

## Disruption of *Ini1* Leads to Peri-Implantation Lethality and Tumorigenesis in Mice

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Received 20 December 2000/Accepted 14 February 2001

**SNF5/INI1 is a component of the ATP-dependent chromatin remodeling enzyme family SWI/SNF. Germ line mutations of *INI1* have been identified in children with brain and renal rhabdoid tumors, indicating that *INI1* is a tumor suppressor. Here we report that disruption of *Ini1* expression in mice results in early embryonic lethality. *Ini1*-null embryos die between 3.5 and 5.5 days postcoitum, and *Ini1*-null blastocysts fail to hatch, form the trophectoderm, or expand the inner cell mass when cultured in vitro. Furthermore, we report that approximately 15% of *Ini1*-heterozygous mice present with tumors, mostly undifferentiated or poorly differentiated sarcomas. Tumor formation is associated with a loss of heterozygosity at the *Ini1* locus, characterizing *Ini1* as a tumor suppressor in mice. Thus, *Ini1* is essential for embryo viability and for repression of oncogenesis in the adult organism.**

The compact nature of chromatin structure presents a barrier to cellular processes that require access to DNA. A number of multiprotein complexes have been identified that share the ability to modify chromatin structure. These include the histone acetyltransferases and deacetylases, complexes which chemically modify the amino-terminal tails of histones by the addition or removal of acetyl groups, respectively, as well as a group of enzymes that utilize the energy derived from ATP hydrolysis to alter nucleosome structure (16, 20, 43, 44, 50). Included among these ATP-dependent chromatin remodeling enzymes is the SWI/SNF family of chromatin modifiers.

SWI/SNF enzymes are large multisubunit enzymes of ~1 to 2 MDa. Yeast SWI/SNF genes were originally identified as being required for mating type switching or sucrose fermentation (4, 32, 42). Later work determined that SWI/SNF genes were required for the induction of a subset of yeast genes and that the SWI2/SNF2 protein possessed a DNA-stimulated ATPase activity (6, 22, 26, 33, 34, 54). Mutations in SWI/SNF genes could be suppressed by mutations altering histone gene expression, histone structure, or nonhistone chromatin proteins, leading to the suggestion that these gene products facilitated transcriptional activation by altering chromatin structure (15, 23, 24).

Human SWI/SNF (hSWI/SNF) complexes contain either the human BRM (hBRM) (hSNF2 $\alpha$ ) or BRG1 (hSNF2 $\beta$ ) homologues of the yeast SWI2/SNF2 ATPase (7, 19, 30). Both yeast and human SWI/SNF complexes have been shown to possess nucleosome remodeling activity in vitro (8, 17, 25). Components of mammalian SWI/SNF complexes have been implicated in a variety of cellular processes, including gene activation and repression, development and differentiation, recombination and repair, and cell cycle control. There is ev-

idence supporting a role for SWI/SNF in gene activation events mediated by nuclear hormone receptors, environmental stress, and viral infection (1, 7, 10, 13, 30). In contrast, SWI/SNF components also were shown to be involved in repression of *c-fos* and some E2F-regulated genes (31, 48). Both BRG1 and hBRM can interact with the retinoblastoma oncoprotein and induce cell cycle arrest, an effect that is abrogated by the association of BRG1 with cyclin E (11, 41, 45, 56). Evidence suggesting a role for hSWI/SNF in recombination and repair was provided by studies demonstrating an interaction of components of the hSWI/SNF complex with BRCA1, which is thought to be involved in DNA damage and repair pathways (3). Furthermore, members of the SWI/SNF complex are targets of viral regulatory proteins upon infection of cells by adenovirus, Epstein-Barr virus, human immunodeficiency virus, and human papillomavirus (18, 27, 28, 53).

The role of SWI/SNF enzymes in whole organisms is unclear. While homozygous disruption of *Brg1* in mouse embryonic carcinoma cells resulted in lethality, disruption of *Brm* expression in mice produced only mild proliferative effects (35, 46). The upregulation of *Brg1* in the *Brm*-deficient mice may provide a compensatory effect; however, one cannot rule out the possibility that these differences are due to distinct functions of *Brm*- or *Brg1*-containing complexes.

SNF5/INI1 is a member of both BRG1- and BRM-containing SWI/SNF complexes (29, 51). INI1 was shown to interact with ALL-1, translocations of which are associated with several types of human acute leukemias (37). Furthermore, *INI1* has been found to be altered in malignant rhabdoid tumors, choroid plexus carcinomas, medulloblastomas, and central primitive neuroectodermal tumors (2, 9, 39, 40, 49). Identification of constitutional mutations in a subset of these tumors indicates that INI1 is a tumor suppressor (2, 40). In an attempt to generate a mouse model that would allow further characterization of the mechanisms of *Ini1* in tumorigenesis and to determine the role of the mammalian SWI/SNF complexes in development, we generated mice deficient for *Ini1* expression.

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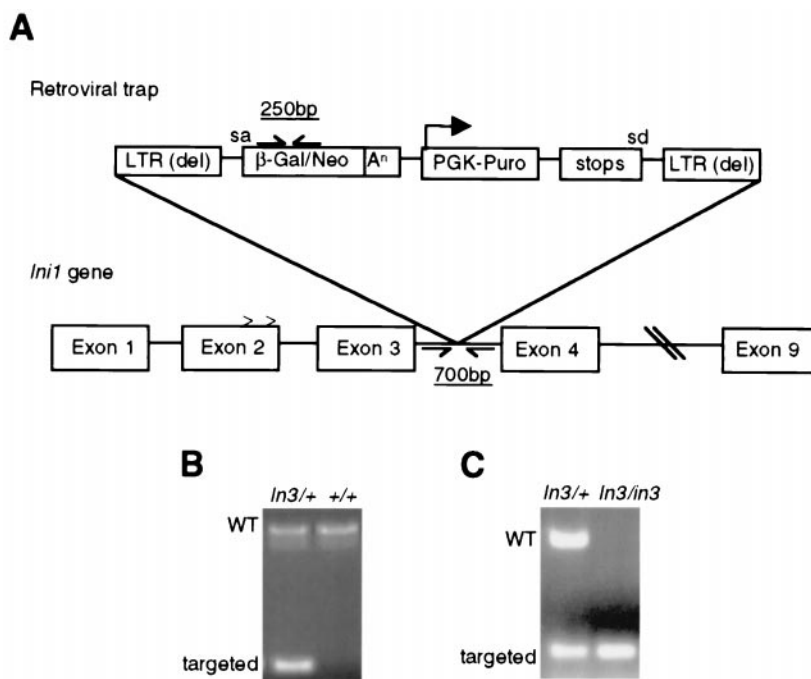


FIG. 1. Disruption of *Ini1*. (A) Targeting strategy for *Ini1*. A retroviral promoter trap vector was inserted in intron 3 of *Ini1*. sa and sd, the splice acceptor site in the  $\beta$ -geo gene cassette and the splice donor site in the puromycin selection marker, respectively. Carets represent the site of alternate splicing. Positions of the primers used for PCR analysis are shown. LTR (del), long terminal repeat deleted; A<sup>p</sup>, polyadenylation signal; Puro, puromycin. (B) Genotyping of *Ini1*-targeted mice by PCR. Genomic DNA was harvested from tails and genotyped as described in Materials and Methods. The size and position of wild-type (WT) and targeted bands are indicated. (C) Genotyping of *Ini1*-targeted embryos. Embryos 6.5 d.p.c. and younger were genotyped by nested PCR. The size and position of wild-type and targeted bands are indicated.

We show that *Ini1*-deficient mice die early in embryogenesis, likely due to an inability of the blastocysts to hatch, implant in the uterus, and continue development. In addition, we report that a subset of the *Ini1*-heterozygous mice present with a variety of tumors in the soft tissues of the head and neck and that loss of heterozygosity at the *Ini1* locus is correlated with tumor formation.

#### MATERIALS AND METHODS

***Ini1* targeting.** Embryonic stem (ES) cells (Omnibank no. OST32815) bearing a retroviral promoter trap that functionally inactivates one allele of *Ini1* were generated as described previously (55). Analysis by rapid amplification of cDNA ends also is described. The site of insertion was determined using sequence analysis.

**Creation of *Ini1*-null mice.** *Ini1*-targeted ES cells were injected into 3.5 days postcoitum (d.p.c.) C57BL/6 blastocysts. Male chimeric mice were mated with wild-type C57BL/6 or 129 females. Germ line transmission of the mutant allele was determined by PCR analysis of tail genomic DNA using the following primers: for *Ini1*, 5'-GCAAGCGCTCTGCCAATTGACC-3' and 3'-CACACCCTATTGTCACTCTGGAA-5';  $\beta$ geo, 5'-CGGTATCGATAAGCTTGATGATC-3' and 3'-GTCAACGCGTCGGACTTACCGC-5'. *Ini1*-heterozygous mice were intercrossed to generate *Ini1*-null mice. Embryos 6.5 d.p.c. and younger were prepared for genotyping by PCR as described previously (47). Nested PCR was done using the above primers for the first round of PCR (29 cycles) and the following intron 3-nested primers for the second round (29 cycles): 5'-GCGTGCGCCACCATGCCTGG-3' and 3'-CTTCTGGAGACTTCACCTACGTCC-5'.

**Blastocyst culture.** Blastocysts from heterozygous intercrosses were flushed from the uteri of *Ini1*<sup>ln3/+</sup> females 3.5 d.p.c. with M15 media (Dulbecco's minimal essential medium, 15% fetal calf serum, 100  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM glutamine, and 1 $\times$  penicillin-streptomycin) and cultured in tissue culture plates for 96 h. Embryo cultures were genotyped as described above.

**$\beta$ -Galactosidase staining of cultured ES cells.** Wild-type AB2.2 ES cells and *Ini1*<sup>ln3/+</sup> ES cells were grown to near-confluency and fixed in 0.5% glutaraldehyde. Cells were then rinsed with phosphate-buffered saline and stained overnight in the dark at room temperature in a solution containing 5 mM potassium

ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub>, and 1 mg of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal)/ml.

**Whole mount staining of embryos for  $\beta$ -galactosidase activity.** Embryos were harvested at various time points postfertilization and fixed in 4% paraformaldehyde for 20 min at 4°C. Embryos were washed and then stained in an X-Gal histochemical reaction mixture (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl<sub>2</sub>, 1 mg of X-Gal/ml) overnight at room temperature. Following staining, embryos were rinsed in phosphate-buffered saline and cleared in 30% sucrose.

**Western analysis of tumor samples.** Control tissues and tumor samples were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 20% glycerol, 1 mM dithiothreitol, 1  $\mu$ g of pepstatin A/ml, 4  $\mu$ g of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride. Extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western analysis for *Ini1* protein was performed as described previously (10).

**Histologic analysis of tumors in mice.** Tumor samples and selected tissues were fixed in 10% buffered formalin phosphate and processed for paraffin embedding as described previously (14). Sections were prepared, stained with hematoxylin and eosin, and examined under a microscope.

#### RESULTS AND DISCUSSION

Mouse ES cells bearing a retroviral promoter trap that functionally inactivates one allele of *Ini1* were constructed as described previously (55). Sequence analysis revealed that the promoter trap was inserted within intron 3 of *Ini1* (Fig. 1A). The beta-galactosidase-neomycin ( $\beta$ -geo) gene fusion cassette within the retroviral insertion has a 5' splice acceptor site; thus,  $\beta$ -geo expression is regulated by the native *Ini1* promoter. We were able to utilize the  $\beta$ -geo gene cassette in a colorimetric assay to determine if *Ini1* is normally expressed in ES cells. *Ini1*-targeted cells stained positive for  $\beta$ -galactosidase activity, indicating that *Ini1* is expressed in ES cells (Fig. 2A). Northern analysis of ES cell total RNA confirmed *Ini1* expression (data

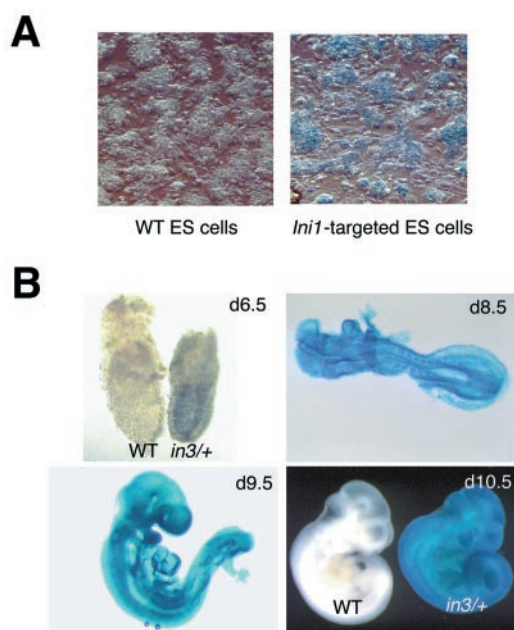


FIG. 2. *Inil* is expressed in ES cells and ubiquitously throughout development. (A)  $\beta$ -galactosidase staining of targeted ES cells showing expression of *Inil*. Wild-type (WT) AB2.2 ES cells were used as a control. (B) Whole mount staining of *Inil* in embryos showing ubiquitous expression of *Inil* at indicated time points. Wild-type embryos at 6.5 and 10.5 d.p.c. are shown as controls.

not shown). Furthermore, sequence data obtained from 5' rapid amplification of cDNA ends analysis of the *Inil*- $\beta$ -galactosidase fusion mRNA revealed that transcripts utilizing either splice donor site in exon 2 spliced into the trap, indicating that both splice variants of *Inil* were inactivated (5).

To determine the role of *Inil* in mammalian development and tumorigenesis, we used the targeted ES cells in blastocyst injection experiments to generate *Inil*-heterozygous (*Inil*<sup>in3/+</sup>) mice. In order to monitor expression of *Inil* during embryogenesis, we performed whole mount staining for  $\beta$ -galactosidase activity in embryos harvested from *Inil*<sup>in3/+</sup> matings at various times during development. We found that *Inil*<sup>in3/+</sup> embryos stained positive in all tissues at all time points examined, including 6.5, 8.5, 9.5, and 10.5 d.p.c., indicating that *Inil* is ubiquitously expressed during embryogenesis (Fig. 2B). *Inil* expression was also detected by Northern analysis in a wide range of adult tissues (35) (data not shown).

Chimeric mice generated from C57BL/6 strain blastocyst injections of the 129 strain-derived ES cells were bred to wild-

TABLE 1. Genotyping of embryos from heterozygous intercrosses<sup>a</sup>

Embryo category	No. of embryos		
	WT	Het	Null
3.5 d.p.c.	7	17	5
In vitro culture	11	14	1 <sup>b</sup>
6.5 d.p.c.	9	23	0
8.5 d.p.c.	4	14	0
13.5 d.p.c.	5	6	0

<sup>a</sup> WT, wild type; Het, heterozygous.

<sup>b</sup> The embryo was dead.

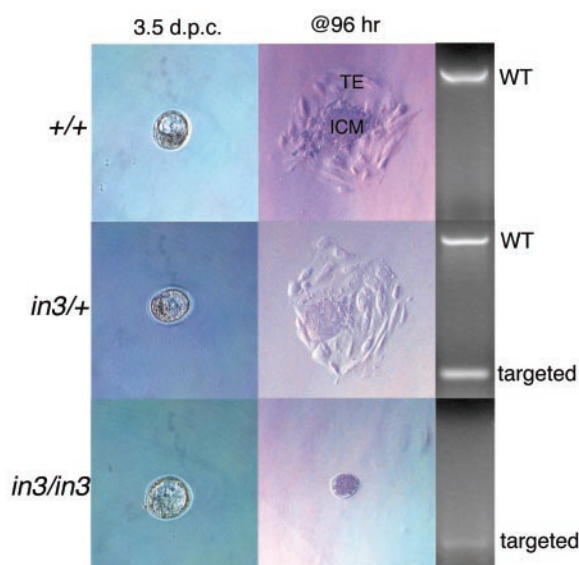


FIG. 3. *Inil*-null mice are early embryonic lethal and fail to hatch in vitro. Blastocysts were harvested from C57BL/6 *Inil*<sup>in3/+</sup> females and plated in culture for 96 h, at which time outgrowths were processed for PCR. Blastocysts are shown before and after culturing. TE, trophectoderm; ICM, inner cell mass.

type C57BL/6 or 129 mice in order to obtain *Inil*<sup>in3/+</sup> mice on either a mixed (C57BL/6  $\times$  129) or pure (129) background. Intercrosses of *Inil*<sup>in3/+</sup> mice in both backgrounds yielded *Inil*<sup>in3/+</sup> offspring and wild-type offspring at a 2:1 ratio (63:26 in the mixed background, 34:17 in the pure background) and no *Inil*-null offspring, indicating that disruption of *Inil* induces embryonic lethality (Fig. 1B). Timed matings of *Inil*<sup>in3/+</sup> mice were performed, and embryos were harvested at various time points in gestation for genotyping via PCR. *Inil*-null embryos could be isolated at 3.5 d.p.c. and were normal in appearance (Fig. 1C and 3). However, no *Inil*<sup>in3/in3</sup> embryos were detected at 6.5 d.p.c. or later (Table 1). Dissection of maternal deciduae at 6.0 to 6.5 d.p.c. revealed no significant increase in the number of embryo reabsorptions, suggesting that *Inil*<sup>in3/in3</sup> lethality occurred between days 3.5 and 5.5 of gestation. These results

TABLE 2. Tumor occurrence

Mouse no.	Age (wk)	Tumor site	Classification
Ini 119	36	Face	Undifferentiated sarcoma
Ini 44	32	Face	Undifferentiated sarcoma
Ini 138	23	Face	Malignant fibrous histiocytoma
Ini 100	23	Face	Malignant fibrous histiocytoma
Ini 180	31	Face	Undifferentiated sarcoma
Ini 26 <sup>a</sup>	24	Face	Malignant fibrous histiocytoma
Ini 262	16	Face	Malignant fibrous histiocytoma
Ini 29 <sup>a</sup>	41	Face	Malignant fibrous histiocytoma
Ini 13 <sup>a</sup>	59	Face/eye	Liposarcoma
Ini 56	52	Neck mass	Lymphoma
Ini 322	21	Brain	Undifferentiated sarcoma
Ini 10	32	Ventral to brain	Undifferentiated sarcoma
Ini 95 <sup>a</sup>	22	Face mass	Malignant fibrous histiocytoma
Ini 127	12	Neck mass	Lymphoproliferative disorder
Ini 328	25	Eye	Undifferentiated sarcoma

<sup>a</sup> 129 strain.



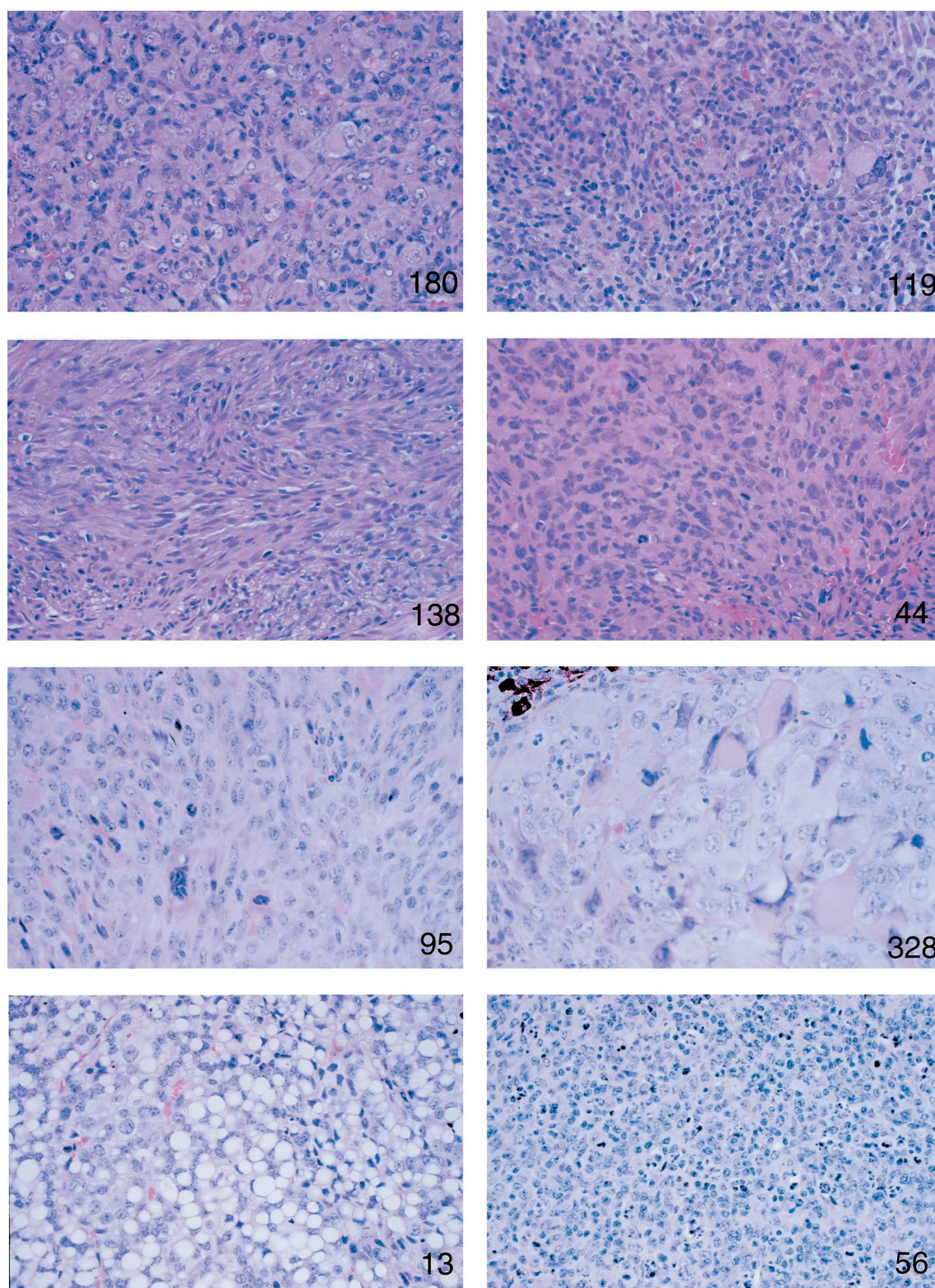


FIG. 4. *Ini1*-heterozygous mice present with various tumors. Microscopic features of different tumors from *Ini1*<sup>in3/+</sup> mice. Paraffin-embedded tumors were sectioned, stained with hematoxylin and eosin, and examined under a microscope at magnification  $\times 75$ . Mouse numbers corresponding to those presented in Table 2 are indicated in each panel.

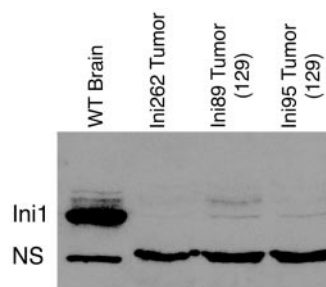


FIG. 5. Loss of heterozygosity at the *Ini1* locus results in tumor formation. Tumor samples and control tissue (wild-type [WT] male brain) were processed for Western analysis as detailed in Materials and Methods. The band corresponding to the Ini1 protein is indicated. NS represents a nonspecific band.

indicate that *Ini1*-null embryos either failed to be implanted into the uterine wall or were implanted and were reabsorbed shortly thereafter. In order to examine further the developmental defect of *Ini1*<sup>in3/in3</sup> embryos, we analyzed the ability of blastocysts from *Ini1*<sup>in3/+</sup> intercrosses to expand in vitro. When 3.5-d.p.c. blastocysts were plated in culture, wild-type and *Ini1*<sup>in3/+</sup> blastocysts hatched from the zona pellucida and were implanted onto the tissue culture plastic. Both wild-type and *Ini1*<sup>in3/+</sup>-implanted embryos formed the trophectoderm and expanded their inner cell mass (ICM). In contrast, no *Ini1*<sup>in3/in3</sup> blastocysts hatched and were implanted in culture (Fig. 3). The results of these experiments suggest that the peri-implantation embryonic lethality of *Ini1*-null mice may be due to a defect in the hatching of the blastocyst from the zona pellucida, an obligate step for implantation of the embryo into the wall of the uterus during normal development. Manual disruption of the zona pellucida of 19 (C57BL/6 × 129) blastocysts harvested from *Ini1*<sup>in3/+</sup> intercrosses did not result in expansion of the *Ini1*-null trophectoderm or ICM during in vitro culture, suggesting that growth of these tissues also is compromised (data not shown). Expression of *Ini1* in ES cells, which are derived from the ICM of 3.5 d.p.c. blastocysts, is consistent with a gene crucial to the peri-implantation or preimplantation stage of embryogenesis.

In humans, loss of *IN11* is correlated with a variety of tumors, the vast majority of which are neuronal or renal in nature. To date, most human malignant rhabdoid tumors and choroid plexus carcinomas examined have deletions and/or mutations in *IN11*, as do a subset of central primitive neuroectodermal tumors and medulloblastomas (39). In mice, we found that approximately 15% of *Ini1* heterozygotes in both the mixed F1 (C57BL/6 × 129) or pure 129 backgrounds presented with tumors. All of these tumors arose in the head or neck regions of the mice, particularly in the soft tissue of the face (Table 2). While 2 of the 15 mouse tumors analyzed thus far had varying degrees of rhabdoid-like cells, none had the characteristic, monomorphous appearance of human rhabdoid tumors. Two *Ini1*<sup>in3/+</sup> mice were found to have a lymphoproliferative disorder or lymphoma originating in an ill-defined region on the neck (Fig. 4). Two-thirds of the tumors originated on the faces of the mice. Interestingly, expression of *Ini1* appears to be elevated during development in the branchial arch and in the frontonasal and maxillary processes (Fig. 2B), structures which contribute to formation of the face. While the majority of the facial tumors were poorly differentiated or

undifferentiated sarcomas and not neuronal in origin, it is possible that the tumors arose in cells derived from neural crest progenitors, since neural crest cells, along with mesodermal cells, coordinate to form the facial primordia (12, 38).

We have analyzed tumors in three representative mice. Northern analysis of total RNA harvested from tumor tissue indicated the presence of wild-type-length *Ini1* message (data not shown). However, Western blot analysis of proteins harvested from these tumors revealed the absence of Ini1 protein in all three samples (Fig. 5). This indicates that loss of heterozygosity at the *Ini1* locus is responsible for tumor formation in the *Ini1*<sup>in3/+</sup> mice.

The mechanism of *Ini1*-mediated tumor suppression is unclear. Other subunits of the human and mouse SWI/SNF chromatin remodeling complexes have been reported to associate with known tumor suppressors, including Rb and Brca1 (3, 11, 48, 56), and several of the SWI/SNF subunits appear to be molecular targets of viral regulators of cell proliferation (18, 27, 28, 53). In addition, one of these subunits, BRG1, recently has been reported to be missing or mutated in a variety of human tumor cell lines, and reintroduction of BRG1 into these tumor cells reverses their transformed morphology (52). These findings suggest a role for chromatin remodeling in regulation of cell growth and/or in tumor suppression.

While this report was in preparation, Roberts et al. and Klochendler-Yeivin et al. published data consistent with our findings (21, 36). The fact that these results are reproducible in knockout lines generated by different targeting strategies confirms the importance of Ini1 in development and tumorigenesis. Klochendler-Yeivin et al. (21) further report in their study that Ini1-deficient embryos can induce the formation of maternal decidua, suggesting that Ini1-deficient embryos undergo hatching and implantation prior to their demise. In contrast, *Ini1*<sup>in3/in3</sup> embryos fail to hatch from the zona pellucida, suggesting that subtle strain variations may influence the precise timing of embryonic lethality. In agreement with these other groups, a percentage of the *Ini1*-heterozygous mice in our colony presented with tumors that contained variable numbers of rhabdoid cells. However, we are hesitant to classify these undifferentiated sarcomas as true rhabdoid tumors, which are described as monomorphous tumors in the human population. Discrepancies between tumor types associated with disruption of *Ini1* in humans and in mice may be due to differences in species-specific differentiation pathways. Regardless, the *Ini1*-heterozygous mice should provide a useful model for studying the general mechanisms involved in tumor suppression by Ini1.

#### ACKNOWLEDGMENTS

We thank J. Castillo and D. Hill for help in preparing the manuscript. We also thank A. Fraire and R. Hesselton for assistance with histopathology.

This work was supported in part by grants from the NIH to A.N.I. and S.N.J. A.N.I. is supported by a Scholar Award from the Leukemia and Lymphoma Society.

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