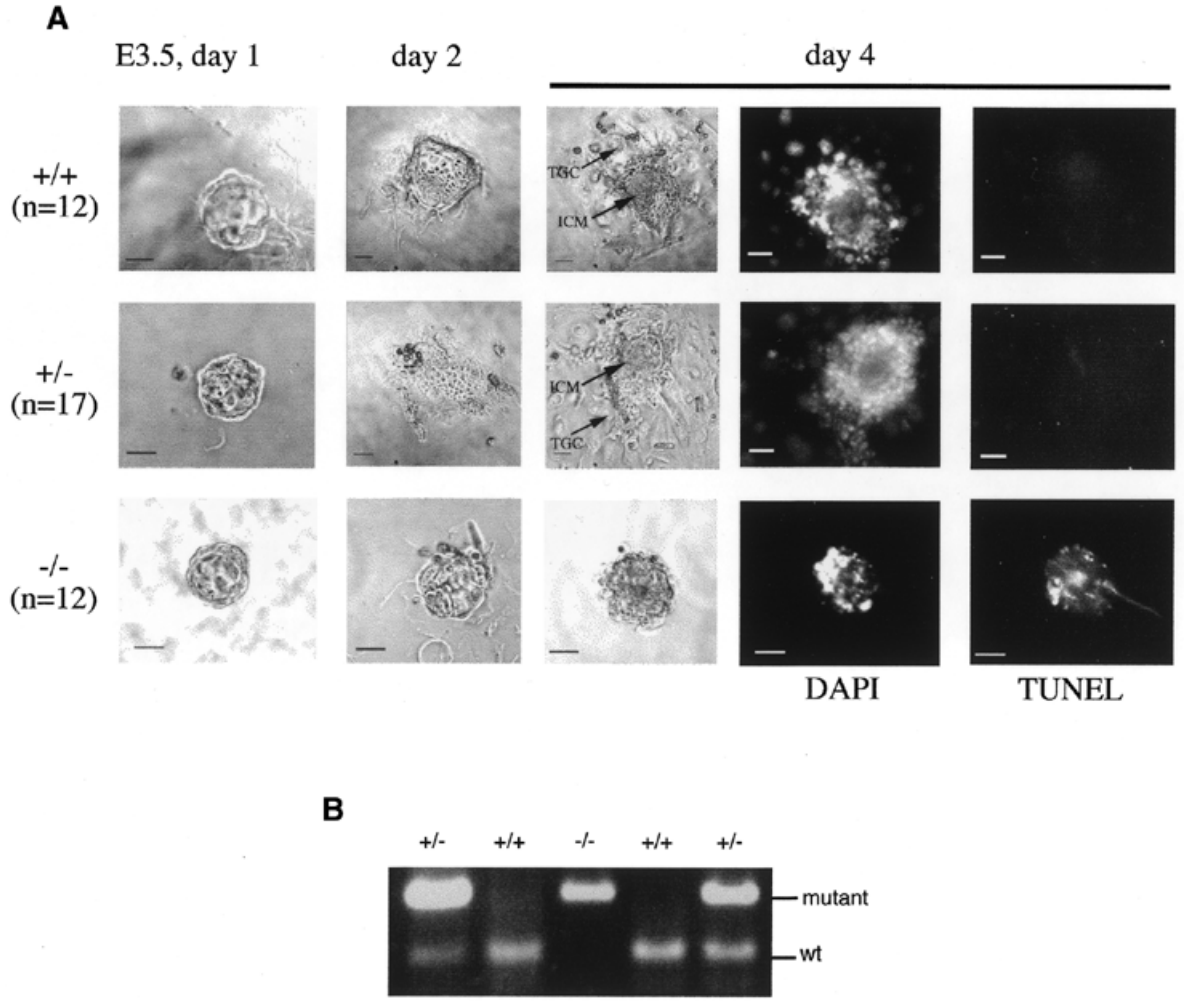


**Fig. 3.** Blastocyst outgrowth and apoptosis studies. (**A**) Day 3.5 p.c. embryos were isolated from *SNF5*$^{+/lacZ}$ intercrosses and cultured in 96-well plates for 4 days. The phase contrast view shows a growing inner cell mass (ICM) node and a single layer of trophoblastic giant cells (TGC) cells in wild-type and heterozygous blastocysts after 1, 2 and 4 days in culture, while homozygous mutants are impaired in both trophectoderm and ICM outgrowth. After 4 days of culture, TUNEL assay was performed. Fluorescein (TUNEL) and 4′-6-diamidino-2-phenylindole (DAPI) fluorescent images are shown. Scale bar, 60 μm. (**B**) Examples of PCR genotyping of cultured blastocysts.

loss results in apoptotic cell death. At the present time we cannot exclude the possibility that SNF5 is required at even earlier times. The survival of mutant embryos to early post-implantation stages might be due to the presence of maternal stores of SNF5.

It has recently been reported that constitutive mutations of *SNF5* predispose to early childhood cancer (rhabdoid predisposition syndrome—RPS) (Sevenet *et al.*, 1999b). Therefore, we monitored *SNF5* heterozygous mice for the potential development of tumors. Careful examination of these animals revealed susceptibility to early onset of cancer. In a population of 124 mice, 32% (40 animals) developed tumors by the age of 15 months, the earliest tumors being detected at 4 months of age (see survival curve in Supplementary data, available at *EMBO reports* Online). The percentage is most likely underestimated since additional heterozygous mice displayed severe wasting symptoms but no macroscopic lesions were detected. Tumor susceptibility was independent of sex or genetic background (C57BL/6 × 129/Sv or inbred 129/Sv). Wild-type littermates (104 mice) did not develop tumors within the 15 month observation period.

Tumors were detected in different locations, but intra-cranial (brain, cerebellum, beneath the optic chiasma; 30%) and paravertebral sites (around the dorsal root ganglia or spinal nerve in the spinal cord; 27%) were very common. We observed additional macroscopic subcutaneous tumors in less common sites (see Supplementary data).

Histological analysis was performed on 37 tumors. In spite of the different locations of the tumors, their microscopic morphology was relatively uniform. They appeared as solid sheets of undifferentiated cells, separated by scanty stroma. The lesions usually consisted of a mixture of polygonal or spindle cells, with large clear pleomorphic nuclei, conspicuous nucleoli and eosinophilic cytoplasm. Mitotic figures were scanty. Giant multinucleated cells were often present. In ~30% of the cases we observed a variable proportion of cells that showed a typical rhabdoid phenotype, with large intracytoplasmic eosinophilic

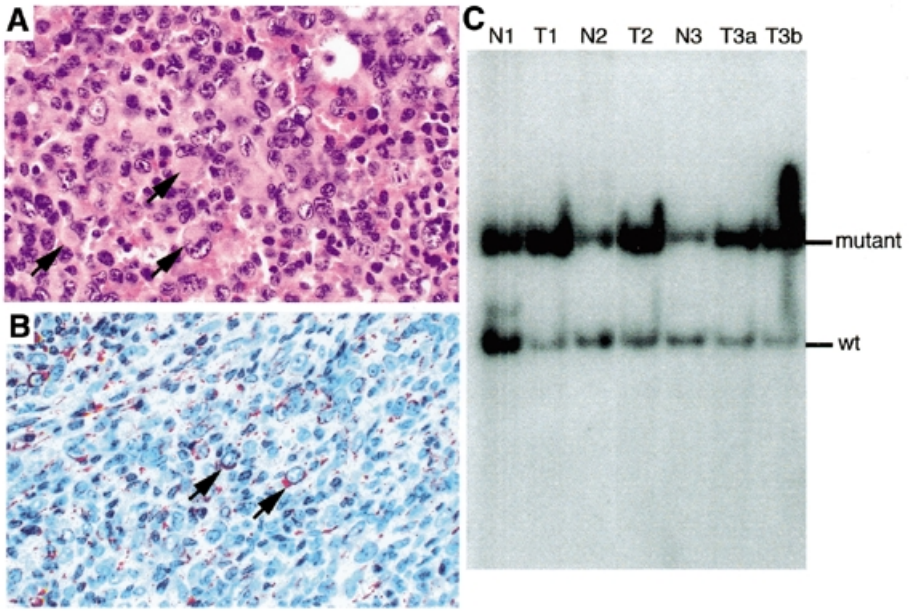


**Fig. 4.** *SNF5* heterozygous mice are predisposed to cancer. (**A**) Microscopic features of tumors (5-month-old mouse; tumor from thoracic wall). Tumor cells appear as polygonal or elongated undifferentiated cells, with large clear pleomorphic nucleus, conspicuous nucleoli and eosinophilic cytoplasm. Some cells (arrows) display the classical appearance of rhabdoid cells: large excentric nucleus, with a prominent nucleolus, and hyalin-like inclusions in the cytoplasm. Hematoxylin–eosin staining, original magnification ×500. (**B**) Vimentin expression in tumor cells (5-month-old mouse; paravertebral tumor). Numerous cells are strongly positive and vimentin is often visible as perinuclear cytoplasmic spots (arrow). Anti-vimentin, hematoxylin counterstain, original magnification ×500. (**C**) Loss of heterozygosity (LOH) analysis. Southern blot analysis of four representative normal (N) and tumor (T) DNA samples prepared from *SNF5*$^{+/-}$ mice. Note the under-representation of the wild-type allele in the tumors; it remains detectable, probably due to contamination by surrounding normal cells. Tumor 1 (T1) was localized beneath the optic chiasma (7-month-old mouse); tumor 2 (T2) was a subcutaneous tumor of the cheek (7-month-old mouse); tumors 3a and 3b (T3a and T3b) developed in the thoracic and abdominal wall in a single 6-month-old animal. Control DNA samples were extracted from normal tissues close to the lesions.

inclusions that displace the nucleus at the periphery of the cell (Figure 4A; Wick *et al.*, 1995). All of the tumors that we analyzed were positive for vimentin staining (19/19) (Figure 4B). Some of the lesions tested showed immunoreactivity for PS100 (14 of 31), NGF-R (p75) (11 of 25) and GFAP (3 of 25), indicating that they could could originate from neural crest-derived cells (see Supplementary data). In summary, on the basis of their morphology and immunophenotype, the tumors were classified as undifferentiated sarcomas with variable rhabdoid features.

To determine whether tumor formation in the *SNF5* heterozygous mutant mice was dependent upon loss of the remaining wild-type *SNF5* allele, as postulated by Knudson's two-hit model (Knudson, 1971), we performed Southern blot analysis on a panel of 10 tumor DNAs (four of them are shown in Figure 4C). Even though the tumor samples still contained some normal cells, the Southern blot showed a substantial loss of the wild-type *SNF5* allele, while surrounding tissues retained both the mutant and wild-type alleles at a 1:1 ratio as expected. These data provide direct evidence that loss of *SNF5* function contributes to tumor formation in the mouse.

The phenotype of the mouse model and human MRT syndrome exhibit a degree of similarity, although we noted some differences with respect to tumor incidence and spectrum. Unlike human patients, where *SNF5*-associated cancer occurs mainly in very young children, *SNF5*$^{+/LacZ}$ mice developed tumors as adults. Furthermore, human kidneys are a frequent site for MRT (Weeks *et al.*, 1989), yet we found no intra-renal macroscopic tumors in our mice. These discrepancies may be explained by species-dependent differences in susceptibility to *SNF5* loss of heterozygousity (LOH), or differences in the growth control pathways of mouse and human cells in particular tissues.

The critical requirement for *SNF5* for cell viability in early development may seem contradictory to the tumorigenesis associated with *SNF5* loss. However, it is likely that SNF5-associated chromatin remodeling complexes participate in multiple transcriptional events and therefore affect numerous cellular processes. *SNF5* inactivation might result in imbalanced transcriptional regulation and give rise to different effects depending on the cell type. Thus, it is conceivable that in some cell lineages *SNF5* disruption is lethal, while in others it impairs differentiation or promotes cell growth. Consistently, it has been shown previously that embryonic carcinoma F9 cells are not viable upon inactivation of *Brg1* (Sumi-Ichinose *et al.*, 1997), whereas some human-derived tumor cell lines (C33 and SW13) proliferate efficiently in the absence of Brg1 and hbrm (Muchardt and Yaniv, 1993). We cannot formally exclude the possibility that SNF5 might have additional functions beyond its role in the SWI/SNF complexes. However, since inactivation of *Brg1*, the major helicase/ATPase subunit of SWI/SNF complex present in early development (LeGouy *et al.*, 1998), also results in very early developmental arrest (Bultman *et al.*, 2000), it is likely that the early lethality of *SNF5*-null embryos is caused by the loss of functional SWI/SNF complexes. Despite the presence of additional chromatin remodeling complexes in mammalian cells, our results strongly suggest an essential and non-redundant function for SWI/SNF in embryonic development. Compared with

knockouts of other transcriptional coactivators, the *SNF5* nullizygous embryos show a similar peri-implantation lethality as embryos deficient for the RNA polymerase II coactivator subunit Srb7 (Tudor *et al.*, 1999). In contrast, disruption of the transcriptional histone acetylases *CBP/p300* or *Gcn5l2* results in later lethality (10.5 days p.c.) (Goodman and Smolik, 2000; Xu *et al.*, 2000).

The molecular mechanism underlying tumor development upon SNF5 deficiency is unknown at present. Several studies imply a role for Brm and Brg1 in Rb-mediated transcriptional repression, in particular, in the repression of genes involved in $G_1$–S progression such as *Cycin E* and *Cyclin A* (Trouche *et al.*, 1997; Zhang *et al.*, 2000). However, it should be emphasized that in both human and mouse, the spectrum of tumors arising in *Rb* and *SNF5* heterozygotes is different. Furthermore, $Rb^{-/-}$ embryos die much later than $SNF5^{-/-}$ embryos (Jacks *et al.*, 1992). Considering that p107 and p130, two closely related pocket proteins, display partial functional redundancy with Rb (Mulligan and Jacks, 1998), we might speculate that the SWI/SNF complex could cooperate with Rb relatives as well, and that mice nullizygous for Rb, p107 and p130 would die at peri-implantation, as observed with $SNF5^{-/-}$ embryos. Nevertheless, the SWI/SNF complexes could be involved in additional growth control checkpoints. Alternatively, cell death resulting from *SNF5* inactivation may create a bias in the tumor spectrum obtained in heterozygous animals. Our studies of the *SNF5* gene provide a novel example of a dual role for a certain number of tumor suppressor genes. These genes are essential for development and organogenesis; they can be inactivated in certain cell types and cause cancer.

## METHODS

**Generation of the *SNF5*-targeted allele.** A 15 kb genomic fragment spanning the promoter region and sequence encoding the N-terminus of murine *SNF5* was isolated from a 129/Sv mouse genomic library, using a human *SNF5* cDNA probe. Genomic fragments of 3.5 kb and 4.5 kb flanking the first two exons of SNF5 were used to generate a targeting construct in which the first two exons were replaced by a NLS-*lacZ* cassette. Briefly, a *Nco*I site was generated at the initiation codon of *SNF5* to allow insertion of the NLS-*lacZ* cassette in frame. A 7 kb *Sal*I fragment corresponding to the 3.5 kb *Sac*I–*Nco*I 5′ flanking region of *SNF5* fused to the *lacZ* reporter gene was inserted upstream of the PGK-*neoR* cassette into a modified pPNT vector containing the 4.5 kb *Xba*I 3′ flanking sequence (Figure 1A). CK35 ES cells (Kress *et al.*, 1998) were electroporated with the *Not*I-linearized vector and clones were selected in 300 μg/ml G418 and 2 μM gancyclovir. Among 200 ES cell colonies, three correctly targeted clones were identified by Southern blot analysis, using 5′ and 3′ external probes located outside the homologous regions of the targeting vector (Figure 1B). Two independent targeted clones were injected into C57BL/6 host blastocysts, and chimeric males were mated to 129Sv and C57BL/6 females to generate mixed background and inbred lines carrying the mutation.

**Genotyping of mouse tails and embryos.** DNA from either mouse tails or post-implantation embryos (E6.5 or E7.5) was directly precipitated with isopropanol after proteinase K treatment. For pre-implantation embryos (E3.5), DNA was prepared by incubation of individual blastocysts in 5 μl of proteinase K buffer (10 mM Tris pH 8.4, 50 mM KCl, 0.01% gelatin + 300 μg/ml proteinase K) for 1 h at 55°C followed by incubation at 95°C for 10 min. The entire DNA isolate was then used directly for PCR. A three-way PCR was performed with a sense primer within the *SNF5* promoter (5′-AAGGAGCCCAGTAGTGACAC-3′), and two reverse primers in the *SNF5* first exon (5′-GCCGATCATGTAGAACTCCC-3′) and in the *lacZ* gene (5′-AAGCGCCATTCGCCATTCAG-3′), generating a wild-type 220 bp product and a mutant 440 bp product, respectively.

**Blastocyst outgrowth study.** Blastocysts generated from heterozygous intercrosses were collected by uterine flush at 3.5 days p.c. and individually cultured for 5 days in gelatin-coated 96-well round bottom plates, in 100 μl Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 μM β-mercaptoethanol and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Gibco-BRL). After photography, cells were scraped off and collected for PCR genotyping as described for 3.5 days p.c. embryos.

**X-gal staining.** Embryos were collected at different gestation stages, fixed for 10 min in 4% paraformaldehyde and washed extensively in phosphate-buffered saline. Whole-mount X-gal staining was performed overnight as described (Thepot *et al.*, 2000).

**Histology and immunohistochemistry.** Tumors were fixed in 10% neutral-buffered formalin, embedded in paraffin and processed routinely for histology. For immunohistochemical study, standard indirect peroxidase or peroxidase–anti-peroxidase methods with amplification by the EnVision+ system (Dako) were used on paraffin sections. Primary antibodies were the following: EPOS monoclonal mouse antibodies against vimentin (Sigma; clone vim 13.2); desmin (Dako; clone 33); neuron-specific enolase (Dako; clone H14); smooth muscle actin (DAKO; clone 1A4); epithelial membrane antigen (clone E29). Rabbit polyclonal antibodies were: multi-cytokeratins (NCL-CKp; Novocastra); PS100 (Dako); myosin (Biogenex); GFAP (Dako); p75 nerve growth factor receptor (DAKO).

**Analysis of *SNF5* loss of heterozygosity.** *SNF5* LOH analysis was performed on DNA isolated from fresh tumor samples. The genotype of the tumor DNA was determined by Southern blotting of *Pst*I-digested DNA and hybridization with the genomic 5′ external probe (Figure 1A).

**Supplementary data.** Supplementary data for this paper are available at *EMBO reports* Online.

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