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THE ROLE OF THE SWI/SNF COMPONENT INI1 IN MAMMALIAN DEVELOPMENT AND TUMORIGENESIS

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This thesis is dedicated to my sister, Lisa, and family friend, Michael, two lives that were much too short. In life, Lisa taught me to have fun and not take everything so seriously. In death, she has taught me that life is too short to take anything, or anyone, for granted. Science is a tough career, but never forget what is truly important in life. Michael succumbed to leukemia at the age of 14. It was a disease he battled for more than half of his life. Through it all he always had a great zest for life. It was because of Michael that I first because interested in research. He has been my inspiration throughout college and graduate school. Whenever things get tough, I think of Michael and the courage he had, and I know I can get through anything.

ABSTRACT

In vivo DNA is compacted tightly, via its association with histones and non-histone proteins, into higher-order chromatin structure. In this state, the DNA is refractory to the cellular factors that require access to DNA. The repressive nature of chromatin is alleviated in part by the action enzymes that modify chromatin structure. There are two major groups of chromatin modifying enzymes: those that post-translationally modify histones by the addition of small chemical moieties and those that utilize the energy derived from ATP hydrolysis to physically disrupt chromatin structure. The SWI/SNF enzyme belongs to this latter group.

The SWI/SNF complex was identified originally in yeast. Several of its subunits are required for the expression of a subset of inducible genes. The ATPase activity is provided by the SWI2/SNF2 protein. In mammals, there are two biochemically separable SWI/SNF complexes that contain either BRG1 or BRM, both homologs of yeast SWI2/SNF2. The yeast and mammalian SWI/SNF complexes are able to disrupt the Dnase I digestion pattern of in vitro assembled mononucleosomes and arrays, as well as facilitate the accessibility of restriction nucleases and transcription factors. The mechanism by which SWI/SNF functions has yet to be elucidated.

SNF5 is a component of the yeast SWI/SNF complex. It is required for sucrose fermentation and mating type switching. The mammalian homolog of

Snf5 is SNF5/INI1. SNF5/INI1 was identified simultaneously by two groups as a protein that shares homology with Snf5 and via a yeast two hybrid assay as a protein that interacts with HIV integrase (INtegrase interactor). INI1 is a component of all mammalian SWI/SNF complexes purified to date.

In humans, mutations and/or deletions in *INI1* are associated with a variety of cancers, including malignant rhabdoid tumors, choroid plexus carcinomas, medullablastomas, primitive neuralectodermal tumors, and some cases of leukemia. Furthermore, constitutional mutations within *INI1* in individuals presenting with these tumors support the role of INI1 as a tumor suppressor.

In this thesis, we show that Ini1 also functions as a tumor suppressor in mice. Approximately 20% of mice heterozygous for *Ini1* present with tumors. Most of these tumors are undifferentiated or poorly differentiated sarcomas with variable rhabdoid features. All tumors examined to date show loss of heterozygosity at the *Ini1* locus. We also show that Ini1 is essential for embryonic development. Mice homozygous-null for *Ini1* die between days 4 and 5.5 post-fertilization due to an inability to adhere to their substratum, form trophectoderm, and expand their inner cell mass.

We further characterize the function of Ini1 in tumor suppression by generating mice heterozygous for both *Ini1* and either *Rb* or *p53*. While heterozygosity at the *Ini1* locus appears to have no effect on the rate of tumorigenesis in *Rb*-heterozygous mice, many of the tumors arising in compound

heterozygous mice present with an altered morphology. This finding suggests that Ini1 may contribute to tumor progression due to loss of Rb. In contrast, mice compound heterozygous for *Ini1* and *p53* show a marked reduction in the rate of tumorigenesis compared to *p53*-heterozygous mice. Furthermore, the tumor spectrum is altered in these compound heterozygous mice. These findings suggest that Ini1 may function normally to repress p53 activity.

Lastly, we show that expression of the Ini1 tumor suppressor itself is regulated tightly. Tissues and cells heterozygous for *Ini1* express roughly equivalent levels of Ini1 protein and mRNA as their wild-type counterparts. We further show that this compensation is mediated by an increase in the rate of transcription from the wild-type *Ini1* allele. Moreover, when exogenous Ini1 is introduced into *Ini1*-heterozygous cells, expression from the Ini1 promoter is reduced. These data indicate that a compensatory mechanism exists to ensure that the steady-state levels of Ini1 are constant.

In summary, research detailed in this thesis has contributed to our understanding of the regulation of Ini1 as well as the role this protein plays in mammalian development and tumor suppression.

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Chapter I

Introduction

A. Chromatin Structure

The core particle of chromatin structure is the nucleosome. Combined data obtained from micrococcal nuclease digestion as well as x-ray and electron crystallography at 7 Å resolution indicate that the nucleosome consists of approximately 146 base pairs of DNA wrapped in 1.8 helical turns around the histone octamer (61, 168, 240). The octamer itself has a $(H3)_2(H4)_2$ tetramer at its center with an H2A-H2B dimer at each end of the DNA path. Each histone has a polypeptide chain fold known as the "histone fold" (9). The histone fold is formed by a long, central α-helix that is flanked on either side by shorter helices and loops that interact with DNA. At the amino terminal end of each histone are 15-30 residues that comprise the "histone tail". The histone tails appear unstructured at this resolution.

The 2.8 Å resolution crystal structure (Figure 1.1) shows that the phosphodiester backbones of the DNA strands on the inner surface of the superhelix contact the octamer every ten base pairs, where the minor groove of the double helix faces inward (144). The amino-terminal tails of both H2B and H3 pass through the gap in the DNA superhelix formed by aligned minor grooves to the outside of the core particle. The H2A and H4 tails pass across the superhelix on the flat faces of the particle to the outside as well. The position of

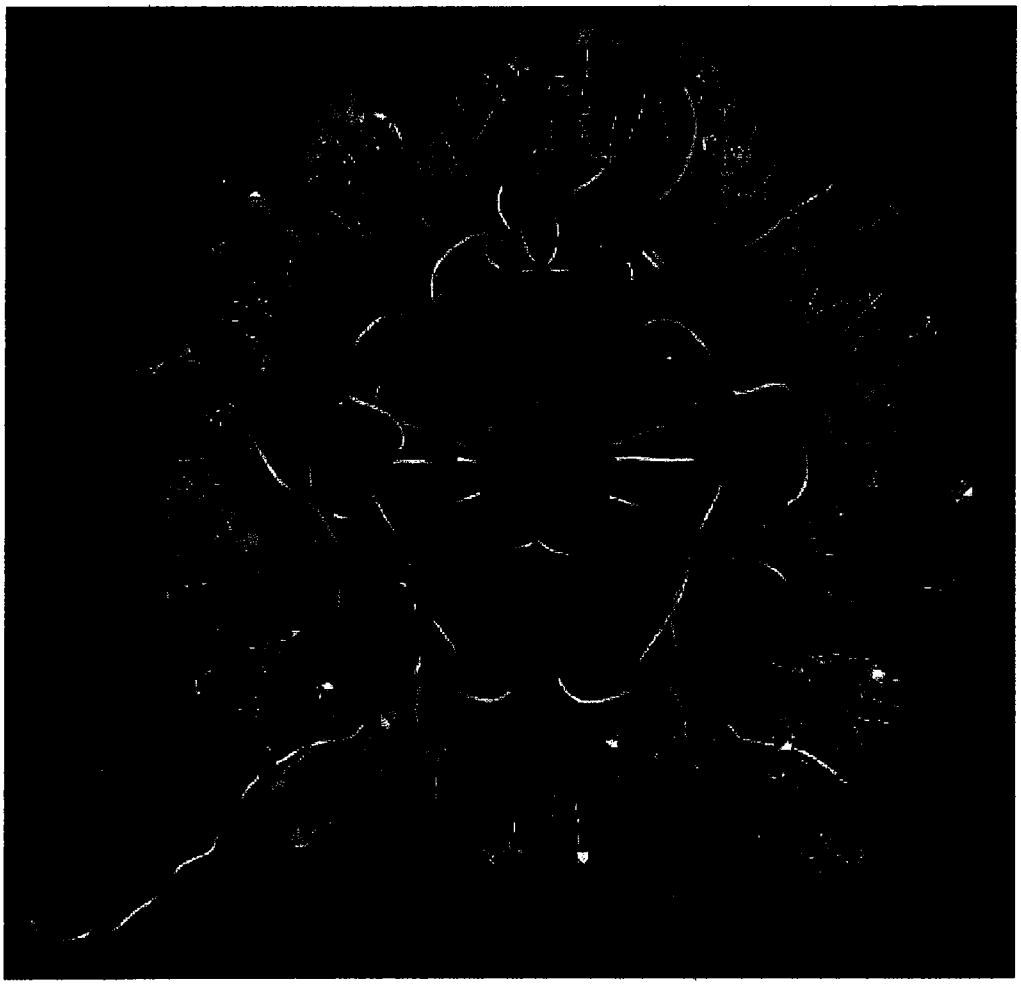


Figure 1.1: 2.8 Å resolution crystal structure of nucleosome core particle (144)

the tails suggests that they are exposed. The 16-25 amino terminal residues of the H4 tail extend into the adjacent nucleosome to interact with the negatively charged face of the H2A-H2B dimer. This interaction may mediate higher order folding.

The 1.9 Å resolution x-ray crystal structure of a nucleosome core particle containing 147 base pairs of DNA shows that water molecules and ions play an important role in nucleosome structure (45). The water molecules serve as hydrogen bond bridges between the histone proteins and DNA. It has been suggested that these bonds diminish the requirement for sequence specificity in nucleosome positioning. Monovalent anions are located in proximity to the DNA phosphodiester backbone and may partially neutralize the electrostatic interaction between histones and DNA. Divalent cations, bound at specific sites in the nucleosome, contribute to histone-histone and histone-DNA interactions between adjacent nucleosomes. As with the histone tail of histone H4, these divalent cations may participate in higher order folding.

The DNA between adjacent nucleosomes is called linker DNA. The histone H1 binds to the linker DNA near one end of the core DNA inside the chromatin fiber (280). Chromatin fibers are composed of arrays of nucleosomes, linker histones, and trans-acting factors. *In vitro*, nucleosomal arrays adopt an extended 10nm diameter or 30nm diameter fiber depending on ionic strength of the medium. Tail-less chromatin fibers can neither fold into 30nm fibers nor form fiber-fiber associations, suggesting that the tails play an important role in higher

order chromatin structure (37). The 30nm fiber is the basic component of both interphase chromatin and mitotic chromosomes; however, the mechanism by which these fibers are packed into the highly condensed, organized structure of the mitotic chromosome is not well understood. Recent data indicate that a macromolecular complex called condensin is required for proper chromosome condensation, but how this complex functions is unclear (43, 87, 224). Furthermore, the core histone tails, but not histone H1, also are required for mitotic chromosome condensation (47).

Chromatin structure generally inhibits the function of transcriptional machinery. The packaging of promoters in nucleosomes prevents the initiation of transcription by bacterial and eukaryotic RNA polymerases *in vitro* (116, 141). *In vivo*, when H4 synthesis is inhibited, several TATA-containing promoters are activated in the absence of their normal activation mechanisms (79). Furthermore, in DNA microarray analysis, nucleosome loss results in activation of 15% of yeast genes, not including the ~40% of the yeast genome that is constitutively active (75, 265).

B. Chemical Modification of Chromatin Structure

The core histone tails, and in some cases the histone H1 tail, are susceptible to a wide range of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, glycosylation, and ADP-ribosylation. The effects of these modifications on gene expression are varied.

While chemical modification of histone tails is not the focus of this thesis, some of these modifications, including histone acetylation/deacetylation, methylation, phosphorylation, and ubiquitination are discussed in this introduction, with a particular emphasis on their links to cancer.

1. Histone Acetylation

Hyperacetylation of histone tails has been correlated with increased gene activity (72, 74, 83, 84, 221). The regions of the histone tails that are acetylated are conserved, often invariant, lysine residues. Mutation of acetylatable lysines in histone H4 of *Saccharomyces cerevisiae* shows that these residues are required for activation of regulated genes. It is believed that the changes in the charge of histone tails resulting from acetylation either weakens histone:DNA contacts, alters histone:histone interactions between neighboring nucleosomes, or disrupts histone:regulatory protein interactions, or a combination of all three (85, 144, 145, 234, 258).

Histone Acetyl Transferases, or HATs, are responsible for the acetylation of histones. They can be divided into two categories: A-type and B-type. B-type HATs are cytoplasmic and likely catalyze acetylation events linked to transport of newly synthesized histones from cytoplasm to nucleus for deposition onto newly replicated DNA (4, 196). A-type HATs include nuclear HATs that likely catalyze transcription-related acetylation events (25). The A-type HAT proteins can be divided, based on sequence, into distinct families that show high sequence

similarity within families but poor to no sequence similarity between families. These families include the GNAT superfamily, the MYST family, the p300/CBP family, the basal transcription factors, and the nuclear receptor cofactors (193).

The GNAT superfamily encompasses the GCN5-related N-acetyltransferases (166). They contain limited sequence homology within four, 15-35 residue, motifs (named A-D). This family includes the prototype GCN5/PCAF, as well as Hat1, Elp3, and Hpa2. The first description linking histone acetyltransferase activity to gene activation came in 1996 with the finding that the *Tetrahymena* histone acetyltransferase A had homology with the yeast GCN5, a known transcriptional activator (24, 25).

The MYST family is named for the founding members: MOZ; Ybf2/Sas3; Sas2; and Tip60. It also includes Esa1, MOF, and Hbo1. Many members of the MYST family contain chromodomains (chromatin organization modifier), protein-protein interaction domains often found in heterochromatin-associated proteins (106). It is possible that these domains serve to target members of the MYST family to chromatin targets. The MYST family has been linked to cancer via the founding member, MOZ (monocytic leukemia zinc finger protein). As its name implies, *MOZ* is an oncogene, translocations of which are involved in certain cases of monocytic leukemia (21, 33, 34). *MOZ* is the human homologue of yeast Ybf2/Sas3, the catalytic subunit of NuA3, a yeast HAT complex that specifically acetylates histone H3 (70, 105, 187). Though MOZ has not been

demonstrated to possess HAT activity, the sequence similarity to Sas3 suggests that it is likely a HAT.

P300 and CBP were isolated independently as factors that interact with adenovirus E1A protein (p300) or with the phosphorylated form of the transcription factor CREB (CBP) (41, 58). Both share sequence similarities and their function is interchangeable *in vitro* (7, 8, 146). Each contains three putative zinc finger regions, a bromodomain (a domain that interacts with acetyl-lysine residues), a HAT domain, and at least two independent regions that interact with multiple transcription factors. They are transcriptional coactivators; they do not bind DNA directly. They interact with many factors including, but not limited to c-jun, c-myb, c-fos, TFIID, MyoD, nuclear hormone receptors, and E2F-1 (12, 44, 60, 101, 102, 108, 152, 169, 197, 273). Their HAT activities are required for their functions in transcriptional activation (11, 152, 170).

The first line of evidence linking misregulation of HAT activity to cancer came from the finding that the adenoviral E1A oncoprotein targets p300/CBP (8, 58). Overexpression of E1A prevents binding of p300/CBP to PCAF and induces entry of cells into S phase (269). The transforming activity of E1A depends on its ability to interact with and sequester p300/CBP, as excess p300/CBP inhibits E1A-mediated cell immortilization.

The gene encoding CBP has been shown to be involved in chromosomal translocations in certain leukemias. In acute myeloid leukemia, the t(8;16)(p11;p13) translocation results in the fusion of *CBP* to the human

oncogene *MOZ* (21). This fusion creates a protein with two HAT domains. Recruitment of this protein by CBP or MOZ cofactors may bring inappropriate HAT activity to target promoters. In addition, two inversions within chromosome 8 that are associated with leukemia fuse *MOZ* to transcriptional intermediary factor 2 (*TIF2*), a p300/CBP interacting protein with intrinsic HAT activity (33, 34). The resulting fusions retain the HAT domains of both proteins.

The t(11;16)(q23;p13) chromosomal translocation, found in many leukemias, fuses *CBP* to *MLL/ALL-1* (209). *MLL/ALL-1* is the human homologue of Drosophila trithorax, a protein that functions during development in maintenance of open chromatin configuration for proper expression of homeotic genes. Additionally, a *MLL-p300* translocation has been described in a patient with AML (94).

There is evidence suggesting that *CBP* is a bona fide tumor suppressor. *CBP* heterozygosity is associated with Rubenstein-Taybi Syndrome (RTS), a human disorder characterized by cranial and digital malformation, mental retardation, hematopoietic abnormalities and higher risk for developing certain types of cancer (155, 180). *CBP* has been targeted in mouse knock out experiments (122, 171). *CBP* heterozygous mice display various developmental defects and develop a high incidence of hematological malignancies, including histiocytic sarcomas and myelogenous and lymphocytic leukemias. Tumorigenesis is correlated with loss of heterozygosity in transformed cells.

The histone acetyltransferase p300 also may be a tumor suppressor. A number of human tumors, including glioblastomas, colorectal cancers, and breast cancer, show loss of heterozygosity of *p300* (65, 160). In a study examining a variety of primary tumors or tumor cell lines for mutations in *p300*, ten of 193 were shown to have loss of function mutations (65). *P300* has been targeted in mouse knock out experiments (271). However, there have been no reported cases of malignancy in *p300* heterozygous mice.

Overexpression of some histone acetyltransferases has been correlated with cancer. The nuclear hormone cofactor ACTR is overexpressed in several breast and ovarian cancers (5). Though it is unclear if this is a cause or effect of these cancers, it is possible that overexpression of ACTR leads to increased activation of target genes which in turn may lead to increased cellular proliferation. Additionally, the RNA polymerase III transcription factor TFIIIC2, which is an acetyltransferase, is overexpressed in ovarian tumors, contributing to the abnormal abundance of pol III transcripts in these tumors (257).

2. Histone Deacetylation

While histone acetylation is associated with gene activation, histone deacetylation is associated with gene repression. In fact, many gene products that were known to act as corepressors were later found to have deacetylase activity. The link between histone deacetylation and gene repression first was demonstrated by the isolation of the human histone deacetylase HDAC1, which

has sequence highly similar to the yeast Rpd3, a known negative regulatory protein (228). Histone deacetylases (HDACs) are categorized, based on homology, into two classes. The first class includes the yeast HDACs Rpd3, Hos1, and Hos2 as well as the mammalian histone deacetylases HDAC1-3, and 9. The second class consists of yeast Hda1 and mammalian HDAC4-8, and 10. Most HDACs are associated in multisubunit complexes; substrate specificity is regulated by components of these complexes.

The mammalian HDAC1 and HDAC2 have been shown to play important roles in cellular growth arrest (46, 118, 147). The multiprotein complex SIN3-HDAC consists of both HDAC1 and HDAC2, along with the scaffolding protein SIN3 and at least eight other proteins (3, 86, 162). This co-repressor complex has been shown to associate with the basic helix-loop-helix-zipper protein Mad and is required for Mad-induced transcriptional repression. The repression mediated by this complex prevents the activation of target genes such as E2F and cdc25, leading to growth arrest in a wide range of cells. HDAC activity appears to be required for the ability of Mad to induce growth arrest, as inhibitors of deacetylase activity partially overcome this effect.

The SIN3-HDAC complex also plays an important role in retinoblastoma tumor suppressor protein (Rb)-mediated repression (reviewed in (80)). Rb controls cellular proliferation by repressing transcription of genes required for progression through G1 and S of the cell cycle. Rb is recruited to target genes via its interaction with the E2F family of transcription factors. Rb represses E2F-

mediated transactivation by two mechanisms; it blocks the E2F transactivation domain and it actively represses E2F promoters. The deacetylase activity of the SIN3-HDAC complex helps to repress E2F-regulated genes.

Certain forms of leukemia are associated with misregulation of SIN3-HDAC activity. RAR is a transcriptional regulator that responds to retinoids and is important for the differentiation of cells into many lineages, especially myeloid lineages (39). RARs recruit the SIN3-HDAC complex, via N-CoR (nuclear receptor corepressor) or SMRT (silencing mediator for retinoid and thyroid receptors), to promoters containing RARE (retinoic acid response element) sequences. In the presence of retinoic acid (RA), the SIN3-HDAC complex is released from RAR allowing the TIF2-CBP HAT complex to bind to a domain on RAR that is masked in the absence of ligand (3, 86, 162, 163). In this manner, retinoic acid is able to induce genes containing RARE sequences.

Chromosomal translocations resulting in the fusion of the *RAR* gene to the gene encoding PML have been associated with some cases of human acute promyelocytic leukemia (APL) (71, 137). The normal function of PML is unclear; however, it is known to homodimerize and to interact with HDACs (153). PML-RAR fusion proteins retain the regions of RAR required for DNA and ligand binding, as well as the regions of PML required for HDAC interaction and homodimerization. Leukemogenesis is believed to result from the dimerization of the fusion proteins and subsequent stronger association with HDACs. HDAC association is maintained at physiological levels of RA, but released at high

levels of ligand. Patients with PML-RAR translocations often go into remission after treatment with pharmacological doses of retinoic acid. In other forms of APL, *RAR* is fused to *PLZF* (promyelocytic leukemia zinc finger) (71, 137). The normal function of PLZF is not known, though it is able to homodimerize and interacts with SIN3-HDAC. The PLZF-RAR fusion protein retains these known abilities of PLZF. The SIN3 protein is not released from PLZF-RAR even at high concentrations of RA, and patients with this translocation are resistant to treatment with pharmacological does of retinoic acid. Interestingly, inhibitors of histone deacetylase activity have been shown to dramatically potentiate retinoid-induced gene activation of RA-sensitive and restore retinoid response of RA-resistant APL cell lines (71, 137). This finding suggests that the RAR fusion proteins mediate leukemogenesis through aberrant chromatin acetylation.

3. Histone Methylation

Lysine histone methyltransferases contain a conserved methyltransferase domain termed a SET [Su(var)3-9, Enhancer-of-zeste, Trithorax] domain (reviewed in (119, 200). To date, not all SET-domain containing proteins have been shown to have methyltransferase activity, though lack of detectable activity may be due to inappropriate assay conditions. The effect of histone methylation on gene activation is varied. The lysine histone methyltransferases are divided into four families: SUV39, SET1, SET2, and RIZ (119, 200).

The SUV39 subfamily includes: Suv39h1, Suv39h2, EuHMTase1, G9a, ESET, and CLLL8. Su(var)3-9 originally was identified in a genetic screen as a suppressor of position effect variegation in Drosophila melanogaster. The SET domain of Su(var)3-9 is the founding member of the SUV39 subfamily of SET domains. The mouse homologues are Suv39h1 and Suv39h2 (186). Though mice deficient for either gene are phenotypically normal, double knockout mice of *Suv39h1/h2* display dramatic genomic instability (176). They are predisposed to cancer and approximately one-third of the mice develops late-onset B-cell lymphoma. A common feature of these tumors is non-segregated chromosomes that are linked via acrocentric regions. These knockout mice have a greatly reduced level of H3 K9 methylation, suggesting that the methyltransferase activity of Suv39h1/h2 is important for suppressing tumorigenesis. The human SUV39H1/2 methyltransferase has been linked to oncogenesis via its interaction with Rb (167). This interaction is required for correct regulation of the gene encoding cyclin E, which is important in cell cycle regulation (172). Many human cancers have mutations in Rb and some of these Rb mutants fail to bind SUV39H1 (167). It is possible that the interaction between Rb and SUV39H1 plays a significant role in tumor suppression.

The SET1 subfamily includes hSET7 and ySET1, both of which have been shown to possess a H3 K4-specific methyltransferase activity (191, 249, 268). Other members of this subfamily have not been shown to have methyltransferase activity. These include the polycomb (PcG) proteins EZH1 and EZH2. Polycomb

genes are a group of genes required to repress homeotic (hox) gene activity. MLL1-3 and ALR, trithorax (trxG) genes that are required to maintain hox gene activity, also belong to the SET1 subfamily. There are many links between members of the SET1 subfamily and cancer. *MLL1* is translocated in many leukemias (10, 275, 281). In fact, over 30 different chromosomal fusions of this region have been observed and all of these fusions lack the SET domain. Additionally, deletions in exon 8 of *MLL1* have been observed in acute lymphoblastic leukemias (138). A partial duplication of *MLL1* has been documented in acute myeloblastic leukemia and gastric carcinoma cell lines (198). It is unclear if these mutations in *MLL1* result in tumorigenesis due to loss of function of the normal *MLL1* product, a gain of function of the fusion proteins, or a combination of both. Another MLL gene product, MLL2 is amplified in some solid tumor cell lines (93). Chromosomal aberrations of the third MLL gene, *MLL3*, are associated with hematological neoplasia and holoprosencephaly, a congenital malformation of the brain and face (225). The polycomb gene *EZH2* is upregulated in tumor cell lines (247). It is localized to a region crucial for malignant myeloid disorders (35), and its SET domain interacts with XNP, which is mutated in different inherent disorders, including ATR-X syndrome (36).

The SET2 subfamily includes NSD1-3, HIF1, AND ASH1. The founding member of this subfamily, the S. cerevisiae SET2 protein, has intrinsic histone methyltransferase activity specific for H3 K36 (217). Members of the mammalian nuclear receptor-binding SET-domain containing (NSD) family contain a SET

domain that is highly related to that of ySET2; however, NSD proteins have yet to be shown to possess methyltransferase activity. NSD1 can enhance androgen receptor (AR)-mediated transactivation in prostate cancer, though it is unclear if this is a cause or result of oncogenesis (254). In the t[5,11](q35;p15.5) translocation in acute myeloid leukemia, *NSD1* is fused to the *NUP98* gene, which encodes a nucleoporin that plays a role in nuclear trafficking (100). In addition, truncations in the SET domain of *NSD1* have been identified in individuals with Sotos syndrome, a familial disorder linked with a predisposition to cancers such as Wilm's tumor, hepatocarcinomas, mixed paratoid tumors, and osteochondromas (123). *NSD2* maps to a region deleted in the Wolf-Hirschhorn syndrome (WHS) critical region (212). Deletions in this region cause WHS, which is characterized by mental retardation and developmental defects. *NSD2* often is found fused to the *IgH* gene in multiple myeloma (149, 212). The third member of the NSD family, *NSD3*, is amplified in several breast cancer cell lines and in primary breast carcinomas (211). In addition, this gene also is found fused to *NUP98* in acute myeloid leukemia (192).

The RIZ subfamily includes RIZ, BLIMP-1, MEL1, PFM1, and MDS1-EVI1. The SET domain of the RIZ protein was the founding member of this subfamily. None of the proteins in this subfamily have been shown to possess methyltransferase activity. The *RIZ* gene encodes for two proteins, RIZ1 and RIZ2, via the use of two alternative promoters (1). RIZ2 is identical to RIZ1 except that it lacks the first 200 amino acids, including the SET domain. RIZ1

expression is reduced or lost in many types of cancer including breast cancer, lung cancer, osteosarcomas, hepatoma, neuroblastoma, and colorectal cancer (1, 92). Frameshift mutations in *RIZ* have been found in 37% of primary tumors of the colon, stomach, endometrium and pancreas (29, 183). Furthermore, mice deficient for RIZ1 are prone to develop diffuse B-cell lymphomas and a broad spectrum of unusual tumors (213). It is interesting to note that *RIZ1*-deficient mice present with similar tumors as mice deficient for the Suv39h1/h2 methyltransferases. The *MDS1-EVI1* gene encodes for two products: the SET-domain-containing MDS1-EVI1 and the EVI1 protein that lacks the SET domain (59). Certain chromosomal rearrangements cause disruption of the MDS-EVI1 protein and activation of the EVI1 protein leading to myeloid leukemia. Furthermore, EVI1 is overexpressed in solid tumors and leukemia (59). Another RIZ subfamily member, *BLIMP-1*, is deleted in B-cell-non-Hodgkin lymphoma (109, 157). *MEL1* is transcriptionally activated by translocation in acute myeloid leukemia (156). Lastly, *PFM1* maps to a tumor suppressor locus on chromosome 12 (270).

Clearly, a large number of the SET-domain-containing proteins play an important role in cell cycle regulation. In fact, misregulation of a number of these proteins has been linked to a variety of cancers. In some cases, tumorigenesis has been linked to the diminished methyltransferase activity of the disrupted gene products. However, not all of the SET-domain proteins have been shown to possess methyltransferase activity. As such, it is not clear if a

methyltransferase activity of all of the described factors is required for their normal activity.

4. Histone Phosphorylation

The core histones and histone H1 undergo phosphorylation on specific serine and threonine residues. Phosphorylation of H3 and H1 are cell cycle regulated, with the highest level of phosphorylation occurring during M-phase (68, 77, 175, 255). Phosphorylation of H1 has been associated with transcriptional activation of the MMTV promoter (130). Phosphorylation of H3 also has been shown to play a role in the transcriptional induction of immediate early genes in mammalian cells (38, 148). H3 residues within the promoter of c-fos and c-myc are rapidly phosphorylated in serum starved cells when the Ras-mitogen activated protein kinase (MAPK) pathway is stimulated by growth factors. Furthermore, the mitotic phosphorylation of H3 also is associated with chromosomal condensation. The condensation of chromosomes during mitosis is essential for the proper transmission of parental genetic information to daughter cells. The aurora kinase family is involved in histone H3 phosphorylation (91). Members of the aurora kinase family are overexpressed in a variety of cancers including colorectal cancers and invasive ductal carcinomas of the breast (18, 226, 227, 279). The mechanism by which overexpression of aurora kinase family members leads to tumorigenesis is unclear; however, this

finding points to the importance of proper regulation of histone phosphorylation in maintaining normal cellular proliferation.

5. Histone Ubiquitination

Histones H2A, H2B, H3, and the linker histone H1 can be reversibly ubiquitinated. The carboxyl-terminus of the ubiquitin molecule is covalently attached via an isopeptide bond to the ε-amino group of lysine. Approximately five to fifteen percent of total H2A and ~1.5% of total H2B present in mammalian cells are monoubiquitinated (114, 135, 136, 256). Ligation of ubiquitin moieties to short-lived proteins tags them for degradation by the 26S proteasome; however, mono-ubiquitinated histones do not appear to be tagged for degradation *in vivo* (201, 264). The biological significance of histone ubiquitination is unclear. Studies suggesting that ubiquitinated histone H2A is associated with transcriptional activation are contrasted by those that suggest ubiquitination of histone H2A result in gene repression (for review see (104)). To date, only one tentative link between misregulation of histone ubiquitination and cancer has been published. The levels of ubiquitinated H2A were found to be highly upregulated in SV-40 transformed human fibroblasts and keratinocytes, suggesting that this modification may play an important role in cell cycle control (242).

C. ATP-dependent Chromatin Remodeling

ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to alter chromatin structure. Every ATP-dependent chromatin-remodeling complex contains an ATPase subunit that is highly conserved across species. Each of the ATPase subunits belongs to the SWI2/SNF2 superfamily of proteins. Based on the homology of the ATPase subunit, these complexes can be classified into three subfamilies: the SWI2/SNF2 subfamily; the ISWI subfamily; and the CHD subfamily (Figure 1.2). While some attention is given to each of these subfamilies, the bulk of discussion concentrates on SWI/SNF, the focus of this thesis.

1. CHD Subfamily

CHD (chromo-helicase-DNA-binding) proteins have a SWI2/SNF2-like helicase/ATPase domain, a DNA-binding domain, and a chromodomain. This subfamily includes S. cerevisiae Chd1, human NURD complexes, Xenopus Mi-2 complex, and Drosophila Mi-2 complex.

The yeast Chd1 has not been found to assemble into a complex, but rather appears to dimerize (232). Chd1 has an ATPase activity that is stimulated by DNA and nucleosomes. Chd1 is able to alter, to some extent, the DNase I digestion pattern of *in vitro* assembled mononucleosomes. Yeast strains bearing chd1-null deletions are viable; however, chd1-null mutants are synthetically lethal

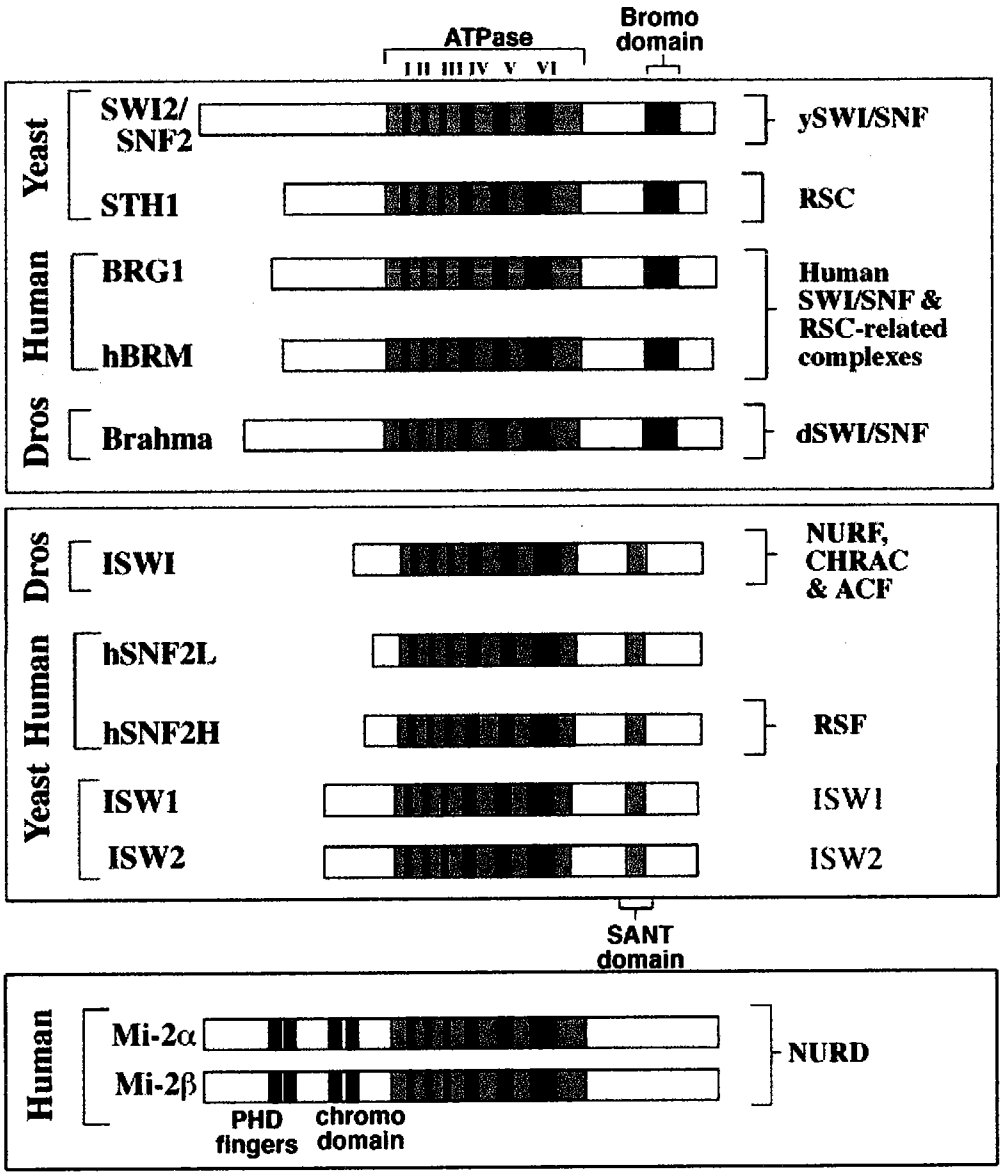


Figure 1.2: Comparison of ATPase subunits of SWI2/SNF2 superfamily (113)

with swi2-null mutants, suggesting that Chd1 and SWI/SNF may share redundant functions. In human cells, a complex possessing both ATP-dependent chromatin remodeling activity and histone deacetylase activity was purified simultaneously by three groups. These complexes were named NURD (nucleosome remodeling and histone deacetylation), NuRD, and NRD (nucleosome remodeling and deacetylating) (231, 267, 277). It is unclear whether these are identical complexes or separate, highly related complexes. They contain one or both of two human CHD proteins, CHD3/Mi-2α and/or CHD4/Mi-2β. CHD3/Mi-2 α and CHD4/Mi-2 β are highly related proteins that are autoantigens in dermatomyositis, a human disease that predisposes 15-30% of those afflicted to cancer (66, 202). Recombinant Mi-2 protein was found to have ATPase activity similar to that of intact NuRD complex (250). The histone deacetylase activity of these complexes is provided by HDAC1 and HDAC2. These complexes also contain either MTA1 or MTA2 (metastasis-associated protein), expression of which correlates with the metastatic potential of several human cancer cell lines and tissues (230). The NuRD complex has been shown to contain two alternatively spliced forms of MBD3 (Methyl-CpG Binding Domain). Furthermore, this complex interacts with MBD2, a protein that is believed to link NuRD to methylated DNA. MBD2 also has been identified as NY-CO-41, a human cancer antigen that is recognized by autoantibodies from some colon cancer patients (248).

2. ISWI Subfamily

Members of the ISWI subfamily contain a subunit that shares homology with the *Drosophila* ISWI (Imitation Switch) protein. These subunits are homologous to Swi2/Snf2 only in their ATPase domain. The ATPase activity is stimulated by nucleosomal DNA.

In *Drosophila*, three ISWI-containing complexes have been identified: NURF (nucleosome remodeling factor), CHRAC (chromatin accessibility complex), and ACF (ATP-utilizing chromatin assembly and remodeling factor) (98, 235, 237, 241). Aside from ISWI, the constituents of these complexes vary. All share the ability to regularly space nucleosome arrays in an ATP-dependent fashion; however, only CHRAC has been shown to increase the accessibility of restriction enzymes to chromatin templates. Drosophila ISWI is essential for cell viability. Interestingly, null and dominant-negative mutations in ISWI resulted in alteration of the structure of the male X-chromosome, suggesting that this factor plays a role in higher order chromatin structure (53).

Two homologues of Drosophila ISWI, Isw1p and Isw2p, have been identified in yeast cells (67, 236). These two subunits are present in distinct complexes. Like Drosophila ISWI, Isw1p and Isw2p possess an ATPase activity that is stimulated by nucleosomal DNA. Isw1p- and Isw2p-containing complexes have an ATP-dependent nucleosome remodeling and spacing activity.

In humans, the Drosophila ISWI-homologue, hSnf2H, has been purified in four, apparently distinct, complexes: RSF (remodeling and spacing factor),

WCRF, ACF, and hCHRAC (19, 133, 134, 184). Like their homologues, these complexes have an ATPase activity that is stimulated by nucleosomal DNA. Furthermore, they remodel and space nucleosomes in an ATP-dependent manner. The RSF complex also has been shown to stimulate transcriptional initiation from a promoter within a nucleosome template. The WCRF and ACF complexes contain WSTF (Williams syndrome transcription factor) protein, which has been found to be mutated in the developmental disorder Williams syndrome.

3. SWI2/SNF2 Subfamily

The SWI2/SNF2 subfamily includes *S. cerevisiae* SWI/SNF, RSC (remodels the structure of chromatin), and INO80.com; *Drosophila* Brahma; and mammalian SWI/SNF. The activity of the ATPase subunit of each of these complexes is stimulated by both DNA and nucleosomes (31, 42, 56, 97, 124, 182). The ATPase subunits also share a C-terminal bromodomain and two other conserved regions of unknown function (260).

a. Ino80.com

The Ino80 protein was identified in yeast based on its homology to the Swi2/Snf2 ATPase (207). Ino80 also has homologues in Drosophila (dINO80) and humans (hINO80). The yeast Ino80 associates with approximately 12 proteins in a complex called Ino80.com. This complex possesses a 3' to 5' DNA helicase activity, though it has yet to be determined if Ino.com is able to alter

chromatin structure. Ino80-null mutants are viable but are sensitive to hydroxyurea, methyl methanesulfonate, ultraviolet light, and ionizing radiation, suggesting a role for Ino80.com in DNA damage response.

b. RSC Complex

The yeast RSC complex contains the ATPase Sth1, a protein that shares high homology with Swi2/Snf2 (31). This complex consists of 15 subunits, some of which share homology with other members of the yeast SWI/SNF complex. Rsc8/Swh3, Rsc6, and Sfh1 are homologs to SWI/SNF subunits Swi3, Swp73, and Snf5, respectively. Unlike the yeast SWI/SNF constituents, members of the RSC complex are required for mitotic growth (32). The RSC complex catalyzes the transfer of histone octamers from one strand of DNA to another (142). It is also able to increase the accessibility of restriction nucleases to nucleosomal templates (140). The remodeled state persists after removal of RSC and ATP, and can be reversed upon re-addition of RSC and ATP.

It is unclear if mammalian cells contain a complex homologous to yeast RSC. The BAF180 subunit of the SWI/SNF-B complex shares homology with three yeast RSC complex subunits, Rsc1, Rsc2, and Rsc4 (266). This has led some to propose that SWI/SNF-B is the mammalian homologue of yeast RSC (164). Furthermore, the mammalian SWI/SNF-B complex localizes to the kinetochores of mitotic chromosomes, suggesting that this complex may play a similar to RSC in cell cycle progression.

c. SWI/SNF complexes

The SWI/SNF complex first was identified in yeast (30, 177). It is comprised of 11 subunits, with the core ATPase subunit encoded by the SWI2/SNF2 gene. None of the members of the yeast SWI/SNF complex are required for viability; however, several of its components originally were isolated as being required for mating type switching (SWI) and sucrose fermentation (SNF) (23, 165, 214). These phenotypes are due to the fact that SWI/SNF is required for induction of the mating type switch gene, HO as well as the SUC2 invertase that is required for sucrose fermentation. The first hint that SWI/SNF plays a role in chromatin remodeling came from the discovery that several mutations that suppressed *swi/snf* phenotypes corresponded to genes encoding histones and non-histone components of chromatin structure (89, 120, 121). SWI/SNF later was shown to alter the DNase I digestion pattern of *in vitro* assembled mononucleosomes, giving credence to the idea that it could directly alter chromatin structure. In addition, the activity of SWI/SNF can facilitate the binding of a number of transcription factors and accessibility to restriction nucleases to nucleosomal DNA templates (28, 42, 139, 239). Data obtained from DNA microarray expression analysis indicate that approximately 5% of yeast genes that are constitutively expressed are dependent on the ATPase activity of SWI/SNF. Interestingly, SWI/SNF appears to be involved in the repression of just as many genes as it activates (90, 222). Recently, SWI/SNF was shown to directly repress transcription of the yeast gene SER3 (151).

Mammalian SWI/SNF complexes contain one of two SWI2/SNF2 ATPase homologues, BRM (SNF2α) or BRG1 (SNF2β) (252). The mammalian SWI/SNF complex is composed of 8-12 subunits, with its composition differing slightly between cell types. Like the yeast counterpart, mammalian SWI/SNF complexes are able to disrupt the DNase I digestion pattern of in vitro assembled mononucleosomes and increase the accessibility of some transcription factors to nucleosomal templates (97, 124).

The mechanism by which SWI/SNF alters chromatin structure is unclear. There is data that have led some to suggest that SWI/SNF either removes or rearranges the H2A-H2B dimers to facilitate remodeling (42, 89, 131, 144, 178, 210). Other studies demonstrate that SWI/SNF is able to induce nucleosome sliding or octamer transfer (103, 181). Lastly, experiments utilizing cross-linking reagents demonstrate that the octamer need not be perturbed in order for SWI/SNF to alter the DNase I cleavage pattern or restriction enzyme accessibility of in vitro assembled mononucleosomes or arrays (6, 22).

Components of mammalian SWI/SNF complexes have been implicated in a variety of cellular processes. SWI/SNF complexes are involved in gene activation events associated with nuclear hormone receptors, environmental stress, and viral infection (2, 13, 40, 48, 64, 159, 174). Mammalian SWI/SNF components also play a role in gene repression events; the repression of c-fos and some E2F-regulated genes are mediated in part by SWI/SNF (161, 233, 251). Furthermore, members of the SWI/SNF complex are targets of viral

regulatory proteins of Epstein-Barr virus, adenovirus, human papilloma virus, and human immunodeficiency virus (HIV) (107, 127, 128, 154, 262).

SWI/SNF complexes play a role in several developmental processes. The induction of genes necessary for muscle differentiation in vitro require SWI/SNF activity (49). Functional SWI/SNF complexes were shown to be required for the alteration of chromatin structure, concomitant with muscle differentiation, in the promoter region of myogenin, a muscle specific transcription factor required for the differentiation process. Mouse knock-out experiments have demonstrated that the SWI/SNF components Brg1, Ini1, and Srg3 play an essential role in murine development, as mice homozygous-null for any of these factors are early embryonic lethal (27, 76, 112, 115, 188).

SWI/SNF constituents associate with a number of known tumor suppressors. Both BRG1 and BRM have been shown to interact with the Rb tumor suppressor and facilitate the repression of certain gene expression events required for entry into S-phase. In fact, BRG1 or BRM is required for Rb-dependent G1 arrest (57, 218, 276). SWI/SNF also has been shown to interact with the breast cancer susceptibility gene product, BRCA1 (20). The ATPase activity of BRG1 is required for the ability of BRCA1 to stimulate p53-mediated transcription, suggesting that SWI/SNF may function in recombination and repair pathways. Ini1 also has been shown to interact with, and facilitate the function of, the growth arrest and DNA-damage inducible protein GADD34, providing another link between SWI/SNF and recombination and repair (263). Moreover,

BRG1 and INI1 interact directly with the p53 tumor suppressor in co-immunoprecipitation experiments (126). This interaction appears to facilitate activation of some p53-responsive genes.

Misexpression of *BRG1* and *BRM* has been found in a number of human tumor cell lines and primary tumors. Expression of *BRG1* and *BRM* is down-regulated or absent in tumor cell lines derived from various tissues, including prostate, lung, and breast (259). In another study, both alleles of *BRG1* were found to be mutated in 2 out of 22 breast carcinoma cell lines examined (50). On the contrary, BRG1 was found to be overexpressed in approximately 60% of gastric carcinomas examined (203).

Results from mouse knock-out experiments reveal variable roles for Brg1 and Brm in tumorigenesis. Mice lacking Brm are viable but show mild proliferative effects, suggesting a role for Brm in the control of cellular proliferation (188). Mice lacking Brg1 are early embryonic lethal (27). Furthermore, a small percentage of mice heterozygous for *Brg1* develop apocrine tumors. However, loss of heterozygosity in the tumors has yet to been demonstrated.

SNF5/INI1 is a core subunit of all mammalian SWI/SNF complexes purified to date (252). It originally was identified based on its homology to the yeast Snf5 protein and by a yeast two-hybrid screen as a protein that interacts with HIV-1 integrase (INtegrase Interactor 1) (107, 158). Bi-allelic deletions or truncating mutations of *INI1* have been shown to be associated with most cases

of malignant rhabdoid tumor, a rare but aggressive pediatric cancer of the soft tissues (16, 17, 51, 194, 238, 245). Mutations in *INI1* also have been found in other neuronal tumors such as choroid plexus carcinomas, medullablastomas, and central primitive neuroectodermal tumors (15, 204). Furthermore, deletions of *INI1* have been reported in chronic phase and blast crisis of chronic myeloid leukemia (69). Recent studies indicate that germ-line mutations in *INI1* predispose afflicted individuals to some of these cancers (204, 229).

Mice lacking Ini1, like those lacking Brg1, are early embryonic lethal (76, 115, 190). Approximately 30% of mice heterozygous for *Ini1* develop undifferentiated or poorly differentiated sarcomas, with variable rhabdoid features, of soft tissues. In these cases, tumor occurrence has been correlated with loss of heterozygosity at the *Ini1* locus. Furthermore, almost 100% of mice bearing a reversibly inactivating conditional allele of *Ini1* present rapidly with tumors. While many of these tumors have variable rhabdoid features, the vast majority are CD8+ T-cell lymphomas (189).

The INI1 protein has no known conserved motifs that may provide insight into its function. While its exact function is not clear, INI1 is able to facilitate HIV proviral DNA integration (107). INI1 also has been shown to repress expression of cyclin D1 in INI1-deficient tumor cells to induce cell cycle arrest (278). Other studies utilizing similar cell lines show that INI1 induces p16ink4a to induce cell cycle arrest (14). INI1 has been shown to interact with ALL-1, translocations of which are involved in human acute leukemias (195). The *Drosophila* homolog of

ALL-1, trithorax, likewise has been shown to interact with the INI1 homolog snr1. Trithorax is a member of the trithorax-polycomb gene family that is required to maintain proper expression of Antennapedia and Bithorax complexes throughout embryogenesis (110). Like *Ini1*-null mice, homozygous snr1 mutants die early during embryogenesis (54). These similarities between snr1 and INI1 hint at the possibility that INI1 may play a role in homeotic gene regulation early in mammalian development.

It is unclear if BRG1 and INI1 function independently as tumor suppressors or function cooperatively via an activity of the SWI/SNF complex. Heterozygous disruption of *Brg1* and *Ini1* in mouse models results in divergent phenotypes. However, while disruption of Ini1 may affect both Brg1 and Brm-containing complexes, it is possible that Brm is able to partially compensate for Brg1-deficiency. Clearly, Brm is unable to compensate for the absence of Brg1 in early development. This may be due to the fact that during early mouse embryonic development, Brg1 and Brm show differences in their levels of expression as well as localization at the blastocyst stage (132). On the contrary, the level of Brm message is comparable to that of Brg1 in adult tissues and many cell lines. In human tumor cell lines lacking BRG1, BRM is able to compensate for BRG1 function in cell cycle arrest mediated by Rb (219). Thus, it is possible that the presence of Brm in Brg1-heterozygous mice is sufficient to maintain the putative tumor suppressor function of SWI/SNF.

While it is possible that the ability of BRG1 and INI1 to function as tumor suppressors depends on their role in the SWI/SNF complex, recent data suggest that INI1 has functions distinct from those of BRG1 and BRM. As mentioned above, cell cycle arrest mediated by Rb depends on the presence of functional BRG1 or BRM. On the contrary, INI1 is not required for the ability of Rb to induce arrest (14, 244). When a constitutively active Rb is introduced into human tumor cell lines lacking INI1, the cells arrest in G1. Therefore, it is possible that the tumor suppressor function of Ini1 is distinct from its function as a member of the SWI/SNF complex. However, before the biological roles of INI1 can be compared to those of BRG1 or BRM, it is first necessary that the functions of INI1 be elucidated.

D. Thesis Aims

The focus of this thesis was the murine SWI/SNF subunit Ini1, with particular emphasis on its role in development and tumorigenesis. Chapter II details the generation and characterization of a murine model deficient for Ini1. The research described in this chapter demonstrated an essential role for Ini1 in murine development, as mice null for Ini1 died early in embryogenesis. Furthermore, mice heterozygous for Ini1 were predisposed to undifferentiated or poorly differentiated tumors, suggesting that Ini1 also plays an important role in tumor suppression.

Chapters III and IV present data aimed at understanding the mechanism by which Ini1 functions as a tumor suppressor. As detailed above, Ini1 has been linked to the tumor suppressors Rb and p53. Results from crosses of Ini1-heterozygous mice to Rb-heterozygous mice (Chapter III) and p53-heterozygous mice (Chapter IV) are described. We show that introduction of Ini1-heterozygosity on a Rb-heterozygous background does not alter the tumor curve from that observed for mice deficient solely for Rb. However, some of the tumors that arose in compound heterozygous mice presented with histology characteristic of more aggressive tumors. Mice heterozygous for Ini1 and p53, however, showed a decreased rate of tumorigenesis compared to p53-heterozgyous mice. In addition, the observed tumor spectrum was skewed.

In Chapter V, the regulation of Ini1 itself is examined. While Ini1 has been shown to play an important role in a diverse set of processes, little has been published as to its regulation. We show here that Ini1 is upregulated in cells and tissues heterozygous for Ini1. This upregulation is mediated by an increase in the rate of transcription from the Ini1 promoter, such that the steady level of Ini1 RNA from one allele in heterozygous cells approximately doubles that from wild-type cells. Furthermore, we provide evidence suggesting that Ini1 is involved in its own regulation.

Lastly, Chapter VI provides a brief discussion, with the aim of tying together the sum of these results to provide a better understanding of the regulation and function of Ini1.

Chapter II

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

Introduction

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 acetyl-transferases 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 (96, 113, 215, 216, 246). Included among these ATP-dependent chromatin remodeling enzymes is the SWI/SNF family of chromatin modifiers.

SWI/SNF enzymes are large multisubunit enzymes of ~1-2 MDa. Yeast SWI/SNF genes were originally identified as being required for mating type switching or sucrose fermentation (23, 165, 214). 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 (30, 117, 125, 177, 179, 272). Mutations in SWI/SNF genes could be suppressed by mutations altering histone gene expression, histone structure, or non-histone chromatin proteins leading to the suggestion that these gene products facilitated transcriptional activation by altering chromatin structure (89, 120, 121).

Human SWI/SNF (hSWI/SNF) complexes contain either the human BRM (hSNF2α) or BRG1 (hSNF2β) homologues of the yeast SWI2/SNF2 ATPase (40, 111, 159). Both yeast and human SWI/SNF complexes have been shown to possess nucleosome remodeling activity *in vitro* (42, 97, 124). 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 evidence supporting a role for SWI/SNF in gene activation events mediated by nuclear hormone receptors, environmental stress, and viral infection (2, 40, 48, 64, 159). In contrast, SWI/SNF components also were shown to be involved in repression of c-fos and some E2F-regulated genes (161, 233). 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 (57, 206, 218, 276). 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 (20). 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 (107, 128, 154, 261).

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 (188, 223). The upregulation of Brg1 in the Brm-deficient mice may provide a compensatory effect; however, one can not 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 (158, 253). INI1 was shown to interact with ALL-1, translocations of which are associated with several types of human acute leukemias (195). Furthermore, *INI1* has been found to be altered in malignant rhabdoid tumors, choroid plexus carcinomas, medullablastomas, and central primitive neuroectodermal tumors (17, 51, 204, 205, 245). Identification of constitutional mutations in a subset of these tumors indicates that the INI1 is a tumor suppressor (17, 205). In an attempt to generate a mouse model that would allow further characterization of the mechanisms of Ini1 in tumorigenesis as well as to determine the role of the mammalian SWI/SNF complexes in development, we generated mice deficient for *Ini1* expression. 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

ES cells (omnibank # OST32815) bearing a retroviral promoter trap that functionally inactivates one allele of *Ini1* were generated as described previously (274). RACE analysis also is described. Site of insertion was determined using sequence analysis.

Creation of *Ini1*-null mice

Ini1-targeted ES cells were injected into 3.5 d.p.c. C57BL/6 blastocysts. Male chimeric mice were mated with wild-type C57BL/6 or 129 females. Germline transmission of the mutant allele was determined by PCR analysis of tail genomic DNA using the following primers: for *Ini1*, 5'-GCAAGCGCTCTGCCAATTG ACC-3', 3'-CACACCCTATTGTCACTCTGGAA-5'; for *βgeo*, 5'-CGGTATCGATA AGCTTGATGATC-3', 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(88). 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', 3'-CTTCTGGAGACTTCACTTAC GTCC-5'.

Blastocyst culture

Blastocysts from heterozygous intercrosses were flushed from the uterus of *Ini1*$^{in3/+}$ females 3.5 d.p.c. with M15 media [DMEM, 15% fetal calf serum, 100 μM β-mercaptoethanol, 2 mM glutamine, and 1X penicillin/streptomycin] and cultured in tissue culture plates for 96 hr. Embryo cultures were genotyped as described above.

β-Galactosidase staining of cultured ES cells

Wild-type AB2.2 ES cells and *Ini1*$^{in3/+}$ ES cells were grown to near confluency and fixed in 0.5% Glutaraldehyde. Cells were then rinsed with PBS and stained overnight in the dark at room temperature in a solution containing 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide, 2mM $MgCl_2$, and 1mg/ml X-gal.

Whole mount staining of embryos for β-galactosidase activity

Embryos were harvested at indicated time points post fertilization and fixed in 4% paraformaldehyde for 20 min at 4°C. Embryos were washed and then stained in X-gal histochemical reaction mix [4mM Potassium Ferrocyanide, 4mM Potassium Ferricyanide, 2mM $MgCl_2$, 1mg/ml X-gal] overnight at room temperature. Following staining, embryos were rinsed in PBS 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 DTT, 1 μg/mL pepstatin A, 4 μg/mL leupeptin, and 1 mM PMSF. Extracts were resolved by SDS-PAGE and western analysis for Ini1 protein was performed as described previously(48).

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(81). Sections were prepared and stained with haematoxylin and eosin and examined under a microscope.

Results and Discussion

Mouse embryonic stem (ES) cells bearing a retroviral promoter trap that functionally inactivates one allele of *Ini1* were constructed as described previously (274). Sequence analysis revealed that the promoter trap inserted within intron 3 of *Ini1* (Figure 2.1). The beta-galactosidase-neomycin (β-geo) gene fusion cassette within the retroviral insertion has a 5' splice acceptor site; thus, β-geo expression is regulated by the native *Ini1* promoter. We were able to utilize the β-geo gene cassette in a colorimetric assay to determine if *Ini1* is

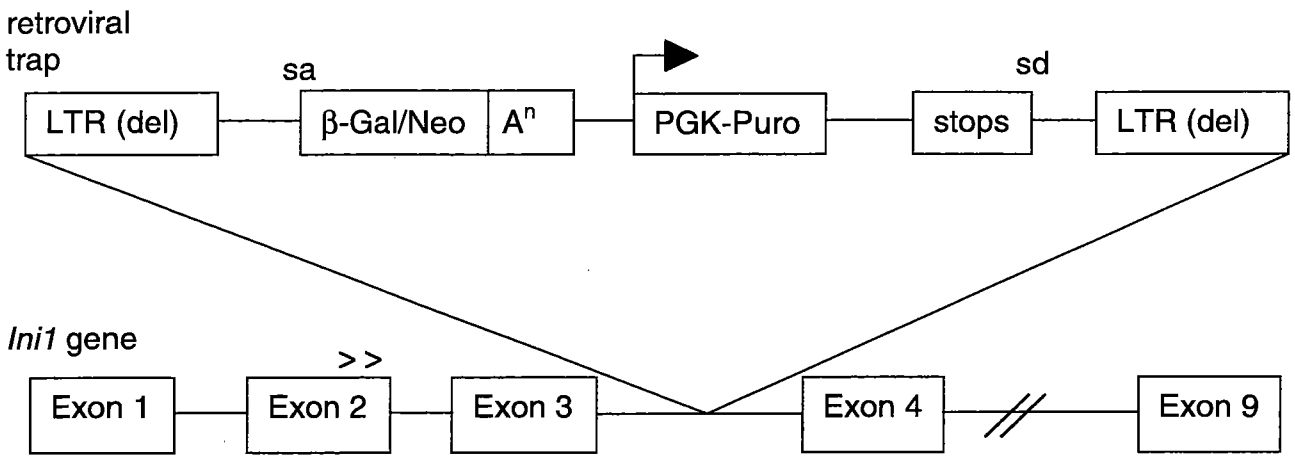


Figure 2.1: Schematic of targeted Ini1 allele

sa and sd represent splice acceptor and splice donator sites, respectively

LTR(del) are the retroviral long terminal repeat promoters with inactivating deletions

β-gal/neo is a β-galactosidase/neomycin gene fusion cassette

PGK-puro is a puromycin cassette under control of a PGK promoter

carets denote site of alternative splicing within the Ini1 gene

normally expressed in ES cells. *Ini1*-targeted cells stained positive for β-gal activity, indicating that *Ini1* is expressed in ES cells (Figure 2.2). Northern analysis of ES cell total RNA confirmed *Ini1* expression (data not shown). Furthermore, sequence data obtained from 5' RACE analysis of the *Ini1*-β-gal fusion mRNA revealed that transcripts utilizing either splice donor site in exon 2 spliced into the trap, indicating that both splice variants of *Ini1* were inactivated (26).

To determine the role of Ini1 in mammalian development and tumorigenesis, we used the targeted ES cells in blastocyst injection experiments to generate *Ini1*-heterozygous ($Ini1^{in3/+}$) mice. In order to monitor expression of *Ini1* during embryogenesis, we performed whole mount staining for β-galactosidase activity in embryos harvested from $Ini1^{in3/+}$ matings at various times during development. We found that $Ini1^{in3/+}$ 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 *Ini1* is ubiquitously expressed during embryogenesis (Figure 2.3). *Ini1* expression was also detected by northern analysis in a wide range of adult tissues (188) (and data not shown).

Chimeric mice generated from C57BL/6 strain blastocyst injections of the 129 strain-derived ES cells were bred to wild-type C57BL/6 or 129 mice in order to obtain $Ini1^{in3/+}$ mice on either a mixed (C57BL/6 x 129) or pure (129) background. Intercrosses of $Ini1^{in3/+}$ mice in both backgrounds yielded $Ini1^{in3/+}$ offspring and wild-type offspring at a 2:1 ratio (63:26 in the mixed background,

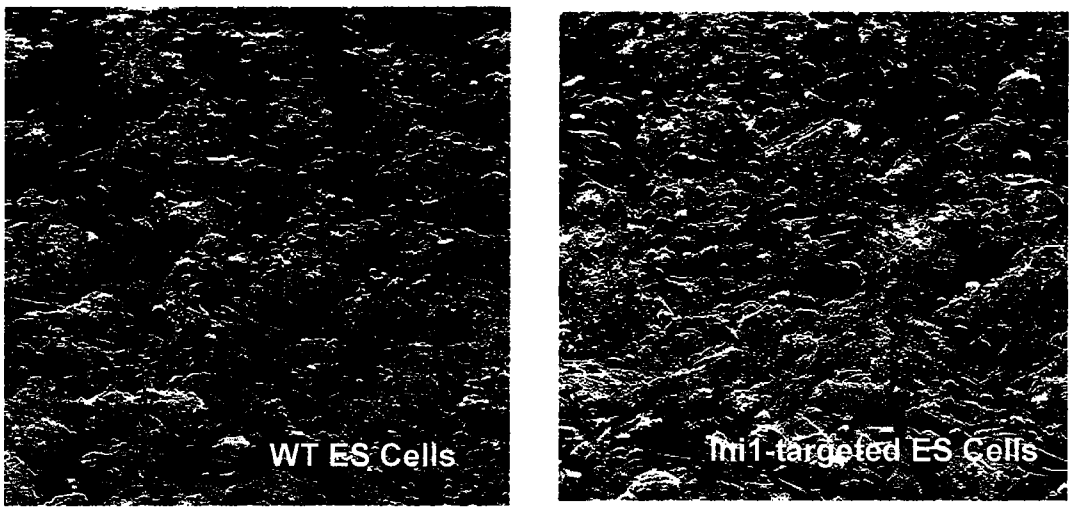


Figure 2.2: Mouse embryonic stem (ES) cells express Ini1

Wild-type and *Ini1*-targeted ES cells were stained for β-gal activity. Because β-gal is under control of the Ini1 promoter, cells that express Ini1 will stain blue.

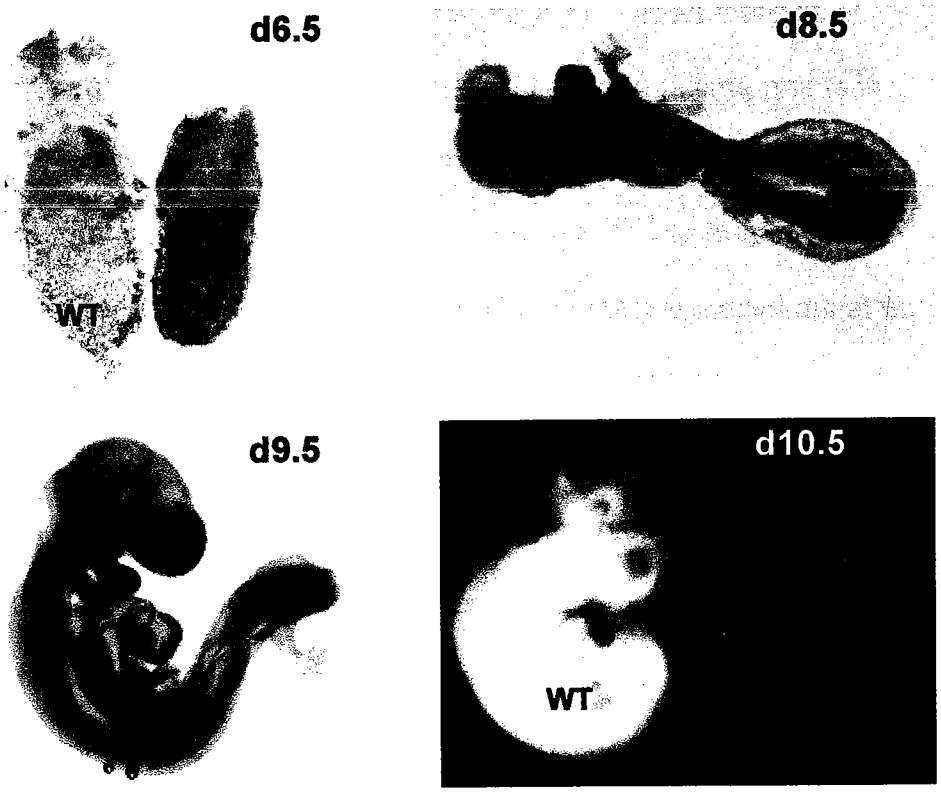
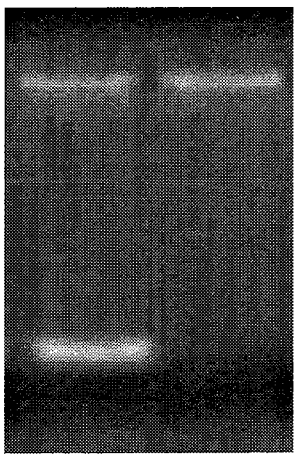


Figure 2.3: Ini1 is ubiquitously expressed throughout embryonic development

Embryos from Ini1-heterozgyote intercrosses were harvested at indicated times and stained for β-gal activity. Targeted cells will stain blue, while wild-type cells will not.

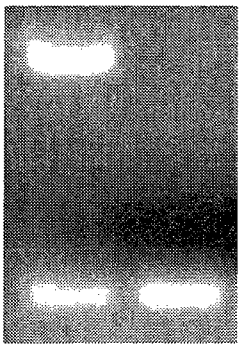
34:17 in the pure background), and no *Ini1*-null offspring, indicating that disruption of *Ini1* induces embryonic lethality (Figure 2.4). Timed matings of *Ini1*$^{in3/+}$ mice were performed and embryos harvested at various time points in gestation for genotyping via PCR. *Ini1*-null embryos could be isolated at 3.5 d.p.c. and were normal in appearance. However, no *Ini1*$^{in3/in3}$ embryos were detected at 6.5 d.p.c. or later (Figure 2.5). Dissection of maternal diciduas at 6.0-6.5 d.p.c. revealed no significant increase in the number of embryo reabsorptions, suggesting that *Ini1*$^{in3/in3}$ lethality occurred between day 3.5 and 5.5 of gestation. These results indicate that *Ini1*-null embryos either failed to implant into the uterine wall or implanted and were reabsorbed shortly thereafter. In order to examine further the developmental defect of *Ini1*$^{in3/in3}$ embryos, we analyzed the ability of blastocysts from *Ini1*$^{in3/+}$ intercrosses to expand *in vitro*. When 3.5 d.p.c. blastocysts were plated in culture, wild-type and *Ini1*$^{in3/+}$ blastocysts hatched from the zona pellucida and implanted onto the tissue culture plastic. Both wild-type and *Ini1*$^{in3/+}$-implanted embryos formed trophectoderm and expanded their inner cell mass (ICM). In contrast, no *Ini1*$^{in3/in3}$ blastocysts hatched and implanted in culture (Figures 2.5 and 2.6). The results of these experiments suggest that the peri-implantation embryonic lethality of *Ini1*-null mice may be due to a defect in 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



	+/+	+/-	-/-	Total
BL6	26	63	0	89
129	17	34	0	51

Figure 2.4: *Ini1*-null mice are embryonic lethal

Ini1-heterzygous mice were intercrossed and progeny genotyped by PCR. As shown above the WT allele generates a 700 bp band while the targeted allele generates a 250 bp band. No *Ini1*-null mice were obtained from these crosses.



d.p.f.	+/+	+/-	-/-
3.5	7	17	3
in vitro	14	25	1(dead)
6.5	9	23	0
8.5	4	14	0
13.5	5	6	0

Figure 2.5: *Ini1*-null embryos die between days 4 and 5.5 post-fertilization

Embryos from *Ini1*-heterozygote intercrosses were harvested at indicated times and genoyped by PCR. While *Ini1*-null embryos were obtained at day 3.5 post-fertilization, no *Ini1*-null embryos were obtained at day 6.5 and beyond.

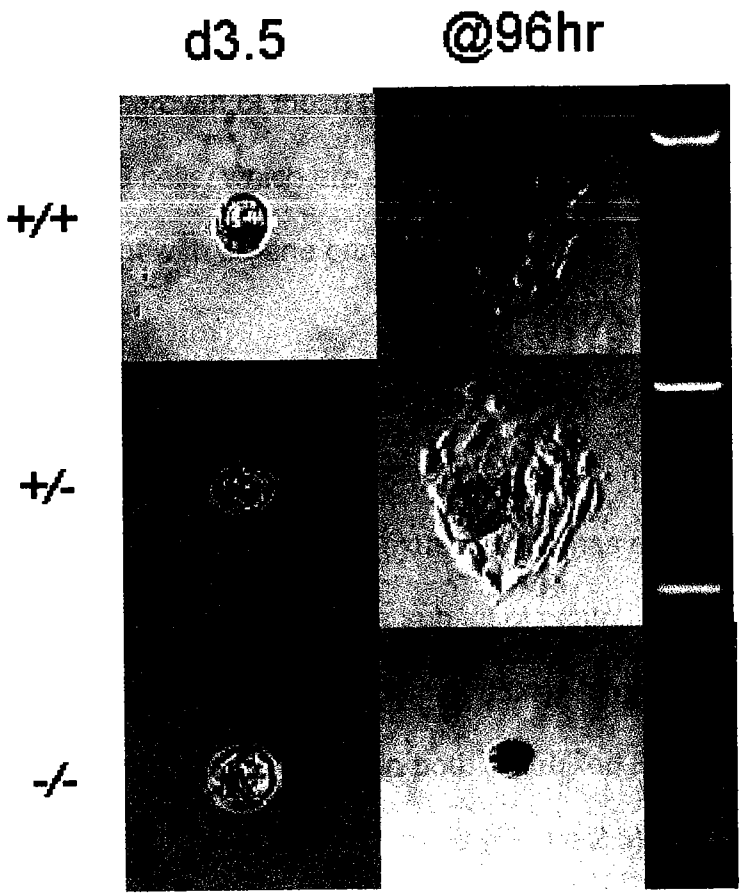


Figure 2.6: *Ini1*-null blastocysts fail to expand in culture

Blastocysts were harvested from *Ini1*-heterozygote intercrosses at 3.5 days post fertilization and plated in culture as described in the materials and methods. At 96 hours after culturing, outgrowths were harvested and genotyped by PCR. Unlike their wild-type and *Ini1*-heterozygous counterparts, *Ini1*-null blastocysts failed to attach to the substratum, form trophectoderm, and expand their inner cell mass.

pellucida of 19 (C57BL/6 x 129) blastocysts harvested from *Ini1*$^{in3/+}$ intercrosses did not result in expansion of *Ini1*-null trophectoderm or ICM during *in vitro* culture, suggesting that growth of these tissues also is compromised (Figure 2.7). 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- or pre-implantation stage of embryogenesis.

In humans, loss of *INI1* 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 *INI1*, as do a subset of central primitive neuroectodermal tumors and medullablastomas (204). In mice, we found that approximately 20% of *Ini1* heterozygotes in both the mixed F1 (C57BL/6 x 129) or pure 129 backgrounds presented with tumors (Figure 2.8). All of these tumors arose in the head or neck region of the mice, particularly in the soft tissue of the face (Figure 2.9). While 2 of the 15 mouse tumors analyzed had varying degrees of rhabdoid-like cells, characterized by their eosinophilic cytoplasms containing speroid perinuclear inclusion bodies and whorls of intermediate filaments, none had the monomorphous appearance of human rhabdoid tumors. Two *Ini1*$^{in3/+}$ mice were found to have a lymphoproliferative disorder or lymphoma originating in an ill-defined region on the neck (Table 2.1 and Figure 2.10). Two-thirds of the tumors originated on the face of the mice. Interestingly, expression of *Ini1* appears to be elevated during development in the branchial arch and in the

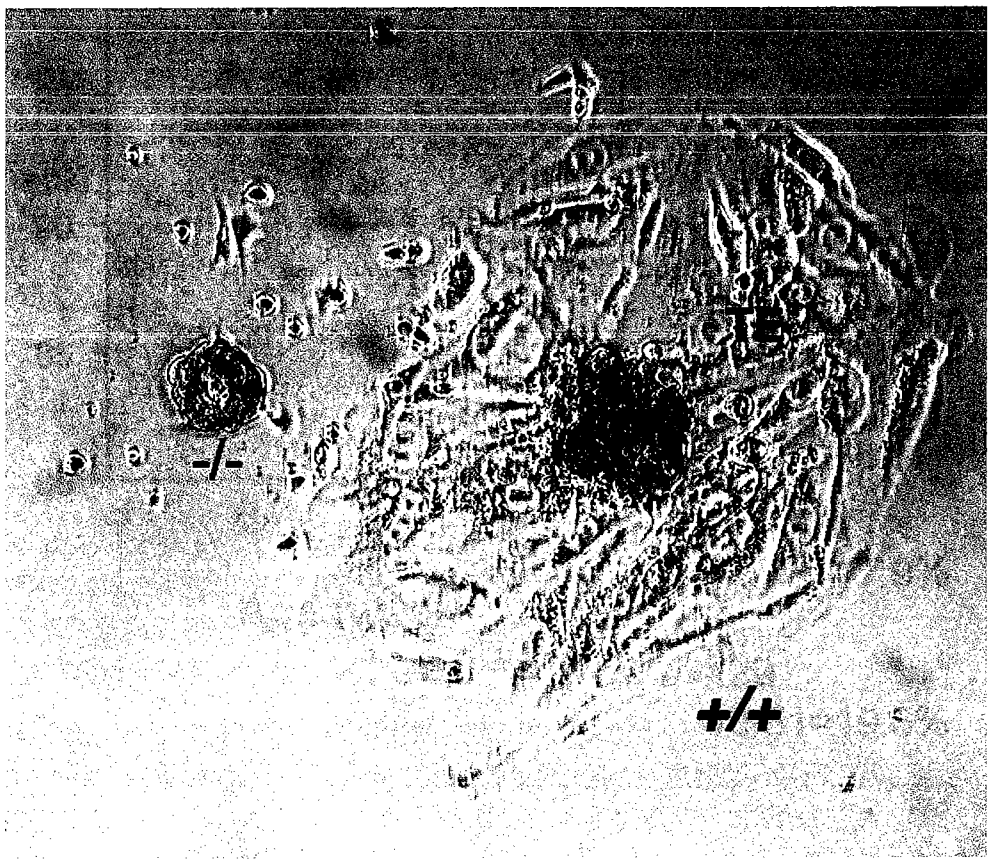


Figure 2.7: *Ini1*-null blastocysts fail to expand in culture when manually hatched

Ini1-heterozygous mice were intercrosses and blastocysts harvested at day 3.5 post-fertilization. Blastocysts were plated in culture and zona pellucidas were removed manually with a fine-pulled needle. After 96 hours in culture, outgrowths were harvested and genotyped by PCR. TE, trophectoderm; ICM, inner cell mass

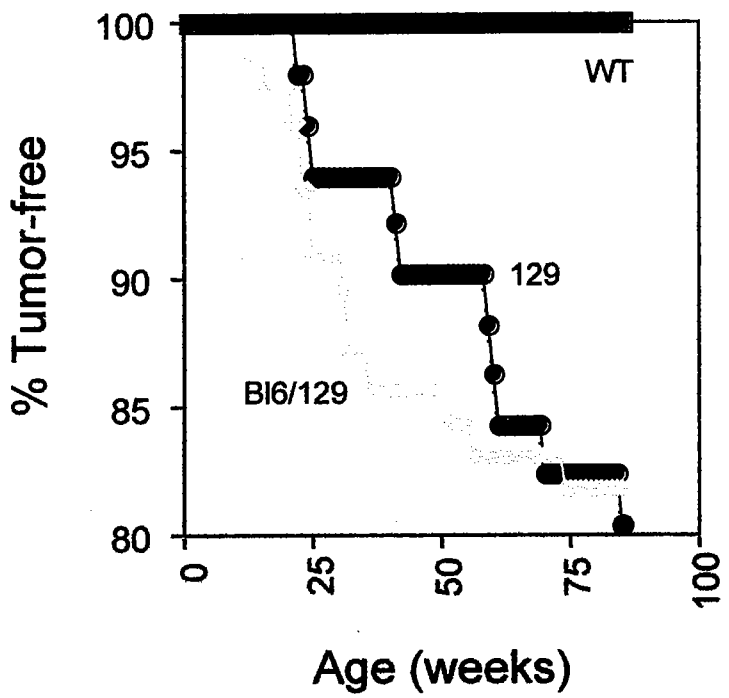


Figure 2.8: Tumor Curve for Ini1-heterozygous mice

Ini1-heterozygous mice on both a purebred and mixed background were monitored for tumor development. Mice presenting with tumors were graphed according to age in weeks.

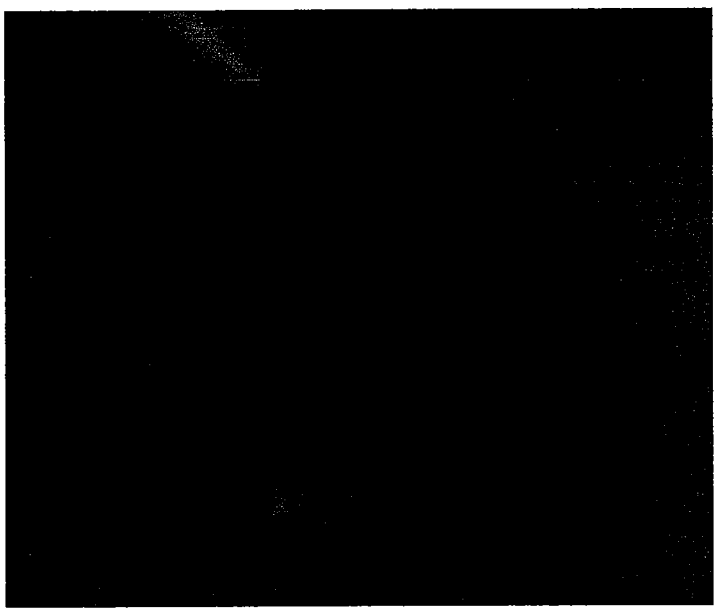


Figure 2.9: *Ini1*-heterozygous mice present with tumors mostly in the head and neck region

Photograph of *Ini1*-heterozygous mouse presenting with facial tumor common in this study.

Table 2.1: Tumor Occurrence			
Mouse	Age (weeks)	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*	24	face	malignant fibrous histiocytoma
Ini 262	16	face	malignant fibrous histiocytoma
Ini 29*	41	face	malignant fibrous histiocytoma
Ini 13*	59	face/eye	liposarcoma
Ini 56	52	neck	lymphoma
Ini 322	21	brain	undifferentiated sarcoma
Ini 10	32	ventral to brain	undifferentiated sarcoma
Ini 95*	22	face	malignant fibrous histiocytoma
Ini 127	12	neck	lymphoproliferative disorder
Ini 328	25	eye	undifferentiated sarcoma
*129 Strain			

Table 2.1: Representation of tumors from Ini1-heterozygous mice

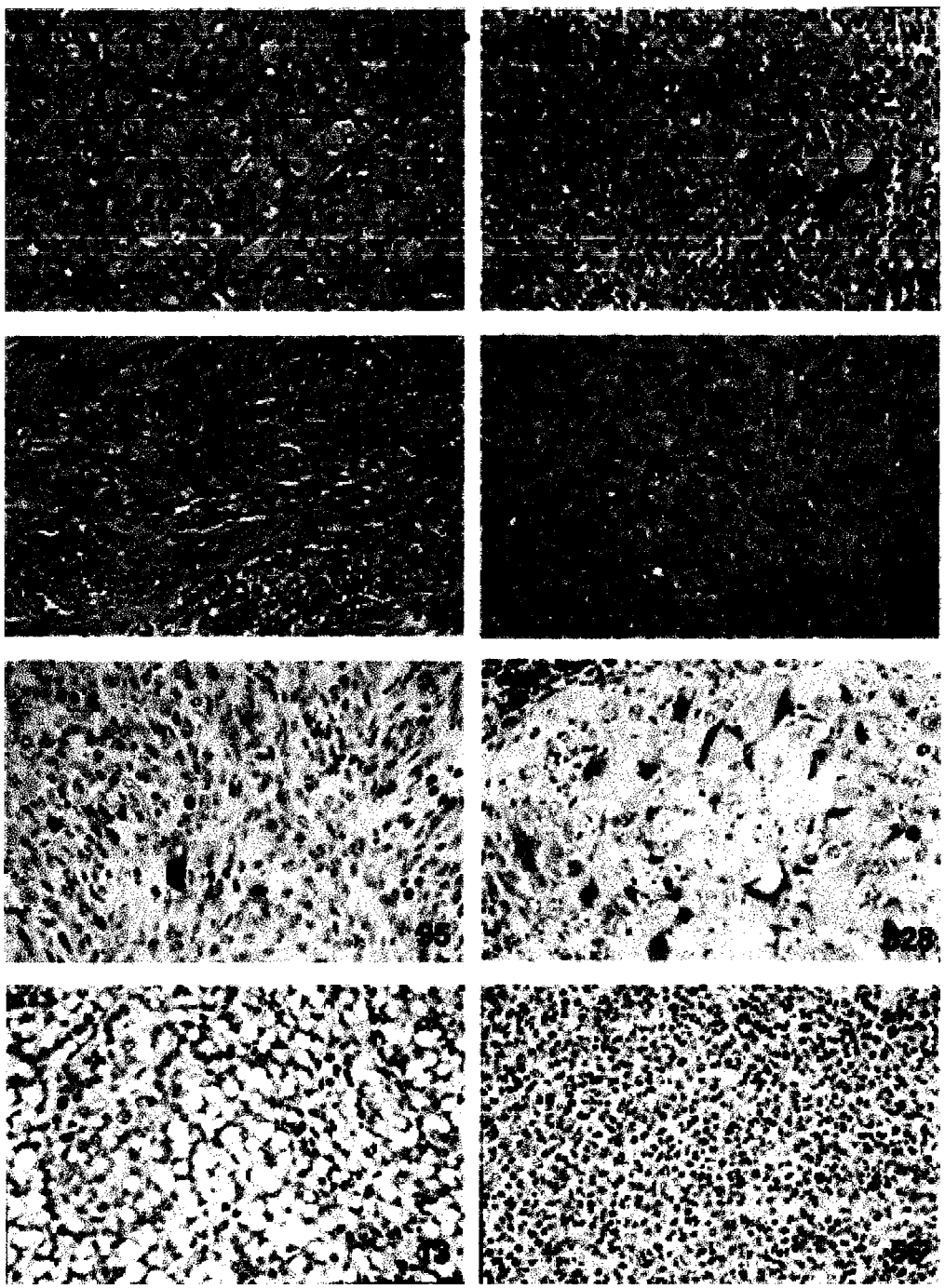


Figure 2.10

Figure 2.10: Tumor sections from *Ini1*-heterozyous mice

Tumor samples were harvested from mice and processed for H&E staining as described in the materials and methods. Most tumors were poorly differentiated or undifferentiated sarcomas with the exception of one liposarcoma (panel 13) and one lymphoma (panel 56). Some tumors show variable rhabdoid features (see arrow in panel 119). Numbers in figure correspond to mice listed in Table 2.1.

frontonasal and maxillary processes (Figure 2.3), structures that 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, as neural crest cells, along with mesodermal cells, coordinate to form the facial primordia (63, 199).

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 absence of Ini1 protein in all three samples (Figure 2.11). This indicates that LOH at the *Ini1* locus is responsible for tumor formation in the $Ini1^{in3/+}$ mice.

The mechanism of *Ini1*-mediated tumor suppression is unclear. Other subunits of the Swi/Snf chromatin remodeling complex have been reported to associate with known tumor suppressors, including Rb and Brca1 (20, 57, 233, 276), and several of the SWI/SNF subunits appear to be molecular targets of viral regulators of cell proliferation (107, 128, 154, 261). 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 (259). These findings suggest a role for chromatin remodeling in regulation of cell growth and/or in tumor suppression.

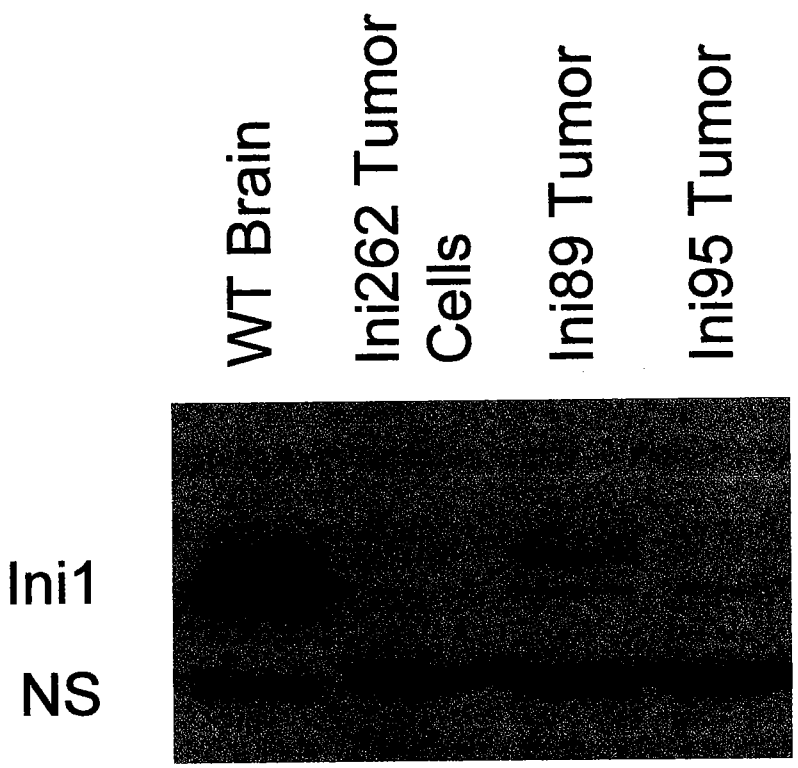


Figure 2.11: Tumors from *Ini1*-heterozygous mice show loss of heterozygosity

Tumor samples and control tissue were processed for Western analysis as described in the materials and methods. As shown above, tumor samples show a marked decrease in expression of Ini1 protein compared to wild-type brain, indicating loss of heterozygosity. NS, non-specific band

While this manuscript was in preparation, Roberts *et al.* and Klochendler-Yeivin *et al.* published data consistent with our findings (115, 190). The fact that these results are reproducible in knock-out lines generated by different targeting strategies confirms the importance of Ini1 in development and tumorigenesis. Klochendler-Yeivin *et al.* further report in their study that Ini1-deficient embryos can induce the formation of maternal dicidua, suggesting that Ini1-deficient embryos undergo hatching and implantation prior to their demise. In contrast, *Ini1*$^{in3/in3}$ 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 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.

Chapter III

Disruption of *Ini1* on a *Rb*-heterozygous Background in Mice Alters Tumor Morphology but Not Tumor Rate

Introduction

INI1 functions as a tumor suppressor in humans and mice. It originally was identified in mammals based on its homology to the yeast Snf5 protein as well as via a yeast two hybrid assay as a protein that interacts with the HIV integrase protein (107, 158). INI1 is a member of all SWI/SNF chromatin remodeling enzymes purified to date (252). The identification of bi-allelic mutations in *INI1* in malignant rhabdoid tumors, an aggressive tumor of the soft tissues, provided the first evidence that INI1 plays a role as a tumor suppressor (245). Mutations in *INI1* were found subsequently in other tumors, such as choroid plexus carcinomas, medullablastomas, central primitive neuroectodermal tumors, and a few cases of leukemia (15-17, 69, 205). The fact that a subset of patients presenting with these tumors had constitutional mutations further supported the role of INI1 as a tumor suppressor in humans (17, 205).

Gene targeting experiments confirmed that Ini1 also functions as a tumor suppressor in mice (76, 115, 190). While *Ini1*-null mice are embryonic lethal, 15-30% of mice heterozygous for *Ini1* present with tumors, all of which are poorly differentiated or undifferentiated tumors with variable rhabdoid features. All tumors examined show loss of heterozygosity at the *Ini1* locus. Furthermore,

almost 100% of mice bearing a reversibly inactivating conditional allele of *Ini1* present rapidly with tumors. While many of these tumors have variable rhabdoid features, the vast majority are CD8+ T-cell lymphomas (189).

Despite the fact that INI1 plays a role in tumor suppression, the mechanism by which it does so has yet to be elucidated. BRG1 and BRM, ATPases that constitute biochemically distinct SWI/SNF complexes, both play a role in growth arrest mediated by the retinoblastoma (Rb) tumor suppressor (57, 218, 276). When Rb is hypophosphorylated (active), it is bound by the histone deacetylase HDAC1and BRG1/BRM, thereby repressing E2F activity (276). E2F is a transcription factor responsible for inducing the expression of a number of genes required for DNA synthesis, such as ribonucleotide reductase, topoisomerase II, DNase polymerase α, and proliferating cell nuclear antigen (PCNA) (52). At the onset of S-phase, coordinated phosphorylation events result in the release of HDAC1 and BRG1/BRM, allowing for the activation of E2F and progression through S-phase.

Unlike wild-type cells, SW13 cells derived from an aprocine tumor lacking BRG1 and BRM do not arrest when a constitutively active form of Rb is introduced (220). Furthermore, a constitutively active form of Rb is not able to arrest 3T3 mouse fibroblasts that express an ATPase-defective BRG1 (221). These data suggest that functional SWI/SNF is required for Rb-mediated arrest. Like BRG1 and BRM, the ability of INI1 to arrest cells depends on the presence of functional Rb (244). However, a constitutively active form of Rb is able to

arrest a variety of INI1-deficient cell lines derived from malignant rhabdoid tumors. It is unclear if the observed difference in Rb activity is due specifically to absence of INI1 versus BRG1/BRM, or if other differences between the various cell lines utilized may be responsible.

To determine if Rb participates in INI1-mediated tumor suppression, we crossed *Ini1*-heterozygous mice to *Rb*-heterozygous mice and monitored the offspring for tumor formation. We found that the tumor curve for mice heterozygous for both Rb and Ini1 was virtually indistinguishable from that for *Rb*-heterozygous mice. Like *Rb*-heterozygous mice, almost all of the compound heterozygous mice succumbed to pituitary adenomas. However, approximately one half of the tumors from the compound heterozygous mice had an atypical histology. These data suggest that loss of Ini1 may contribute to the aggressiveness of pituitary adenomas associated with loss of Rb.

Materials and Methods

Mouse strains

Ini1-heterozygous mice on a mixed (C57/Bl6 X 129) background and *Rb*-heterozygous mice were described previously (76, 129). Tail DNA from mice was genotyped for *Ini1* as described. *Rb* genotyping was done by PCR using the following primers: 5'aattgcggccgcatctgcatcatctttatcgc, 5'cccatgttcggtcctag, and 5'gaagaacgagatcagcag. An annealing temperature of 53°C was used.

Histologic analysis of tumors in mice

Mice were sacrificed when they presented with visible tumors or signs of wasting. Tumor samples and selected tissues were fixed in 10% buffered formalin phosphate and processed for paraffin embedding as described previously (81). Sections were prepared and stained with haematoxylin and eosin and examined under a microscope.

Results and Discussion

We previously described the generation and characterization of *Ini1*-heterozygous mice (76). These *Ini1*-heterozygous mice were mated to *Rb*-heterozygous mice (described in (129)) and compound heterozygous progeny were intercrossed. Resultant progeny were genotyped for *Ini1* and *Rb*. No *Ini1*-null or *Rb*-null mice were obtained (Table 3.1), regardless of the genetic background, indicating that loss of Rb does not rescue lethality due to absence of Ini1 and vice-versa.

Because *Ini1*-null and *Rb*-null mice are embryonic lethal (77, 131), only four *Ini1/Rb* genotypes were available for tumor analysis: wild-type (*Ini1+/+Rb+/+*), *Rb*-heterozygous (*Ini1+/+Rb+/-*), *Ini1*-heterozgyous (*Ini1+/-Rb+/+*), and compound heterozygous (*Ini1+/-Rb+/-*). As reported previously (77), wild-type mice did not present with tumors during the course of this study. Also described previously (131), all mice heterozygous for *Rb* and wild-type for *Ini1* presented with pituitary adenomas by 80 weeks of age. Furthermore,

Genotype	# Expected	Actual
Ini1+/+Rb+/+	2	4
Ini1+/+Rb+/-	4	7
Ini1+/+Rb-/-	2	0
Ini1+/-Rb+/+	4	10
Ini1+/-Rb+/-	9	13
Ini1+/-Rb-/-	4	0
Ini1-/-Rb+/+	2	0
Ini1-/-Rb+/-	4	0
Ini1-/-Rb-/-	2	0

Table 3.1: Absence of *Rb* does not rescue *Ini1*-null embryonic lethality and vice-versa

Mice compound heterozygous for *Ini1* and *Rb* were intercrossed and their progeny were genotyped as described in materials and methods. As shown, no *Ini1*-null or *Rb*-null mice were obtained.

approximately 20% of mice heterozygous for *Ini1* and wild-type for *Rb* presented with poorly differentiated or undifferentiated sarcomas with variable rhabdoid features. These tumors were described in our previous study (77). Interestingly, the tumor curve for *Ini1+/-Rb+/-* mice was similar to that observed for *Rb+/-* mice (Figure 3.1). The vast majority of the compound heterozygous mice (24 of 25) presented with tumors by 80 weeks of age. Most of these tumors were pituitary adenomas, with the exception of one tumor, which presented in the neck (Table 3.2 and data not shown).

Upon histological analysis of a number of the tumors from *Ini1+/-Rb+/-* mice, it became clear there were morphological differences from the typical pituitary adenomas that *Rb+/-* mice develop. All of the 12 pituitary adenomas examined from Ini1+/+Rb+/- mice had a histology that was uniform in appearance (Figure 3.2). The tumor cells were small with centric nuclei and an apparently low mitotic index. While many of the pituitary adenomas from the *Ini1+/-Rb+/-* mice were indistinguishable from those from *Rb+/-* mice, approximately one half (8 of 15 analyzed) of the tumors from the *Ini1+/-Rb+/-* presented with an altered phenotype. As shown in Figure 3.2, these tumors had a less uniform appearance. There were many large cells with acentric nuclei. In addition, the gross mitotic index appeared higher than tumors from the *Rb+/-* counterparts. Often, these tumors appeared to have 2-3 different populations cells: one population that resembled a typical pituitary adenoma and one or more with an atypical histology.

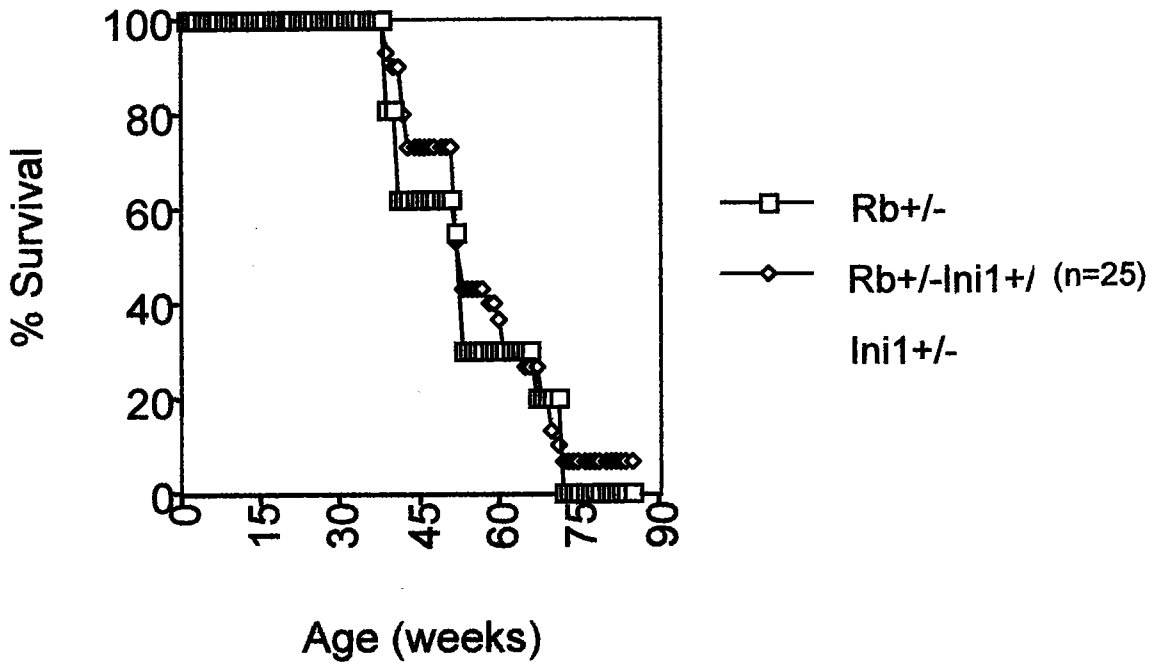


Figure 3.1: Heterozygosity of *Ini1* does not alter tumor curve of *Rb*-heterozygous mice

Survival curve for *Ini1+/-Rb+/-* mice was generated during this study. Tumor curve for *Ini1* was described previsouly (77). Data for *Rb+/-* mice of same strain as compound heterozygous mice were provided by Andrew Koff.

Mouse	Age (weeks)	Tumor Characterization
RI133e	104	c/w pituitary adenoma
R255	52	c/w necrotic pituitary adenoma
R214	73	cellular poorly differentiated malignant tumor (malignant pituitary adenoma)
R235	60	probable pituitary adenoma with foci of atypical or malignant histology (↑ nuclear size, pleomorphism, apoptotic cells, mitoses)
R212	58	c/w pituitary adenoma with slightly atypical histology (pleomorphism, apoptotic cells)
RI24	70	pituitary adenoma, and 2) malignant tumor with foci of large tumor cells with rhabdoid features
RI112e	52	probable pituitary adenoma with focally atypical or malignant histology
R210	68	c/w pituitary adenoma
RI18	67	small aggregates of tumor cells with slightly atypical histology
RI1	72	c/w pituitary adenoma
RI25	52	c/w necrotic pituitary adenoma

Table 3.2: Representation of tumors from Ini1+/-Rb+/- mice

Tumors showing atypical morphology are noted

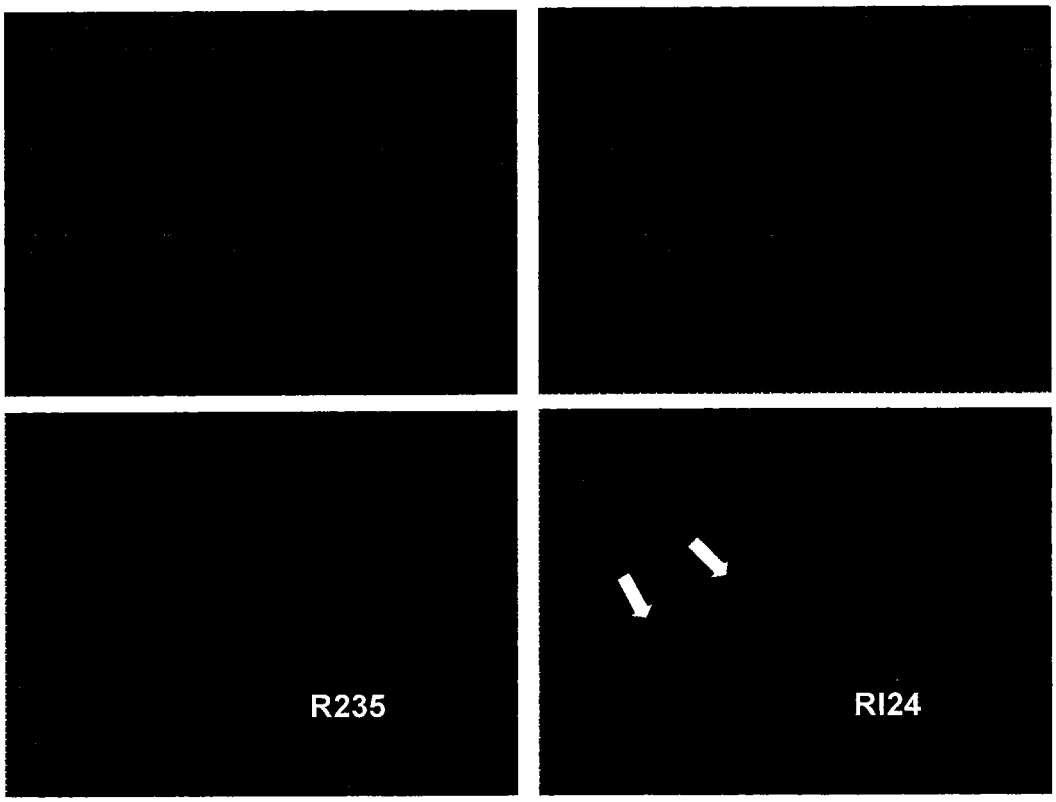


Figure 3.2: Approximately one half of pituitary adenomas from *Ini1+/-Rb+/-* mice have an altered morphology

Tumor samples from *Ini1+/-Rb+/-* mice were processed for H&E staining as described in the materials and methods. H&E stained tumor slides for *Rb+/-* mice were provided by Andrew Koff. Arrows denote large, rhabdoid-like cells.

We were interested in determining the state of Ini1 in these atypical tumors. It has been well documented that pituitary adenomas from *Rb+/-* mice show loss of heterozygosity at the *Rb* locus (131, 175); therefore, loss of Rb alone could not account for the altered phenotype in the tumors from the compound heterozygous mice. To date, no pituitary adenomas have been described in *Ini1+/-* mice. One possibility is that the pituitary tumors arose when the wild-type *Rb* allele was lost and the subsequent loss of *Ini1* in some of the tumors resulted in the development of a distinct tumor type. To determine if the more aggressive population of tumor cells arose due to loss Ini1, we are in the process of doing immunohistochemistry for Ini1 in the atypical tumors.

Discussion

Rb and Ini1 are tumor suppressors required for viability and prevention of oncogenesis. While Rb and Ini1 have been linked to cell cycle arrest via the SWI/SNF chromatin remodeling complex, the direct interactions between Ini1 and Rb are less clear. Functional BRG1 is required for the ability of a constitutively active form of Rb to arrest cells; however, INI1 is dispensable for this function of Rb (218, 244). On the contrary, the ability of INI1 to arrest cells is dependent on the presence of functional Rb. These data present conflicting conclusions as to whether or not Rb and Ini1 are functionally linked. In an attempt to discern the biological significance of any potential interactions between these two tumor suppressors, we crossed *Ini1*-heterozygous and *Rb*-

heterozygous mice. As mice homozygous null for *Ini1* or *Rb* are embryonic lethal, we were able to characterize tumor progression only in the compound heterozygous mice.

Mice that are deficient for Rb and either p27 or p53, two tumor suppressors that are on intersecting pathways with Rb, develop tumors at a faster rate than mice deficient for either tumor suppressor alone (82, 173). We report here that the tumor rate in mice heterozygous for both *Rb* and *Ini1* was unaltered from that for *Rb*-heterozygous mice. This finding suggests that neither Rb nor Ini1 require the other to mediate tumor suppression.

Interestingly, approximately one half of the pituitary adenomas that develop in the *Ini1+/-Rb+/-* mice have a morphology that is uncharacteristic of those that normally develop in *Rb+/-* mice. As mentioned above, these tumors appear to have two to three subpopulations of cell types. At least one population is similar to that which is seen in typical pituitary adenomas. In contrast, the other population(s) has a morphology that is similar in appearance to tumors that develop in *Ini1*-heterozygous mice. Specifically, large cells reminiscent of rhabdoid cells are visible in these tumors (Figure 3.2, arrow).

To date, we have yet to identify any pituitary adenomas from *Rb*-heterozygous mice that have an altered morphology similar to that described for some of the tumors that arose in *Ini1+/-Rb+/-* mice. Furthermore, we never observed any pituitary tumors, or neuronal tumors, in mice heterozygous for only *Ini1*. Therefore, it is likely that Rb and Ini1 are both disrupted in the altered

pituitary adenomas. We are currently trying to determine if Ini1 is indeed lost in these tumors.

The significance of the altered morphology of the pituitary adenomas in *Ini1+/-Rb+/-* mice is unclear. While their non-uniform morphology suggests that these tumors are more aggressive, there is no correlation in this study between tumor morphology and the age of the mouse at the time of sacrifice. Of course, as it is not feasible to visually monitor progression of these tumors, it is possible that they proliferated more quickly after developing the altered morphology. It will be interesting to determine if any of the tumor cells lost Ini1 expression and whether or not absence of Ini1 expression correlates with morphology. Regardless, though absence of both Ini1 and Rb does not appear to accelerate tumorigenesis, Ini1 does contribute to the tumor morphology associated with loss of Rb.

As mentioned above, BRG1 is required for the ability of Rb to growth arrest cells. *Brg1*-null mice are early embryonic lethal; however, a small percentage of mice heterozygous for *Brg1* present with tumors (27). It would be interesting to determine if loss of Brg1 alters the rate of tumorigenesis in *Rb*-heterozygous mice. Such experiments may determine if Ini1 functions independently of SWI/SNF.

Chapter IV

Heterozygosity at the *Ini1* Locus Inhibits Tumor Formation in *p53*-heterozygous Mice and Alters Tumor Spectrum

Introduction

INI1 is a component of all SWI/SNF chromatin remodeling enzymes purified to date. It originally was identified in mammals based on its homology to the yeast Snf5 protein as well as via a yeast two hybrid assay as a protein that interacts with the HIV integrase protein (107, 158). INI1 has been well established as a tumor suppressor in humans and mice. *INI1* is mutated in a number of human tumors, including rhabdoid tumors, choroid plexus carcinomas, medullablastomas, central primitive neuroectodermal tumors, and a few cases of leukemia (15-17, 69, 205). Furthermore, constitutional mutations in *INI1* have been identified in some of these tumors (17, 205).

Ini1 also functions as a tumor suppressor in mice (76, 115, 190). While *Ini1*-null mice are embryonic lethal, 15-30% of mice heterozygous for *Ini1* present with tumors, all of which are poorly differentiated or undifferentiated tumors with variable rhabdoid features. All tumors examined show loss of heterozygosity at the *Ini1* locus. Furthermore, almost 100% of mice bearing a reversibly inactivating conditional allele of *Ini1* present rapidly with tumors. While many of these tumors have variable rhabdoid features, the vast majority are CD8+ T-cell lymphomas (189).

While it is apparent that INI1 is a potent tumor suppressor, the mechanism by which it exerts its effect is unclear. The p53 tumor suppressor is one of the most studied transcription factors to date. It is mutated or deleted in a wide variety of human tumors, including those of the head and neck (143). Interestingly, most of the tumors that present in *Ini1*-heterozygous do so in the head and neck region. Furthermore, Ini1 has been linked indirectly to p53 via the SWI/SNF complex, which has been shown to co-purify from human cells with the breast cancer susceptibility protein BRCA1 (20). An ATPase-active BRG1 is required for the ability of BRCA1 to transactivate a p53-dependent reporter construct. A more recent study demonstrated that BRG1 and INI1 interact with p53 via co-immunoprecipitation assays (126). This study also presented data from transfection experiments that suggested SWI/SNF is necessary for the activation of p53-mediated transcription.

To determine if Ini1 and p53 cooperate to suppress tumorigenesis, we crossed mice heterozygous for *Ini1* to mice heterozygous for *p53* and monitored the progeny for tumor progression. To our surprise, mice heterozygous for both tumor suppressors presented with significantly fewer tumors than mice heterozygous for *p53* alone. In addition, the tumor distribution in compound heterozygous mice was different from that in *p53*-heterozygous mice. These data suggest that Ini1 and p53 functionally interact *in vivo*; however, contrary to previously published work, it appears that Ini1 may be involved in the repression of p53-responsive genes.

Materials and Methods

Mouse strains

Ini1-heterozygous mice on a mixed (C57/Bl6 X 129) background and *p53*-heterozygous mice were described previously (76, 99). *Ini1* genotyping was done on tail DNA as described (77). Genotyping for *p53* was done by Southern blot on EcoRI digested tail DNA (100).

Blastocyst culture

Blastocysts from *Ini1+/-p53-/-* intercrosses were flushed at 3.5 days post fertilization with M15 media [DMEM, 15% fetal calf serum, 100 μM β-mercaptoethanol, 2 mM glutamine, and 1X penicillin/streptomycin] and cultured in tissue culture plates for 96 hr. Outgrowths were genotyped for *Ini1* as described previously (76).

Histologic analysis of tumors in mice

Mice were sacrificed when they presented with visible tumors or signs of wasting. Tumor samples and selected tissues were fixed in 10% buffered formalin phosphate and processed for paraffin embedding as described previously (81). Sections were prepared and stained with haematoxylin and eosin and examined under a microscope.

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 DTT, 1 μg/mL pepstatin A, 4 μg/mL leupeptin, and 1 mM PMSF. Extracts were resolved by SDS-PAGE and western analysis for Ini1 protein was performed as described previously(48). The p53 western was performed using Ab-7 from Oncogene.

Results

The generation and characterization of *Ini1*-heterozygous mice and *p53*-heterozgyous mice has been described previously (76, 99). We crossed the individual heterozygous mice to generate mice heterozygous for both *Ini1* and *p53*. It has been established that *p53*-null mice are viable, while *Ini1*-null mice are early embryonic lethal. To determine if loss of *p53* rescues embryonic lethality due to absence of Ini1, compound heterozygous mice were intercrossed. No *Ini1*-null mice were obtained, indicating that absence of p53 is not sufficient to rescue *Ini1*-null lethality (Table 4.1).

Mice null for the breast cancer susceptibility gene Brca1 are early embryonic lethal. However, in the absence of p53, the day of lethality for *Brca1*-null embryos is extended from days 7.5 post-fertilization to days 9.5 post-fertilization (78). As mentioned above, functional BRG1 is required for the ability of BRCA1 to transactivate a p53-dependent reporter construct, providing a link

Genotype	Expected	Actual
Ini1+/+p53+/+	2	4
Ini1+/+p53+/-	5	8
Ini1+/+p53-/-	2	4
Ini1+/-p53+/+	5	8
Ini1+/-p53+/-	10	11
Ini1+/-p53-/-	5	4
Ini1-/-p53+/+	2	0
Ini1-/-p53+/-	5	0
Ini1-/-p53-/-	2	0

Table 4.1: Absence of *p53* does not rescue *Ini1*-null embryonic lethality

Ini1+/-p53+/- mice were intercrossed and resultant progeny genotyped. As shown, no *Ini1*-null mice were obtained.

between p53 and SWI/SNF pathways (128). To determine if absence of p53 also allows for an extended survival of *Ini1*-null embryos, *Ini1+/-p53-/-* mice were intercrossed and the embryos harvested at day 3.5 post fertilization. These blastocysts were plated in culture and outgrowths were genotyped 72 hours later. *Ini1+/+p53-/-* and *Ini1+/-p53-/-* blastocysts adhered to the tissue culture substratum, formed trophectoderm, and expanded their inner cell masses (data not shown). In contrast, *Ini1-/-p53-/-* blastocysts did not undergo these processes, indicating that *Ini1-/-* embryos are unable to progress beyond the blastocyst stage in vitro even in the absence of p53.

Because *p53*-null mice present with tumors particularly fast (100), a change in the rate of tumor progression is better observed in *Ini1*-heterozygous mice on a *p53*-heterozygous background rather than a *p53*-null background. Therefore, we followed *Ini1+/- p53+/-* mice for tumor development and compared this rate to those obtained for *Ini1*-heterozygous mice and *p53*-heterozygous mice. To our surprise, the rate of tumorigenesis was reduced in the compound heterozygous mice compared to *p53*-heterozygous mice. While all of the Ini+/+p53+/- mice presented with tumors by 125 weeks of age, only 12 of the 48 Ini1+/-p53+/- mice presented with tumors (Figure 4.1). Furthermore, as shown in Figure 4.2, the distribution of tumor spectrums in compound heterozygous mice was altered relative to *p53*-heterozygous mice.

Two lymphomas that presented in different *Ini1+/-p53+/-* mice were harvested for Western analysis. As shown in Figure 4.3, both of these tumors

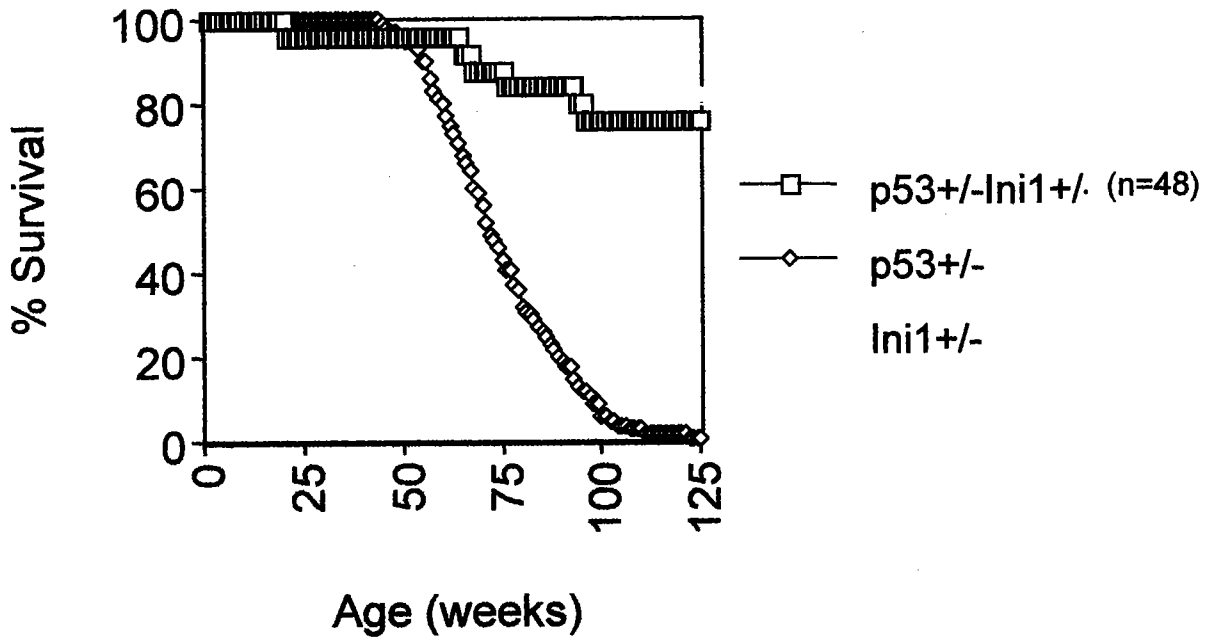


Figure 4.1: *Ini1+/-p53+/-* mice present with fewer tumors than *p53+/-* mice

Mice showing signs of tumorigenesis or wasting were sacrificed and graphed according to age in weeks. Curves for p53+/- mice and Ini1+/- mice are consistent with those published (77,100)

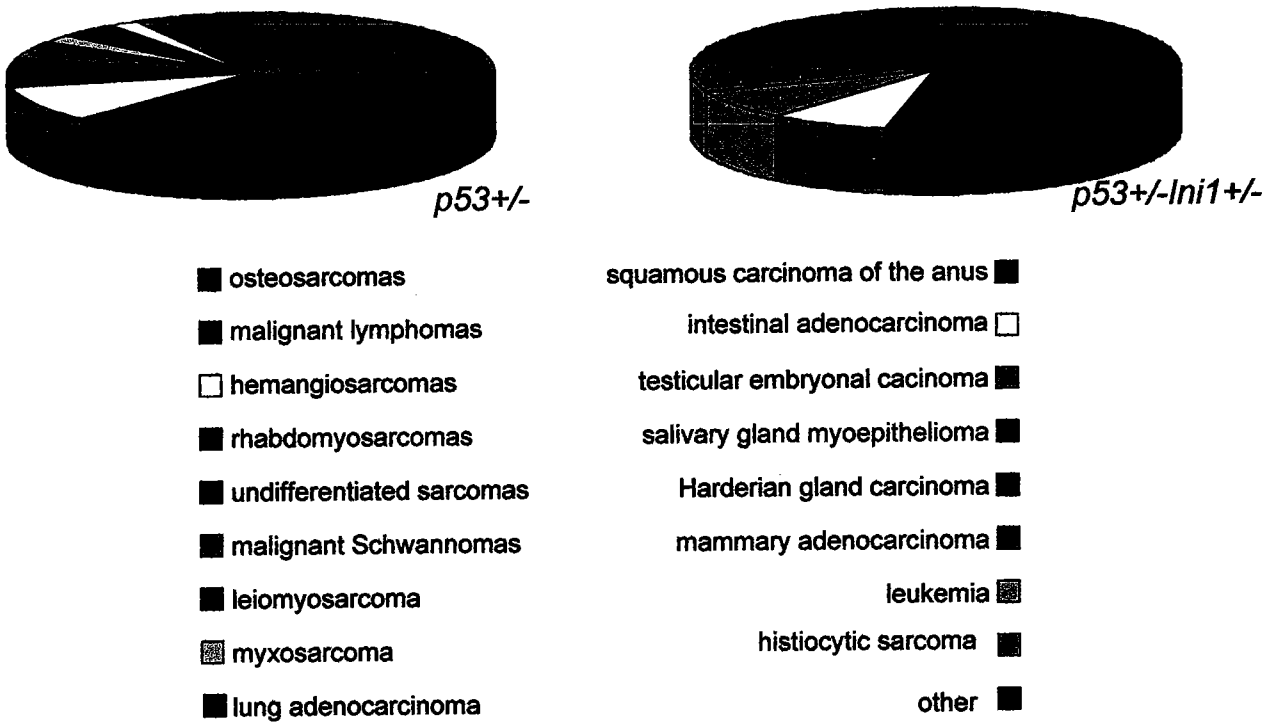


Figure 4.2: Altered tumor spectrum in *Ini1+/-p53+/-* mice

The percentage of tumor types was graphed for *p53+/-* mice and *Ini+/-p53+/-* mice. Each color represents the same tumor type in both pie graphs.

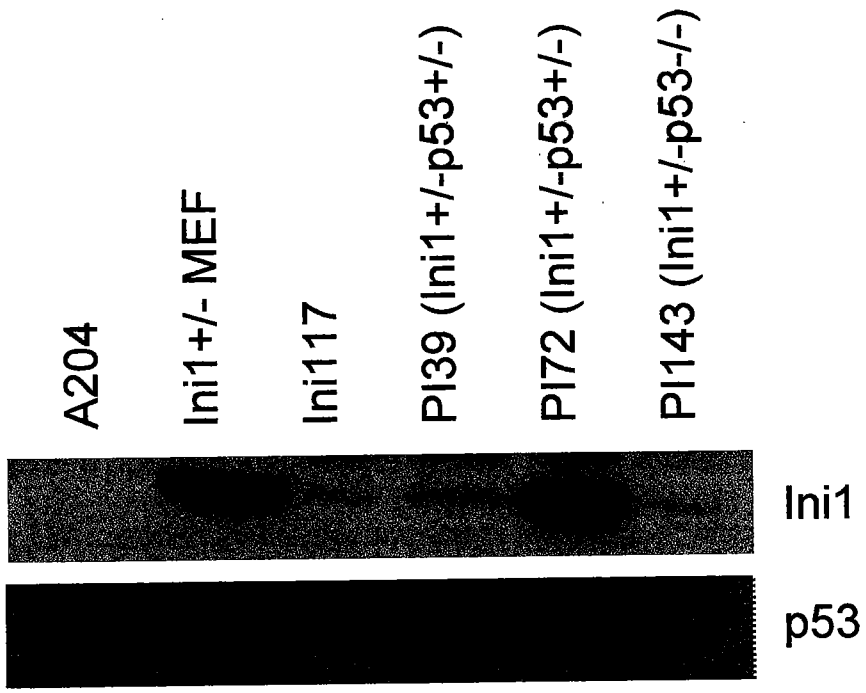


Figure 4.3: Analysis of Ini1 and p53 protein levels in tumors from *Ini1+/-p53+/-* mice

Tumor samples and cells were processed for Western analysis as described in the materials and methods. A204 is an Ini1-deficient cell line derived from a human malignant rhabdoid tumor. Ini117 is a tumor sample derived from an *Ini1*-heterozygous mouse.

lost expression of p53 protein, while only one showed loss of Ini1. Interestingly, the mouse that presented with the lymphoma devoid of both p53 and Ini1 also had the onset of a lymph tumor in the mandible region, similar to tumors described previously in *Ini1*-heterozygous mice.

Discussion

We crossed *Ini1*-heterozygous mice to *p53*-heterozygous mice in an attempt to dissect a potential mechanism by which Ini1 suppresses tumorigenesis. We show here the occurrence of a significant latency in the rate of tumorigenesis in mice heterozygous for both *Ini1* and *p53* compared to mice heterozygous for *p53* alone. We further show an alteration in the spectrum of observed tumors. Though we examined only a few tumor samples from the compound heterozygous mice, both lost expression of p53, while only one lost expression of Ini1.

Heterozygosity for the tumor suppressor *p53* is sufficient to result in tumor formation in mice without the need for loss of heterozygosity (243). Tumorigenesis likely is due to the fact that cells heterozygous for *p53* have reduced levels of p53 protein, and hence reduced p53 activity. Our findings suggest that Ini1 may be involved in the repression of p53 activated genes, such that cells heterozygous for Ini1 are better able to cope with the reduced levels of p53. Accordingly, p53 would have to be lost in order for tumorigenesis to occur. This hypothesis appears to be inconsistent with data published previously, in

which SWI/SNF was shown to be involved in the activation of *p53*-mediated transcription (126). However, those studies entailed transfection assays in transformed cells and the mode of action of SWI/SNF *in vivo* may be different.

It will be interesting to determine the rate of tumorigenesis in mice heterozygous for *Ini1* and null for *p53*. These results would indicate whether or not the presence of p53 is required for the observed suppression of tumorigenesis. Additioanlly, we are interested in characterizing the biochemical interactions between Ini1 and p53. Studies are currently underway utilizing Ini1 and p53-targeted mouse embryonic fibroblasts to examine the growth characteristics and DNA damage response of cells heterozygous for both of these tumor suppressors.

Chapter V

Compensation of the Ini1 Tumor Suppressor

Introduction

Tumor suppressors play important roles in regulating cellular proliferation and preventing oncogenesis in higher eukaryotes. Gene targeting experiments in mice have facilitated the identification and further study of a number of tumor suppressors. Generally, when mice heterozygous or homozygous-null for a specific gene present with tumors, the factor encoded by that gene is classified as a tumor suppressor.

INI1 is a component of the mammalian SWI/SNF chromatin remodeling enzymes. It was identified simultaneously by two groups as a protein that shares homology to the yeast Snf5 protein as well as via a yeast two hybrid assay as a protein that interacts with the HIV integrase protein (107, 158). The first evidence implicating INI1 as a tumor suppressor arose when Versteege et al. identified bi-allelic mutations in *INI1* in malignant rhabdoid tumors, an aggressive tumor of the soft tissues (245). Mutations in *INI1* were found subsequently in other tumors, such as choroid plexus carcinomas, medullablastomas, central primitive neuroectodermal tumors, and a few cases of lymphomas (15-17, 69, 205). Identification of constitutional mutations in a subset of these tumors further supported the role of INI1 as a tumor suppressor in humans (17, 205).

Gene targeting experiments confirmed that Ini1 also functions as a tumor suppressor in mice (76, 115, 190). While *Ini1*-null mice are embryonic lethal, 15-30% of mice heterozygous for *Ini1* present with tumors, all of which are poorly differentiated or undifferentiated tumors with variable rhabdoid features. All tumors examined show loss of heterozygosity at the *Ini1* locus. Furthermore, almost 100% of mice bearing a reversibly inactivating conditional allele of *Ini1* present rapidly with tumors (189). The vast majority of these tumors have been classified as CD8+ T-cell lymphomas.

It is interesting to note the difference in tumor penetrance between *Ini1*-heterozygous mice and the conditional Ini1 mice. These findings indicate that Ini1 must be lost for tumorigenesis to occur. It has been documented that heterozygosity for the tumor suppressor *p53* is sufficient to result in tumor formation in mice without the need for loss of heterozygosity (243). Tumorigenesis likely is due to the fact that cells heterozygous for p53 have reduced levels of p53 protein. Furthermore, other tumor suppressors, such as p53, Caveolin-1, Fhit, TαR-II (Transforming Growth Factor-α Type II Receptor), and Sno, also exhibit a marked reduction in protein and/or RNA levels in heterozygous mice (55, 62, 95, 185, 208). In these cases, haploinsufficiency is correlated with developmental defects and/or tumorigenesis. In contrast, we report here that mouse tissues and cells heterozygous for *Ini1* had levels of Ini1 protein and RNA equivalent to those in WT tissues and cells. It is possible that

this compensation of Ini1 is responsible for the low tumor penetrance in *Ini1*-heterozygous mice.

Because regulation of Ini1 plays an important role in mammalian viability, we were interested in further characterizing the method by which Ini1 message is compensated. We show here that the rate of transcription from the *Ini1* promoter is increased in *Ini1*-heterozygous cells, accounting for the increase in steady-state message levels. Furthermore, we find that exogenous expression of Ini1 results in a decrease in expression from the endogenous *Ini1* promoter, indicating that Ini1 plays a role in the regulation of its own promoter. These findings extend our current knowledge of the regulation of this tumor suppressor.

Materials and Methods

RT-PCR

Indicated tissues were harvested from one WT mouse and two *Ini1*-heterozygous mice and homogenized in Trizol (Life Technologies). RNA was isolated following manufacturer's protocol. Reverse transcription was performed using random hexamers and Mu-MLV RT (Invitrogen) with 2 μg of RNA for each tissue. The following oligos were used for amplification: Ini1, 5'gcgtcaagtttcagctggagg and 5'caccagcacctcaggctgtgacgc; GAPDH, 5'gcagtggcaaagtggagattgt and 5'cacggccatcacgccacagctt; 18S rRNA, 5'acttggataactgtggtaattc and 5'aattaccgcggctgctggcacc. Sample amplifications

were done by real-time PCR to ascertain the linear range for each primer set. Band intensities were quantitated utilizing ImageQuant software.

Northern analysis

RNA was isolated from WT and *Ini1*-heterozygous mouse ES cells and MEFs using Trizol. For each sample, 10 μg of mRNA was electrophoresed on a formaldehyde gel and transferred to GeneScreen plus membrane. Hybridization was performed at 50°C in 10% Dextran Sulfate, 1.5X SSPE, 1% SDS, 0.5% Milk, and 625 μg/mL yeast RNA extract. Blots were washed twice, 20 minutes each with 10%SDS, 2X SSC at 50°C and once 15 minutes with 1%SDS, 0.2X SSC at 50°C. Blots were then exposed to a PhosphoImage screen and quantitated using ImageQuant software.

Western analysis

Indicated tissues were homogenized in NP-40 Lysis Buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 20% Glycerol containing 1 mM DTT, 4 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 mM PMSF) and briefly sonicated. Samples were centrifuged at 4°C for 10 minutes at 10000 X g. Protein extracts were transferred to fresh Eppendorf tubes and 50 μg of each sample was electrophoreses on SDS-polyacrylamide gels, then transferred to nitrocellulose membranes. Antibodies against Ini1, Brg1, Brm, and Tubulin have been described previously (48, 76). Blots were developed using Amersham ECL

reagent, exposed to autoradiograph film, which were then scanned and quantitated using ImageQuant software.

Half-Life Assays

WT and *Ini1*-heterozygous MEFs were grown to 80% confluency prior to addition of 10 ug/mL Actinomycin D (Sigma). Cells were harvested at 2 hour intervals between 0-12 hours after addition of Actinomycin D and again for a final time-point at 24 hours. Samples were processed for RNA using Trizol reagent (Life Technologies) following manufacturer's protocol. Reverse transcription was performed using random hexamers and Mu-MLV RT (Invitrogen) with 2 ug of RNA for each time-point. Ini1, GAPDH, and 18S rRNA were amplified utilizing real-time PCR (MJ Research) with Qiagen SYBR-green reagents. Primers used are described above. The quantity of starting material for each product at each time-point was determined using software provided with real-time PCR machine. Ini1 and GAPDH are graphed relative to 18S rRNA. Results shown are the average of two experiments done in triplicate for each time-point. Time 0 was arbitrarily set to 1, and the remaining time-points were adjusted accordingly.

Nuclear runon transcription assay

Nuclear runon transcription assays were done as described previously by Schübeler and Bode (www.biomednet.com). Wild-type and *Ini1*-heterozygous mouse embryonic fibroblasts (~4 X 10^6) were collected by trypsination into 10 mL

conicals and centrifuged at room temperature at 200 X g for 5 minutes. Cell pellets were washed once with phosphate-buffered saline and collected by centrifugation at 200 X g for 5 minutes at 4ºC. 5 mL of cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40) were added to the cell pellets, which then were vortexed at low speed and incubated on ice for 3 minutes. Nuclei were collected by centrifugation at 500 X g for 5 minutes at 4ºC. These lysis steps were repeated three more times. The final nuclear pellets were resuspended in 100 µL nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and either used directly or frozen in liquid N_2 and stored at

-80ºC.

Nuclei suspensions were mixed with 30 µL of 5X runon buffer (25 mM Tris-HCl, pH8, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM each of ATP, GTP, and CTP, and 100 µCi α-^{32}P-UTP) and incubated at 30ºC for 30 minutes. DNA was digested by the addition of 15 units of RNase-free DNaseI and incubation at 30ºC for 15 minutes. RNA was isolated using Trizol reagent. Reaction mixtures (~150 µL) were transferred to 2 mL Eppendorf tubes containing 1.5 mL of Trizol and shaken for 2 minutes. Then, 300 µL $CHCl_3$ were added and the mixtures were shaken for another 5 minutes. Samples were centrifuged at 12000 X g for 15 minutes at 4ºC. The aqueous phase was transferred to a fresh Eppendorf tube containing 800 µL of isopropanol, mixed for 5 minutes at room temperature, and RNA pelleted by centrifugation at 12000 X g for 15 minutes at 4ºC. RNA pellets

were washed twice with 75% ethanol, air dried, and dissolved in 100 µL water. Incorporation of ^{32}P was determined by Cerenkov counting on a 1 µL aliquot.

For hybridization, plasmid fragments of interest were dot blotted manually to GeneScreen plus membrane. NaOH and EDTA were added to linearized cDNAs corresponding to exons 1-3 of Ini1, full-length GAPDH, and empty vector control to a final concentration of 0.4 M and 10 mM, respectively. Samples were boiled for 10 minutes, chilled on ice and then added in 2 µL aliquots to membrane that had been cut to size and wetted in dH20. The membrane then was rinsed in 2X SSC and DNA cross-linked using a Stratalinker. Prehybridization was done for at least 2 hours at 60ºC in 1% SDS, 10% dextran sulfate, 1.4 M NaCl, 325 µg/mL each of salmon sperm DNA and yeast RNA extract. Equivalent amounts of runon RNA (~3 X 10^6 counts/min) from *WT* and Ini1-heterozygous cell extracts were added to blot and hybridization was performed for 16-42 hours. Blots then were washed: 2X SSC (2 X 5 min, RT); 2X SSC, 1% SDS (15 min, 65ºC); 2X SSC (5 min, RT); 2X SSC, 10 µg/mL RNase A (10 min, 37ºC); 2X SSC (5 min, RT); 0.1X SSC (3 X 5 min, RT) and exposed to PhosphoImage screen. Hybridization was quantitated using ImageQuant software.

Beta-galactosidase assay

BOSC cells were transfected with retroviral pBABE vector or retroviral pBABE vector encoding a FLAG-epitope-tagged Ini1 using Fugene reagent

according to manufacturer's protocol. After 48 hours, the transfected BOSC cell supernatants were used to infect Ini1-heterozygous mouse embryonic fibroblasts at 80%. 48 hours post-infection, blasticidin (10 ug/mL) was added to the MEFs to select for infected cells. After 48 hours of selection, adherent cells were washed with 1X PBS twice. Residual PBS was removed and sufficient volume of 1X Reporter Lysis Buffer (Promega) to cover the cells. Cells were incubated at room temperature for 15 minutes and then transferred to an Eppendorf tube. Tubes were centrifuged at top speed for 2 minutes at 4ºC and supernatants transferred to fresh tubes. An equal volume of 2X assay buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/mL ONPG) was added to each cell lysate and incubated at room temperature until the first sample turned a faint yellow. Reactions were stopped with 0.6M Sodium Carbonate and absorbances read at 420 nm.

Results

We previously generated mice heterozygous for *Ini1* utilizing an inactivating retroviral insertion vector (76). During the course of this study, we observed that various tissues from *Ini1*-heterozygous mice had levels of Ini1 protein equivalent to those from wild-type (WT) tissues (Figure 5.1a). To determine if compensation of Ini1 was occurring at the level of protein regulation or mRNA regulation, we looked at steady state levels of Ini1 mRNA in various tissues from WT and *Ini1*-heterozygous mice by RT-PCR. The number of

amplification cycles used was determined to be in the linear range by real-time PCR (data not shown). As shown in Figure 5.1b, WT and *Ini1*-heterozygous tissues contain roughly equivalent levels of Ini1 message, indicating that modulation of Ini1 mRNA accounts for the observed compensation. We next looked at the levels of Ini1 protein and message in WT and *Ini1*-heterozygous mouse embryonic stem (ES) cells and mouse embryonic fibroblasts (MEFs). Similar to results obtained for mouse tissues, cells heterozygous for *Ini1* had roughly equivalent levels of Ini1 protein and RNA as WT cells (Figure 5.2).

We hypothesized that compensation of Ini1 mRNA was achieved by either an increase in the stability of Ini1 mRNA or an increase in the rate of transcription from the *Ini1* promoter. We first compared the half-life of Ini1 mRNA in WT cells to that of Ini1 mRNA in *Ini1*-heterozygous cells. Cells were exposed to 10 ug/ml Actinomycin D and then harvested at various intervals until 24 hours. Ini1 and GAPDH mRNA decay was monitored by real-time PCR. As shown in Figure 5.3, the decay rate of Ini1 message in *Ini1*-heterozygous cells is the same as that in WT cells, suggesting that Ini1 compensation is not mediated by an increase in Ini1 mRNA stability. This is not surprising since Ini1 message appears to be long-lived in MEFs.

We next looked at the rate of transcription of Ini1 in WT and *Ini1*-heterozygous cells by a run-on transcription assay. Newly synthesized RNA from WT and *Ini1*-heterozygous cells was labeled in vitro with ^{32}P-UTP and used to probe cDNAs immobilized on a membrane. As described in our previous

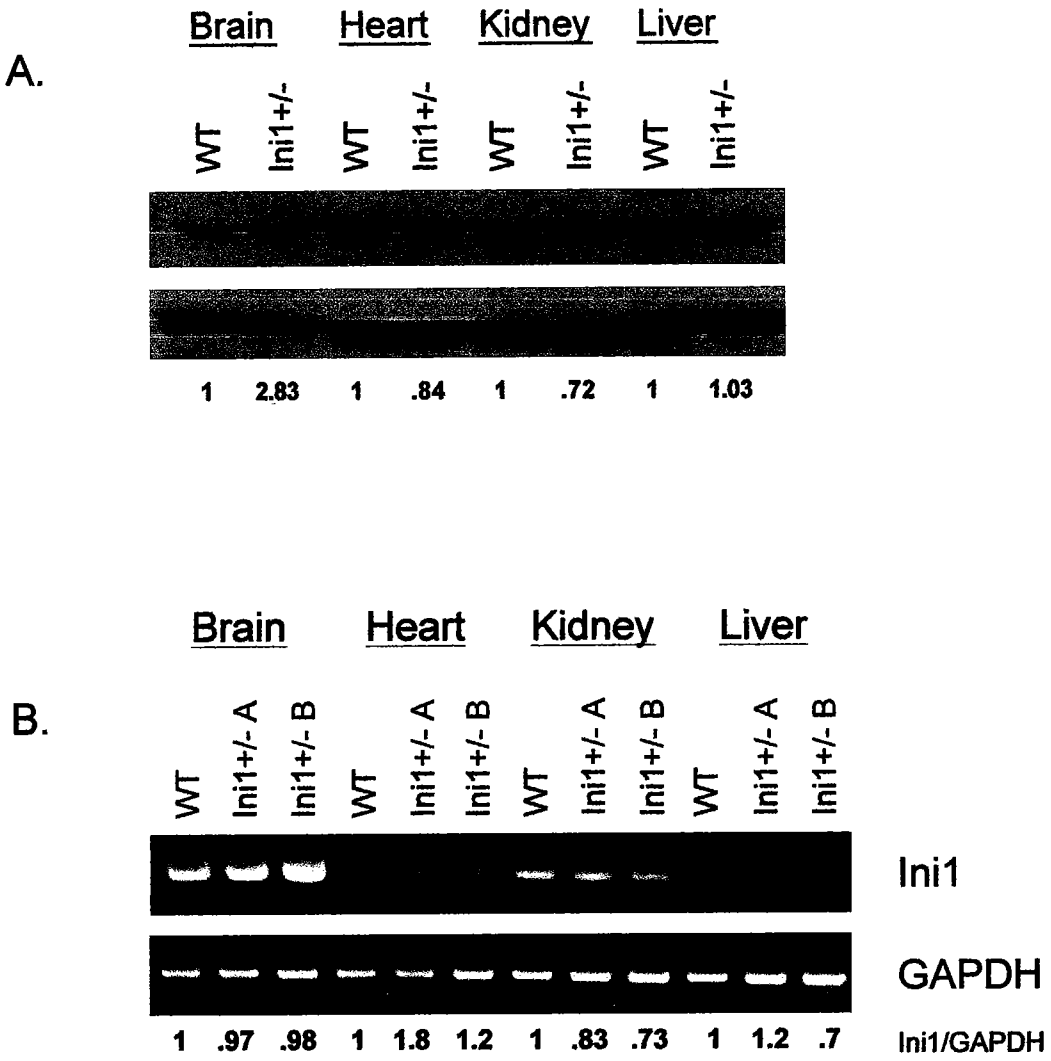


Figure 5.1: Tissues from *Ini1*-heterozygous mice have similar levels of protein and mRNA as wild-type mice

Indicated tissues from wild-type mice and *Ini1*-heterozygous mice were harvested and processed for Western analysis (A.) or RT-PCR (B.) as described in the materials and methods. The intensity of each protein or cDNA band was determined using ImageQuant software.

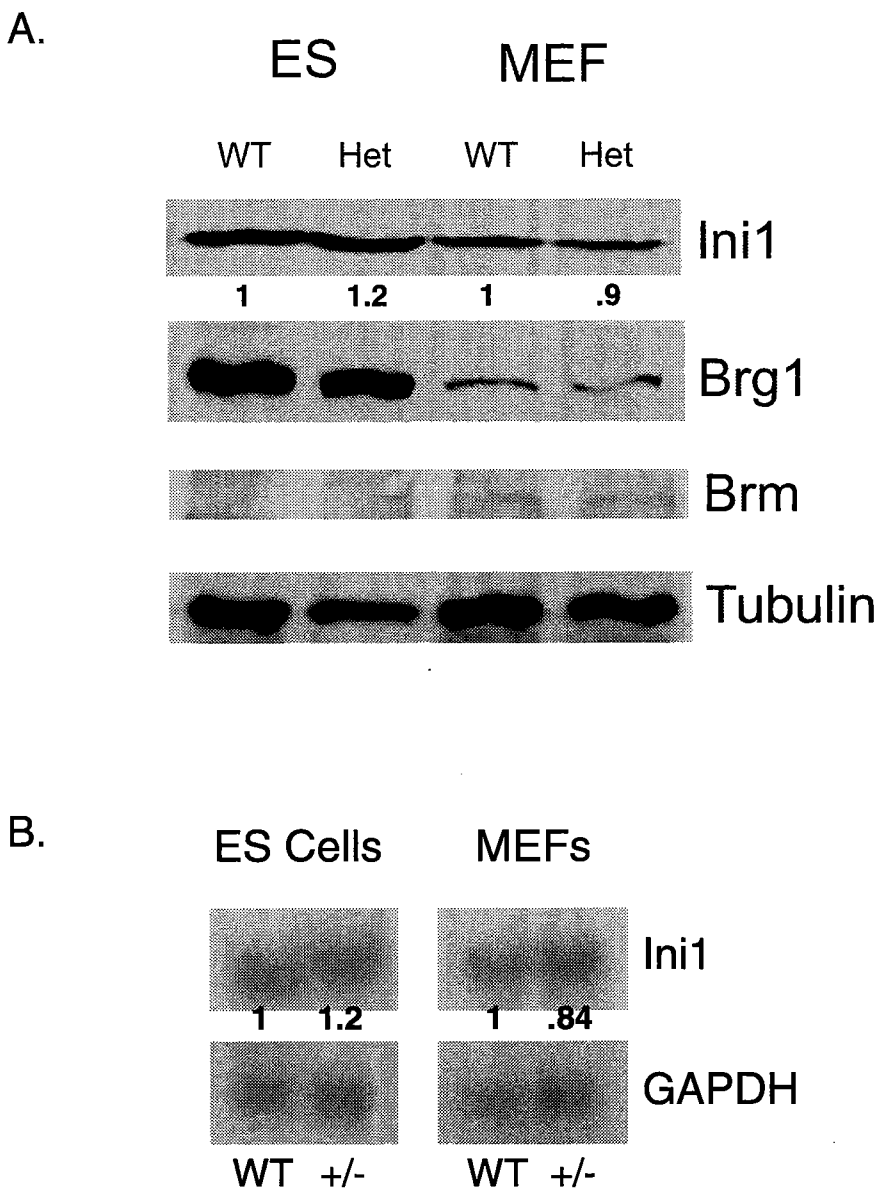
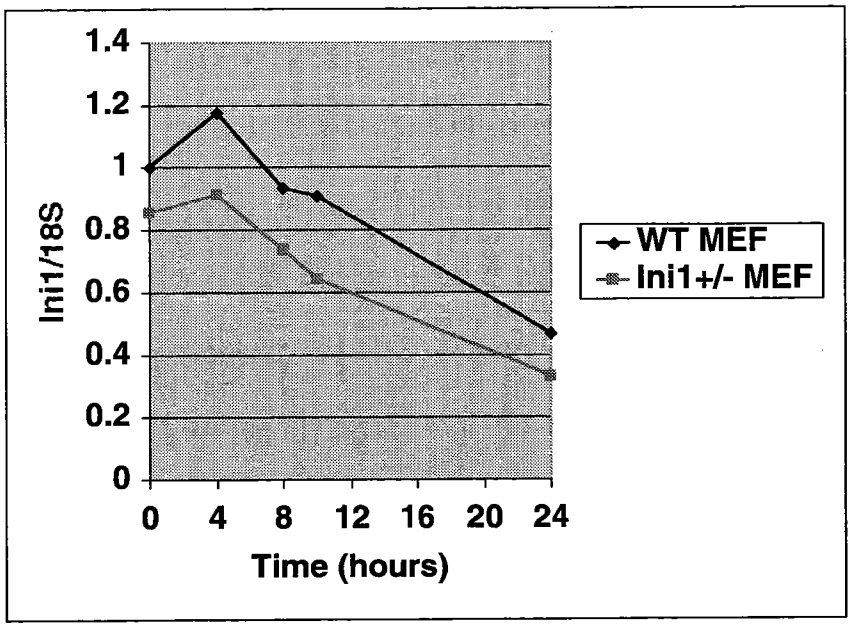


Figure 5.2: *Ini1*-heterozygous cells have similar levels of Ini1 protein and RNA as wild-type cells

Wild-type and *Ini1*-heterozygous ES cells or MEFs were processed for Western analysis (A.) or Northern analysis (B.) as described in the materials and methods. Intensity of each protein or RNA band was determined using ImageQuant software.

A.



B.

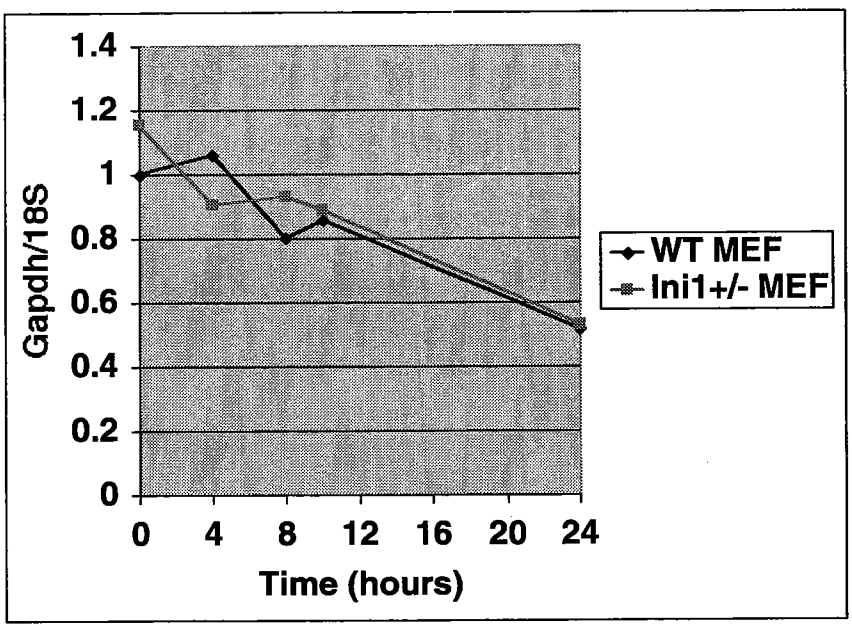


Figure 5.3: The decay rate of Ini1 message is not altered in *Ini1*-heterozygous cells

Wild-type and *Ini1*-heterozygous cells were exposed to Actinomycin D and harvested at various time-points thereafter. Message levels of Ini1 and GAPDH were determined by real-time PCR and graphed relative to 18 rRNA.

manuscript, Ini1-heterozygous cells contain one wild-type allele of *Ini1* as well as an allele of *Ini1* that is disrupted by the insertion of a retroviral vector within intron 3. This insertion results in the synthesis of two fusion transcripts: one containing exons 1-3 of Ini1 fused to β-galactosidase and another containing puromycin fused to the remaining exons of Ini1. In order to identify only those transcripts generated from the Ini1 promoter, a cDNA of Ini1 corresponding to exons 1-3 was used in the run-on assay. GAPDH cDNA and empty plasmid vector were used as controls. After hybridization and extensive washing, blots were exposed to a PhosphoImage screen, scanned, and quantitated utilizing ImageQuant software. As illustrated in Figure 5.4, *Ini1*-heterozgous cells contain almost double the amount of radiolabeled Ini1 RNA as WT cells. Thus, the rate of transcription from the *Ini1* promoter is increased in *Ini1*-heterozygous cells, accounting for the observed compensation.

To determine if Ini1 plays a role in the regulation of its own transcription, we exogenously expressed Ini1 in the *Ini1*-targeted MEFs and examined the effect on *Ini1*-promoter activity. We utilized the fact that these cells contain a β-galactosidase gene cassette under the control of the *Ini1* promoter. The activity of the *Ini1* promoter was monitored in *Ini1*-heterozygous cells infected with either a retrovirus encoding Ini1 or the empty retroviral vector control by assaying for β-galactosidase activity using a spectrophotometric assay described in the Materials and Methods. We found that cells infected with Ini1 exhibited reduced β-galactosidase activity as compared to cells infected with empty retroviral vector

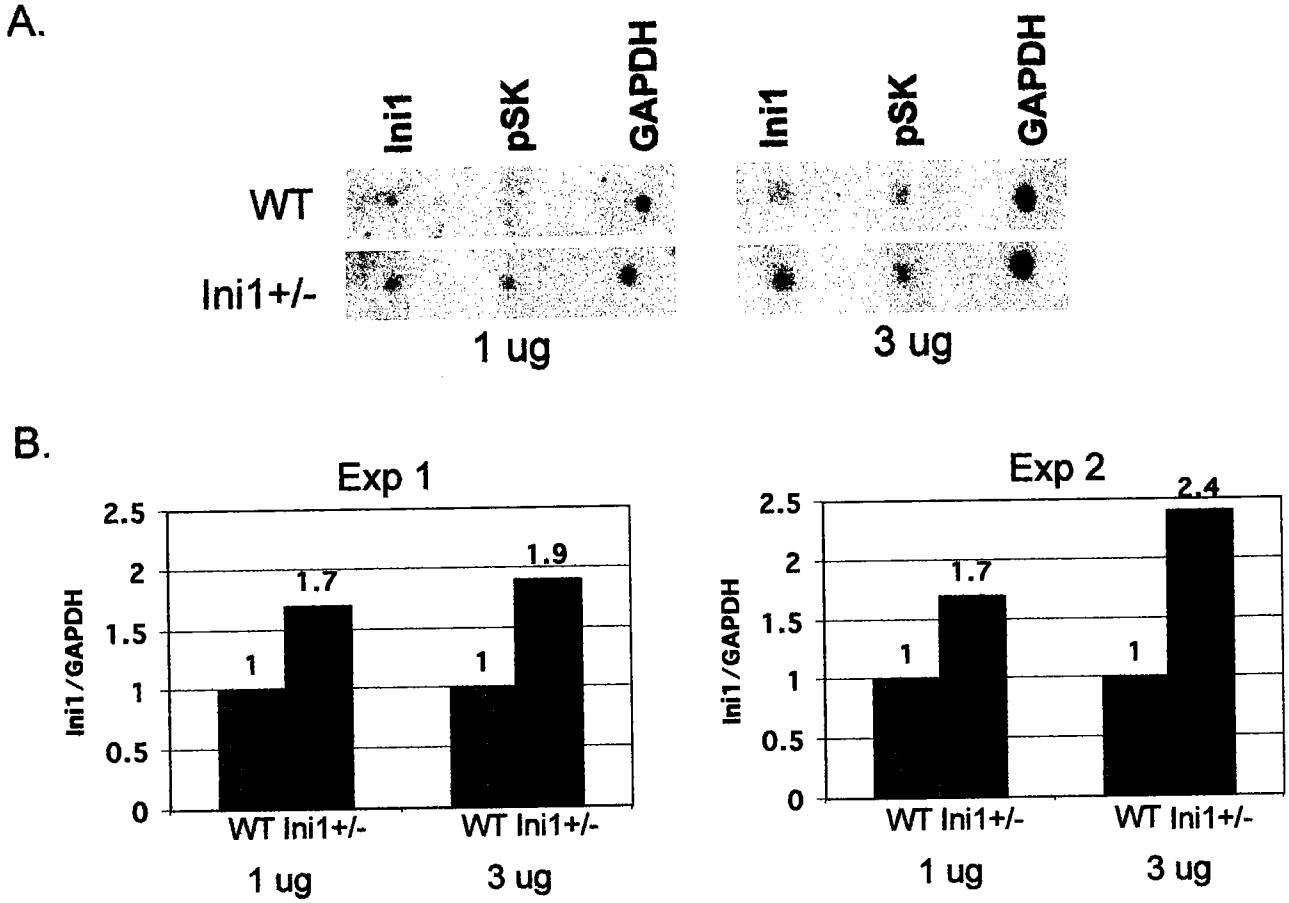


Figure 5.4: The rate of transcription of Ini1 is increased in *Ini1*-heterozgyous cells

A. Nuclear run-on assays were performed as described in the materials and methods. Representative blots demonstrating increased levels of newly synthesized Ini1 RNA in *Ini1*-heterozygous cells.

B. Graphical representation of two nuclear run-on experiments. The intensity of Ini1 relative to GAPDH was arbitrarily set to 1 for WT samples and the intensity of Ini1/GAPDH for *Ini1*-heterozygous samples was set accordingly.

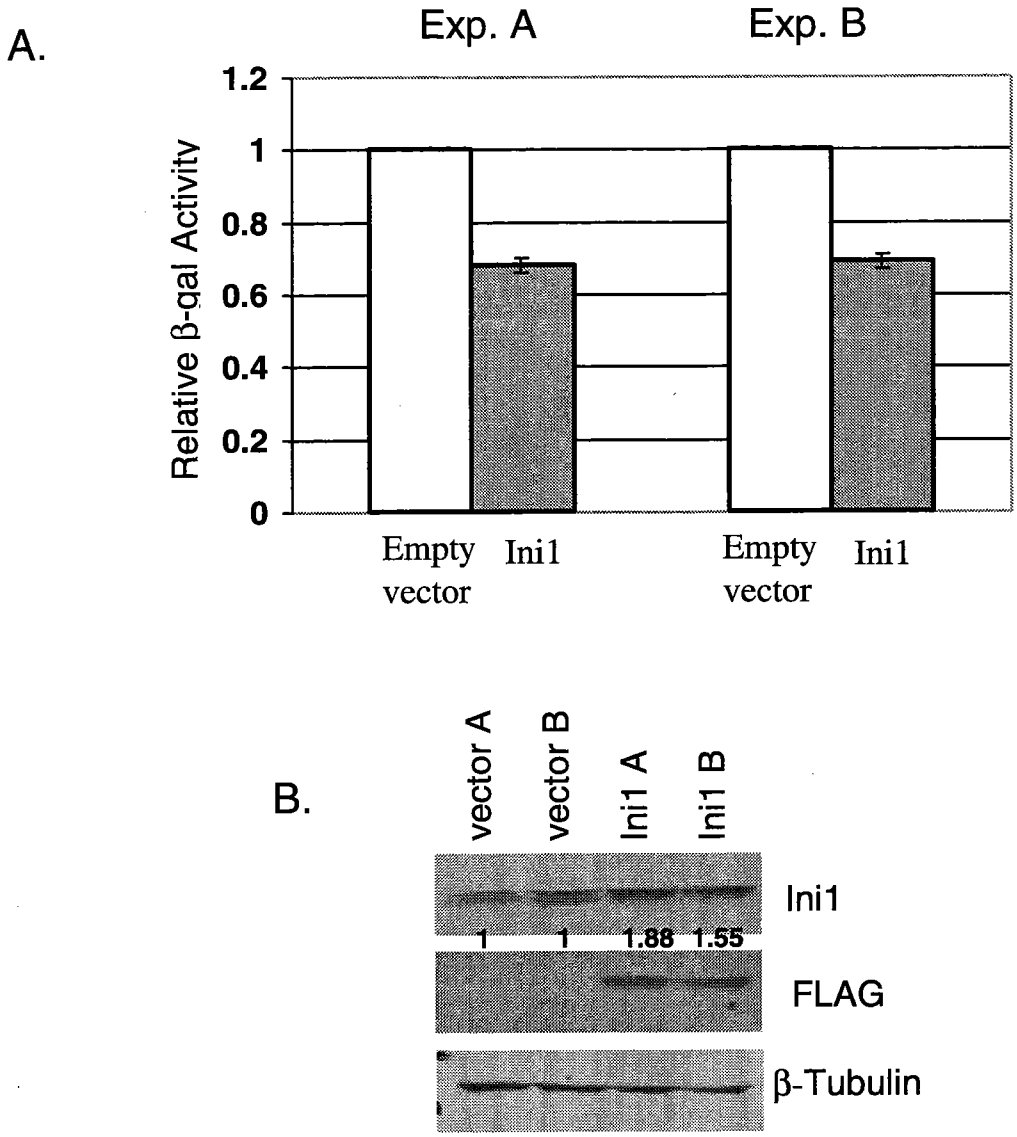


Figure 5.5: The expression level of Ini1 is tightly regulated

A. Ini1-heterozygous cells were infected with a retroviral vector encoding Ini1 or an empty retroviral vector. Relative β-gal activity was determined as described in the materials and methods. The graph represents results from two experiments done in triplicate.

B. Expression of FLAG epitope-tagged Ini1 in cells infected with Ini1-encoding retroviral vector.

(Figure 5.5). Because we measurined activity of the β -galactosidase protein, which is very stable, it is likely that transcription from the *Ini1* promoter is reduced even further than the reduction observed for β -galactosidase activity. Thus, it is clear that Ini1 plays a role, direct or indirect, in the regulation of its own transcription.

Discussion

Here we report that the mammalian tumor suppressor Ini1 is upregulated in *Ini1*-heterozygous cells by an increase in the rate of transcription from the Ini1 promoter. Furthermore, Ini1 appears to play a role in the regulation of its own promoter as exogenous expression of Ini1 results in decreased activity from the *Ini1* promoter. The observed transcriptional compensation for *Ini1* heterozygosity likely contributes to the low tumor penetrance in *Ini1*-heterozygous mice, as the increase in transcription rate from the single functional allele prevents potentially deleterious effects due to a decreased level of Ini1.

In *Drosophila*, dosage compensation is achieved by increasing the rate of transcription of genes on the single X chromosome in males to approximately double that of genes on each of the two X chromosomes in females. In mammals, few cases involving a compensating increase in transcriptional rate have been documented. It has been postulated that dosage compensation of the X chromosome in Drosophila males originally evolved on a gene to gene basis (150). As such, it is reasonable that gene dosage compensation in mammals

also exists on a gene to gene basis. It is likely that such events have not been characterized previously in mammals since phenotypic differences should not be observed in heterozygous individuals when the targeted gene is upregulated. In the case of Ini1, the non-targeted allele must be disrupted before any phenotypic difference is observed.

Clearly, it would be interesting to determine the exact mechanism by which the rate of transcription of Ini1 is increased in *Ini1*-heterozygous cells. To date, the promoter of Ini1 has not been defined. A database search for known consensus binding sites of transcription factors within the region upstream of the Ini1 start site yielded no obvious candidates that may be responsible for the observed upregulation. It should be noted that while dosage compensation in Drosophila has been studied extensively, the exact mechanism by which this compensation occurs has yet to be elucidated.

Chapter VI

Summary

It has become apparent that INI1 plays a critical role in mammalian development and tumor suppression. Work presented in this thesis has shown that Ini1 is essential for murine embryogenesis. Mice homozygous-null for Ini1 fail to adhere to substratum, form trophectoderm, and expand their inner cell mass. Because embryonic lethality occurs so early, it is difficult to determine the function of Ini1 at this stage of development. Data from another group show that Ini1 is maternally deposited in oocytes (115). These maternal stores of Ini1 can be detected up to day 3.5 post fertilization, near the time at which null embryos die. It is possible that Ini1 is required for general cell viability rather than for a specific function at a determined developmental time-point. This is supported further by the fact that conditional Ini1-knockout mice die shortly after inactivation of Ini1 expression (189).

We also have shown that mice heterozygous for *Ini1* are predisposed to poorly differentiated or undifferentiated sarcomas with variable rhabdoid features. All of the tumors examined show loss of heterozygosity at the *Ini1* locus. Although the *Ini-1* heterozygous mice do not recapitulate completely the human tumors associated with mutations in *Ini1*, they have provided a useful model system for addressing the function of Ini1 in suppressing tumorigenesis.

Ini1 may function as a tumor suppressor by interacting with other known tumor suppressors. We focused on Rb and p53 because there were existing data in the literature linking these tumor suppressors to SWI/SNF and/or INI1. Most of these data, however, were generated using transformed cell lines. We were able to address the *in vivo* functions of Ini1 by utilizing the *Ini1*-heterozgyous mice in crosses with mice heterozygous for either *Rb* or *p53.*

Data we obtained suggest that Ini1 and Rb do not cooperate to suppress tumorigenesis. This is consistent with transfection experiments in which INI1 is not required for the ability of Rb to growth arrest cells derived from human malignant rhabdoid tumors (244). However, it appears that Ini1 may contribute to the progression of Rb-deficient pituitary tumors, as mice heterozygous for both Rb and Ini1 present with tumors that have a morphology altered from that of typical pituitary adenomas.

When we generated mice heterozygous for both *Ini1* and *p53*, we were hoping to discover that these two factors share overlapping pathways in suppressing tumorigenesis. On the contrary, we found that Ini1 may be involved in the repression of p53-responsive genes, such that p53 activity is upregulated in *Ini1*-heterozygous cells. This was a surprising discovery in lieu of the fact that previous data suggested that SWI/SNF is required for p53-mediated transcriptional activation (20, 126). However, it is important to keep in mind that these previous studies were done using transfection assays in transformed cells.

It will be interesting to further explore the function of Ini1 on p53 activity in mouse embryonic fibroblasts with targeted deletions in *Ini1* and/or *p53*.

The promoter of *INI1* has yet to be identified and as such, little is known about the regulation of this gene. As presented in this thesis, we were able to show that the expression of Ini1 is regulated to ensure that relatively constant levels of Ini1 are present at a given time. Therefore, when one allele of *Ini1* is disrupted, the functional allele is transcriptionally upregulated. Likewise, when Ini1 is expressed exogenously, the endogenous alleles are down-regulated. The fact that Ini1 is compensated in heterozygous cells and tissues has implications on other aspects of this thesis. For instance, this compensation may explain why only 20% of mice heterozygous for *Ini1* present with tumors, as loss of heterozygosity may be required for tumor development. Furthermore, care will have to be given to interpretations of the crosses described above. If the steady-state levels of Ini1 protein are equivalent in wild-type and Ini1-heterozygous cells, why is there a difference in the rate of tumorigenesis between *Ini1+/+p53+/-* mice and *Ini1+/-p53+/-* mice? One possibility is that the compensation is not exact. In fact, we found that Ini1 mRNA levels in *Ini1*-heterozygous cells were approximately 85% of those in wild-type cells. Another possibility is that the compensation of Ini1 is disrupted in *p53*-heterozygous tissues. Preliminary Western analysis suggests that this is not the case. Lastly, it is possible that the mechanism by which Ini1 is upregulated also affects p53 or other factors that may play a role in stimulating p53 activity.

Of course, there is much yet to learn about the normal activities of INI1. Continued research hopefully will dissect the pathways in which INI1 plays a role, both in cell viability and tumor suppression. It also will be important to determine if INI1 functions solely in the context of the SWI/SNF complexes, or if it has a distinct role.

Chapter VII

References

1. **Abbondanza, C., N. Medici, V. Nigro, V. Rossi, L. Gallo, G. Piluso, A. Belsito, A. Roscigno, P. Bontempo, A. A. Puca, A. M. Molinari, B. Moncharmont, and G. A. Puca.** 2000. The retinoblastoma-interacting zinc-finger protein RIZ is a downstream effector of estrogen action. Proc Natl Acad Sci U S A **97:**3130-5.
2. **Agalioti, T., S. Lombardas, B. Parekh, J. Yie, T. Maniatis, and D. Thanos.** 2000. Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell **103:**667-678.
3. **Alland, L., R. Muhle, H. J. Jou, J. Potes, L. Chin, N. Schreiber-Agus, and R. A. DePinho.** 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature (London) **387:**49-55.
4. **Allis, C. D., L. G. Chicoine, R. Richman, and I. G. Schulman.** 1985. Deposition-related histone acetylation in micronuclei of conjugating Tetrahymena. Proc Natl Acad Sci U S A **82:**8048-52.
5. **Anzick, S. L., J. Kononen, R. L. Walker, D. O. Azorsa, M. M. Tanner, X.-Y. Guan, G. Sauter, O.-P. Kallioniemi, J. M. Trent, and P. S. Meltzer.** 1997. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science **277:**965-968.
6. **Aoyagi, S., G. Narlikar, C. Zheng, S. Sif, R. E. Kingston, and J. J. Hayes.** 2002. Nucleosome remodeling by the human SWI/SNF complex requires transient global disruption of histone-DNA interactions. Molecular and Cellular Biology **22:**3653-3662.
7. **Arany, Z., D. Newsome, E. Oldread, D. M. Livingston, and R. Eckner.** 1995. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature **374:**81-4.
8. **Arany, Z., W. R. Sellers, D. M. Livingston, and R. Eckner.** 1994. E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. Cell **77:**799-800.
9. **Arents, G., and E. N. Moudrianakis.** 1995. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimeriztion. Proc. Natl. Acad. Sci. USA **93:**11174-11179.
10. **Ayton, P. M., and M. L. Cleary.** 2001. Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. Oncogene **20:**5695-707.
11. **Bannister, A. J., and T. Kouzarides.** 1996. The CBP co-activator is a histone acetyltransferase. Nature **384:**641-643.
12. **Bannister, A. J., T. Oehler, D. Wilhelm, P. Angel, and T. Kouzarides.** 1995. Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are

required for CBP induced stimulation in vivo and CBP binding in vitro. Oncogene **11:**2509-14.

13. **Belandia, B., R. L. Orford, H. C. Hurst, and M. G. Parker.** 2002. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. EMBO J **21:**4094-103.
14. **Betz, B. L., M. W. Strobeck, D. N. Reisman, E. S. Knudsen, and B. E. Weissman.** 2002. Re-expression of hSNF5/INI1/BAF47 in pediatric tumor cells leads to G1 arrest associated with induction of p16ink4a and activation of RB. Oncogene **21:**5193-203.
15. **Biegel, J. A., B. Fogelgren, J. Y. Zhou, C. D. James, A. J. Janss, J. C. Allen, D. Zagzag, C. Raffel, and L. B. Rorke.** 2000. Mutations of the INI1 rhabdoid tumor suppressor gene in medulloblastomas and primitive neuroectodermal tumors of the central nervous system. Clin Cancer Res **6:**2759-63.
16. **Biegel, J. A., L. Tan, F. Zhang, L. Wainwright, P. Russo, and L. B. Rorke.** 2002. Alterations of the hSNF5/INI1 Gene in Central Nervous System Atypical Teratoid/Rhabdoid Tumors and Renal and Extrarenal Rhabdoid Tumors. Clin Cancer Res **8:**3461-3467.
17. **Biegel, J. A., J. Y. Zhou, L. B. Rorke, C. Stenstrom, L. M. Wainwright, and B. Fogelgren.** 1999. Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. Cancer Res **59:**74-9.
18. **Bischoff, J. R., L. Anderson, Y. Zhu, K. Mossie, L. Ng, B. Souza, B. Schryver, P. Flanagan, F. Clairvoyant, C. Ginther, C. S. Chan, M. Novotny, D. J. Slamon, and G. D. Plowman.** 1998. A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. **17:**3052-3065.
19. **Bochar, D. A., J. Savard, W. Wang, D. W. Lafleur, P. Moore, J. Cote, and R. Shiekhattar.** 2000. A family of chromatin remodeling factors related to Williams syndrome transcription factor. Proc. Natl. Acad. Sci. U.S.A. **97:**1038-43.
20. **Bochar, D. A., L. Wang, H. Beniya, A. Kinev, Y. Xue, W. S. Lane, W. Wang, F. Kashanchi, and R. Shiekhattar.** 2000. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer [In Process Citation]. Cell **102:**257-65.
21. **Borrow, J., A. M. Shearman, V. P. Stanton, Jr., R. Becher, T. Collins, A. J. Williams, I. Dube, F. Katz, Y. L. Kwong, C. Morris, K. Ohyashiki, K. Toyama, J. Rowley, and D. E. Housman.** 1996. The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat Genet **12:**159-67.
22. **Boyer, L. A., X. Shao, R. H. Ebright, and C. L. Peterson.** 2000. Roles of the histone H2A-H2B dimers and the (H3-H4)(2) tetramer in nucleosome remodeling by the SWI-SNF complex. J Biol Chem **275:**11545-52.
23. **Breeden, L., and K. Nasmyth.** 1987. Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. Cell **48:**389-397.

24. **Brownell, J. E., and C. D. Allis.** 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in *Tetrahymena* macronuclei. Proceedings of the National Academy of Sciences, USA **92:**6364-6368.
25. **Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, and C. D. Allis.** 1996. Tetrahymena histone acetyltransferase A: A homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell **84:**843-851.
26. **Bruder, C. E., J. P. Dumanski, and D. Kedra.** 1999. The mouse ortholog of the human SMARCB1 gene encodes two splice forms [In Process Citation]. Biochem Biophys Res Commun **257:**886-90.
27. **Bultman, S., T. Gebuhr, D. Yee, C. La Mantia, J. Nicholson, A. Gilliam, F. Randazzo, D. Metzger, P. Chambon, G. Crabtree, and T. Magnuson.** 2000. A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. Mol. Cell **6:**1287-95.
28. **Burns, L. G., and C. L. Peterson.** 1997. The yeast SWI/SNF complex facilitates binding of a transcriptional activator to nucleosomal site in vivo. Molecular and Cellular Biology **17:**4811-4819.
29. **Buyse, I. M., G. Shao, and S. Huang.** 1995. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. Proc Natl Acad Sci U S A **92:**4467-71.
30. **Cairns, B. R., Y.-J. Kim, M. H. Sayre, B. C. Laurent, and R. D. Kornberg.** 1994. A multisubunit complex containing the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* gene products isolated from yeast. Proceedings of the National Academy of Sciences, USA **91:**1950-1954.
31. **Cairns, B. R., Y. Lorch, Y. Li, M. Zhang, L. Lacomis, H. Erdjument-Bromage, P. Tempst, J. Du, B. Laurent, and R. D. Kornberg.** 1996. RSC, an essential, abundant, chromatin-remodeling complex. Cell **87:**1249-1260.
32. **Cao, Y., B. R. Cairns, R. D. Kornberg, and B. C. Laurent.** 1997. Sfh1p, a component of a novel chromatin-remodeling complex, is required for cell cycle progression. Mol Cell Biol **17:**3323-34.
33. **Carapeti, M., R. C. Aguiar, J. M. Goldman, and N. C. Cross.** 1998. A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. Blood **91:**3127-33.
34. **Carapeti, M., R. C. Aguiar, A. E. Watmore, J. M. Goldman, and N. C. Cross.** 1999. Consistent fusion of MOZ and TIF2 in AML with inv(8)(p11q13). Cancer Genet Cytogenet **113:**70-2.
35. **Cardoso, C., C. Mignon, G. Hetet, B. Grandchamps, M. Fontes, and L. Colleaux.** 2000. The human EZH2 gene: genomic organisation and revised mapping in 7q35 within the critical region for malignant myeloid disorders. Eur J Hum Genet **8:**174-80.
36. **Cardoso, C., S. Timsit, L. Villard, M. Khrestchatisky, M. Fontes, and L. Colleaux.** 1998. Specific interaction between the XNP/ATR-X gene

product and the SET domain of the human EZH2 protein. Hum Mol Genet **7:**679-84.

37. **Carruthers, L. M., and J. C. Hansen.** 2000. The core histone N-termini function independently of linker histones during chromatin condensation. J. Biol. Chem. **275:**37825-37290.
38. **Chadee, D. N., M. J. Hendzel, C. P. Tylipski, C. D. Allis, D. P. Bazett-Jones, J. A. Wright, and J. R. Davie.** 1999. Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. J Biol Chem **274:**24914-20.
39. **Chambon, P.** 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. **10:**940-954.
40. **Chiba, H., M. Muramatsu, A. Nomoto, and H. Kato.** 1994. Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila Brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. Nucleic Acids Research **22:**1815-1820.
41. **Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature **365:**855-9.
42. **Côté, J., J. Quinn, J. L. Workman, and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265:**53-60.
43. **Cubizollez, F., V. Legagneux, R. Le Guellec, C. Isabelle, R. Uzbekov, C. Ford, and K. Le Guellec.** 1998. pEg7, a new *Xenopus* protein required for mitotic chromosome condensation in egg extracts. J. Cell. Biol. **143:**1437-1446.
44. **Dai, P., H. Akimaru, Y. Tanaka, D. X. Hou, T. Yasukawa, C. Kanei-Ishii, T. Takahashi, and S. Ishii.** 1996. CBP as a transcriptional coactivator of c-Myb. Genes Dev **10:**528-40.
45. **Davey, C. A., D. F. Sargent, K. Luger, A. W. Maeder, and T. J. Richmond.** 2002. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol **319:**1097-113.
46. **Davie, J. R., and D. N. Chadee.** 1998. Regulation and regulatory parameters of histone modifications. J. Cell. Biochem. **30:**203-213.
47. **de la Barre, A. E., V. Gerson, S. Gout, M. Creavan, C. D. Allis, and S. Dimitrov.** 2000. Core histone amino-termini play an essential role in mitotic chromosome condensation. EMBO J. **19:**379-391.
48. **de la Serna, I. L., K. A. Carlson, D. A. Hill, C. J. Guidi, R. O. Stephenson, S. Sif, R. E. Kingston, and A. N. Imbalzano.** 2000. Mammalian SWI-SNF complexes contribute to activation of the hsp70 gene. Mol Cell Biol **20:**2839-51.
49. **de la Serna, I. L., K. A. Carlson, and A. N. Imbalzano.** 2001. Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. Nature Genetics **27:**187-90.

50. **DeCristofaro, M. F., B. L. Betz, C. J. Rorie, D. N. Reisman, W. Wang, and B. E. Weissman.** 2001. Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. J. of Cell. Physiology **186:**136-145.

51. **DeCristofaro, M. F., BLBetz, W. Wang, and B. E. Weissman.** 1999. Alteration of hSNF5/INI1/BAF47 detected in rhabdoid cell lines and primary rhabdomyosarcomas but not Wilms' tumors. Oncogene **18:**7559-7565.

52. **DeGregori, J., T. F. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transription factor include DNA synthesis and G1/S regulatory genes. Molecular and Cellular Biology **15:**4215-4224.

53. **Deuring, R., L. Fanti, J. A. Armstrong, M. Sarte, O. Papoulas, M. Prestel, G. Daubresse, M. Verardo, S. L. Moseley, M. Berloco, T. Tsukiyama, C. Wu, S. Pimpinelli, and J. W. Tamkun.** 2000. The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. Mol Cell **5:**355-65.

54. **Dingwall, A. K., S. J. Beek, C. M. McCallum, J. W. Tamkun, G. V. Kalpana, S. P. Goff, and M. P. Scott.** 1995. The Drosophila snr1 and brm proteins are related to yeast SWI/SNF proteins and are components of a large protein complex. Molecular Biology of the Cell **6:**777-791.

55. **Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley.** 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature **356:**215-21.

56. **Du, J., I. Nasir, B. K. Benton, M. P. Kladde, and B. C. Laurent.** 1998. Sth1p, a saccharomyces cerevisiae Snf2p/Swi2p homolog, is an essential ATPase in RSC and differs from Snf/Swi in its interactions with histones and chromatin-associated proteins [In Process Citation]. Genetics **150:**987-1005.

57. **Dunaief, J. L., B. E. Strober, S. Guha, P. A. Khavari, K. Ålin, J. Luban, M. Begemann, G. R. Crabtree, and S. P. Goff.** 1994. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell **79:**119-130.

58. **Eckner, R., M. E. Ewen, D. Newsome, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev **8:**869-84.

59. **Fears, S., C. Mathieu, N. Zeleznik-Le, S. Huang, J. D. Rowley, and G. Nucifora.** 1996. Intergenic splicing of MDS1 and EVI1 occurs in normal tissues as well as in myeloid leukemia and produces a new member of the PR domain family. Proc Natl Acad Sci U S A **93:**1642-7.

60. **Ferreri, K., G. Gill, and M. Montminy.** 1994. The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc Natl Acad Sci U S A **91:**1210-3.
61. **Finch, J. T., L. C. Lutter, D. Rhodes, A. S. Brown, B. Rushton, M. Levitt, and A. Klug.** 1977. Structure of nucleosome core particles of chromatin. Nature **269:**29-36.
62. **Fong, L. Y., V. Fidanza, N. Zanesi, L. F. Lock, L. D. Siracusa, R. Mancini, Z. Siprashvili, M. Ottey, S. E. Martin, T. Druck, P. A. McCue, C. M. Croce, and K. Huebner.** 2000. Muir-Torre-like syndrome in Fhit-deficient mice. Proc Natl Acad Sci U S A **97:**4742-7.
63. **Francis-West, P., R. Ladher, A. Barlow, and A. Graveson.** 1998. Signalling interactions during facial development. Mechanisms of Development **75:**3-28.
64. **Fryer, C. J., and T. K. Archer.** 1998. Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. Nature **393:**88-91.
65. **Gayther, S. A., S. J. Batley, L. Linger, A. Bannister, K. Thorpe, S. F. Chin, Y. Daigo, P. Russell, A. Wilson, H. M. Sowter, J. D. Delhanty, B. A. Ponder, T. Kouzarides, and C. Caldas.** 2000. Mutations truncating the EP300 acetylase in human cancers. Nat Genet **24:**300-3.
66. **Ge, Q., D. S. Nilasena, C. A. O'Brien, M. B. Frank, and I. N. Targoff.** 1995. Molecular analysis of a major antigenic region of the 240-kD protein of Mi-2 autoantigen. J. Clin. Invest. **96:**1730-1737.
67. **Gelbart, M. E., T. Rechsteiner, T. J. Richmond, and T. Tsukiyama.** 2001. Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. Mol. Cell. Biol. **21:**2098-106.
68. **Goto, H., Y. Tomono, K. Ajiro, H. Kosako, M. Fujita, M. Sakurai, K. Okawa, A. Iwamatsu, T. Okigaki, T. Takahashi, and M. Inagaki.** 1999. Identificaion of a novel phosphorylation site on histone H3 coupled with mitotic chromsome condensation. 274.
69. **Grand, F., S. Kulkarni, A. Chase, J. M. Goldman, M. Gordon, and N. C. Cross.** 1999. Frequent deletion of hSNF5/INI1, a component of the SWI/SNF complex, in chronic myeloid leukemia. Cancer Res **59:**3870-4.
70. **Grant, P. A., L. Duggan, J. Côté, S. M. Roberts, J. E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C. D. Allis, F. Winston, S. L. Berger, and J. L. Workman.** 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes and Development **11:**1640-1650.
71. **Grignani, F., S. De Matteis, C. Nervi, L. Tomassoni, V. Gelmetti, M. Cioce, M. Fanelli, M. Ruthardt, F. F. Ferrara, I. Zamir, C. Seiser, F. Grignani, M. A. Lazar, S. Minucci, and P. G. Pelicci.** 1998. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature (London) **391:**815-818.

72. **Gross, D. S., and W. T. Garrard.** 1988. Nuclease hypersensitive sites in chromatin. Annual Review of Biochemistry **57:**159-197.
73. **Grosveld, F.** 1999. Activation by locus control regions? Curr Opin Genet Dev **9:**152-7.
74. **Grunstein, M.** 1997. Histone acetylation in chromatin structure and transcription. Nature (London) **389:**349-352.
75. **Grunstein, M.** 1990. Histone function in transcription. Annual Review of Cell Biology **6:**643-678.
76. **Guidi, C. J., A. T. Sands, B. P. Zambrowicz, T. K. Turner, D. A. Demers, W. Webster, T. W. Smith, A. N. Imbalzano, and S. N. Jones.** 2001. Disruption of Ini1 leads to peri-implantation lethality and tumorigenesis in mice. Mol Cell Biol **21:**3598-603.
77. **Gurley, L. R., J. A. D'Anna, S. S. Barham, L. L. Deaven, and R. A. Tobey.** 1978. Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. Eur J Biochem **84:**1-15.
78. **Hakem, R., J. L. de la Pompa, A. Elia, J. Potter, and T. W. Mak.** 1997. Partial rescue of Brca1 (5-6) early embryonic lethality by p53 or p21 null mutation. Nature Genetics **16:**298-302.
79. **Han, M., and M. Grunstein.** 1988. Nucleosome loss activates yeast downstream promoters in vivo. Cell **55:**1137-1145.
80. **Harbour, J. W., and D. C. Dean.** 2000. The Rb/E2F pathway: Expanding roles and emerging paradigms. Genes Dev. **14:**2393-2409.
81. **Harvey, M., M. J. McArthur, C. A. M. Jr., J. S. Butel, A. Bradley, and L. A. Donehower.** 1993. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nature Genetics **5:**225-229.
82. **Harvey, M., H. Vogel, E. Y.-H. P. Lee, A. Bradley, and L. A. Donehower.** 1995. Mice deficient in both *p53* and *Rb* develop tumors primarily of endocrine origin. Cancer Research **55:**1146-1151.
83. **Hebbes, T. R., A. W. Thorne, A. L. Clayton, and C. Crane-Robinson.** 1992. Histone acetylation and globin gene switching. Nucleic Acids Research **20:**1017-1022.
84. **Hebbes, T. R., A. W. Thorne, and C. Crane-Robinson.** 1988. A direct link between core histone acetylation and transcriptionally active chromatin. EMBO Journal **7:**1395-1402.
85. **Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, and M. Grunstein.** 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: A molecular model for the formation of heterochromatin in yeast. Cell **80:**583-592.
86. **Heinzel, T., R. M. Lavinsky, T.-M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W.-M. Yang, G. Brard, S. D. Ngy, J. R. Davie, E. Seto, R. N. Elsenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcription al repression. Nature (London) **387:**43-48.

87. **Hirano, T., and T. J. Mitchison.** 1994. A heterodimeric coil-coil protein required for mitotic chromosome condensation *in vitro*. Cell **79:**449-458.
88. **Hiroyuki Takai, K. T., Noboru motoyama, Yohji A. Minamishima, Hiroyasu Nagahama, Tadasuke Tsukiyama, Kyoji Ikeda, Keiko Nakayama, Makoto Nakanishi, and Kei-ichi Nakayama.** 2000. Aberrant cell cycle checkpoint function and early embryonic death in *Chk1*$^{-/-}$ mice. Genes & Development **14:**1439-1447.
89. **Hirschhorn, J. N., S. A. Brown, C. D. Clark, and F. Winston.** 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes and Development **6:**2288-2298.
90. **Holstege, F. C., E. G. Jennings, J. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young.** 1998. Dissecting the regulatory circuitry of a eukaryotic genome. Cell **95:**717-28.
91. **Hsu, J. Y., Z. W. Sun, X. Ki, M. Reuben, K. Tatchell, D. K. Bishop, J. M. Grushcow, R. Kin, M. M. Smith, and C. D. Allis.** 2000. Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell **102:**279-291.
92. **Huang, S.** 1999. The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers. Front Biosci **4:**D528-32.
93. **Huntsman, D. G., S. F. Chin, M. Muleris, S. J. Batley, V. P. Collins, L. M. Wiedemann, S. Aparicio, and C. Caldas.** 1999. MLL2, the second human homolog of the Drosophila trithorax gene, maps to 19q13.1 and is amplified in solid tumor cell lines. Oncogene **18:**7975-84.
94. **Ida, K., I. Kitabayashi, T. Taki, M. Taniwaki, K. Noro, M. Yamamoto, M. Ohki, and Y. Hayashi.** 1997. Adenoviral E1A-associated protein p300 is involved in acute myeloid leukemia with t(11;22)(q23;q13). Blood **90:**4699-704.
95. **Im, Y. H., H. T. Kim, I. Y. Kim, V. M. Factor, K. B. Hahm, M. Anzano, J. J. Jang, K. Flanders, D. C. Haines, S. S. Thorgeirsson, A. Sizeland, and S. J. Kim.** 2001. Heterozygous mice for the transforming growth factor-beta type II receptor gene have increased susceptibility to hepatocellular carcinogenesis. Cancer Res **61:**6665-8.
96. **Imbalzano, A.** 1998. ATP dependent chromatin remodelers: Complex complexes and their components. Critical Reviews in Eukaryotic Gene Expression **8:**225-255.
97. **Imbalzano, A. N., H. Kwon, M. R. Green, and R. E. Kingston.** 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. Nature **370:**481-485.
98. **Ito, T., M. Bulger, M. J. Pazin, R. Kobayashi, and J. Kadonaga, T.** 1997. ACF, an I-SWI containing and ATP utilizing chromatin assembly and remodeling factor. Cell **90:**145-155.

99. **Jacks, T., L. Remington, B. O. Williams, E. M. Schmitt, S. Halachmi, R. T. Bronson, and R. A. Weinberg.** 1994. Tumor spectrum analysis in p53-mutant mice. Curr. Biol. **4:**1-7.
100. **Jaju, R. J., C. Fidler, O. A. Haas, A. J. Strickson, F. Watkins, K. Clark, N. C. Cross, J. F. Cheng, P. D. Aplan, L. Kearney, J. Boultwood, and J. S. Wainscoat.** 2001. A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood **98:**1264-7.
101. **Janknecht, R., M. A. Cahill, and A. Nordheim.** 1995. Signal integration at the c-fos promoter. Carcinogenesis **16:**443-50.
102. **Janknecht, R., and T. Hunter.** 1996. Transcription. A growing coactivator network. Nature **383:**22-3.
103. **Jaskelioff, M., M. G. I, C. L. Peterson, and C. Logie.** 2000. SWI-SNF-mediated nucleosome remodeling: role of histone octamer mobility in the persistence of the remodeled state. Mol Cell Biol **20:**3058-68.
104. **Jason, L. J. M., S. C. Moore, J. D. Lewis, G. Lindsey, and J. Ausio.** 2002. Histone uiquitination: a tagging tail unfolds? BioEssays **24:**166-174.
105. **John, S., L. Howe, S. T. Tafrov, P. A. Grant, R. Sternglanz, and J. L. Workman.** 2000. The something about silencing protein, Sas3, is the catalytic subunit of NuA3, a yTAF(II)30-containing HAT complex that interacts with the Spt16 subunit of the yeast CP (Cdc68/Pob3)-FACT complex. Genes Dev **14:**1196-208.
106. **Jones, D. O., I. G. Cowell, and P. B. Singh.** 2000. Mammalian chromodomain proteins: their role in genome organisation and expression. Bioessays **22:**124-37.
107. **Kalpana, G. V., S. Marmon, W. Wang, G. R. Crabtree, and S. P. Goff.** 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science **266:**2002-2006.
108. **Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell **85:**403-14.
109. **Keller, A. D., and T. Maniatis.** 1991. Identification and characterization of a novel repressor of beta-interferon gene expression. Genes Dev **5:**868-79.
110. **Kennison, J. A.** 1995. The polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function. Annual Review of Genetics **29:**289-303.
111. **Khavari, P. A., C. L. Peterson, J. W. Tamkun, and G. R. Crabtree.** 1993. BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. Nature **366:**170-174.
112. **Kim, J. K., S. O. Huh, H. Choi, K. S. Lee, D. Shin, C. Lee, J. S. Nam, H. Kim, H. Chung, H. W. Lee, S. D. Park, and R. H. Seong.** 2001. Srg3, a

mouse homolog of yeast SWI3, is essential for early embryogenesis and involved in brain development. Mol. Cell. Biol. **21**:7787-95.

113. **Kingston, R., and G. Narlikar.** 1999. ATP dependent remodeling and acetylation as regulators of chromatin fluidity. Genes and Development **13**:2339-2352.
114. **Kleinschmidt, A. M., and H. G. Martinson.** 1981. Structure of nucleosome core particles containing uH2A (A24). Nucleic Acids Res. **9**:2423-2431.
115. **Klochendler-Yeivin, A., L. Fiette, J. Barra, C. Muchardt, C. Babinet, and M. Yaniv.** 2000. The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression. EMBO Rep **1**:500-6.
116. **Knezetic, J. A., and D. S. Luse.** 1986. The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro. Cell **45**:95-104.
117. **Kodaki, T., K. Hosaka, J. Nikawa, and S. Yamashita.** 1995. The SNF2/SWI2/GAM1/TYE3/RIC1 gene is involved in the coordinate regulation of phospholipid synthesis in Saccharomyces cerevisiae. J Biochem (Tokyo) **117**:362-8.
118. **Koipally, J., A. Renold, J. Kim, and K. Georgopoulos.** 1999. Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. EMBO J. **18**:3090-3100.
119. **Kouzarides, T.** 2002. Histone methylation in transcriptional control. Current Opinion in Genetics & Development **12**:198-209.
120. **Kruger, W., and I. Herskowitz.** 1991. A negative regulator of HO transcription, SIN1 (SPT2), is a nonspecific DNA binding protein related to HMG1. Molecular and Cellular Biology **11**:4135-4146.
121. **Kruger, W., C. L. Peterson, A. Sil, C. Coburn, G. Arents, E. N. Moudrianakis, and I. Herskowitz.** 1995. Amino acid substitutions in the structured domains of histones H3 and H4 partially relieve the requirement of the yeast SWI/SNF complex for transcription. Genes and Development **9**:2770-2779.
122. **Kung, A. L., V. I. Rebel, R. T. Bronson, L. E. Ch'ng, C. A. Sieff, D. M. Livingston, and T. P. Yao.** 2000. Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. Genes Dev **14**:272-7.
123. **Kurotaki, N., K. Imaizumi, N. Harada, M. Masuno, T. Kondoh, T. Nagai, H. Ohashi, K. Naritomi, M. Tsukahara, Y. Makita, T. Sugimoto, T. Sonoda, T. Hasegawa, Y. Chinen, H. A. Tomita Ha, A. Kinoshita, T. Mizuguchi, K. Yoshiura Ki, T. Ohta, T. Kishino, Y. Fukushima, N. Niikawa, and N. Matsumoto.** 2002. Haploinsufficiency of NSD1 causes Sotos syndrome. Nat Genet **30**:365-6.

124. **Kwon, H., A. N. Imbalzano, P. A. Khavari, R. E. Kingston, and M. R. Green.** 1994. Nucleosome disruption and enhancement of activator binding by a human SWI/SNF complex. Nature **370:**477-481.
125. **Laurent, B. C., I. Treich, and M. Carlson.** 1993. The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation. Genes Dev. **7:**583-591.
126. **Lee, D., J. W. Kim, T. Seo, S. G. Hwang, E.-J. Choi, and J. Choe.** 2002. SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. The Journal of Biological Chemistry **277:**22330-22337.
127. **Lee, D., C. Lim, T. Seo, H. Kwon, H. Min, and J. Choe.** 2002. The viral oncogene HPV E7 deregulates transcriptional silencing by BRG-1 via molecular interactions. J Biol Chem.
128. **Lee, D., H. Sohn, G. V. Kalpana, and J. Choe.** 1999. Interaction of E1 and hSNF5 proteins stimulates replication of human papillomavirus DNA. Nature **399:**487-91.
129. **Lee, E. Y., C. Y. Chang, N. Hu, Y. C. Wang, C. C. Lai, K. Herrup, W. H. Lee, and A. Bradley.** 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature **359:**270-271.
130. **Lee, H. L., and T. K. Archer.** 1998. Prolonged glucocorticoid exposure dephosphorylates histone H1 and inactivates the MMTV promoter. Embo J **17:**1454-66.
131. **Lee, K. M., S. Sif, R. E. Kingston, and J. J. Hayes.** 1999. hSWI/SNF disrupts interactions between the H2A N-terminal tail and nucleosomal DNA. Biochemistry **38:**8423-9.
132. **LeGouy, E., E. M. Thompson, C. Muchardt, and J. P. Renard.** 1998. Differential preimplantation regulation of two mouse homologues of the yeast SWI2 protein. Dev Dyn **212:**38-48.
133. **LeRoy, G., A. Loyola, W. S. Lane, and D. Reinberg.** 2000. Purificatin and characterization of a human factor that assembles and remodels chromatin. J. Biol. Chem. **275:**14787-90.
134. **LeRoy, G., G. Orphanides, W. S. Lane, and D. Reinberg.** 1998. Requirement of RSF and FACT for transcription of chromatin templates in vitro [see comments]. Science **282:**1900-4.
135. **Levinger, L., J. Barsoum, and A. Varshavsky.** 1981. Two-dimenstiolnal hybridization mapping of nucleosomes. Comparison of DNA and protein patterns. J. Mol. Biol. **146:**287-304.
136. **Levinger, L., and A. Varshavsky.** 1980. High-resolution fractionation of nucleosomes: minor particles, "whiskers" and separation of nucleosomes containing and lacking A24 semihistone. Proc. Natl. Acad. Sci. USA **77:**3244-3248.
137. **Lin, R. J., L. Magy, S. Inoue, W. Shao, W. H. Miller, Jr., and R. M. Evans.** 1998. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature (London) **391:**811-814.

138. **Lochner, K., G. Siegler, M. Fuhrer, J. Greil, J. D. Beck, G. H. Fey, and R. Marschalek.** 1996. A specific deletion in the breakpoint cluster region of the ALL-1 gene is associated with acute lymphoblastic T-cell leukemias. Cancer Res **56:**2171-7.
139. **Logie, C., and C. L. Peterson.** 1997. Catalytic activity of the yeast SWI/SNF complex on reconstituted nucleosome arrays. EMBO Journal **16:**6772-6782.
140. **Lorch, Y., B. R. Cairns, M. Zhang, and R. D. Kornberg.** 1998. Activated RSC-nucleosome complex and persistently altered form of the nucleosome. Cell **94:**29-34.
141. **Lorch, Y., J. W. LaPointe, and R. D. Kornberg.** 1987. Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. Cell **49:**203-210.
142. **Lorch, Y., M. Zhang, and R. D. Kornberg.** 1999. Histone octamer transfer by a chromatin-remodeling complex. Cell **96:**389-92.
143. **Louis B. Harrison, R. B. S., and Waun Ki Hong.** 1999. Head and Neck Cancer. Lippincott-Raven Publishers.
144. **Luger, K., A. W. Mäder, R. K. Richmond, D. F. Sargent, and T. J. Richmond.** 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:**251-260.
145. **Luger, K., and T. J. Richmond.** 1998. The histone tails of the nucleosome. Current Opinion in Genetics and Development **8:**140-146.
146. **Lundblad, J. R., R. P. Kwok, M. E. Laurance, M. L. Harter, and R. H. Goodman.** 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature **374:**85-8.
147. **Luo, R. X., A. A. Postigo, and D. C. Dean.** 1998. Rb interacts with histone deacetylase to repress transcription. Cell **92:**463-473.
148. **Mahadevan, L. C., A. C. Willis, and M. J. Barratt.** 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. Cell **65:**775-83.
149. **Malgeri, U., L. Baldini, V. Perfetti, S. Fabris, M. C. Vignarelli, G. Colombo, V. Lotti, S. Compasso, S. Bogni, L. Lombardi, A. T. Maiolo, and A. Neri.** 2000. Detection of t(4;14)(p16.3;q32) chromosomal translocation in multiple myeloma by reverse transcription-polymerase chain reaction analysis of IGH-MMSET fusion transcripts. Cancer Res **60:**4058-61.
150. **Marin, I., M. L. Siegal, and B. S. Baker.** 2000. The evolution of dosage-compensation mechanisms. Bioessays **22:**1106-14.
151. **Martens, J. A., and F. Winston.** 2002. Evidence that Swi/Snf directly represses transcription in S. cerevisiae. Genes Dev **16:**2231-6.
152. **Martinez-Balbas, M. A., U. M. Bauer, S. J. Nielsen, A. Brehm, and T. Kouzarides.** 2000. Regulation of E2F1 activity by acetylation. Embo J **19:**662-71.

153. **Melnick, A., and J. D. Licht.** 1999. Deconstructing a disease: RAR alpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood **93:**3167-3215.
154. **Miller, M. E., B. R. Cairns, R. S. Levinson, K. R. Yamamoto, D. A. Engel, and M. M. Smith.** 1996. Adenovirus E1A specifically blocks SWI/SNF-dependent transcriptional activation. Molecular and Cellular Biology **16:**5737-5743.
155. **Miller, R. W., and J. H. Rubinstein.** 1995. Tumors in Rubinstein-Taybi syndrome. Am J Med Genet **56:**112-5.
156. **Mochizuki, N., S. Shimizu, T. Nagasawa, H. Tanaka, M. Taniwaki, J. Yokota, and K. Morishita.** 2000. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. Blood **96:**3209-14.
157. **Mock, B. A., L. Liu, D. LePaslier, and S. Huang.** 1996. The B-lymphocyte maturation promoting transcription factor BLIMP1/PRDI-BF1 maps to D6S447 on human chromosome 6q21-q22.1 and the syntenic region of mouse chromosome 10. Genomics **37:**24-8.
158. **Muchardt, C., C. Sardet, B. Bourachot, C. Onufryk, and M. Yaniv.** 1995. A human protein with homology to *Saccharomyces cerevisiae* SNF5 interacts with the potential helicase hbrm. Nucleic Acids Research **23:**1127-1132.
159. **Muchardt, C., and M. Yaniv.** 1993. A human homologue of *Saccharomyces cerevisiae SNF2/SWI2* and *Drosophila brm* genes potentiates transcriptional activation by the glucocorticoid receptor. EMBO J. **12:**4279-4290.
160. **Muraoka, M., M. Konishi, R. Kikuchi-Yanoshita, K. Tanaka, N. Shitara, J. M. Chong, T. Iwama, and M. Miyaki.** 1996. p300 gene alterations in colorectal and gastric carcinomas. Oncogene **12:**1565-9.
161. **Murphy, D. J., S. Hardy, and D. A. Engel.** 1999. Human SWI-SNF component BRG1 represses transcription of the c-fos gene. Mol Cell Biol **19:**2724-33.
162. **Nagy, L., H.-Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans.** 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell **89:**373-380.
163. **Nagy, L. e. a.** 1999. Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev. **13:**3209-3216.
164. **Neely, K. E., A. H. Hassan, C. E. Brown, L. Howe, and J. L. Workman.** 2002. Transcription activator interactions with multiple SWI/SNF subunits. Mol. Cell. Biol. **22:**1615-25.
165. **Neigeborn, L., and M. Carlson.** 1984. Genes affecting the regulation of *SUC2* gene expression by glucose repression in *saccharomyces cerevisiae*. Genetics **108:**845-858.

166. **Neuwald, A. F., and D. Landsman.** 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. Trends Biochem Sci **22:**154-5.
167. **Nielsen, S. J., R. Schneider, U. M. Bauer, A. J. Bannister, A. Morrison, D. O'Carroll, R. Firestein, M. Cleary, T. Jenuwein, R. E. Herrera, and T. Kouzarides.** 2001. Rb targets histone H3 methylation and HP1 to promoters. Nature **412:**561-5.
168. **Noll, M., and R. D. Kornberg.** 1977. Action of micrococcal nuclease on chromatin and the location of H1. J. Mol. Biol. **109:**393-404.
169. **Oelgeschlager, M., R. Janknecht, J. Krieg, S. Schreek, and B. Luscher.** 1996. Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. Embo J **15:**2771-80.
170. **Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani.** 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell **87:**953-959.
171. **Oike, Y., A. Hata, T. Mamiya, T. Kaname, Y. Noda, M. Suzuki, H. Yasue, T. Nabeshima, K. Araki, and K. Yamamura.** 1999. Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. Hum Mol Genet **8:**387-96.
172. **Owa, T. e. a.** 2001. Cell cycle regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents. Curr. Med. Chem. **8:**1487-1503.
173. **Park, M. S., J. Rosai, H. T. Nguyen, P. Capodieci, C. Cordon-Cardo, and A. Koff.** 1999. p27 and Rb are on overlappig pathways suppressing tumorigenesis in mice. Proc. Natl. Acad. Sci. USA **96:**6382-6387.
174. **Pattenden, S. G., R. Klose, E. Karaskov, and R. Bremner.** 2002. Interferon-γ-induced chromatin remodeling at the CIITA locus is BRG1 dependent. EMBO J **21:**1978-1986.
175. **Paulson, J. R., and S. S. Taylor.** 1982. Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. J. Biol. Chem. **257:**6064-6072.
176. **Peters, A. H., D. O'Carroll, H. Scherthan, K. Mechtler, S. Sauer, C. Schofer, K. Weipoltshammer, M. Pagani, M. Lachner, A. Kohlmaier, S. Opravil, M. Doyle, M. Sibilia, and T. Jenuwein.** 2001. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell **107:**323-37.
177. **Peterson, C. L., A. Dingwall, and M. P. Scott.** 1994. Five *SWI/SNF* gene products are components of a large multiprotein complex required for transcriptional enhancement. Proc. Natl. Acad. Sci. U.S.A. **91:**2905-2908.
178. **Peterson, C. L., and J. W. Tamkun.** 1995. The SWI-SNF complex: A chromatin remodeling machine? Trends in Biochemical Sciences **20:**143-146.

179. **Peterson, L., and I. Herskowitz.** 1992. Characterization of the yeast *SWI1*, *SWI2*, and *SWI3* genes, which encode a global activator of transcription. Cell **68:**573-583.
180. **Petrij, F., R. H. Giles, H. G. Dauwerse, J. J. Saris, R. C. Hennekam, M. Masuno, N. Tommerup, G. J. van Ommen, R. H. Goodman, D. J. Peters, and et al.** 1995. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature **376:**348-51.
181. **Phelan, M. L., G. R. Schnitzler, and R. E. Kingston.** 2000. Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases [In Process Citation]. Mol Cell Biol **20:**6380-9.
182. **Phelan, M. L., S. Sif, G. J. Narlikar, and R. E. Kingston.** 1999. Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Mol Cell **3:**247-53.
183. **Piao, Z., W. Fang, S. Malkhosyan, H. Kim, A. Horii, M. Perucho, and S. Huang.** 2000. Frequent frameshift mutations of RIZ in sporadic gastrointestinal and endometrial carcinomas with microsatellite instability. Cancer Res **60:**4701-4.
184. **Poot, R. A., G. Dellaire, B. B. Hulsmann, M. A. Grimaldi, D. F. Corona, P. B. Becker, W. A. Bickmore, and P. D. Varga-Weisz.** 2000. HuCHRAC, a human ISWI chromatin remodeling complex contains hACF1 and two novel histone-fold proteins. EMBO J. **19:**3377-87.
185. **Razani, B., J. A. Engelman, X. B. Wang, W. Schubert, X. L. Zhang, C. B. Marks, F. Macaluso, R. G. Russell, M. Li, R. G. Pestell, D. Di Vizio, H. Hou, Jr., B. Kneitz, G. Lagaud, G. J. Christ, W. Edelmann, and M. P. Lisanti.** 2001. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem **276:**38121-38.
186. **Rea, S. e. a.** 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature **406:**593-599.
187. **Reifsnyder, C., J. Lowell, A. Clarke, and L. Pillus.** 1996. Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nat Genet **14:**42-9.
188. **Reyes, J. C., J. Barra, C. Muchardt, A. Camus, C. Babinet, and M. Yaniv.** 1998. Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). EMBO J **17:**6979-91.
189. **Roberts, C. W., M. M. Leroux, M. D. Fleming, and S. H. Orkin.** 2002. Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene Snf5. Cancer Cell **2:**415-25.
190. **Roberts, C. W. M., S. A. Galusha, M. E. McMenamin, C. D. M. Fletcher, and S. H. Orkin.** 2000. Haploinsufficiency of Snf5 (integrase interactor 1) predisposes to malignant rhabdoid tumors in mice. PNAS **97:**13796-13800.
191. **Roguev, A., D. Schaft, A. Shevchenko, W. W. Pijnappel, M. Wilm, R. Aasland, and A. F. Stewart.** 2001. The Saccharomyces cerevisiae Set1

complex includes an Ash2 homologue and methylates histone 3 lysine 4. Embo J **20:**7137-48.

192. **Rosati, R., R. La Starza, A. Veronese, A. Aventin, C. Schwienbacher, T. Vallespi, M. Negrini, M. F. Martelli, and C. Mecucci.** 2002. NUP98 is fused to the NSD3 gene in acute myeloid leukemia associated with t(8;11)(p11.2;p15). Blood **99:**3857-60.
193. **Roth, S. Y., J. M. Denu, and C. D. Allis.** 2001. Histone acetyltransferases. Annu. Rev. Biochem. **70:**81-120.
194. **Rousseau-Merck, M. F., I. Versteege, I. Legrand, J. Couturier, A. Mairal, O. Delattre, and A. Aurias.** 1999. hSNF5/INI1 inactivation is mainly associated with homozygous deletions and mitotic recombinations in rhabdoid tumors. Cancer Res **59:**3152-6.
195. **Rozenblatt-Rosen, O., T. Rozovskaia, D. Burakov, Y. Sedkov, S. Tillib, J. Blechman, T. Nakamura, C. M. Croce, A. Mazo, and E. Canaani.** 1998. The C-terminal SET domains of ALL-1 and TRITHORAX interact with the INI1 and SNR1 proteins, components of the SWI/SNF complex. Proceedings of the National Academy of Sciences, USA **95:**4152-4157.
196. **Ruiz-Carrillo, A., L. J. Wangh, and V. G. Allfrey.** 1975. Processing of newly synthesized histone molecules. Science **190:**117-28.
197. **Sartorelli, V., J. Huang, Y. Hamamori, and L. Kedes.** 1997. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol Cell Biol **17:**1010-26.
198. **Schichman, S. A., M. A. Caligiuri, Y. Gu, M. P. Strout, E. Canaani, C. D. Bloomfield, and C. M. Croce.** 1994. ALL-1 partial duplication in acute leukemia. Proc Natl Acad Sci U S A **91:**6236-9.
199. **Schilling, T. F.** 1997. Genetic analysis of craniofacial development in the vertebrate embryo. BioEssays **19:**459-468.
200. **Schneider, R., A. J. Bannister, and T. Kouzarides.** 2002. Unsafe SETs: histone lysine methyltransferases and cancer. TRENDS in Biochemical Sciences **27:**396-402.
201. **Seale, R. L.** 1981. Rapid turnover of the histone-ubiquitin conjugate, protein A24. Nucleic Acids Res. **9:**3151-3158.
202. **Seelig, H. P., I. Moosbrugger, H. Ehrfeld, T. Fink, M. Renz, and E. Genth.** 1995. The major dermatomyositis-specific Mi-2 autoantigen is a presumed helicase involved in transcriptional activation. Arthritis Rheum. **38:**1389-1399.
203. **Sentani, K., N. Oue, H. Kondo, K. Kuraoka, J. Motoshita, R. Ito, H. Yokozaki, and W. Yasui.** 2001. Increased expression but not genetic alteration of BRG1, a component of the SWI/SNF complex, is associated with the advanced stage of human gastric carcinomas. Pathobiology **69:**315-320.
204. **Sévenet, N., A. Lellouch-Tubiana, D. Schofield, K. Hoang-Xuan, M. Gessler, D. Birnbaum, C. Jeanpierre, A. Jouvet, and O. Delattre.** 1999.

Spectrum of hSNF5/INI1 somatic mutations in human cancer and genotype-phenotype correlations. Hum Mol Genet **8:**2359-68.

205. **Sévenet, N., E. Sheridan, D. Amram, P. Schneider, R. Handgretinger, and O. Delattre.** 1999. Constitutional mutations of the *hSNF5/INI1* gene predispose to a variety of cancers. Am. J. Hum. Genet. **65:**1342-1348.
206. **Shanahan, F., W. Seghezzi, D. Parry, D. Mahony, and E. Lees.** 1999. Cyclin E associates with BAF155 and BRG1, components of the mammalian SWI-SNF complex, and alters the ability of BRG1 to induce growth arrest. Mol Cell Biol **19:**1460-9.
207. **Shen, X., G. Mizuguchi, A. Hamiche, and C. Wu.** 2000. A chromatin remodelling complex involved in transcription and DNA processing. Nature **406:**541-4.
208. **Shinagawa, T., H. D. Dong, M. Xu, T. Maekawa, and S. Ishii.** 2000. The sno gene, which encodes a component of the histone deacetylase complex, acts as a tumor suppressor in mice. Embo J **19:**2280-91.
209. **Sobulo, O. M., J. Borrow, R. Tomek, S. Reshmi, A. Harden, B. Schlegelberger, D. Housman, N. A. Doggett, J. D. Rowley, and N. J. Zeleznik-Le.** 1997. MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16)(q23;p13.3). Proc Natl Acad Sci U S A **94:**8732-7.
210. **Spangenberg, C., K. Eisfeld, W. Stunkel, K. Luger, A. Flaus, T. J. Richmond, M. Truss, and M. Beato.** 1998. The mouse mammary tumour virus promoter positioned on a tetramer of histones H3 and H4 binds nuclear factor 1 and OTF1. J Mol Biol **278:**725-39.
211. **Stec, I., G. J. van Ommen, and J. T. den Dunnen.** 2001. WHSC1L1, on human chromosome 8p11.2, closely resembles WHSC1 and maps to a duplicated region shared with 4p16.3. Genomics **76:**5-8.
212. **Stec, I., T. J. Wright, G. J. van Ommen, P. A. de Boer, A. van Haeringen, A. F. Moorman, M. R. Altherr, and J. T. den Dunnen.** 1998. WHSC1, a 90 kb SET domain-containing gene, expressed in early development and homologous to a Drosophila dysmorphy gene maps in the Wolf-Hirschhorn syndrome critical region and is fused to IgH in t(4;14) multiple myeloma. Hum Mol Genet **7:**1071-82.
213. **Steele-Perkins, G., W. Fang, X. H. Yang, M. Van Gele, T. Carling, J. Gu, I. M. Buyse, J. A. Fletcher, J. Liu, R. Bronson, R. B. Chadwick, A. de la Chapelle, X. Zhang, F. Speleman, and S. Huang.** 2001. Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. Genes Dev **15:**2250-62.
214. **Stern, M. J., R. Jensen, and I. Herskowitz.** 1984. Five *SWI* genes are required for expression of the *HO* gene in yeast. J. Mol. Biol. **178:**853-868.
215. **Sterner, D. E., and S. L. Berger.** 2000. Acetylation of histones and transcription-related factors. Microbiol. Mol. Biol. Rev. **64:**435-59.
216. **Strahl, B. D., and C. D. Allis.** 2000. The language of covalent histone modifications. Nature **403:**41-5.

217. **Strahl, B. D., P. A. Grant, S. D. Briggs, Z. W. Sun, J. R. Bone, J. A. Caldwell, S. Mollah, R. G. Cook, J. Shabanowitz, D. F. Hunt, and C. D. Allis.** 2002. Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol Cell Biol **22:**1298-306.

218. **Strobeck, M., W., K. E. Knudsen, A. F. Fribourg, M. F. DeCristoforo, B. E. Weissman, A. N. Imbalzano, and E. S. Knudsen.** 2000. BRG-1 is required for Rb mediated cell cycle arrest. Proceedings of the National Academy of Sciences, USA **97:**7748-7753.

219. **Strobeck, M. W., D. N. Reisman, R. W. Gunawardena, B. L. Betz, S. P. Angus, K. E. Knudsen, T. F. Kowalik, B. E. Weissman, and E. S. Knudsen.** 2002. Compensation of BRG-1 function by Brm: insight into the role of the core SWI-SNF subunits in retinoblastoma tumor suppressor signaling. J Biol Chem **277:**4782-9.

220. **Strobeck, M. W., D. N. Reisman, R. W. Gunawardena, B. L. Betz, S. P. Angus, K. E. Knudsen, T. F. Kowalik, B. E. Weissman, and E. S. Knudsen.** 2002. Compensation of BRG-1 function by Brm: insight into the role of the core SWI-SNF subunits in retinoblastoma tumor supprssor signaling. J. Biol. Chem. **277:**4782-9.

221. **Struhl, K.** 1998. Histone acetylation and transcriptional regulatory mechanisms. Genes and Development **12:**599-606.

222. **Sudarsanam, P., V. R. Iyer, P. O. Brown, and F. Winston.** 2000. Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **97:**3364-9.

223. **Sumi-Ichinose, C., H. Ichinose, D. Metzger, and P. Chambon.** 1997. SNF2β-BRG1 is essentail for the viability of F9 murine embryonal carcinoma cells. Molecular and Cellular Biology **17:**5976-5986.

224. **Sutani, T., T. Yuasa, T. Tomonaga, N. Dohmae, K. Takio, and M. Yanagida.** 1999. Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4. Genes Dev. **13:**2271-2283.

225. **Tan, Y. C., and V. T. Chow.** 2001. Novel human HALR (MLL3) gene encodes a protein homologous to ALR and to ALL-1 involved in leukemia, and maps to chromosome 7q36 associated with leukemia and developmental defects. Cancer Detect Prev **25:**454-69.

226. **Tanaka, T., M. Kimura, K. Matsunaga, D. Fukada, H. Mori, and Y. Okano.** 1999. Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. Cancer Res. **59:**2041-2044.

227. **Tatsuka, M., H. Katayama, T. Ota, T. Tanaka, S. Odashima, F. Suzuki, and Y. Terada.** 1998. Multinuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells. Cancer Res. **58:**4811-4816.

228. **Taunton, J., C. A. Hassig, and S. L. Schreiber.** 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science **272:**408-411.

229. **Taylor, M. D., N. Gokgoz, I. L. Andrulis, T. G. Mainprize, J. M. Drake, and J. T. Rutka.** 2000. Familial posterior fossa brain tumors of infancy secondary to germline mutation of the hSNF5 gene. Am. J. Hum. Genet. **66:**1403-1406.

230. **Toh, Y., S. D. Pencil, and G. L. Nicolson.** 1994. A novel candidate metastasis-associated gene, *mta1*, differentially expressed in highly metastatic mammary adenocarcinoma cell lines. cDNA cloning, expression and protein analyses. J. Biol. Chem. **269:**22958-22963.

231. **Tong, J. K., C. A. Hassig, G. R. Schnitzler, R. E. Kingston, and S. L. Schreiber.** 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature **395:**917-21.

232. **Tran, H. G., D. J. Steger, V. R. Iyer, and A. D. Johnson.** 2000. The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. Embo J **19:**2323-31.

233. **Trouche, D., C. Le Chalony, C. Muchardt, M. Yaniv, and T. Kouzarides.** 1997. Rb and hbrm cooperate to repress the activation functions of E2F1. Proceedings of the National Academy of Sciences, USA **94:**11268-11273.

234. **Tse, C., T. Sera, A. P. Wolffe, and J. C. Hansen.** 1998. Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III [In Process Citation]. Mol Cell Biol **18:**4629-38.

235. **Tsukiyama, T., C. Daniel, J. Tamkun, and C. Wu.** 1995. *ISWI*, a member of the *SWI2/SNF2* ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. Cell **83:**1021-1026.

236. **Tsukiyama, T., J. Palmer, C. C. Landel, J. Shiloach, and C. Wu.** 1999. Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae. Genes Dev **13:**686-97.

237. **Tsukiyama, T., and C. Wu.** 1995. Purification and properties of an ATP-dependent nucleosome remodeling factor. Cell **83:**1011-1020.

238. **Uno, K., J. Takita, K. Yokomori, Y. Tanaka, S. Ohta, H. Shimada, F. H. Gilles, K. Sugita, S. Abe, M. Sako, K. Hashizume, and Y. Hayashi.** 2002. Aberrations of the hSNF5/INI1 gene are restricted to malignant rhabdoid tumors or atypical teratoid/rhabdoid tumors in pediatric solid tumors. Genes Chromosomes Cancer **34:**33-41.

239. **Utley, P. T., J. Côté, T. Owen-Hughes, and J. L. Workman.** 1997. SWI/SNF stimulates the formation of disparate activator-nulceosome complexes but is partially redundant with cooperative binding. Journal of Biological Chemistry **272:**12642-12649.

240. **van Holde, K. E., B. R. Shaw, D. Lohr, T. M. Herman, and R. T. Kovacic.** 1975. Amsterdam: North Holland.

241. **Varga-Weisz, P. D., M. Wilm, E. Bonte, K. Dumas, M. Mann, and P. B. Becker.** 1997. Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. Nature (London) **388:**598-602.
242. **Vassilev, A. P., H. H. Rasmussen, E. I. Christensen, S. Nielsen, and J. E. Celis.** 1995. The levels of ubiquitinated histone H2A are highly upregulated in transformed human cells: partial colocalization of uH2A cluster and PCNA/cyclin foci in a fraction of cells in S-phase. J. of Cell Sci. **108:**1205-1215.
243. **Venkatachalam, S., Y. P. Shi, S. N. Jones, H. Vogel, A. Bradley, D. Pinkel, and L. A. Donehower.** 1998. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. Embo J **17:**4657-67.
244. **Versteege, I., S. Medjkane, D. Rouillard, and O. Delattre.** 2002. A key role of the hSNF5/INI1 tumour suppressor in the control of the G1-S transition of the cell cycle. Oncogene **21:**6403-12.
245. **Versteege, I., N. Sevenet, J. Lange, M. F. Rousseau-Merck, P. Ambros, R. Handgretinger, A. Aurias, and O. Delattre.** 1998. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature **394:**203-6.
246. **Vignali, M., A. H. Hassan, K. E. Neely, and J. L. Workman.** 2000. ATP-dependent chromatin-remodeling complexes. Mol Cell Biol **20:**1899-910.
247. **Visser, H. P., M. J. Gunster, H. C. Kluin-Nelemans, E. M. Manders, F. M. Raaphorst, C. J. Meijer, R. Willemze, and A. P. Otte.** 2001. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol **112:**950-8.
248. **Wade, P. A., A. Gegonne, P. L. Jones, E. Ballestar, F. Aubry, and A. P. Wolffe.** 1999. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation [see comments]. Nat Genet **23:**62-6.
249. **Wang, H., R. Cao, L. Xia, H. Erdjument-Bromage, C. Borchers, P. Tempst, and Y. Zhang.** 2001. Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol Cell **8:**1207-17.
250. **Wang, H. B., and Y. Zhang.** 2001. Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. Nucleic Acids Res **29:**2517-21.
251. **Wang, S., B. Zhang, and D. V. Faller.** 2002. Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. Embo J **21:**3019-28.
252. **Wang, W., J. Côte, Y. Xue, S. Zhou, P. A. Khavari, S. R. Biggar, C. Muchardt, G. V. Kalpana, S. P. Goff, M. Yaniv, J. L. Workman, and G. R. Crabtree.** 1996. Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. EMBO Journal **15:**5370-5382.
253. **Wang, W., Y. Xue, S. Zhou, A. Kuo, B. R. Cairns, and G. R. Crabtree.** 1996. Diversity and specialization of mammalian SWI/SNF complexes. Genes and Development **10:**2117-2130.

254. **Wang, X., S. Yeh, G. Wu, C. L. Hsu, L. Wang, T. Chiang, Y. Yang, Y. Guo, and C. Chang.** 2001. Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells. J Biol Chem **276:**40417-23.

255. **Wei, Y., L. Yu, J. Bowen, M. A. Gorovsky, and C. D. Allis.** 1999. Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. Cell **97:**99-109.

256. **West, M. H. P., and W. M. Bonner.** 1980. Histone 2B can be modified by the attachment of ubiquitin. Nucleic Acids Res. **8:**4671-4680.

257. **Winter, A. G., G. Sourvinos, S. J. Allison, K. Tosh, P. H. Scott, D. A. Spandidos, and R. J. White.** 2000. RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. Proc. Natl. Acad. Sci. U.S.A. **97:**12619-24.

258. **Wolffe, A. P., and J. J. Hayes.** 1999. Chromatin disruption and modification. Nucleic Acids Res **27:**711-20.

259. **Wong, A. K., F. Shanahan, Y. Chen, L. Lian, P. Ha, K. Hendricks, S. Ghaffari, D. Iliev, B. Penn, A. M. Woodland, R. Smith, G. Salada, A. Carillo, K. Laity, J. Gupte, B. Swedlund, S. V. Tavtigian, D. H. Teng, and E. Lees.** 2000. BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Research **60:**6171-7.

260. **Workman, J. L., and R. E. Kingston.** 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annual Review of Biochemistry **67:**545-579.

261. **Wu, D. Y., G. V. Kalpana, S. P. Goff, and W. H. Schubach.** 1996. Epstein-Barr virus nuclear protein 2 (EBNA2) binds to a component of the human SNF-SWI complex, hSNF5/Ini1. Journal of Virology **70:**6020-6028.

262. **Wu, D. Y., A. Krumm, and W. H. Schubach.** 2000. Promoter-specific targeting of human SWI-SNF complex by Epstein-Barr virus nuclear protein 2. J Virol **74:**8893-903.

263. **Wu, D. Y., D. C. Tkachuck, R. S. Roberson, and W. H. Schubach.** 2002. The human SNF5/INI1 protein facilitates the function of the growth arrest and DNA damage-inducible protein (GADD34) and modulates GADD34-bound protein phosphatase-1 activity. J Biol Chem **277:**27706-15.

264. **Wu, R. S., K. W. Kohn, and W. M. Bonner.** 1981. Metabolism of ubiquitinated histones. J. Biol. Chem. **256:**5916-5920.

265. **Wyrick, J. J. e. a.** 1999. Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature **402:**418-421.

266. **Xue, Y., J. C. Canman, C. S. Lee, Z. Nie, D. Yang, G. T. Moreno, M. K. Young, E. D. Salmon, and W. Wang.** 2000. The human SWI/SNF-B chromatin-remodeling complex is related to yeast Rsc and localizes at kinetochores of mitotic chromosomes. Proc. Natl. Acad. Sci. USA **97:**13015-13020.

267. **Xue, Y., J. Wong, G. T. Moreno, M. K. Young, J. Cote, and W. Wang.** 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities [In Process Citation]. Mol Cell **2:**851-61.

268. **Yang, L., L. Xia, D. Y. Wu, H. Wang, H. A. Chansky, W. H. Schubach, D. D. Hickstein, and Y. Zhang.** 2002. Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. Oncogene **21:**148-52.

269. **Yang, X.-J., V. V. Ogryzko, J.-I. Nishikawa, B. H. Howard, and Y. Nakatani.** 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature **382:**319-324.

270. **Yang, X. H., and S. Huang.** 1999. PFM1 (PRDM4), a new member of the PR-domain family, maps to a tumor suppressor locus on human chromosome 12q23-q24.1. Genomics **61:**319-25.

271. **Yao, T. P., S. P. Oh, M. Fuchs, N. D. Zhou, L. E. Ch'ng, D. Newsome, R. T. Bronson, E. Li, D. M. Livingston, and R. Eckner.** 1998. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell **93:**361-72.

272. **Yoshimoto, H., and I. Yamashita.** 1991. The GAM1/SNF2 gene of Saccharomyces cerevisiae encodes a highly charged nuclear protein required for transcription of the STA1 gene. Molecular and General Genetics **228:**270-280.

273. **Yuan, W., G. Condorelli, M. Caruso, A. Felsani, and A. Giordano.** 1996. Human p300 protein is a coactivator for the transcription factor MyoD. Journal of Biological Chemistry **271:**9009-9013.

274. **Zambrowicz, B., G. Friedrich, E. Buxton, S. Lilleberg, C. Person, and A. Sands.** 1998. Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells. Nature **392:**608-11.

275. **Zeleznik-Le, N. J., A. M. Harden, and J. D. Rowley.** 1994. 11q23 translocations split the "AT-hook" cruciform DNA-binding region and the transcriptional repression domain from the activation domain of the mixed-lineage leukemia (MLL) gene. Proc Natl Acad Sci U S A **91:**10610-4.

276. **Zhang, H. S., M. Gavin, A. Dahiya, A. A. Postigo, D. Ma, R. X. Luo, J. W. Harbour, and D. C. Dean.** 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell **101:**79-89.

277. **Zhang, Y., G. LeRoy, H. P. Seelig, W. S. Lane, and D. Reinberg.** 1998. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell **95:**279-89.

278. **Zhang, Z. K., K. P. Davies, J. Allen, L. Zhu, R. G. Pestell, D. Zagzag, and G. V. Kalpana.** 2002. Cell cycle arrest and repression of cyclin D1 transcription by INI1/hSNF5. Mol Cell Biol **22:**5975-88.

279. **Zhou, H., J. Kuang, L. Zhong, W. L. Kuo, J. W. Gray, A. Sahin, B. R. Brinkley, and S. Sen.** 1998. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet. **20:**189-193.

280. **Zhou, Y. B., S. E. Gerchman, V. Ramakrishnan, A. Travers, and S. Muyldermans.** 1998. Position and orientation of the globular domain of linker histone H5 on the nucleosome. Nature **395:**402-5.

281. **Ziemin-van der Poel, S., N. R. McCabe, H. J. Gill, R. Espinosa, III, Y. Patel, A. Harden, P. Rubinelli, S. D. Smith, M. M. LeBeau, J. D. Rowley, and et al.** 1991. Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc Natl Acad Sci U S A **88:**10735-9.

Appendix A

Preliminary Data

The Role of Brg1 in Early Mammalian Development

In yeast, the activity of the SWI/SNF enzyme is required for the expression of the mating type switch gene HO and the SUC2 invertase required for sucrose fermentation (23, 167, 216). Not surprisingly, there are no mammalian homologs of these genes. As such, one of the major thrusts of our lab has been to identify the mammalian gene targets of SWI/SNF.

We have been interested particularly in the gene regulation events occurring during mammalian embryogenesis that may require SWI/SNF activity. Mice homozygous-null for the ATPase subunit, *Brg1*, of the mammalian SWI/SNF complex are early embryonic lethal, with death occurring between days 3.5 and 6.5 post-fertilization (27). Because lethality occurs so early, it is not possible to generate mouse embryonic fibroblasts lacking Brg1. Likewise, *Brg1*-null embryonic stem (ES) cells are not viable, precluding the ability to study the *in vitro* differentiation of cells lacking Brg1 protein. To circumvent this obstacle, we generated mouse ES cells that constitutively expressed a dominant negative version of human BRG1 and examined the ability of these cells to differentiate *in vitro*.

Mouse embryonic stem cells are pluripotent cells that can differentiate into a variety of lineages. When plated in methylcellulose, ES cells will differentiate

into the three germ layers (endoderm, mesoderm, and ectoderm), muscle lineages, hematopoietic lineages, and neuronal lineages. The gene regulation events that occur throughout the differentiation process can be monitored by RT-PCR for various lineage-specific factors. We found that mouse ES cells expressing dominant negative BRG1 were defective in their ability to differentiate. However, preliminary experiments indicate that the expression of only a subset of germ layer markers was altered, suggesting that the inability of these cells to differentiate properly was not due to a global defect in transcription. Rather, it appears that SWI/SNF activity regulates the expression of specific factors required for mammalian development.

Materials and Methods

Generation of Cell Lines

AB2.2 mouse embryonic stem cells were electroporated with a linearized plasmid bearing a FLAG epitope-tagged, dominant-negative version of BRG1 (K798R) under control of a retroviral LTR. Puromycin-resistant clones were harvested for Western analysis for FLAG as described previously (49). Of 85 puromycin-resistant colonies screened, only one expressed significant levels of dominant-negative BRG1 protein. 3E3 is a FLAG-expressing clone, while 1E1 is a clone that was puromycin resistant but did not express detectable levels of FLAG-tagged protein.

Differentiation of ES Cells

ES cells of interest were grown to no more than 60% confluency on mitotically inactivated fibroblasts engineered to express leukemia inhibitory factor (LIF). At day 0 of differentiation ES cells were trypsinized and resuspended in DMEM with 15% FCS and 150 μM monothiolglycerol (MTG). Cells were plated in 1% methylcellulose media in DMEM + 15% FCS + 150 μM MTG at 20,000 cells/ 60 mm Petri dish.

RT-PCR

At indicated times, cells were harvested and processed for RNA using Trizol (Life Technologies), following the manufacturer's protocol. For each time-point, 1mg of RNA was subjected to reverse transcription using AMV-RT (Gibco) and oligo dT. Germ layer and control markers were amplified using the following primers: Hprt, F, 5′ GCTGGTGAAAAG GACCTCTCGAAGTG-3′, R, 5′-ATGGCCACAGG ACTAGAACACCTGC-3′, internal (I), 5′-CAAAGCCTAAGATGAGCGCAAGTT G-3′, Ta 55 °C; Sox2, F, 5′-GTTACCTCTTCCTCCCACTCCAG-3′, R, 5′-CCCG CCCTCCCCGCCGCCCTCA G-3′, I, 5′-GAGGGCTGGACTGCGAACTGGAGA A-3′, Ta 58 °C; Brachyury, F, 5′-TGCTGCCTGTGAGTCATAAC-3′, R, 5′-ACCA GGTGCTATATATTGCC-3′, I, 5′-GCTGGGAGCTCAGTTCTTTCGAGGC-3′, Ta 54 °C; Hnf1, F, 5′-TTCTAAGC TGAGCCAGCTGCAGACG-3′, R, 5′-GCTGAGGT TCTCCGGCTCTTTCAGA-3′, I, 5′-TGACACGGATGACGATGGGGAAGAC-3′,

Ta 62 °C; Hnf4, F, 5´-ACACGT CCCCATCTGAAG-3´, R, 5´-CTTCCTTCTTCAT GCCAG-3´, I, 5´-TCGAGCTGTG ACGGCTGCAAGGGGT-3´, Ta 55 °C. PCR products were resolved on 2% agarose gels, transferred to nylon membrane (GeneScreen), and probed with the indicated, radiolabeled internal primers. Erythrocyte markers were amplified using the following primers: β major globin, F, 5'-CACAACCCCAGAAACAGACA-3', R, 5'-CTGACAGATGCTCTCTTGGG-3'; ε^{Y2} globin, F, 5'-GGAGAGTCCATTAAGAACCTAGACAA-3', R, 5'-CTGTCAATTC ATTGCCGAAGTAC-3'; βH1 globin, F, 5'-CTCAAGGAGACCTTTGCTCA-3', R, 5'-AGTCCCCATGGAGCTAAAGA-3'. Products were resolved on 2% agarose gels and stained with ethidium bromide.

Results

In order to disrupt SWI/SNF activity, a mouse embryonic stem cell line was generated that constitutively expressed an ATPase-defective form of human BRG1 (DN-BRG1) under control of a retroviral LTR (Figure A.1). The ATPase-inactive form of BRG1 previously was shown to interact with other components of the SWI/SNF complex in 3T3 cell lines (50), suggesting that the mutant protein depletes functional SWI/SNF complexes.

The DN-BRG1 expressing 3E3 cells were plated in methylcellulose medium to induce differentiation. As controls, wild-type AB2.2 ES cells as well as a cell line (1E1), derived from a clone that had been electroporated but did not express detectable levels of DN-BRG1 protein, were differentiated. When

A.



B.

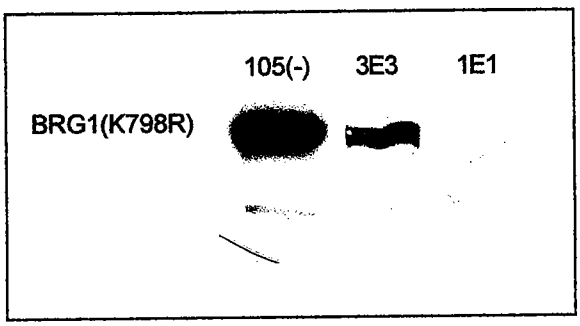


Figure A.1. 3E3 ES cells constitutively express dominant negative BRG1.

A. Comparison of conserved ATPase domain of mammalian BRG1 and yeast SWI2. Lysine boxed is mutated to arginine in the dominant negative BRG1. **B.** Western analysis showing expression of FLAG-tagged DN-BRG1 in 3E3 but not 1E1 cell lines. 105(-) is a 3T3 cell line that inducible expressed DN-BRG1.

differentiated *in vitro*, ES cells form clusters called embryoid bodies. As shown in Figure A.2, WT ES cells and 1E1 cells formed embryoid bodies that developed normally with time. However, while 3E3 embryoid bodies were indistinguishable from those of WT and 1E1 cells at day 3 post-differentiation, they failed to develop significantly beyond day 3. The defect was more severe than just a delay in the differentiation process as the 3E3 cells remained morphologically the same from day 7 to day 14 post-differentiation.

Embryoid bodies were harvested at days 3, 5, 10, and 12, and processed for RNA. The endodermal markers Hnf1 and Hnf4, the mesodermal marker Brachyury, and the ectodermal marker Sox2 were amplified and subjected to Southern blot analysis for visualization. Hprt was amplified as a loading control. As shown in Figure A.3, the expression patterns of Hnf1 and Sox2 in ES cells expressing DN-BRG1 were similar to those in wild-type ES cells. However, the expression patterns of Hnf4 and Brachyury were altered in the DN-BRG1 ES cells. The expression of Brachyury was delayed in the 3E3 cells, suggesting that SWI/SNF is required for proper kinetics of Brachyury expression. In contrast, Hnf4 was expressed earlier in the 3E3 cells, suggesting that SWI/SNF normally plays a role in the repression of this gene.

We also examined the ability of the 3E3 cells to differentiate into erythroid lineages. In murine cells there are four globin genes: two fetal globins, ε^{y2} and βH1, and two adult globins, β-major and β-minor, all of which are under control of a common locus control region (Figure A.4). After 12 days of differentiation in

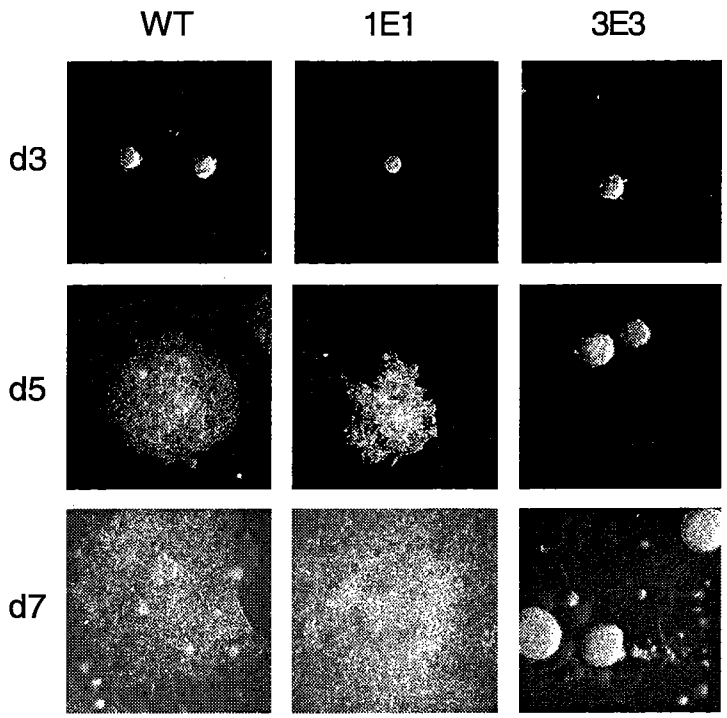


Figure A.2. ES cells expressing dominant negative BRG1 are severely impaired in their ability to differentiate.

Wild-type AB2.2 ES cells, 1E1 (negative control) ES cells, and 3E3 (DN-BRG1) ES cells were differentiated in methylcellulose medium, harvested at indicated time-points and photographed under phase microscopy.

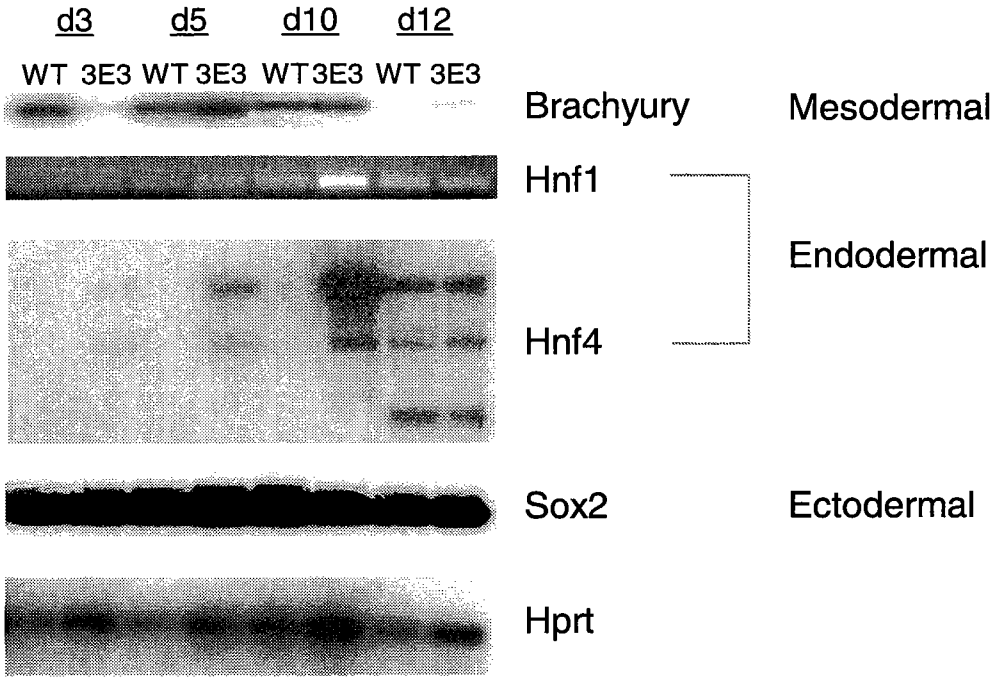


Figure A.3. The express of a subset of germ layer markers is altered in the presence of dominant negative BRG1.

Wild-type and 3E3 ES cells were differentiated in methylcellulose, harvested at indicated time-points, and processed for RNA. RT-PCR and Southern blot hybridization were performed for listed markers using primers described in the Materials and Methods.

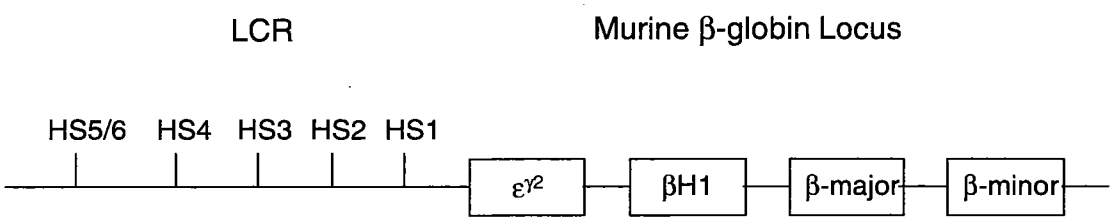


Figure A.4. Murine β-globin locus.

Cartoon illustrating murine b-globin locus. LCR: locus control region. HS1-6: DNase I hypersensitive sites.

methylcellulose, embryoid bodies express all four globins. Therefore, we harvested wild-type and 3E3 embryoid bodies at day 12 of differentiation and examined the expression levels of ε^{y2}, βH1, and β-major globins. As shown in Figure A.5, the levels of the fetal globins were decreased in the 3E3 cells, while the adult β-major globin was unaltered.

Discussion

Mice homozygous null for *Brg1* are embryonic lethal between days 3.5 and 6.5 post-fertilization (27). Due to the complexity of harvesting embryos at these stages in development, it is difficult to elucidate the pathways in which SWI/SNF functions *in vivo*. However, it is possible to utilize *in vitro* differentiation of mouse embryonic stem cells to examine more easily the requirement for SWI/SNF activity in specific gene regulation events. Although *Brg1*-null ES cells are not viable, we were able to disrupt SWI/SNF activity by constitutively expressing a dominant negative version of BRG1. It is interesting to note that only one of the 85 clones screened expressed DN-BRG1 protein at significant levels, suggesting that there is a requirement for a basal level of SWI/SNF activity to ensure cellular viability. In fact, further attempts to generate an ES cell line that expresses the dominant negative BRG1 have been unsuccessful.

Upon differentiation of the ES cells, we found that there were striking morphological differences between the embryoid bodies derived from wild-type or control ES cells and those derived from ES cells expressing DN-BRG1. The

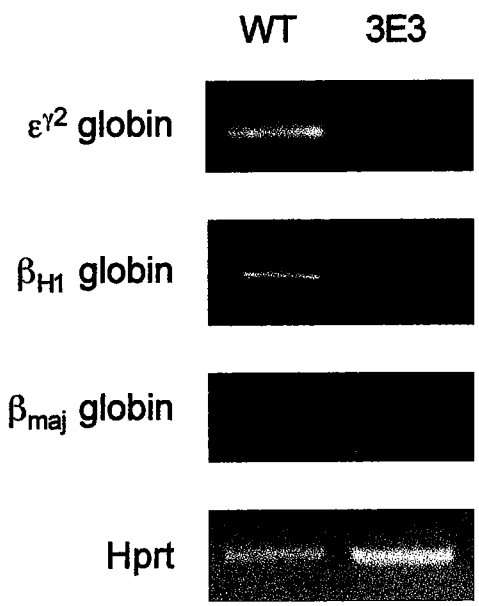


Figure A.5. The expression of fetal β-globins is inhibited in the presence of dominant negative BRG1.

Wild-type and 3E3 ES cells were plated in methylcellulose. At day 12 of differentiation, embryoid bodies were harvested for RNA. RT-PCR was done using primers described in the Materials and Methods.

development of embryoid bodies that expressed DN-BRG1 was severely retarded. We examined the expression pattern of a selection of germ layer markers in an attempt to equate a defect in SWI/SNF activity to the observed morphological differences. Interestingly, the expression of a subset, but not all, of the germ layer markers was altered. Specifically, the endoderm marker Hnf4 was expressed at much earlier time-points in the embryoid bodies derived from DN-BRG1 ES cells compared to those derived from wild-type counterparts. This finding suggests that SWI/SNF normally functions to inhibit Hnf4 at early time-points in mammalian development. The possibility that SWI/SNF inhibits Hnf4 expression is not surprising since data obtained from DNA microarray expression analysis indicate that SWI/SNF appears to be involved in the repression of just as many genes as it activates (224). Furthermore, mammalian SWI/SNF components also have been shown to play a role in the repression of c-fos and some E2F-regulated genes (165, 235, 253).

The expression pattern of the mesoderm marker Brachyury also was altered. In particular, the expression kinetics of Brachyury appeared to be delayed in the DN-BRG1 embryoid bodies. The subtle changes in Brachyury expression may be due to the fact that SWI/SNF activity is inhibited partially and not completed abrogated. However, it is interesting to consider the possibility that SWI/SNF may regulate subtle changes in gene expression that have the potential to lead to drastic phenotypic abnormalities in development when misregulated.

We also examined the effect of DN-BRG1 on the expression of the beta-globin genes ε^{y2}, βH1, and β-major. It is interesting to note that expression of the fetal globins ε^{y2} and βH1 was inhibited while expression of the adult β-major globin was unaffected in the presence of DN-BRG1. This is particularly intriguing since all of the beta-globin genes are under the regulation of the same locus control region. It is unclear how SWI/SNF differentially modulates the expression of the various globin genes. As depicted in Figure A.4, there are several DNase I hypersensitive (HS) sites located in the beta-globin LCR (73). The HS site that is more prevalent at a given point in development depends on which beta-globin is expressed more highly, suggesting that the chromatin structure within the beta-globin LCR may be differentially remodeled. One of the future aims of this study will be to examine the effect of DN-BRG1 on the sensitivity of these various sites to DNase I.

There are several experiments we would like to conduct to complete this study. First, we would like to generate another clone that expresses dominant negative BRG1 to corroborate the results presented here. Alternatively, ES cell lines could be generated that express an inducible DN-BRG1. It also would be of interest to examine the expression of more germ layer markers to determine if there is a link between the genes regulated by SWI/SNF. The potential also exists to enrich the embryoid bodies for erythrocytes using erythropoietin in the differentiation medium, muscle cells using DMSO, and/or neuronal cells using retinoic acid. By examining the effect of disrupting SWI/SNF activity on the

differentiation of a variety of cell lineages, we will gain a more complete understanding of the processes in which SWI/SNF functions.

Appendix B

Preliminary Results

Hydroxyl Radical Footprinting of SWI/SNF-Remodeled Mononucleosomes

The mechanism by which SWI/SNF alters chromatin structure is unclear. Early data led some to suggest that SWI/SNF either removes or rearranges the H2A-H2B dimers to facilitate remodeling (180). More recent studies demonstrate that SWI/SNF is able to induce nucleosome sliding or octamer transfer (104, 183). It has been postulated that SWI/SNF induces nucleosome sliding by first inducing a twist in the DNA that is then diffused throughout the nucleosomal DNA. However, experiments utilizing nucleosomes that contain branched DNA as a steric block to twisting and a nick that allows the dissipation of torsional stress within the nucleosome indicate that a twist-diffusion mechanism is not required for remodeling by SWI/SNF (6). Furthermore, cross-linking reagents demonstrate that the octamer need not be perturbed in order for SWI/SNF to alter the DNase I cleavage pattern or restriction enzyme accessibility of *in vitro* assembled mononucleosomes or arrays (22).

Digestion of *in vitro* assembled, rotationally phased mononucleosomes with DNase I yields a 10 base pair ladder when resolved on a denaturing polyacrylamide gel. This cleavage pattern is altered when the mononucleosomes are pre-incubated with SWI/SNF in the presence of ATP, suggesting a fairly drastic effect of SWI/SNF activity (43, 98, 126). However,

DNase I is a large enzyme that needs to bind DNA to cleave and as such may contribute to the alteration of the mononucleosomes in response to SWISNF activity. In contrast, hydroxyl radicals are small chemical moieties that do not bind DNA. Therefore hydroxyl radical cleavage may provide a more accurate depiction of the of SWI/SNF activity on mononucleosomes.

We monitored SWI/SNF activity by utilizing hydroxyl radical digestion of mononucleosomal templates. Unlike the DNase I digestion pattern, the hydroxyl radical digestion pattern of rotationally-phased mononucleosomes was unaltered in the presence of SWI/SNF and ATP. This finding suggests that the mechanism of remodeling by the SWI/SNF enzymes may be more subtle than currently believed.

Materials and Methods

Mononucleosome assembly

Mononucleosomes were assembled by salt dilution, as described previously (98), using a 154 base pair EcoRI/RsaI fragment corresponding to the *Xenopus borealis* somatic 5S RNA gene. The assembly reaction included 0.45 µg of ^{32}P-labeled DNA fragment (end-labeled by Klenow fill-in with [^{32}P]-dATP at the EcoRI end), 5 µg of HaeIII-digested pUC18 DNA, and 8.6 µg of core histone octamers purified from HeLa cell nuclei. Assembled mononucleosomes were purified from unincorporated DNA over a 5 mL, 5-30% glycerol gradient containing 50 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 mg/mL BSA.

Cleavage reactions

For both DNase I and hydroxyl radical cleavage, 0.3 ng (1.2X10-10 M) of labeled nucleosomes were added to reactions of 25 μL total volume containing 12 mM HEPES, pH 9, 60 mM KCL, 7 mM $MgCl_2$, 15% glycerol, 0.5 μg BSA, 10 mM Tris-HCl, pH 7.5, 0.6 mM DTT, and 0.06 mM EDTA. Where indicated reactions also contained 100-400 ng of SWI/SNF purified from HeLa cell nuclei as described previously (98) and 0.02 mM ATP. Reactions were incubated at 30°C for 30 minutes. For DNase I cleavage, RQ1 DNase I was added to naked DNA at 0.01 units and to nucleosomes at 0.1 units for 2 minutes at room temperature. Reactions were stopped with 2 μL of 0.5 M EDTA. For hydroxyl radical digestion, 3.5 μL each of 10 mM (1 mM for naked DNA) Fe-EDTA, 200 mM (20 mM for naked DNA) sodium ascorbate, and 30% (3% for naked DNA) H_2O_2 were added to the sides of the tubes, tapped in, and incubated for 3 minutes at room temperature. Reactions were stopped with 3.5 μL of 50% glycerol, 10 mM EDTA. Samples were prepared for electrophoresis as described previously (98) and resolved on an 8% polyacrylamide/urea gel.

Results and Discussion

A 154 base pair 5S rRNA fragment from *Xenopus borealis* was assembled into mononucleosomes, incubated in the presence of SWI/SNF +/- ATP, and digested with DNase I or hydroxyl radicals. While the DNase I cleavage pattern was altered in the presence of SWI/SNF + ATP, the hydroxyl radical cleavage

pattern remained unaltered (Figure B.1). DNase I cleavage was done in the presence of the hydroxyl radical components to verify that SWI/SNF was not inactivated (data not shown).

The hydroxyl radical cleavage data suggest that SWI/SNF is not drastically altering the mononucleosome structure. The nucleosomal DNA appears to maintain its rotational phasing; however, at this time we cannot rule out the possibility that the octamer has shifted 10 base pairs, or increments thereof, along the length of the DNA. Another possibility is that the SWI/SNF enzyme weakens the histone-DNA contacts. The observed alteration of the DNase I footprint may be a consequence of the DNase I enzyme itself binding to the "remodeled" nucleosome further perturbing the nucleosome structure. Hydroxyl radical digestion of mononucleosomes +/- SWI/SNF in the presence of a DNase I mutant (H252Q) that is able to bind DNA but is cleavage defective will be done to determine if binding of DNase I alters the digestion pattern. Weakening of histone-DNA contacts also would allow the binding of transcription factors or restriction endonucleases to the altered DNA. However, the mechanism by which SWI/SNF may weaken the histone-DNA contacts has yet to be determined.

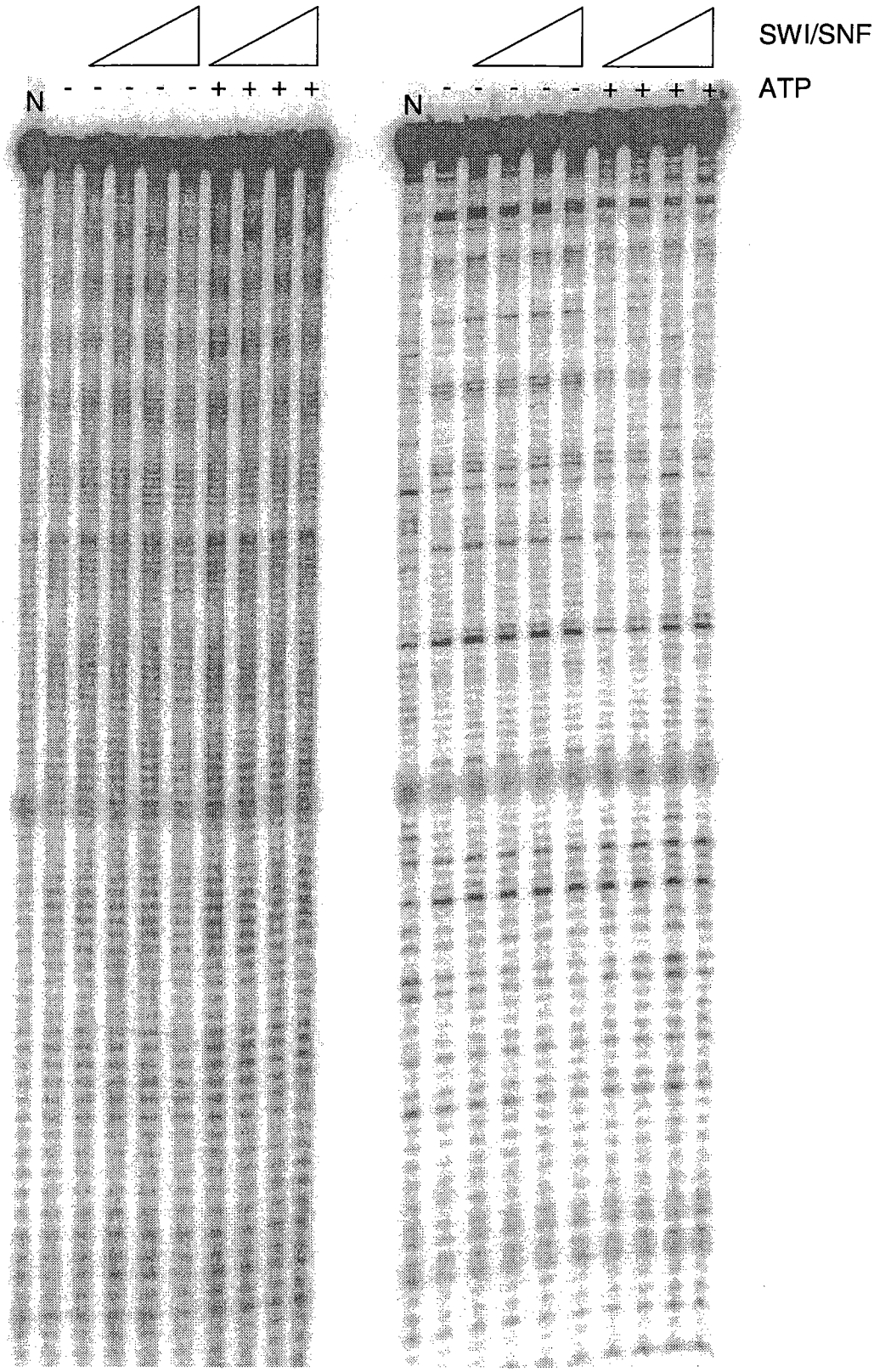

SWI/SNF
ATP
N
- - - - - + + + +
N
- - - - - + + + +

Figure B.1. SWI/SNF does not alter hydroxyl radical cleavage pattern of rotationally phased mononucleosomes.

A 154 base pair fragment from the Xenopus borealis 5S rRNA gene was end-labeled with ^{32}P and assembled into mononucleosomes *in vitro*. Mononucleosomes were digested with DNase I or hydroxyl radicals after incubation with SWI/SNF +/- ATP. Purified DNA was resolved on an 8% denaturing gel.