Characterization of the Tumor Suppressor Capabilities of SWI/SNF Complex Member BAF155 in Cancer Cell Lines and Cooperation of SNF5 and p53 Pathways

Jessica Nachel DelBove

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Approved by:

Bernard E. Weissman, PhD

Terry Van Dyke, PhD

William Coleman, PhD

William Funkhouser, MD, PhD

David Threadgill, PhD

ABSTRACT

JESSICA NACHEL DELBOVE: Characterization of the Tumor Suppressor Capabilities of SWI/SNF Complex Member BAF155 and Cooperation of the SNF5 and p53 Pathways
(Under the direction of Bernard E. Weissman, PhD)

Tumorigenesis is often attributed to aberrant gene expression leading to altered cell cycle control, abnormal differentiation, and inefficient DNA repair. The activity of chromatin remodeling complexes is vital to maintaining proper control of such gene expression. The SWI/SNF chromatin remodeling complex is responsible for remodeling up to 6% of the human genome, with many of those genes known to be associated with cell cycle control. Therefore, impaired or defective activity of this complex could encourage tumor development. Little is known, however, of how SWI/SNF accomplishes those tasks and the differing roles of the distinct members of the complex. Composed of more than ten members, several components are now known to have tumor suppressive roles, as their absence correlates with tumorigenic phenotypes. We propose also that core SWI/SNF member, BAF155, is also likely to be involved in controlling tumor progression. I have utilized two carcinoma cell lines lacking endogenous BAF155 expression to explore the role of BAF155 in cell cycle control and found that re-expression of BAF155 in these cells leads to a reduction in cell number due to replicative senescence. These BAF155 null cells were also found to be sensitive to Rb-mediated cell cycle arrest. This data imply a role for SWI/SNF member BAF155 in cell cycle control and in turn, tumor progression. To

determine the pathways in which SWI/SNF core member SNF5 suppresses tumor progression we utilized mouse models to explore the relationship between the p53 pathway and that of SNF5/INI1 in controlling tumor progression and found that while double heterozygous mice still develop p53 wild type SNF5 null rhabdoid tumors similar to their SNF5+/- littermates, SNF5 loss of heterozygosity is accelerated on a p53+/- background leading to reduced latency and increased penetrance of p53 null SNF5 null rhabdoid tumors in alternate anatomical locations as well as the formation of lymphomas. The resulting rhabdoid tumors were found to express a pattern of markers similar to the human rhabdoid phenotype, solidifying this model as an appropriate recapitulation of human rhabdoid tumors. These studies shed light on the specific roles of SWI/SNF chromatin remodeling complex in tumor suppression.

ACKNOWLEDGEMENTS

Although I alone have shouldered the responsibility to complete this work, and only my name appears under the title, many of my friends and family have been invaluable in the completion of this undertaking. The unconditional support of my close friends and family was both necessary and sufficient for overcoming the various obstacles encountered in such a task.

I would specifically like to acknowledge my parents: Peter P. DelBove and Keith & Connie Grubbs for their extraordinary patience, and unending encouragement, as well as being inspiring examples. Thank you so much.

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SCLC	small cell lung cancer
NSCLC	non-small cell lung cancer
MRT	malignant rhabdoid tumor
IHC	immunohistochemistry
NSCLC	non small cell lung cancer
BAF	BRG1- associated factor
BRM	brahma
SWI/SNF	SWItch & Sucrose Non Fermenting
RB	Retinoblastoma
BrdU	Bromodeoxyuridine
MEFs	Mouse Embryonic Fibroblasts
CPC	Choroid Plexus Carcinoma
HDAC	Histone deacetylase
HAT	Histone aceyltransferase
Cdk	cyclin dependent kinase
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
IP	immunoprecipitation
ChIP	chromatin immunoprecipitation
PCR	polyermerase chain reaction
TSG	tumor suppressor gene
Het	heterozygous/ heterozygote

LOH	loss of heterozygosity
HIV	human immunodeficiency virus
HPV	human papilloma virus
BRCA1	breast cancer 1 gene
SANT	Swi3, Ada2, N-coR, TFIIB
SWIRM	SWI3, RSC3, and Moira
SRG3	Swi3 related gene (BAF155)
GEM	Genetically engineered mouse
TMA	Tissue micro array
TCR	T-cell antigen receptor
ICM	Inner cell mass

CHAPTER 1

I. Introduction

As patterns of genetic aberrations became evident in various neoplasms, cancer came to be considered a genetic disease. The more this most complex of pathologies is studied; we realize it is as much epigenetic as it is genetic. Genetically, cancer is propagated in large part by the aberrant expression of genes involved in cell cycle control. Behind the scenes however, the decision of when, where, and if these genes are expressed is determined by epigenetic factors. Chromatin remodeling complexes, like epigenetic mechanisms, operate behind the scenes responding to the epigenetic signals that coordinate appropriate gene expression patterns from development to death and every cell division in between. One of the best characterized chromatin remodeling complexes is the SWI/SNF complex. Although affecting only a small percentage of the genome, SWI/SNF is involved in remodeling the chromatin of cancer.

A. Epigenetic control of gene expression

The primary sequence of a gene is critical to determining its fate, but this one-dimensional view is only part of what determines a gene's physiological function. The initiation and timing of a gene's expression is often determined epigenetically. Epigenetics refers to any change in gene function that occurs in the absence of a change in DNA sequence. Although reversible, epigenetic changes are often stable through cell division (Laird, 2005). Epigenetic changes involve the regulation of chromatin structure and DNA

accessibility and can therefore, have a defining role in controlling gene transcription. There are two main categories of epigenetic change: DNA methylation and histone modification (which includes acetylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deiminatin, proline isomerization, as well as histone substitution). DNA methylation is the heritable methylation of cystosine residues of CpG dinucleotides and correlates with transcriptional repression (Bestor, 1998). DNA methylation is essential for survival and normal development (Lie, 1992, Okano, 1999, Jackson-Grusby,2001) as well as having a role in a variety of cellular processes from genomic imprinting, to X chromosome inactivation, tissue-specific gene expression, cell differentiation, and aging.

Histone modification also has profound effects on gene expression patterns. By disrupting chromatin contacts or affecting the recruitment of nonhistone proteins to chromatin, these modifications orchestrate the ordered recruitment of enzyme complexes to manipulate DNA. Mechanistically, histone modification is the alteration of the N-terminal tails of histones leading to a perturbation of the affinity between the histones and DNA. The process can be covalent or non covalent. Covalent modifications such as acetylation, methylation, and phosphorylation may be able to directly alter the physical properties of the chromatin fiber, leading to a cascade of effects on higher-order structures. Acetylation specifically neutralizes the basic charge of the lysine, which may lead directly to an unfolding of chromatin. The repressive state of chromatin structure is usually associated with hypoacetylation and hypomethylation of particular residues, and the permissive configuration with localized phospho-hyperacetlyation and localized hypermethylation. It is the job of histone modifying enzymes, as part of a larger system of transcription control involving chromatin remodeling complexes, to read this ‘histone code’ and unlock the chromatin,

exposing target genes. Precision is necessary in the targeting of chromatin for expression of a specific gene, especially those involved in cell cycle regulation. The area of chromatin must be found, opened, and transcribed within seconds. It is the epigenetic histone code that allows for speed and precision.

Chromatin remodeling complexes are targeted or drawn to areas of chromatin according to this histone code of acetylation and methylation. If a gene is aberrantly targeted or repressed, aberrant expression patterns will follow. Such an error could occur due to aberrant methylation or acetylation patterns- the histone code itself. The most common error of this type is aberrant transcriptional silencing of genes associated with DNA hypermethylation of promoter region CpG islands. An example of DNA hypermethylation of a gene leading to tumorigenesis is *p16* ^{INK4A}. Hypermethylation of the *p16*^{INK4A} promoter leads to a loss of function and is observed during progression of tumors such as lung cancer as early as preneoplastic lesions (Belinsky, 1998 & 2004; Nuovo, 1999). The effects of an epigenetic error in this one gene are catastrophic for the cell and ultimately the individual. The loss of p16^{INK4A} function allows the emergence of genomic instability (Foster, 1998; Kiyono, 1998) and may also directly allow for additional epigenetic silencing of other genes (Reynolds, 2006) causing a snowball effect to rapid tumorigenesis. Alternatively, due to the histone code itself being in error, a gene could be aberrantly transcribed or repressed due to a misreading of the code or lack of follow through, if all complexes and required agents were not properly recruited to a target gene. For example, this could mean aberrant targeting by transcription factors or lack of proper chromatin remodeling by chromatin remodeling complexes.

The above scenario illustrates the intimate connection between the various epigenetic mechanisms, chromatin remodeling and cancer, the details of which are beginning to be outlined. For example, the nucleosomal remodeling complex NuRD interacts with DNA methylation binding protein (MBD2) to direct the complex to methylate DNA (Zhang, 1999). The catalytic subunits of the chromatin remodeling complex SWI/SNF, BRG1 and BRM, are able to interact with acetylated histones via their bromo domains (Dhalluin, 1999; Hudson, 2000). SWI/SNF member BRM was also shown to associate with the methylated DNA binding protein MeCP2, culminating in gene silencing (Harikrishnan, 2005). It is becoming increasing clear the interrelatedness of the epigenetic, chromatin remodeling, and gene transcription fields are to one another and to the development and progression of cancer. Our study concentrates on the tumor suppressive activities of the chromatin remodeling arm of gene expression control, via the SWI/SNF chromatin remodeling complex.

B. The human SWI/SNF chromatin remodeling complex.

There are several classes of ATPase chromatin remodeling complexes, but SWI/SNF is the quintessential ATP-dependent nucleosomes remodeling complex, initially linked to chromatin structure through the isolation of mutations in histone-encoding genes by Kingston in 1996 and Perez-Martin in 1998. These original experiments into the yeast function and specific dysfunctions, lead to their discovery and name: mating type SWItching and Sucrose-Non-Fermenting to yield SWI/SNF (Winston, 1992). In 1999, Kingston and co-workers further showed that the ATP-dependent action of SWI/SNF causes the disruption of nucleosome structure, leading them to propose the current model of SWI/SNF action- in vivo

Figure 1-1

Schematic of chromatin remodeling by the SWI/SNF chromatin remodeling complex by octomer transfer or octamer sliding.

Image adapted from: Roberts, C. and S. H. Orkin (2004). "The SWI/SNF Complex-Chromatin and Cancer." Nature Reviews Cancer **4**: 132-142.

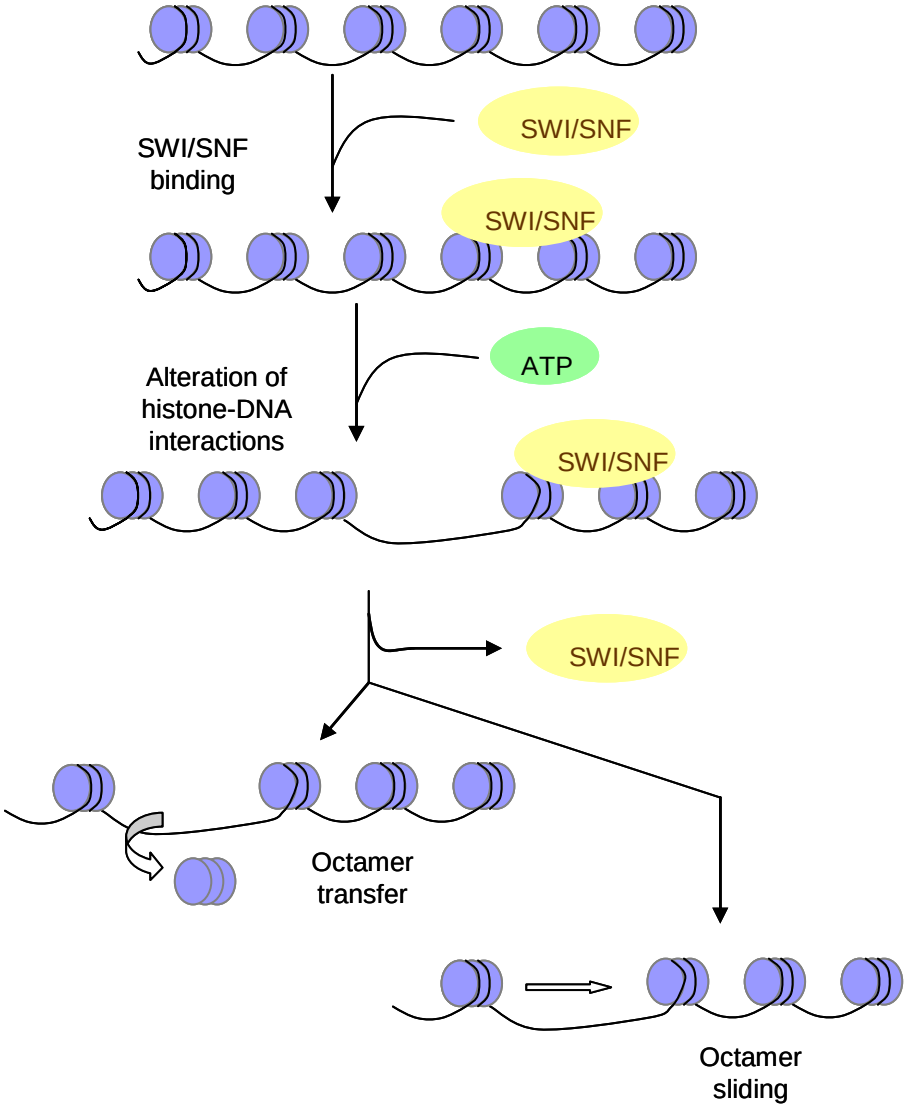

SWI/SNF
binding
SWI/SNF
SWI/SNF
Alteration of
histone-DNA
interactions
ATP
SWI/SNF
SWI/SNF
Octamer
transfer
Octamer
sliding

Figure 1-1

disruption of chromatin structure at promoters to facilitate the binding of transcription factors. The complex accomplishes this task by physically altering the nucleosomes structure, displacing it to expose target DNA.

SWI/SNF is now established as being evolutionarily conserved from yeast to man, where it maintains a constant core of BRG1 or BRM (Brahma), with SNF5/INI1, and BAF155/170, as well as several other variable members to make a 2 megadalton, 8-12 member complexes. The inclusion of specific non-core members is tissue- and developmental stage-specific (Wang, 1996). Loss of any member leads to nearly identical phenotypes in yeast, likely due to a requirement of all members to have a fully functional complex (Winston, 1992, Kingston 1999). Further yeast studies revealed, via microarray, that the complex is involved in the transcriptional regulation of up to 5% of genes interspersed throughout the genome, repressing more than activating (Sudarsanam, 2000). It is believed that the unique, variable SWI/SNF subunits are involved in targeting and structural functions of the complex, while the core is responsible for the actual remodeling. For example, the actin-like subunit BAF53 is likely involved with anchoring the complex to the nuclear matrix. In mammalian cells several variants of the complex exist: SWI/SNF-A (BAF), SWI/SNF-B (PBAF), and E-BAF complexes (see Figure 1-2).

These complexes are similar but have distinct components and regulate mutually exclusive target genes (Wang 2003). These unique functions may be due to the distinct members in the differing complexes. Structural domains involved in protein-protein interactions are present in BAF250a/ARID1A/p270 and BAF250b/ARID1B, BAF180, BAF170, BAF155, BAF60, BAF53, and BAF47SNF5/INI1. Various different sequence-dependent and sequence-independent DNA binding domains can be found in BAF250,

BAF180, BAF170, BAF155, and BAF57. We know BAF57, only found in higher eukaryotes, is a critical modulator of the androgen receptor –whose activity is required for prostate development, growth, and survival (Link, 2005). Nagl et al has demonstrated the necessity of BAF250 in specific proliferation control steps, the loss of which leads to carcinogenesis. BAF60c has been demonstrated to be essential in the developing heart in mice (Lickert, 2004).

Although variation among complexes leads to specificity and variable phenotypic disorders upon loss or mutation of specific complex members, the theme of dysfunction remains a lack of proper chromatin remodeling, which is ultimately the responsibility of the SWI/SNF core. There is variation in the multiple SWI/SNF complexes among the non-core as well as core members, although it is always a combination of BRG1 (or BRM) with SNF5/INI1 and BAF155/BAF170. BRG1 and BRM are the larger of the core members and contain the ATPase domains to fuel the remodeling. SNF5, on the other hand, is the smallest of all SWI/SNF members and is an established tumor suppressor gene connected with human rhabdoid tumors. BAF155 and BAF170 have high sequence homology and do not yet have distinct identified roles in the complex. As mentioned previously, these subunits form a chromatin remodeling complex involved in remodeling 5% of the genome. This is a small but very important 5% as many of the genes affected by the SWI/SNF remodeling are cell cycle related. Proper cell cycle regulation touches nearly every important moment in the

Figure 1-2:

Schematic of the various mammalian SWI/SNF complexes. The complexes differ by catalytic subunit either BRG1 or BRM, accompanied by varying smaller subunits. Precise combinations depend on cell type, developmental stage, and other micro-environmental effectors.

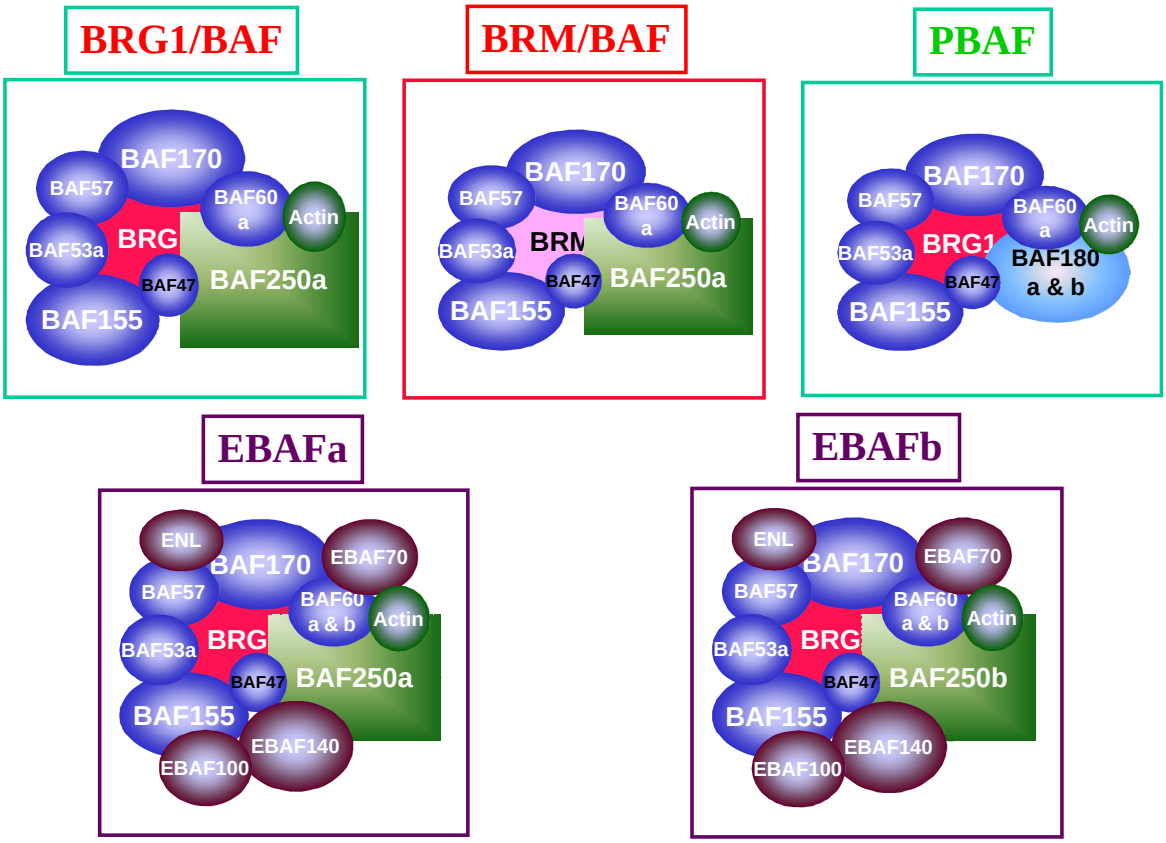

BRG1/BAF
BAF170
BAF57
BAF60
a
Actin
BAF53a
BRG
BAF47
BAF250a
BAF155
BRM/BAF
BAF170
BAF57
BAF60
a
Actin
BAF53a
BRM
BAF47
BAF250a
BAF155
PBAF
BAF170
BAF57
BAF60
a
Actin
BAF53a
BRG1
BAF180
a & b
BAF47
BAF155
EBAFa
ENL
BAF170
EBAF70
BAF57
BAF60
a & b
Actin
BAF53a
BRG
BAF47
BAF250a
BAF155
EBAF140
EBAF100
EBAFb
ENL
BAF170
EBAF70
BAF57
BAF60
a & b
Actin
BAF53a
BRG
BAF47
BAF250b
BAF155
EBAF140
EBAF100

Figure 1-2

life of a cell: development, differentiation, replication, repair, age-related senescence, and apoptosis. The following segments will outline specific functions correlated with SWI/SNF activity.

1. SWI/SNF in normal cellular processes: Development and aging

The coordination of precise cell division or death signals, proper growth and differentiation during development, as well as normal growth and aging are understandably dependent on proper epigenetic control. It is known that changes in gene expression during embryonic development are accompanied by reorganization of the chromatin. There are several lines of evidence implying this reorganization is one of the roles of the SWI/SNF complex. The fundamental function of a chromatin remodeling complex is to remodel nucleosomes, so it is logical that the consequences would be dire upon loss of function of a core member. This is indeed the case, as loss of any core member in mice is not compatible with life, demonstrating the importance of the SWI/SNF complex to development. Given the role of these genes as part of an epigenetic complex, it is not surprising that large-scale aberrant gene expression leading to death results with their loss. The majority of what is known of SWI/SNF's role in development concerns these core members. Although the strongest evidence for SWI/SNF's role in development does stem from the core members, there are studies showing roles for various other subunits of the complex; BAF57 in androgen-dependent proliferation (Link 2005), BAF60c in heart development (Lickert, 2004), BAF180 in cardiac chamber maturation (Wang 2004), and BAF200 in regulation of expression of selective interferon-responsive genes (Yan, 2005). The following will outline

correlations between SWI/SNF core members and the development and differentiation of neural and lymph tissue.

While homozygous loss of any core member is embryonic lethal, a subset of the heterozygotes develop neural tube defects manifesting as exencephaly (Bultman, 2000; Kim, 2001; Roberts, 2000). BRG1 has surfaced as having several vital roles in the development of this and multiple other tissue types. Seo demonstrated in 2005 that BRG1 is specifically required for cell cycle exit and neuronal differentiation of neural progenitor cells. Loss of BRG1 expression has been shown in vitro to abrogate MKK6-dependent induction of muscle gene expression (Simone, 2004) And a lack of BRG1 in developing T cells leads to a complete lack of CD8 and CD4 positive T cells, as well as leading to a Bcl-2 induced apoptosis (Chi, 2003). Double positive T cell apoptosis is also linked to expression levels of a second core member, BAF155. Although each core member will be detailed more extensively later, briefly: Srg3 (mouse BAF155) is expressed at higher levels in the thymus than other surrounding developing tissues, where it is involved in T-cell apoptosis susceptibility. Sensitivity to glucocorticoids in T cells can be modified by altering the expression levels of Srg3. As outlined further in the section on BAF155 below, it also appears to have an important role in the inhibition pathway of glucocorticoid-mediated apoptosis by T-cell antigen receptor (TCR) (Ko, 2004). SNF5 has been implicated in the survival of hematopoietic cells as well, from mouse studies where SNF5 loss coincides with bruising and intestinal hemorrhage so severe it leads to the death of these mice (Roberts, 2002).

In keeping with a similar theme of SWI/SNF involvement in cellular processes involving growth control and development, so too is that SWI/SNF is involved in aging, at

least in the form of cellular senescence. If any of the three main core members BRG1, SNF5, or BAF155 are re-introduced into cell lines lacking endogenous expression, senescence is observed, demonstrated by growth arrest, expression of senescence marker β-galactosidase, and often a flat cell morphology can also be observed (Dunaief, 1994; Hendricks, 2004; Kang, 2004; Shanahan, 1999). Studies done by Yaniv and colleagues as well as the Roberts lab have reinforced this role for SNF5 by showing SNF5 can produce replicative senescence in mouse embryonic fibroblasts (MEFs) in culture upon loss of SNF5 (Roberts, 2002; Klochendler-Yeivin, 2006). While core member BRG1 was shown to be essential to the proper differentiation of specific tissues, its counterpart, BRM, seems to be associated with the scheduled aging of specific tissues. The normal regenerative capacity of the liver is lost in older mice coinciding with the arrival of an apparent repressive complex involving BRM (Iakova, 2003). Several of the above mentioned and other developmental, differentiation, cell cycle interactions can be seen schematically in Figure 1-3.

2. Involvement of SWI/SNF in cell cycle control pathways:

An obvious link exists between development, aging, and cancer. All are intimately associated with precise and coordinated cell cycle control, albeit in a pathologic way with cancer. Although SWI/SNF does not directly control the cell cycle, there is great circumstantial evidence the complex is involved with the transcriptional regulation of several entities that do play key roles in cell cycle progression. The clearest of these links is with the Rb pathway.

Figure 1-3:

Example of the many pathways that target the mammalian SWI/SNF complex.

Adapted from: Simone, C. (2006). "SWI/SNF: The Crossroads Where Extracellular Signaling Pathways Meet Chromatin." Journal of Cellular Physiology **207**: 309-314.

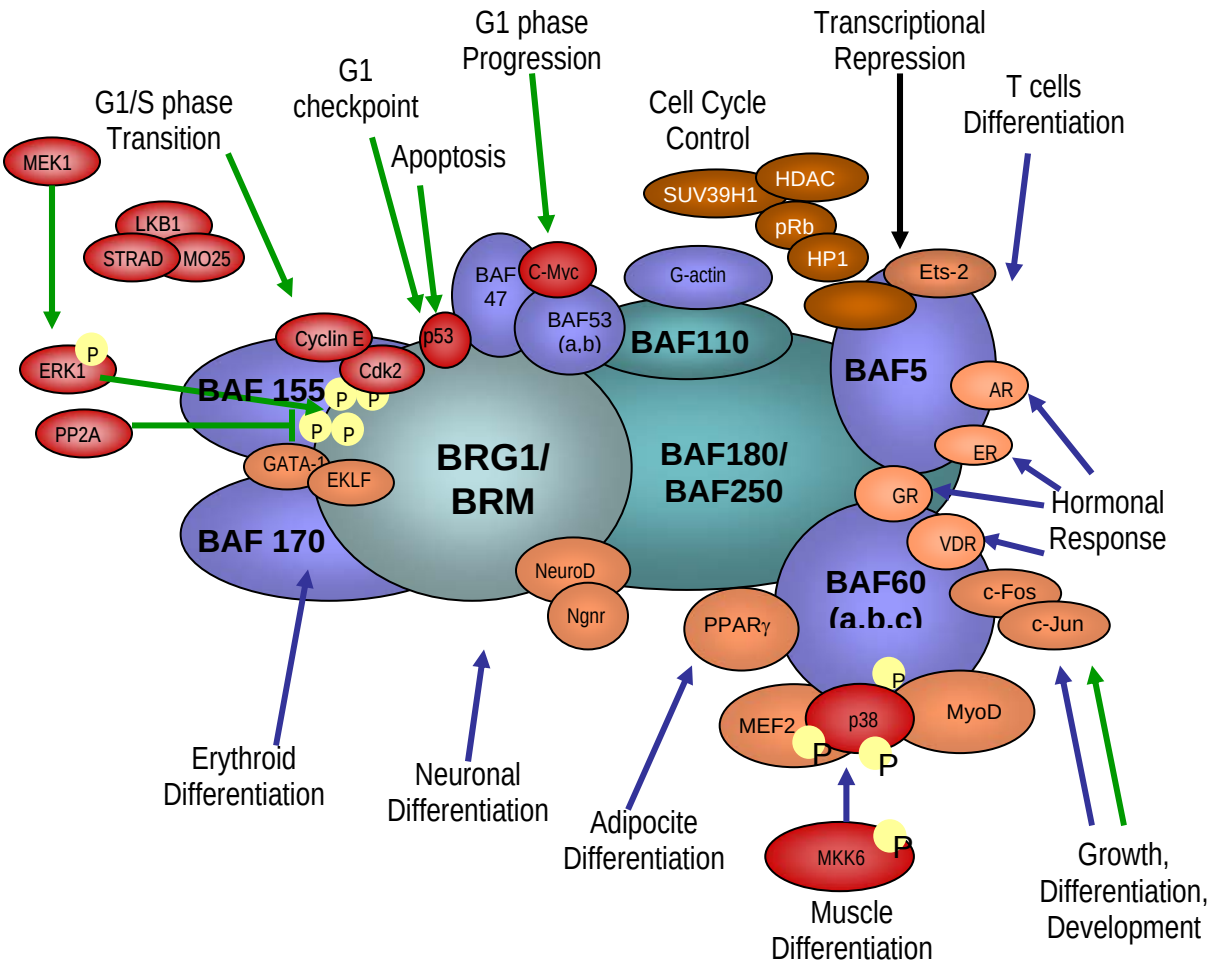

G1 phase
Progression
Transcriptional
Repression
G1/S phase
Transition
G1
checkpoint
Cell Cycle
Control
T cells
Differentiation
Apoptosis
MEK1
LKB1
STRAD
MO25
SUV39H1
HDAC
pRb
HP1
Ets-2
BAF
47
C-Myc
G-actin
BAF53
(a,b)
BAF110
BAF5
p53
Cyclin E
Cdk2
ERK1
P
BAF 155
PP2A
GATA-1
EKLF
BRG1/
BRM
BAF180/
BAF250
AR
ER
GR
VDR
Hormonal
Response
BAF 170
NeuroD
Ngnr
BAF60
(a,b,c)
c-Fos
c-Jun
PPARγ
MEF2
p38
MyoD
MKK6
Erythroid
Differentiation
Neuronal
Differentiation
Adipocite
Differentiation
Muscle
Differentiation
Growth,
Differentiation,
Development

Figure 1-3

a) The Rb pathway.

The retinoblastoma protein, pRb, is one of the major regulators of the cell cycle. It is responsible for controlling the G1/S transition and progression through S phase. In its hypophosphorylated state, Rb associates in early G1 with the E2F transcription factors to recruit HDACs, causing the repression of Cyclins A and E involved in cell cycle progression. Subsequently in late G1, phosphorylation of the Rb pocket results from accumulated Cyclin D/Cdk4/6 complexes, which in turn leads to the displacement of the HDAC activities and eventually to release and activation of E2F, and cell cycle progression.

b) SWI/SNF and the Rb pathway

Several studies have demonstrated a need for components of the SWI/SNF remodeling complex for the proper functioning of the Rb pathway. A physical interaction between Rb and SWI/SNF was originally discovered in a yeast two-hybrid screen using Rb as bait which demonstrated an interaction between Rb and BRG1 or BRM (Dunaief, 1994). The Zhao lab further demonstrated an interaction between BRG1 and Rb by showing that BRG1 can upregulate p21. This induced the hypophosphorylation of pRb, activating pRb (Kang, 2004). Since then, more functional assays have implicated a necessity for BRG1/BRM in the control exerted by pRb on the G1/S transition. The Goff lab has demonstrated a functional requirement for BRM or BRG1 for Rb induction of growth arrest (Dunaief, 1994; Strober, 1996), corroborated by several others groups (Zhang, 2000; Dunaief, 1994; Reisman 2002; Strobeck, 2000a and 2002; Trouche, 1997). One example, seen in SW13 cells which lack BRG1 and BRM, where growth arrest is not induced upon

exposure to a constitutively active hypophosphorylated Rb unless BRG1 or BRM are also expressed (Strobeck, 2000a,b). The physical interaction between BRG1/Brm and Rb family members was further demonstrated by Strober (1996) and Trouche (1997) with both BRG1 and BRM able to bind p107 and p130. Zhang et al (2000) further showed a requirement for SWI/SNF in the coordinated expression of Cyclin E and A during late G1 and S phases via BRG1's association with Rb. This finding was supported by the observation that cells growth-arrested by BRG1 and pRb accumulated in S phase and not G1. Cyclin E has been linked to SWI/SNF via an Rb-independent interaction with phosphorylated BRG1 and BAF155 (Shanahan 1999), as discussed in further detail in the section on BAF155. Some of the key points in the relationship of Rb, SWI/SNF, and the cell cycle are diagramed below in Figure 1-4.

c) The p53 pathway

The statistic usually quoted to show the significance of p53 loss in human cancer development is that it is lost, mutated, or otherwise inactivated in most human cancers. While this is indeed the case, it is the fact that the p53 pathway has failed in its response to successfully halt the cell cycle due to DNA damage that underlies its role in human cancer. Cells are routinely exposed to DNA damaging agents, some endogenous, by the induction of mismatches, crosslinks or strand breaks. DNA damage of one sort or the other is often the first step to genomic instability, which has also been considered a hallmark of human neoplasms. Without functioning guardians of the genome like p53, such DNA

Figure 1-4

Schematic depicting the relationship between the SWI/SNF complex, RB, and cell cycle control.

Adapted from: Roberts, C. and S. H. Orkin (2004). "The SWI/SNF Complex-Chromatin and Cancer." Nature Reviews Cancer **4**: 132-142.

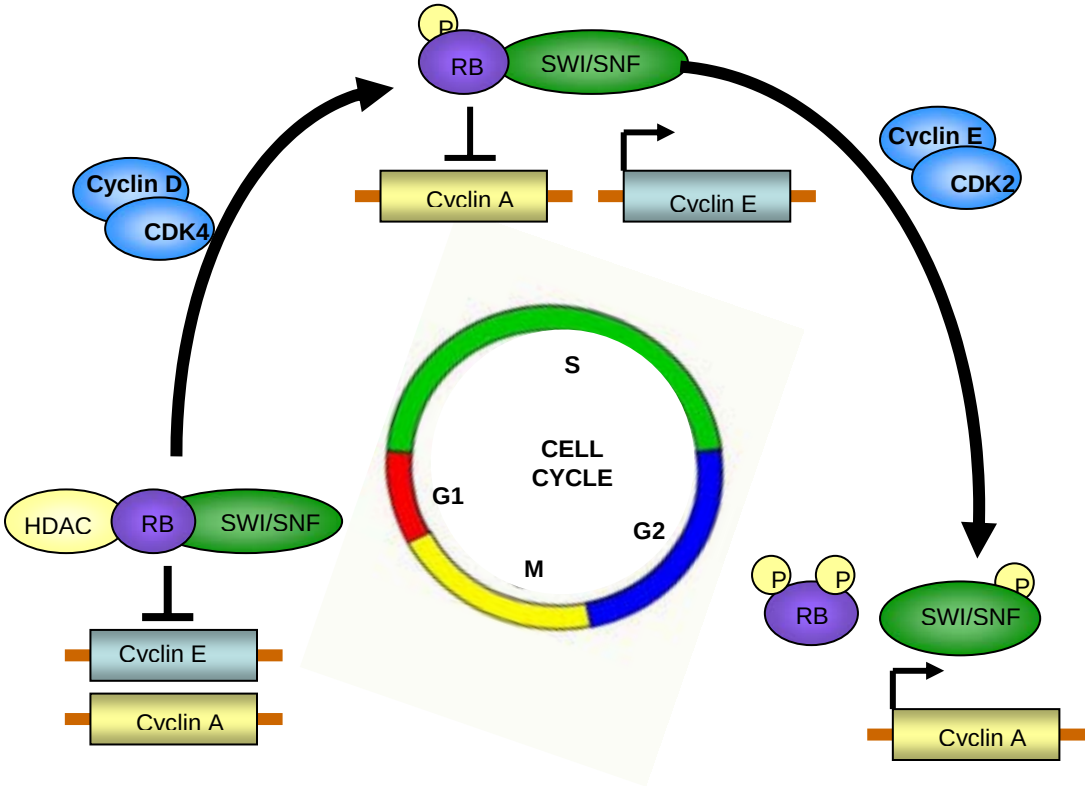

P
RB
SWI/SNF
Cyclin A
Cyclin E
Cyclin E
CDK2
Cyclin D
CDK4
S
CELL
CYCLE
G1
G2
M
HDAC
RB
SWI/SNF
Cyclin E
Cyclin A
P
P
RB
P
SWI/SNF
Cyclin A

Figure 1-4

damage or instability leads to aberrant gene expression of oncogenes or loss of expression tumor suppressors. If not repaired correctly, these lesions can be particularly mutagenic. Double strand breaks, for example, can recombine inappropriately driving gross chromosomal instability. The appropriate response of a cell to such damage is to attempt to repair or undergo apoptosis. Apoptosis is not always due to catastrophe, it is also signaled as part of normal development in some structures, or to protect the organism from self-reacting immune cells. As an adult, one of the most important roles of apoptosis is in removing cells with compromised genetic integrity on the road to malignant transformation.

The agent often employed in carrying out the choice of cell suicide is the p53 tumor suppressor protein (Vousden, 2002). Once stabilized, the p53 protein may induce apoptosis if the damage is deemed irreparable. However, at times where the cell may effectively repair the injuries, the appropriate response may be cell cycle arrest. The arrest can allow the cell a pause to temporarily or permanently prevent the perpetuation of the recognized genetic damage in a cell, be it DNA damage repair defects, hypoxia, or aberrant proliferative signals. The decision to arrest or apoptose is not fully defined but seems to depend largely on cell type and environment (Vousden, 2000; Vogelstein, 2000; Levine, 1997; Hansen, 1997; Bates, 1996). It is this loss of a functional checkpoint that facilitates genetically unstable cells to propagate and continue on to malignant transformation, not the loss of p53 directly, per se. As mentioned previously, upon DNA damage, p53 becomes stabilized and accumulates in the nucleus. The build up may, according the cell type and stimuli, up regulate p21, which will in turn halt the phosphorylation of pRb by cdk2/cyclinE. This will leave pRb in the hypophosphorylated state, not allowing the progression of the cell cycle.

Figure 1-5: The p53 signaling pathway.

Adapted from images on the Sigma-Aldrich website:
http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Signaling/Scientific_Resources/Pathway_Slides___Charts/The_p53_Signaling_Pathway.html

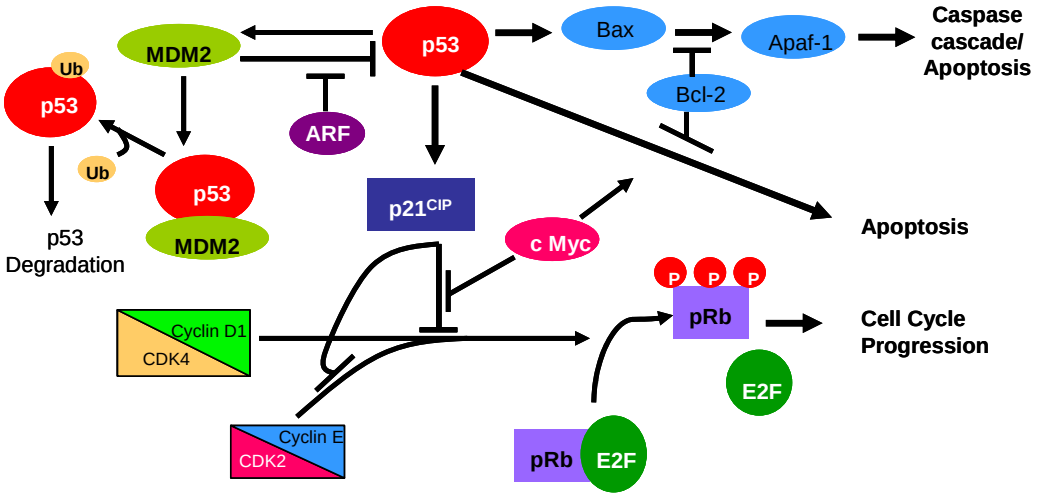

MDM2
p53
Bax
Apaf-1
Caspase
cascade/
Apoptosis
Ub
p53
Bcl-2
ARF
Ub
p53
MDM2
p21CIP
c Myc
Apoptosis
p53
Degradation
P
P
P
pRb
Cell Cycle
Progression
Cyclin D1
CDK4
E2F
Cyclin E
CDK2
pRb
E2F

Figure 1-5

Alternatively, the increase in p53 in the nucleus could lead to Bax induction of the caspase cascade leading to apoptosis. These, and other specific pathways involved, can be seen schematically in Figure 1-5.

d) SWI/SNF and the p53 pathway

The exact mechanisms by which SWI/SNF affects cell cycle control is not understood. However abundant evidence leaves no doubt unfettered cell growth may result without fully functional SWI/SNF. Regulation or even association with key cell cycle 'gatekeepers' such as p53 may be the key. Lee et al demonstrated in 2002 through ChIP analysis that hSNF5 and BRG1 are recruited to the p53-dependent promoter, p21 and directly activate p21 expression. Overexpression of either SNF5 or BRG1 in a dominant negative form inhibited p53-mediated cell growth suppression and apoptosis. Furthermore, binding of BRG1 to BRCA1, as shown by Bochar et al in 2000, is required for BRCA1 stimulation of p53-dependent transcription. The SWI/SNF complex likely has the severe effects on development and cancer due to the aberrant chromatin remodeling of at least one gene important to cell cycle control. The p53 tumor suppressor gene is just such a gene. Our studies were designed to shed light on the potential role of the SWI/SNF complex on p53 tumor suppression *in vivo.*

3. Involvement of SWI/SNF in cancer.

The role of SWI/SNF in cancer appears certain, if not well defined. A complex so intertwined in the fabric of epigenetic control of cell cycle regulatory genes is also inseparable from tumorigenesis. Several SWI/SNF complex members are required for proper

functioning of important cell cycle control processes in development, aging, and normally growing cells as outlined above. It is inevitable that patterns surface between lost or malfunctioning complex members and human neoplasms. This is the case with SWI/SNF core members BRG1 and SNF5. More detail will be given in following sections of SNF5 but a brief description of SNF5 and BRG1 in cancer follows. SNF5 is the first SWI/SNF complex member to be dubbed a bona fide tumor suppressor gene, being the distinguishing characteristic in the diagnosis of malignant rhabdoid tumors (MRTs). SNF5 loss is found in a majority human MRTs whether due to germline or sporadic etiology (Biegel, 2002; MacDonald 2003; Reddy, 2005). While it seems SNF5 has a role in development as well as pediatric tumors, loss of BRG1 seems to affect adult tumorigenesis. Although loss of BRG1 in primary tumors is not widespread, it has been noted in several lung cancer cell lines as well as some oral cancers (Reisman, 2003; Gunduz, 2005). Approximately ten percent of human cancer cell lines screen display a mutation or complete loss of BRG1, including pancreatic, breast, lung, and prostate cancer cell lines (DeCristofaro, 2001; Wong, 2000). Loss of both BRG1 and BRM is found in approximately 30% of human non-small cell lung cancer cell lines and 10% of primary tumors. The lack of BRG1 or BRM seems to correlate with a poor prognosis in these patients (Reisman, 2003). Loss of these members may not even be necessary to lose function, aberrant BRM cellular localization has correlated with poor prognosis in lung adenocarcinoma patients (Fukuoka, 2004). This strong link is furthered by the appearance of breast adenocarcinoma-like glandular tumors in BRG1 heterozygote mice (Bultman, 2000). BRM heterozygotes do not readily form tumors but display the phenotypic symptom of being generally larger in size, compared to wild type littermates (Reyes, 1998). Less is known of the third core member, BAF155. It is found in

an area of chromosome 3 (3p) that has been reported lost in primary carcinomas. While widespread loss of BAF155 expression in primary tumors has not been reported, we have found 2 carcinoma cell lines lacking BAF155 expression. Core members SNF5 and BAF155 are the focus of my dissertation studies and are discussed in more depth below.

C. The SNF5 tumor suppressor gene

Cancer has a very well defined, even primary, genetic component, but as we are beginning to see, epigenetics plays perhaps as important a role in cancer development and progression. SNF5 loss combines the genetic and epigenetic fields in the understanding of the mutual importance of these two in controlling cell cycle progression and tumorigenesis. The majority of MRTs have lost the SNF5 allele, but otherwise typically have a completely normal karyotype (Douglass, 1990). The aggressive nature of these tumors may be explained by extensive 'epigenetic instability'.

SNF5 appears important for the SWI/SNF complex for its control of the cell cycle, via p16 and p21 transcriptional regulation (Chai, 2005). SNF5 first came to be considered involved in human cancer progression with the discovery of loss of heterozygosity of the area of chromosome 22 where SNF5 maps (22q11.2) (Biegel, 1990 & 1996; Rosty, 1998). SNF5 was later found to be lost in the majority of human rhabdoid tumors (Versteege, 1998). The discovery of SNF5 as an important molecule in association with devastating disease processes was not limited to cancer. SNF5's INI1 designation came from its involvement in HIV integration (Kalpana, 1994). For association with other SWI/SNF subunits it was also called BAF47 (BRG1-associated factor of 47 kD) by Wang et al. Also known as SMARCB1, it will be referred to as SNF5 throughout this document. The smallest of the

SWI/SNF subunits, the human protein contains three conserved domains: two repeats (repeat 1 and repeat 2) and a C-terminal coiled-coil domain. There is near 100% conservation of SNF5 from mouse to man, although the human SNF5 protein exists as two splice forms, one containing a lysine rich domain not present in the short isoform (SNF5-sp). The first repeat domain has been show to be critical for the interaction of SNF5 with several factors: BRM, c-MYC, HPV-E1, and HIV-IN (Muchardt, 1995; Lee, 1999; Cheng, 1999). Repeat 2 is required for SNF5 interaction with BRM and GADD34 (Wu, 2002), and possibly as a nuclear export signal (NES). The coiled-coil domain has an unusual anti-export signal (Cosma, 1999). It is most often the repeat domains that are lost in human cancer patients (Biegel 1999 & 2000; DeCristofaro, 1999; Savla, 2000; Sevenet, 1999a, b; Versteege, 1998).

1. **The role of SNF5 in development.**

As outlined earlier, the complex as a whole has a role in development as well as cancer, and this holds true for the SNF5 subunit as well. SNF5's links to development have revealed themselves in neural and hematopoietic development. As mentioned earlier, complete loss of SWI/SNF core members BRG1, BAF155, or SNF5 results in embryonic lethality – so an obvious role in development is implied. A subset of heterozygotes of these studies all display neural tube defects. In the case of SNF5, the tube defects may be due to the observed deficiency of RHO activity essential for neural crest cell delamination from the dorsal neural tube (Medjkane, 2004; Liu, 2001; Edelman, 1998). The hematopoietic necessity of SNF5 is best demonstrated in mouse studies of conditionally null mice or tissues. Loss of SNF5 expression in hematopoietic cells in the bone marrow of these mice leads to their death in a rapid fashion (Roberts, 2002). The same study showed a less

penetrant conditional SNF5 mutation that affected approximately half of all tissues (except brain) and left these mice predisposed to CD8+ T cell lymphomas.

2. **The role of SNF5 in tumor formation.**

Pediatric neoplasms have always been emotionally devastating cancers and in the case of rhabdoid tumors, even more so, as these tumors are particularly aggressive, killing over 80% of affected children with a median age of onset of 11 months. Recognized as such, as early as 1978, it was described as a unique sarcomatous variant of Wilm's tumor with very aggressive clinical behavior (Beckwith, 1978). The same group later identified the kidney tumors as distinct and dubbed them 'malignant rhabdoid tumor of the kidney' due to its morphological resemblance to rhabdomyoblasts, but with a lack of ultrastructural evidence for skeletal muscle differentiation (Haas, 1981). Extrarenal rhabdoid tumors are now recognized as typical as well, in as diverse locations as the liver, chest wall, and CNS (Gonzalez-Crussi, 1982; Lunch, 1983; Tsuneyoshi, 1985). These tumors have always presented a diagnostic challenge given the largely undifferentiated although heterogeneous appearance, polyphenotypic immunoprofiles, and similarities to similar tumor types. The designation of rhabdoid has relied on the histological appearance of large epithelioid cells with eccentric eosinophilic cytoplasm, vesicular nuclei, prominent nucleoli, and whorled bundles of keratin positive intermediate filaments. False diagnoses were frequent, leading to unsuccessful treatments. The discovery of deletions and mutations at 22q11.2 involving SNF5/INI1 has since facilitated diagnosis and treatment choices (Versteege, 1998; Biegel, 1999; Sevenet 1999a&b; Rousseau-Merck, 1999). The genetic mutation in MRTs is usually

specific to SNF5, not chromosome or genome wide losses or instability, implying it is the specific loss of SNF5 that allows these tumors to form.

If there was any question of the causal relationship of SNF5 loss and rhabdoid formation, that was put to rest by several groups showing a predisposition to the formation of rhabdoid tumors in *Snf5*$^{+/-}$ mice, up to 30% penetrance by 15 months (Roberts, 2000; Klochendler-Yeivin, 2000, Guidi, 2001). As mentioned above, while nullizygotes were embryonic lethal, dying at the peri-implantation stage, conditional SNF5 null mutants lead to death within 3 weeks of SNF5 loss due to hematopoietic failure. A reversible conditional mutant that causes only partial penetrance of loss was able to avoid the bone marrow failure and death experienced with the fully penetrant conditional mutation. These mice also experienced a fully penetrant phenotype, developing CD8+ T cell lymphomas or rhabdoid tumors by 11 weeks (Roberts, 2002). The 100% penetrance observed by 11 weeks of SNF5 loss brings SNF5 to the top of the list of deadly genetic mutations in cancer, as studied in mice. Loss of either tumor suppressor gene p53, or p16^{INK4A} leads to tumor development at median ages of 20 weeks and 38 weeks, respectively (Sharples, 2001; Donehower, 1992).

Attention then turned to how SNF5 suppresses tumor formation, i.e. what pathways were involved. It had been established that BRG1 and BRM both bind RB and cooperate in RB-mediated growth suppression and repressor activity (Dunaief, 1994; Reisman, 2002; Strobeck, 2000b; Strober, 1996; Trouche, 1997; Zhang, 2000). With the knowledge that RB is one of the most commonly lost tumor suppressor genes in human cancer, and the fact that it was not known whether inactivation of SNF5 in tumors disrupts Rb function, it was questioned if SNF5 functioned through the Rb pathway. Exploring the SNF5-Rb pathway connection, Tsikitis et al has recently shown that cyclin D1 absence allows *Snf5*$^{+/-}$ mice to

survive tumor free, and have also found cyclin D1 positive cells in primary mouse and human rhabdoid tumors, suggesting cyclin D1 is a mediator of rhabdoid tumorigenesis. Fujisawa, 2005 also found overexpression of cyclin D1 in several primary human rhabdoid tumors, but not in tumors lacking SNF5 inactivation, supporting Tsikitis et al's findings. Interaction of SNF5 with c-Myc, Cyclin D1, and p21 led to the question of cooperation between these two tumor suppressors physiologically. Guidi et al showed in 2006 that loss of the Rb pathway is epistatic to loss of SNF5 in tumor formation, although the interesting finding of SNF5 null pituitary tumors has created questions of Rb and SNF5 interaction in the pituitary. More recently, a similar cross of mice haploinsufficient for SNF5 as well as losing the pRb family via a cross with TgT_{121} mice (a truncated SV40 large T antigen that inactivates pRb and family members 107, and p130) revealed cooperation between SNF5 and the Rb family in tumor suppression as loss of both significantly accelerates MRT formation (Chai, 2007). Although every step involved in the SNF5 pathway to tumor suppression is not yet known, it seems fairly certain the Rb pathway is manipulated.

Given the paradoxical role of SNF5 as tumor suppressor and cell survival gatekeeper, it is important to determine if other known cancer signaling pathways are involved. Aberrant proliferation can lead to Rb inactivation and subsequent apoptosis. As discussed above, the p53 signaling pathway plays a major role in regulating apoptosis (Debbas, 1993; Lowe, 1994; Macleod, 1996; Morgenbesser, 1994; Howes, 1994; Sabbatini, 1995; Wagner, 1994). Logically, the aberrant proliferation creates a selective pressure to lose p53 for tumor survival and proliferation. This has actually been demonstrated by Symonds et al where epithelial cell tumorigenesis occurs in response to pRb pathway inactivation, where normally Rb inactivation of the choroid plexus epithelium would result in p53-dependent apoptosis

(Lu, 2001). In fact, it is well documented that most human tumors harbor inactivation of both Rb and p53 (Weinberg, 1995). It was this suspicion that has lead Roberts and co-workers and Yaniv and colleagues to investigate the interaction of SNF5 and p53 tumor suppressors in rhabdoid tumorigenesis. Klochendler-Yeivin et al (2006) demonstrated p53 nullizygosity indeed accelerated rhabdoid tumor formation in SNF5 heterozygous mice – with complete penetrance within 19 weeks (from 28 weeks on wild type p53 background). Neither the tumor spectrum, nor anatomical locations of the resultant rhabdoid tumors, were altered. Although the above mentioned study, like the SNF5 null mutant mouse studies, are extreme and less likely to represent a true physiologic state, they do support the theory put forth earlier that p53 inactivation allows the SNF5 null cells to progress unfettered to aggressive rhabdoid tumors. To observe a more physiologically likely and therefore more relevant representation of any effects of combined SNF5 and p53 loss, the study presented here reveals an indication of the cooperation between these two very important tumor suppressor pathways.

3. **Mechanism of SNF5 tumor suppression.**

Once established as a classic tumor suppressor gene, attention has turned to determining the mode of tumor suppression of SNF5. At least one major mode of the tumor suppressive abilities of SNF5 appears to be, indirectly or otherwise, through control of the cell cycle. Binding to c-MYC, BRCA1, and p53 links SNF5 to multiple cell cycle control pathways and other tumor suppressor pathways (Hill, 2004; Lee, 2002; Bochar, 2000, Gartel, 2003). c-MYC is involved in controlling regulation of cell growth, genomic stability, angiogenesis, cell transformation, differentiation, and apoptosis, and aberrant expression is

found in many tumor types (Amati, 2001). c-MYC is involved in E2F pathway induction, specifically by nullifying the block on cyclins E and D1 by p21, which is furthered by pRb phosphorylation and subsequently S phase progression. A dominant negative form of SNF5 abrogates the transactivation function of c-Myc (Cheng, 1999).

The link of SNF5 to the Rb pathway continues with the fact that cells lacking SNF5 often exhibit polyploidy in a subset of cells, that are no longer detected upon reexpression of SNF5 (Vries et al, 2006). This so-called 'ejection' of poly- and aneuploid cells was accomplished by SNF5 by transcriptionally regulating members of the p16/pRb/E2F pathway in SNF5 null, MRT-derived cell lines, upon re-expression. The induction of the Rb pathway was exemplified by the G1 arrest, cellular senescence, and apoptosis seen in MRT cell lines upon SNF5 re-expression (Ae, 2002; Betz, 2002; Versteege, 2002; Zhang 2002), that was determined to be due to direct transcriptional activation of $p16^{INK4A}$ (Oruetxebarria, 2004), as well as E2F and cyclin D1. This was supported by work done in our lab as well, where re-expression of SNF5 led to promoter binding of p16, p21, and increased levels of p18 in a rhabdoid cell line (Chai, 2005) accompanying a flat cell morphology, growth arrest and β-galactosidase and PML expression (Betz, 2002; Versteege, 2002; Oruetxebarria, 2004).

When SNF5 is inactivated in primary mouse embryonic fibroblasts, immediate and widespread changes in ~5-10% of the genome can be observed. Microarray analysis of $SNF5^{+/+}$ as opposed to $SNF5^{-/-}$ mouse embryonic fibroblasts identified over 1200 effected genes, 978 repressed in SNF5 deficient MEFs and only 322 activated genes (Isakoff, 2005). Specifically, the SNF5 deficient MEFs showed a decrease of $p16^{INK4A}$, and a 'highly significant' increase in E2F targets. SNF5 deficient MEFs have been observed to arrest upon SNF5 re-expression, which may seem to contradict this apparent genetic push toward cell

cycle progression. The cell cycle arrest MEFs experienced upon re-expression of SNF5 is accompanied by apoptosis, polyploidy, and an increase in p53, as well as a 2-3 fold increase in transactivational induction of p53-targets *Mdm2* and *Puma* (a pro-apoptotic Bcl-2 family member) (Klochendler-Yeivin, 2006). Therefore, it is possible that an attempt was made at cell cycle progression by these damaged cells, triggering the p53 apoptotic/arrest pathway. This was corroborated in mouse studies where both SNF5 and p53 were knocked out conditionally leading to tumor formation in less than one month of age compared to 11weeks in the conditional SNF5 null comparison. SNF5 was still considered to be the 'dominant' phenotype in these mice however, because the tumor type seen is that of SNF5 deficient mice, not the tumor spectrum in the p53 knockout mice. Again, as previously mentioned, because SNF5 binds to the p21 promotor when re-expressed in MRT cells (Chai, 2005) and the association of SNF5 and BRG1 with p53 (Lee, 2002) is lost in the absence of SNF5, it does not seen surprising that the aggressive MRTs grow rampant when both SNF5 and p53 tumor suppressors are lost. The mouse study presented here supports this theory that p53 loss allows SNF5 deficient cells to form tumors unfettered, leading to an increase in SNF5 null type tumors at a higher rate than that seen on a wild type p53 background.

D. SWI/SNF core member BAF155

BAF155 first came into focus when it was identified as a gene highly expressed in the thymus compared to the periphery by subtractive hypbridization (Jeon, 1997). It has since been found to be evolutionarily conserved and therefore, as with the other conserved subunits, has multiple names: *Moira* in *Drosophila*, Swi3 in yeast, Srg3 (Swi3-related gene) in the mouse, and BAF155 (BRG1-associated factor 155kD) in humans – and will referred to

as BAF155 in this document unless specifically referencing another species. BAF155 is also one of the core subunits of the SWI/SNF chromatin remodeling complex. Its precise function beyond that is still coming into focus. Several clues hint at a role for BAF155 in maintaining the complex itself, as outlined below. The protein itself has several highly conserved domains: the SANT (SWI3, ADA2, N-CoR, and TFIIIB) domain, the SWIRM (SWI3, RSC8, and MOIRA) domain, and the leucine-zipper motif. A schematic diagram of BAF155 protein domains can be seen in Figure 1-4. SANT domains in general tend to be associated with transcriptional activating proteins and was reported in the case of BAF155, to be important to the interaction of BAF155 with fellow SWI/SNF core member BRM in Drosophila (Crosby, 1999). More recently, BRG1 was shown to interact directly with the SANT domain of SRG3 in mice (Sohn, 2007). Chen et al 2005 showed that BAF155's leucine zipper motif is vital to its interaction and stabilization of SWI/SNF subunit BAF57. The leucine zipper motif also allows BAF155 to exist as a homodimer or to heterodimerize with its close homolog in the complex, BAF170 (Wang, 1996b). The SWIRM domain of SRG3 was shown to be responsible for the direct interaction with BAF60a, and SNF5, which is stabilized by this interaction (Sohn, 2007). It is this combination of interactions that hints that BAF155 may be a scaffold protein whose primary function in the complex is stability. This implies a potential loss of function for the entire SWI/SNF complex if individual members cannot be stabilized due to loss of BAF155.

Figure 1-6: Schematic of the protein domains of SWI/SNF core member BAF155.

Image adapted from: Chen, J. and T. Archer (2005). "Regulating SWI/SNF Subunit Levels via Protein-Protein Interactions and Proteasomal Degradation: BAF155 and BAF170 Limit Expression of BAF57." Mol Cell Biol **25**(20): 9016-9027.

Figure 1-6

Although no statistics are published concerning the loss of BAF155 in primary tumors, loss of a large area of chromosome 3 where BAF155 maps (3p21) is lost in many lung and other adenocarcinomas. Several 3p deletions are detected in nearly 100% of small (SCLC) and greater than 90% of non-small cell lung cancers (NSCLCs) ensuring the presence of at least one tumor suppressor gene on the short arm of chromosome 3 (Zabarovsky, 2002). Other than the two carcinoma cell lines discovered in our lab that lack BAF155 protein expression, as described here, there are no further links of BAF155 to cancer. BAF155 may resemble fellow core member BRM in this respect, with defined importance in development and differentiation more than in tumorigenesis. The Seong lab has pioneered studies into BAF155's role in mouse development with several papers showing the absolute requirement for a full complement of the SWI/SNF core members, as when BAF155 expression is deficient, embryonic lethality results (Kim, 2001) in the mouse (which makes it very different from BRM-null mice). The BAF155 null embryos were lost soon after decidualization by defects in the inner cell mass (ICM) and the primitive endoderm. This is a similar phenotype observed with loss of other SWI/SNF core members SNF5 and BRG1 who experience neural tube defects resulting in embryonic lethatlity of nullzygous mice and exencephaly of heterozygotes, as detailed earlier. On a cellular level, they also showed the need for BAF155 for proper development of T-cells, as with BRG1.

Clonal deletion of immature T cells, which are nonfunctional or self-reactive, is crucial for maintenance of the normal immune system. Glucocorticoids (GCs) have been suggested to trigger this apoptosis of T cells and eliminate developing thymocytes failing to differentiate properly (Vacchio, 1999; Ashwell, 2000; Pazirandeh, 2002). Loss of Srg3 expression in thymomas decreases the apoptosis induced by glucocorticoids (GCs),

suggesting the necessity of BAF155 in the GC-induced apoptosis during T-cell development (Kim, 2001). It was further found that the sensitivity afforded developing T cells to GC-induced apoptosis by SNF5 is inhibited by down regulation of Srg3 by T-cell antigen receptor (TCR) signaling (Ko, 2004). This TCR repression of Srg3 expression was largely mediated through activation of the Ras/MEK/ERK/ or PI3K pathway. Hong et al (2004), reported BAF155's involvement in a positive feedback regulatory loop with androgen receptor (AR) in developing mouse prostate. This function of BAF155 involved no other members of the SWI/SNF complex and was fully functional in its participation in this regulation of AR in BRG1/BRM deficient C33A cells. Recently, BAF155 was shown to affect insulin sensitivity by regulation of insulin-dependent gene expression via interaction with ADD1/SREBP1c, a transcription factor proposed to mediate regulation of insulin-dependent gene expression (Lee, 2007).

As alluded to earlier, BAF155, along with fellow core member BRG1, becomes phosphorylated as the G2/M checkpoint is reached. It is believed this interaction stabilizes the core to ensure proper functioning of the complex at this checkpoint. Precisely how these cell cycle checkpoints function is not yet fully defined. It is known, however, that Cyclin E-cdk2 complexes play an essential and rate-limiting role in the transition between G1 and S phase (Owen-Hughes, 1995; Resnitzky, 1994; Tsai, 1992; van den Heuvel, 1993) although the specific downstream targets of Cyclin E used to carry out the activity are poorly understood. It is also known that Cyclin E is essential even in the absence of Rb suggesting another rate-limiting substrate exists for cyclin E-cdk2. Emma Lees and co-workers have demonstrated an association between BAF155 with Cyclin E and BRG1 that, possibly among other functions, impedes the ability of BRG1 to induce growth arrest (Shanahan, 1999).

E. Specific aims of this dissertation

The importance of epigenetic and related chromatin remodeling complexes to cancer has established itself in the scientific consciousness; however the exact mechanism of how they function has not come into focus. In some cases, like that of the SWI/SNF complex, the specific function of every member of the complex is not yet well understood. It is known SWI/SNF affects the transcription of approximately 5% of the genome, but which 5%, and how that 5% is targeted is still largely unknown. Understanding the function and association of each subunit will shed light on the targeting and exact function of the complex as a whole, and ultimately allow for attempts at correction when the system malfunctions. The study presented here is designed to better define and shape the role of two core subunits of the SWI/SNF chromatin remodeling complex and their roles in tumor suppression. It seems clear that while the BRG1 and SNF5 core members of SWI/SNF play a role in development and cancer, the involvement of BAF155 has not been characterized. We present here, in the first aim, evidence of the potential of SWI/SNF core complex member BAF155 as a tumor suppressor.

While the tumor suppressor status of SNF5 is well established, its mechanism of tumor suppression is not. A vital part of understanding the function of a tumor suppressor is in knowing the cell cycle pathways affected by its loss. The second aim presented here attempts to determine if there exists a cooperative relationship between tumor suppressor and cell cycle regulator p53 and SWI/SNF complex core member SNF5.

CHAPTER 2

MATERIALS AND METHODS

A. Cell lines.

All cell lines were grown at 37°C in a 5% CO_2 incubator with RPMI 1640 medium base supplemented with 10% FBS with the exception of SNUC2B which was grown in 20% FBS. Cell lines listed were acquired from ATCC unless noted otherwise. Cell lines referred to in following chapters but listed here have been previously published and are detailed in the referenced publication. SKOV3, SNUC2B (from Eric Knudsen lab, University of Cincinnati, OH), Normal human fibroblasts (NHF), U2OS (Trevor Archer lab, NIEHS, RTP, NC), MCF-7, and HepG2.

B. Transfections.

Transfections of expression vectors were carried out by the Lipofectamine 2000 (Invitrogen) or Effectine (Qiagen) method according to the manufacturer's instructions. Selection, when used, was begun 24 hours post transfection by adding neo to the nutrient media for the described amounts of time, usually 10-14 days post transfection. Expression vectors used can be seen on table 2-1

C. Western blots

Whole protein was extracted from trypsinized subconfluent cells washed once in PBS. Total proteins were extracted using 8 mol/L urea as described previously. Nuclear

Table 2-1. : Content and source of expression vectors used in the examination of BAF155 loss and function.

Table 2-1:

Plasmid	Content of plasmid	Source
pcDNA3/BAF155-V5	V5 tagged full length BAF155	Kind gift of Dr. Trevor Archer (Chen, 2005)
pCMV/myc/nuc/ BAF155-Δ2	Myc tagged truncation mutant BAF155	Kind gift of Dr. Trevor Archer (Chen, 2005)
H2Bgfp	GFP	Kind gift of Dr. Erik Knudsen
β-gal control vector	β-galactosidase (control) CMV promoter	Open Biosystems RHS3708
pCMV	Empty vector control	Kind gift of Dr. Trevor Archer
pEGFPBAF155	c-terminus-GFP tagged full length wild-type BAF155. CMV promoter, no selection	Gift of Dr. Erik Knudsen

extracts were obtained by first washing the cells before they were pelleted, incubated with hypotonic buffer for 10 min on ice followed by manual homogenation (glass douncer) and centrifugation. Protease inhibited nuclear extract buffer was then added to the homogenized pellet before sonication and a second homogenation. Cell extract was then centrifuged for 30 min at 4^{o}C before clear supernatant is dialyzed for 4.5 hours to pH 7.9. Protein concentration was quantified by the Bio-Rad protein assay (Bio-Rad Labs, Hercules, CA) per manufacturer's instructions. Proteins (30ug) were separated by electrophoresis on 4-20% or 7.5% SDS-polyacrylamide gels (Cambrex, Rockland, ME) and electrotransferred onto Immobilon-P membranes (Millipore, Billerica, MA) according to the manufactures' instructions for Biomax ML film (Kodak, Cedex, France). Commercial antibodies used are detailed in table 2-3

D. DNA extraction from cell lines for sequencing

DNA was extracted using the Qiagen DNeasy kit for extraction and purification of genomic DNA. For cDNA, mRNA was first extracted with Qiagen RNeasy kit, then RT-MMLV kit was used to reverse transcribe cDNA according the manufacture's instructions.

E. Sequencing

Sequencing primners. were designed according to known BLAST sequences of BAF155 to amplify several sections of several hundred base pairs each of cDNA from genomic DNA. Sequencing of individual exons was done as well accoding to the primers in the table below. BAF155 sequencing samples were submitted to UNC-CH sequencing

facility according to their instructions. Details of all primers used can be found on table 2-3

F. Bromodeoxyuridine staining.

Inhibition of BrdU incorporation was performed as previously described (Strobeck, 2002). Briefly, cells were treated with 1:1000 BrdU (Amersham Biosciences) 36 hours after transfection and stained with 1:50 anti-BrdU antibody (Accurate Chem & Sci. Corp.) 12 hours later. BrdU-positive cells were counted in at least 100 GFP-positive cells. Error bars (standard deviation) were calculated from at least three independent experiments.

G. Protein Stability

Lactacystin Treatment was as follows. Lactacystin (3-(4-iodophenyl)-2-mecapto-(Z)-2-propenoic acid) (#426100 from Calbiochem) was dissolved in dimethysulfoxide (DMSO) with the final concentration of DMSO that cells were exposed to being no greater than 0.2%. Lactacystin/DMSO mix or DMSO alone (as a control for the effects of DMSO) was added to the standard media for each cell line and remained on cells for the times indicated in each figure (4-32 hours) at which time 8 molar urea protein extractions were carried out and protein ran out via Western blot.

Table 2-2 : Primer sequences for primers used in sequencing of BAF155 gene in cDNA as well as genomic DNA of SKOV3 and SNUC2B cell lines and primer sequences for genotyping of mice

Table 2-2:

Name	Primer sequence (forward)	Primer sequence (reverse)
122F	CGCAGGCCTAGCTGTTTATC	
290F		GTGCAGCTTCTTCAGTTCC
1F1	ACGACGGGCTGCGACGATG	
2F2	TTGGTGCAGAACAATTGTTTGACC	
1R1		CACCGAATCCAGCTGGGGAC
2R2		CAAGAATGTGACATAAGGCGC
9R1		CGTGGAGGTTCCCTGCATC
7R		GAGTGCTGGTGTGCCTGTTG
16R		TCCTAAGATGTTTCCTGGCATTG
16F	CAATGCCAGGAAACATCTTAGGA	
3F	GCCAGTCAGAAGTCCAGAAAGAAG	
660F	CACATTTACCCATATTCTTCCTCAC	
1001R		TGCTTTTCTATCTCTTCTTTCTGGA
1167F	CCAACACCTGTACCCAATATAGAAG	
2822R		GGAAACTATCATGGACAGAGAGAAA
425F	CAAGTATAAAAATGAACAGGGATGG	
1562R		ATACTCTTGGGGGTTTAGACGATAC
859F	TGGACACTGATATTTTCAATGAATG	
744R		AATGCACTAACACTTGCTTCTCTTT
2496F	GAAAAGGAACAGGATAGTGAAGTGA	
1191R		TCTTTCTTTAGGTTCACATTTTTGG
2833R		TCTAGAGCTTCTTTCTCTCTGTCCA
2496R		TCACTTCACTATCCTGTTCCTTTTC
1538F	GTATCGTCTAAACCCCCAAGAGTAT	
1866R		AGTAAATGTCAGTACGGAGACCAAA
1812F	CAGATGCTAAATTTTCCTGAGAAAA	
122R		TCGATAAACAGCTAGGCCTGCG
3340R		CTGCATCTTCCAGGCTAAGG

290R		TTCCTGGAACTGAAGAAGCTGCACC
3123R		CTGCAACAGTGGGAATCATG
545F	ATCTCGAATGGATCGTAATG	
EXON	**FORWARD PRIMER**	**REVERSE PRIMER**
Exon 1	TTGCATATTCGTGGTTCTAAATTC	GAGGCCAGCTGCCGCCT
Exon 16	GCACATGAAGTTGATTCTTTAG	CAACCCCTAAATGGCATAAC
Exon17	AGAGGCTAACTCAGCCATTT	GCCATTTGTTACTATCCAGTC
Exon 18	CTGAGCTCCATCTTCAGCAT	ACTAAGATGTGCTGGATTGC
Exon 19	CTGAGCTCCATCTTCAGCAT	GGTGACACACAGCGAGACC
Exon 20	TCTTGTTACCTGACATGAAAGC	ATGAATGTAACGGCTGGTAA
Exon 21	AAGTTAGATTGCTGTTCCAT	TATACTGATCATTGTACTCACT
Exon 22	CAGACACTCTCACAGCTGTA	GGTCTGAAAGGCATGCTATC
Exon 23	CGAGCTGTTCTCTACAAGTCAC	AAGTGTAGCCACCAGCATTA
Exon 24	GAGAGTGGGCACGCTAGTA	CAATACGAGCATTCATGCCT
Exon 25	AGCTTGAATGATCTTGTACC	TGGAAAAGTGACCAAGGTT
Exon 26	CTGGTGTCATTTCTTCCACT	CCCTAAATCTCACATGCCTAC
Exon 27	GTTGATCCCTTTCTTACACCC	AACGGACCCTGAGATAATGC
Exon 28	TCCAGCAATAACGAGTTACTC	CACGGCTTGGAGCTGTGAG
Product	**Forward primer**	**Reverse primer**
Wild type SNF5	CAGGAAAATGGATGCAACTAAGAT	CACCATGCCCCCACCTCCCCTACA
Mutant SNF5	GGCCAGCTCATTCCTCCCACTCAT	CACCATGCCCCCACCTCCCCTACA
Wild type p53	ACAGCGTGGTGGTACCTTAT	TATACTCAGAGCCGGCCT
Mutant p53	TCCTCGTCGTTTACGGTATC	TATACTCAGAGCCGGCCT

H. Immunoprecipitation

Cells were lysed in high-salt buffer (20mM Tris-HCl [pH 7.5], 400 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing a protease inhibitor cocktail. A whole-cell extract (500 ug) was first diluted with an equal volume of dilution buffer (lysis buffer without NaCl), brought to 500ul by adding IP buffer (lysis buffer with 200 mM NaCl), and incubated with approximately 3 to 5 ug of antibody at 4^{o}C overnight. The incubation was continued for an additional 2 hours after the addition of 20ul of protein A/G plus-agarose bead slurry (Santa Cruz Biotechnology, Santa Cruz, CA). Agarose beads were washed four times with IP buffer and eluted into 40ul gel loading buffer by heating at 95^{o}C for 5 min. The bound proteins were separated by SDS-PAGE.

I. Northern blotting

Expression of BAF155 mRNA was determined by standard Northern blot analysis. Total cellular RNA was extracted using the Qiagen RNeasy kit. Fifteen micrograms of total RNA were run on 1% agarose/formaldehyde gels and were gravity transferred to positively charged nylon membranes. The membranes were stained for ribosomal 28s proteins for equal loading. BAF155 probes were ^{32}P-radiolabeled by random primer extension, hybridized to membranes, and subsequently rinsed to remove nonspecific radioactivity. Hybridization bands were visualized by autoradiography.

J. Real-time PCR

Real-time quantitative reverse transcriptase polymerase chain reaction: Total RNA was isolated from each cell line using the Qiagen RNA extract kit (Qiagen, Valencia, CA)

according to manufacturer's protocol and analyzed by the TaqMan (Applied Biosystems, Foster City, CA) quantitative real-time reverse-transcription PCR using β-actin as a reference gene in each reaction.. Primers used were as follows: BAF155: CACCCCAGCCAGGTCAGAT (forward) & TGCAACAGTGGGAATCATGC (reverse); BAF170: GAGAAGCACTGGAGTATCAGA (forward) & ATCTCCGCATACTTCAGCTG (reverse). The probes used for BAF155 and BAF170 were CAGGCCCAGGTTCCATGATGCCCG and CAGCAGCTCCTGGCCGACAGACAAG respectively.

K. Apoptosis detection - TUNEL Assay

Apoptosis via DNA fragmentation was measured using the "In Situ Cell Death Detection Kit, TMR red" from Roche (catalog # 12 156 792 910). The kit was used according to manufacturer's instructions following transfection with plasmids, at times post-transfection as indicated in each figure. At least 200 cells were counted in each of at least 3 independent experiments for each condition.

L. Senescence detection via β-galactasidase

Senesence-associated β-galactocidase was carried out as previously described (Dimri et al 1995) with modifications of Kramer et al 2001. Briefly cells were washed with PBS, fixed for 3 minutes with 3% formaldehyde/PBS, washed three times with PBS, and incubated overnight in a 37^{o}C incubator in SA-β-gal solution (5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM mgCl2, and 1mg/ml 5-bromo-4-chloro-3-

indolyl-β-D-galactoside (X-Gal in PBS). Plates were visualized with bright field microscopy on a Zeiss 1M 35 microscope.

M. Generation and Maintenance of mouse colony

Snf5$^{+/-}$ mice were originally generated by Dr. Charlie Roberts as described in: Robersts, 2000. The *p53*$^{+/-}$ mice were originally generated by Dr. Tyler Jacks as described in Jacks,1994. Mice were monitored twice a week for 24 months or until a tumor was observed or evidence of a tumor (paralysis, swelling, extreme lethargy, etc).

N. Histology of mouse tissues

Tumor samples and selected tissues were fixed in 10% buffered formalin phosphate and processed for paraffin embedding. Slides are cut and left unstained for future immunohistochemistry or stained for eosin and hematoxilin by UNC's Histopathology Lab.

O. Immunohistochemistry

IHC analysis carried out using the Vectastain *Elite* ABC Kit from Vector Laboratories (PK-6100), following manufacturer's directions. Antibody diluent buffer from Biomeada. Antigen retrieval citra plus solution from BioGenex (cat # HK081-5K). Liquid DAB substrate system from Innovex biosciences (product # NB314SBD). The immunohistochemical staining of the mouse tissue array for p53 was carried out by The Laboratory of Experimental Pathology Immunohistochemistry Group at NIEHS, RTP, NC. All antibodies used are included in the table below.

P. DNA extraction for mouse genotyping

Genomic DNA was extracted from mouse toes and tumors for genotyping using the Qiagen DNeasy Kit for animal tissues. DNA was extracted/purified from vectors using plasmid prep kits from Qiagen followed by enzymatic digestion at 37^{o}C for one hour.

Q. Genotyping

All PCR recactions were carried out using EasyStart 0.5mL PCR tubes (MolecularBioProducts #6022). The following additions were made to the EasyStart tube: 1-3ul of DNA extracted from mouse toes, taq DNA polymerase, specific primers for the mutant or wild type allele, and water. Primer details can be found on table 2-2

R. Antibodies

All antibodies were used per manufacture's instructions unless otherwise specified in figure legend or table 2-3. Detail of antibodies used for western blot and immunohistochemistry can be seen on table 2-3

Table 2-3: Description and source of antibodies used for western blot and immunohistochemistry

Table 2-3:

ANTIBODY	Brief Description	Source
BAF155 (H-76)	Rabbit, to AA 889-913	Santa Cruz sc-10756
BAF155 (DXD7)	Mouse monoclonal, to AA 591-608. does not react with BAF170	Sigman B5186
Actin	Rabbit polyclonal,	Sigma A2066
BRG1 (J1)	rabbit polyclonal	Kind gift of W. Wang
BRG1 (G-7)	mouse monoclonal	Santa Cruz sc-17796
BRM	Rabbit polyclonal	Abcam ab15597
SNF5	Rabbit polyclonal	Gift of Anthony Imbalzano
SNF5	Mouse monoclonal	Transduction Labs, 612110
BAF57	Rabbit polyclonal	Gift of Karen Knudsen, Univ. of Cincinnati, OH
BAF170	Rabbit polyclonal	Gift of W. Wang
Vimentin	Rabbit polyclonal	Biomeda V2009
B220/CD45R	Mouse monoclonal	BD Pharmingen 550286
CD3	Rabbit polyclonal	Cell Marque CMC363
S100	Rabbit polyclonal	DAKO Z0311
P53 (CM5)	Rabbit polyclonal	Novacastra Labs NCL-p53-CM5p
GFP	Mouse monoclonal	Santa Cruz (sc-9996)
c-jun	Rabbit polyclonal	Oncogene Science PC07
Ubiquitin	Mouse monoclonal	Santa Cruz sc-8017
Cyclin E (M-20)	Rabbit polyclonal	Santa Cruz sc-481
c-myc (9E10)	Mouse monoclonal	Santa Cruz sc-40
V5	Mouse monoclonal	Invitrogen R961-25
PAI-1	Mouse monoclonal	Oncogene IM29L
PML	Mouse monoclonal	Santa Cruz sc-966
PARP	Rabbit polyclonal	Roche 1 835 238
P21 (WAF1)	Mouse monoclonal	Oncogene OP64

CHAPTER 3

Identification of an Additional Core Member of the SWI/SNF Complex, BAF155/SMARCC1, as a Human Tumor Suppressor Gene

A. Abstract

Recent studies have established that two core members of the SWI/SNF chromatin remodeling complex, BRG1 and SNF5/INI1, possess tumor-suppressor activity in human and mouse cancers. While the third core member, BAF155, has been implicated by several studies as having a potential role in tumor development, evidence for its tumor suppressor activity has remained unclear. Therefore, we screened a large number of human tumor cell lines for BAF155 deficiency. We identified 2 cell lines, SNUC2B colon carcinoma and SKOV3 ovarian carcinoma, displaying a complete loss of protein expression while maintaining normal levels of mRNA expression. The SKOV3 cell line possesses a hemizygous 4bp deletion that results in an 855AA truncated protein, while the cause of the loss of BAF155 expression in the SNUC2B cell line remains unknown. However, the lack of detectable BAF155 expression did not prevent assembly of the SWI/SNF complex, or effect sensitivity to Rb-mediated cell cycle arrest. Re-eexpression of full length but not a truncated form of BAF155 in the two cancer cell lines leads to reduced proliferation and colony forming ability. This arrest appears to result from induction of $p21^{WAF1/CIP1}$ expression followed by replicative senescence and not apoptosis. Collectively, these data suggest that loss of BAF155 expression represents another mechanism for inactivation of SWI/SNF

complex activity in the development in human cancer. Furthermore, loss of BAF155 causes a slightly different phenotype than loss of either BRG1 or SNF5/INI1. Finally, our results indicate that the c-terminus proline-glutamine rich domain plays a critical role in the tumor suppressor function of this protein.

B. Introduction

A new era of cancer research is underway with the realization that the initiation and regulation of cancer involves more than the identification of oncogenes or tumor suppressor genes. The field of epigenetics has exploded in recent years with multiple studies demonstrating the involvement of DNA methylation and chromatin modification in cancer development. Both mechanisms regulate gene transcription by controlling the access of transcription factors to DNA. Chromatin disorders have been implicated in the devastating effects of solid tumors as well as myeloid leukemia, Rubinstein-Taybi Syndrome, and malignant rhabdoid tumors.

The proper functioning of the SWI/SNF chromatin remodeling complex is vital to appropriate cell cycle control and tumor suppression. Despite the seemingly small (5%) amount of genes whose regulation the complex affects, they are widely dispersed throughout the genome with more repression than activation (Sudarsanam, 2000). The ubiquitously expressed multi-unit complex is composed of a small core including BRG1 or BRM, SNF5/INI1/BAF47, BAF155, BAF170 and variable associated complex members depending on cell type and stage of development. Most core members are vital to life and essential to development as homozygous knockout mice show embryonic lethality (Bultman, 2000; Reyes, 1998; Kim, 2001).

Either BRG1 or BRM serve as the catalytic subunit of the complex. Approximately ten percent of human cancer cell lines show mutations or deletions of these genes (Reisman, 2003). Another core member, SNF5/BAF47/INI1, is an established tumor suppressor gene that is deleted in almost all MRTs (Biegel, 1999). The absence of SNF5 protein provides the diagnostic marker for these cancers. Genetically engineered mice also provide evidence for the tumor suppressor activity of these genes. Mice heterozygous for *BRG1* develop tumors resembling breast adenocarcinomas (Bultman, 2000), while heterozygous *SNF5* mice develop rhabdoid tumors histologically similar to their human counterparts (Roberts, 2000; Klochendler-Yeivin, 2000; Guidi, 2001). Several studies have implicated that the SWI/SNF complex acts as a tumor suppressor via its role in cell cycle regulation. The SWI/SNF complex can control cellular proliferation by its association with known cell cycle checkpoint genes, such as cyclin E, p21, p53, and p16 (Hill, 2004; Lee, 2002; Bochar, 2000, Gartel, 2003).

The unique contribution of BAF155, another core member, to the complex remains ill-defined. Initially isolated as a gene highly expressed in thymus/low in periphery, BAF155 actually shows ubiquitous expression similar to the rest of the SWI/SNF complex. Also known as SWI3 in yeast, SRG3 (SWI3 related gene) in mouse, and MOIRA in drosophila, BAF155 has been implicated to have a significant role in development. In mice, SRG3 is essential to early embryogenesis, as well as having a specific requirement for brain development and T-cell differentiation (Kim, 2001). SRG3 also can be induced by androgen and subsequently transactivate AR in the prostate (Hong, 2004). Reduction of SRG3 leads to a significant inhibition of GC-induced apoptosis (Vacchio, 1999; Ashwell, 2000; Pazirandeh, 2002). BAF155 has also been implicated in the regulation of cyclin E activity

(Shanahan, 1999). Little is known, however, of the role of BAF155 in cancer related functions.

This study sheds light on the potential tumor-suppressor functions of BAF155 by characterizing 2 human tumor cell lines that lack BAF155 expression. Our studies demonstrate that reexpression of exogenous full length BAF155 induces senescence in these cell lines. In contrast, exogenous expression of BAF155 on 2 human cell lines with endogenous expression had no little or no effect on cell growth. Furthermore, truncation of the c-terminus of BAF155 caused a significant loss of its tumor suppression activity. Our results establish another member of the SWI/SNF complex as a tumor suppressor gene that may contribute to its regulation of the cell cycle and cellular senescence.

C. Results

1. Loss of BAF155 expression in two carcinoma cell lines.

To gauge the frequency of BAF155 loss as well as to generate a cell culture model system to study BAF155 functions, we screened >100 human cancer cell lines by western blot for BAF155 expression. We identified two carcinoma cell lines lacking BAF155 protein (Figure 3-1A): SKOV3, an ovarian carcinoma cell line, and SNUC2B, a colorectal carcinoma cell line. Initial screening of nuclear extracts from these cells showed that other complex members, including BRG1 and SNF5, were present. We also screened whole cell extracts to determine whether BAF155 still appeared in the cytoplasm. We did not observe BAF155 expression under any protein extraction conditions in these two carcinoma cell lines. Both cell lines contained a full complement of other SWI/SNF complex members, although SNUC2B's BRG1 ATPase domain has two point mutations (Wong, 2000).

2. Loss of BAF155 in two carcinoma cells lines is not due to loss of gene expression or proteasome degradation.

To determine the reason for the lack of BAF155 protein expression in the two deficient cell lines, we assessed mRNA levels by northern blot analysis. As shown in Figure 3-1B, both cell lines expressed comparable levels of BAF155 mRNA to BAF155 positive cell lines. Because of the strong homology between BAF155 and BAF170, we confirmed these results with real time PCR. We did not observe any reduction in BAF155 or BAF170 expression by this method (Figure 3-1C).

We next determined whether the absence of BAF155 protein resulted from increased protein degradation. Cells were treated with lactacystin for up to 36 hours (inhibiting the degradation of ubiquitinated proteins), whole proteins was harvested and analyzed by western blot for BAF155 expression. Expression of c-jun, a protein with a rapid degradation rate, was included as a positive control. As shown in Figure 3-1D, c-jun protein levels increased after lactacystin treatment, accompanied by an increase in ubiquinated proteins. However, we did not observe reexpression of the BAF155 protein.

3. **Ovarian carcinoma cell line SKOV3 posesses a truncation mutant BAF155**.

Upon sequencing individual exons of BAF155 in the two cells lines, SKOV3 revealed a 4 base pair deletion in exon 24 leading to a stop codon at 2566bp (figure 3-2). This mutation would yield a truncated protein of 855 amino acids, instead of the full length protein of 1105AA. The loss of the last 250 amino acids would remove the leucine zipper

and proline-glutamine rich domains as well as the antibody binding site. Therefore, we cannot rule out production of a truncated protein by the SKOV3 cell line. The primary

Figure 3-1: Two carcinoma cell lines SKOV3 and SNUC2B lack BAF155 protein expression.

(A) Western blot loaded with 30ug of nuclear extract from cell lines, actin-related BAF53 serves as the loading control.

(B) Northern blot (B) and Quantitative PCR (C) analysis reveals both cell lines lacking BAF155 expression retain comparable amounts of BAF155 and BAF170 RNA levels as other cancer cell lines. A) thirty micrograms of mRNA of each cell line were analyzed by northern blot and probed with BAF155 cDNA probe. 18S and 28S bands serve as loading control indicators.

(C) Both cell lines lacking BAF155 protein expression were analyzed quantitatively for amounts of BAF155 and BAF170. Graphed values are relative quantities as compared to actin.

(D) BAF155 protein is not degrated by the ubiquitin-proteasome pathway in either carcinoma cell line lacking BAF155 expression. Increased time under lactacystin treatment leads to a measurable accumulation of proteins degraded by the ubiquitin-proteasome pathway as demonstrated with c-jun. Mock treatment with DMSO revealed the accumulation is due specifically to the lactacystin treatment/ degradation inhibition

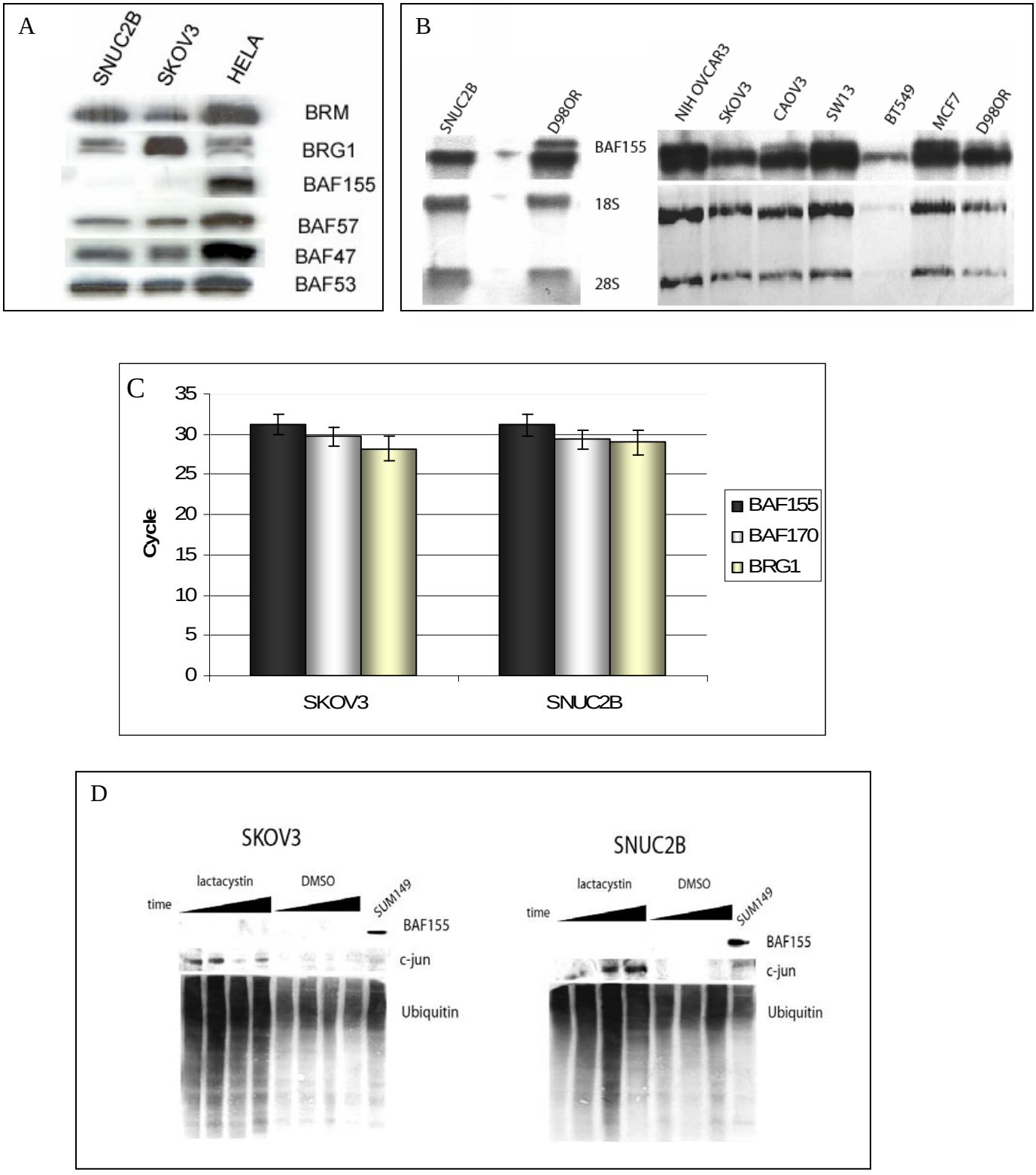

A
SNUC2B
SKOV3
HELA
BRM
BRG1
BAF155
BAF57
BAF47
BAF53
B
SNUC2B
D98OR
NIH OVCAR3
SKOV3
CAOV3
SW13
BT549
MCF7
D98OR
BAF155
18S
28S
C
35
30
25
20
15
10
5
0
Cycle
SKOV3
SNUC2B
BAF155
BAF170
BRG1
D
SKOV3
lactacystin
DMSO
time
SUM149
BAF155
c-jun
Ubiquitin
SNUC2B
lactacystin
DMSO
time
SUM149
BAF155
c-jun
Ubiquitin

FIGURE 3-1

Figure 3-2: Sequence of exon 24 of SKOV3 ovarian carcinoma cell line lacking protein expression.

Individual exons were amplified by PCR, purified and sent to UNC-CH Genome Analysis Facility for analysis. This 4 bp deletion mutation leads to a aberrant stop codon at 2666-2669, yeilding an 855 amino acid truncated BAF155 protein. Result was confirmed three times. The black sequence represents the published BAF155 wild type sequence. The blue sequence represents the sequence of exon 24 of BAF155 genomic DNA in the SKOV3 cell line.


GAAGAAAAGGAGACTGAAGAGAACAAAGAACTCACTGATACATGTAAAGAA
E E K E T E E N K E L T D T C K E
GAAGAAAAGGAGACTGAAGAGAACAAAGAACTCACTGATACATGTAAAGAG
E E K E T E E N K E L T D T C K E
AGAGAAAGTGATACTGGGAAGAAGAAAGTAGAACATGAAATTTCCGAAGGA
R E S D T G K K K V E H E I S E G
AAAGTGATACTGGGAAGAAGAAAGTAGAACATGAAATTTCCGAAGGAAATG
K V I L G R R K STOP
AATGTTGCCACAGCCGCAGCAGCTGCTCTTGCCTCAGCGGCTACCAAAGCCA
N V A T A A A A A L A S A A T K A
TTGCCACAGCCGCAGCAGCTGCTCTTGCCTCAGCGGCTACCAAAGCCAAGC
AG
K

FIGURE 3-2

sequence of SNUC2B has not yielded a mutation. We are currently sequencing the untranslated regions of mRNA to lookfor potential abnormalities in protein translation signals.

4. BAF155-induced growth inhibition of carcinoma cells via senescence.

We next assessed the effects on cell growth after reexpression of BAF155 in the cell lines lacking endogenous expression. We used expression vectors that coded for either a full-length protein or one with a 332 amino acid deletion of the c-terminus (Figure 3-3A). We have previously shown that the c-terminal deletion abrogates BAF155's ability to regulate the stability of another complex member, BAF57 (Chen, 2005). We first examined the effects of BAF155 reexpression on DNA synthesis after 48 hours by measuring BrdU incorporation into nuclei. Re-expression of wild type and mutant tagged-BAF155 protein (Figure 3-3B) resulted in different outcomes for the BAF155-expressing Hela and U2OS cell lines versus the BAF155 deficient SKOV3 and SNUC2B cell lines (Figure 3-3C). As shown in Figure 3-3C, BAF155 null cell lines were inhibited for BrdU incorporation in response to full-length wild type BAF155 transfection with no inhibition with mutant BAF155. In contrast, expression of either form of BAF155 in the Hela or U2OS cells had no effect (Figure 3-3C). These results indicated that a block in cell cycle progression was induced within 48 hours following BAF155 expression.

We then assessed BAF155 effects upon long-term proliferation by a colony-forming assay. Colony forming assays showed a marked reduction in colony forming ability in BAF155 null cells after wild type BAF155 reexpression, but not when transfected with mutant BAF155 (Figure 3-3D). No effect on colony forming ability was seen in Hela cells

endogenously expressing wild type BAF155, although a partial reduction of colony forming ability was observed in U2OS cells.

To determine if the inhibition of growth was due to a senescent or apoptotic response, we investigated typical markers of each of these events in both cell lines at several time points following reexpression of BAF155. No increase in fragmented DNA particles, characteristic of early to middle apoptosis, was seen via TUNEL staining at 2, 4, or 7 days following BAF155 expression (Figure 3-4). In contrast, approximately 15-20% of the BAF155-deficient cell line expressed the senescence marker, ß-galactosidase by 10 days (data not shown). These results are similar to those observed upon reexpression of BRG1 in BRG1/BRM-deficient human tumor cell lines (Dunaief, 1994; Shanahan, 1999; Strober, 1996; Wong, 2000; Hendricks, 2004).

Figure 3-3: Demonstration of BAF155 growth arrest ability.

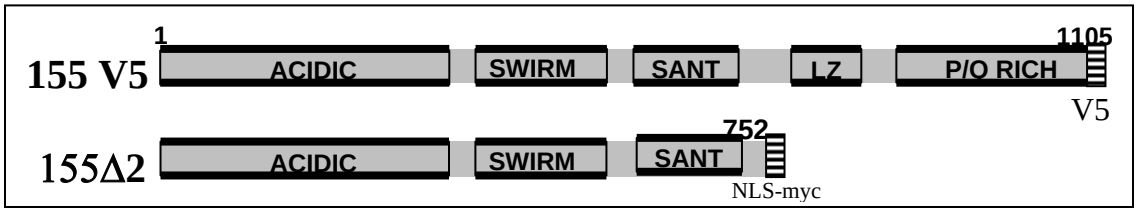
(A) Schematic of BAF155 expression vector contents used for re-expression studies in this and subsequent figures. Note the striped boxes indicate the identifiable tag for each insert. The delta-2 mutant BAF155 also has added a nuclear localization signal.

(B) Western blot demonstrating expression of tagged full length BAF155 (V5) as well as mutant BAF155 (Δ2) 2 days post-transfection. 30ug whole cell extract was loaded. Actin acts as loading control

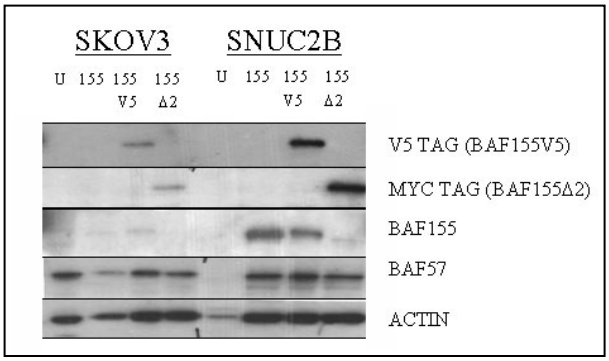
(C) BrdU incorporation: Cells were co-transfected at a 1:10 ratio of GFP: vector of interest, one of the following: empty vector control, full length BAF155-V5 tag, truncation mutant BAF55 Δ2. Bar values are a ratio of GFP positive cells to BrdU positive cells. Results normalized to empty vector control amounts. Values graphed are an average of 3 independent experiments.

(D) Results of the 14 day colony forming assay. Cells were carried under selection for 12 days beginning 2 days post-transfection. Bar values were calculated by a ratio of stained cells present at day 14 to the empty vector control transfected cells for each cell type.

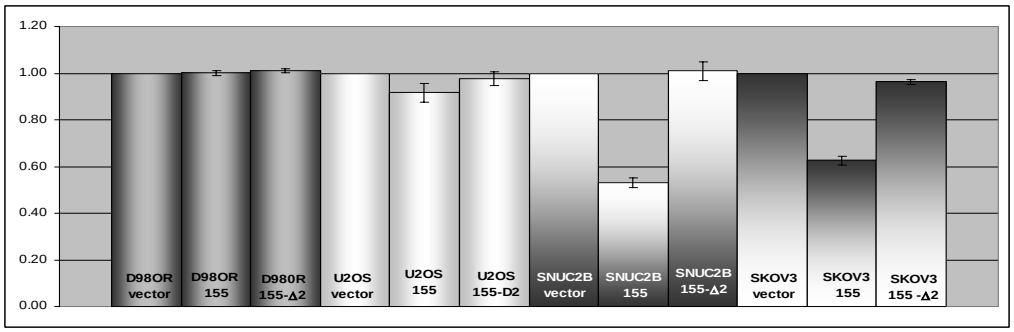
A



B

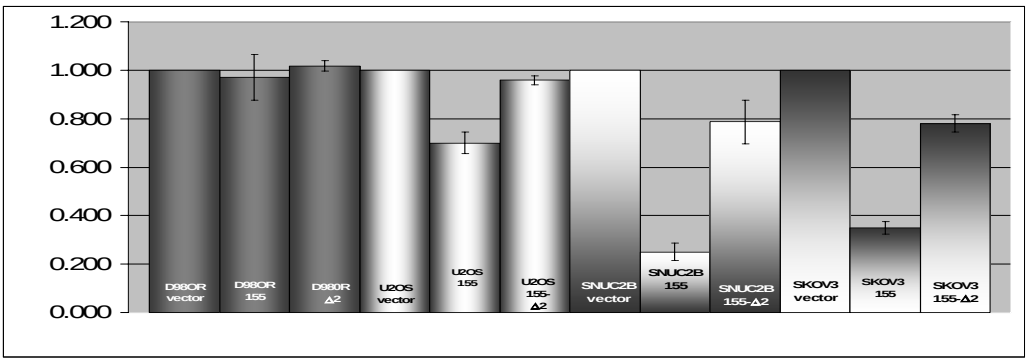


C



Ratio of BrdU incorporation normalized to control

D



colony density normalized to control

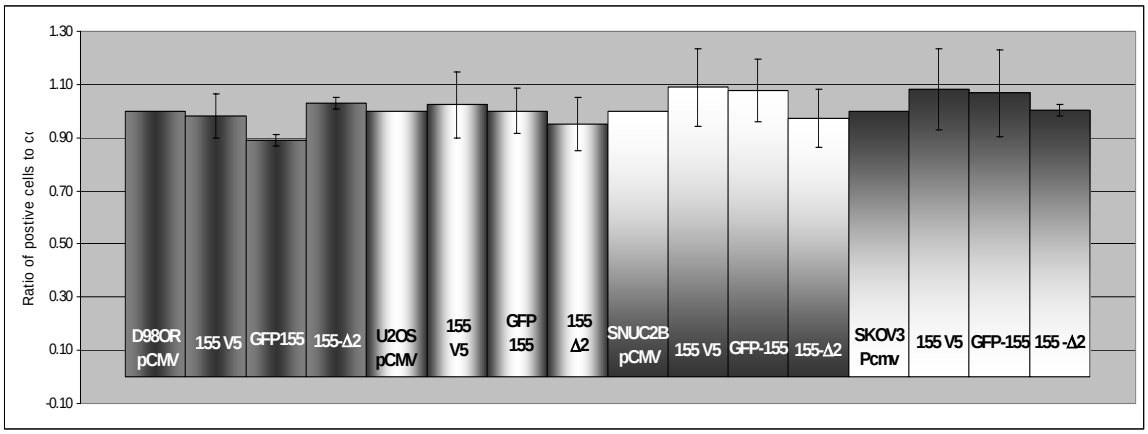
Figure 3-3

5. **The SWI/SNF complex still forms in cells lacking BAF155 expression and retains the ability to support Rb-mediated cell cycle arrest**.

We next asked whether the absence of BAF155 affected formation of the complex. The core components, as well as BAF57, still co-immunoprecipitated in cells with or without BAF155 expression (Figure 3-5A and data not shown). We then determined whether the complex could still contribute to Rb-mediated cell cycle arrest in cells without BAF155 protein. Both cell lines were found to be Rb sensitive, arresting in G0, following $p16^{INK4A}$ expression (Figure 3-5C). Upon infection with $p16^{INK4A}$ encoding adenovirus (demonstrated in Figure 3-5B), accumulation of SKOV3 cells can be in G0/G1 (Figure 3-5D). BrdU labeling reveals these cells never reach S phase (Figure 3-5C). This data shows the complex can still form in

Figure 3-4: TUNEL Apoptosis detection.

Cells were analyzed 48 hours post co-transfection with GFP and one of the following: empty vector control, V5 tagged full length BAF155, or myc tagged Δ2 truncation mutant BAF155. A ratio of TUNEL positive (broken DNA ends = apoptosing) cells to GFP positive cells (presumed to express BAF155 as well) were averaged and normalized to empty vector control cell.



Ratio of positive cells normalized to control

FIGURE 3-4

Figure 3-5

(A) SWI/SNF complex subunits associate with one another despite lack of BAF155. western blot following immunoprecipitation with antibodies listed above lanes and western blot probed by antibodies listed to the right of each lane.

(B) SKOV3 and SNUC2B cells were infected with GFP or p16ink4a encoding adenviruses. Western blot run from protein extracted 36 hours later**.**

(C) 36 hours post infection with GFP or $p16^{ink4A}$ encoding adenviruses cells were analyzed for incorporation of BrdU and graphed as a ratio of BrdU positive cells in the GFP control cells for each cell line.

(D) Cells were infected with GFP or $p16^{ink4A}$ encoding adenoviruses for 36 hours, cells were harvested, fixed with ethanol, and stained with propidium iodide (PI). Cell cycle distribution was then determined by flow cytometry. DNA content is plotted against cell number.

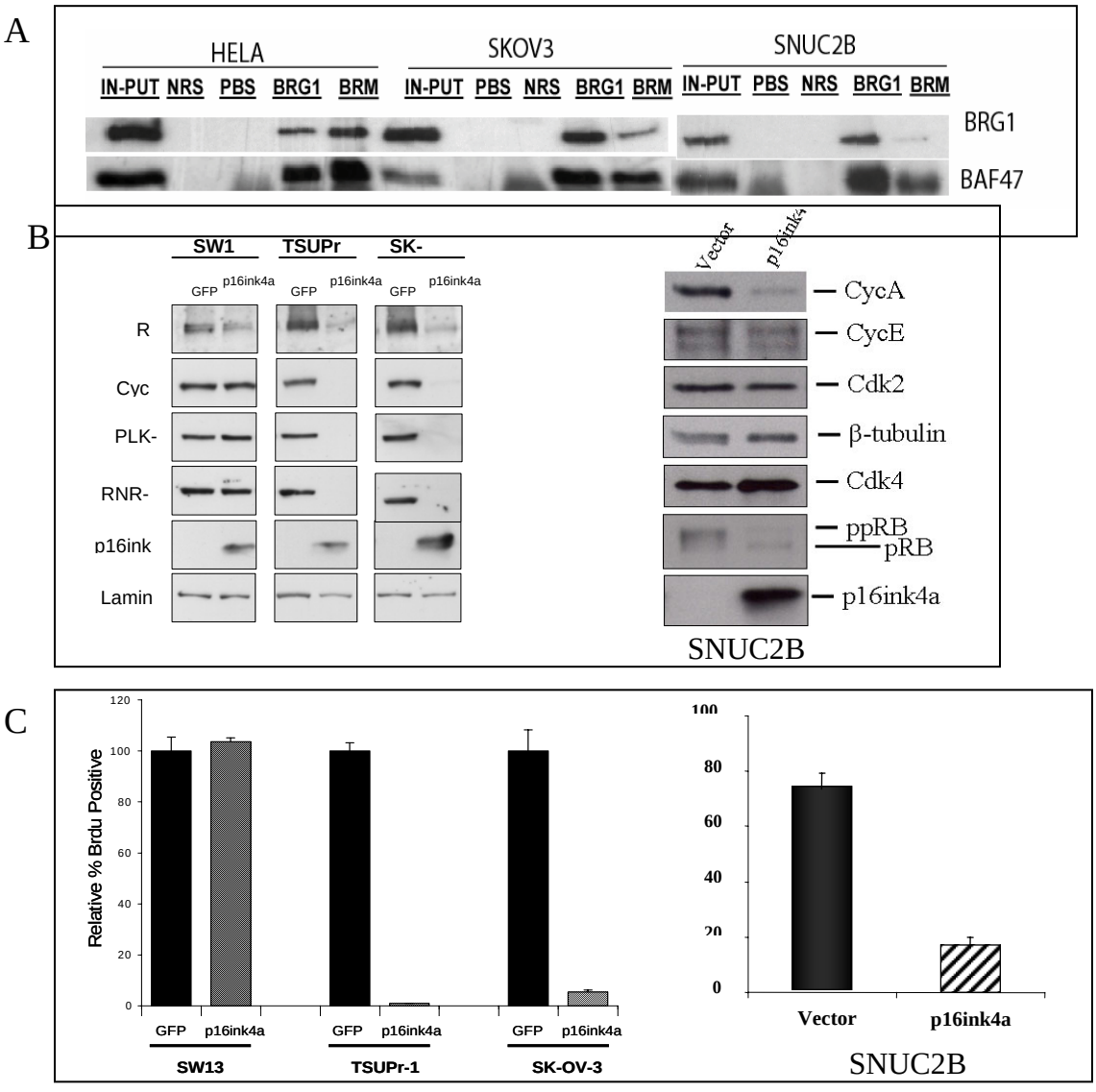

A
HELA
SKOV3
SNUC2B
IN-PUT NRS PBS BRG1 BRM
IN-PUT PBS NRS BRG1 BRM
IN-PUT PBS NRS BRG1 BRM
BRG1
BAF47
B
SW1
TSUPr
SK-
GFP p16ink4a
R
Cyc
PLK-
RNR-
p16ink
Lamin
Vector
p16ink4
CycA
CycE
Cdk2
β-tubulin
Cdk4
ppRB
pRB
p16ink4a
SNUC2B
C
Relative % Brdu Positive
GFP p16ink4a
SW13
TSUPr-1
SK-OV-3
Vector
p16ink4a
SNUC2B
D
(Singlets)
G0/G1
S
G2/M
SK-OV-3 -GFP
SK-OV-3 –p16ink4a

FIGURE 3-5

cells lacking BAF155 and maintain Rb-mediated cell cycle arrest. This result implies Rb mediated cell cycle arrest does not depend on the presence of full length BAF155.

D. Discussion

The previously conceived notion of cancer etiology being nature OR nurture has been replaced with the knowledge that carcinogenesis often includes both genetic and environmental factors. This paradigm becomes more obvious as the field of epigenetics comes into focus to reveal its importance in cancer development and progression. The chromatin remodeling arm of epigenetic modification is carried out by many diverse complexes, including the SWI/SNF complex. In yeast, a fully-functional SWI/SNF complex includes all complex members (Laurent, 1991; Peterson and Herskowitz, 1992) and the loss of any component causes subtle changes in complex activities. In mammalian cells, the SWI/SNF complex associates with and possibly regulates cell-cycle control genes including pRb (Dunaief, 1994), other RB family members (Fryer and Archer, 1998; Singh., 1995; Strober., 1996; Trouche, 1997), BRCA1 (Bochar, 2000), p21 (Chai, 2006) and Cyclins E and A (Zhang, 2000). A loss of any SWI/SNF member could contribute to the tumorigenicity of a cell given these connections to Rb function as well as other cell cycle regulators. This is indeed true for SWI/SNF complex member SNF5/INI1, as loss of SNF5 activity appears to abrogate the Rb signaling pathway through a block in $p16^{INK4A}$ induction or increased expression of cyclin D1.

In this report, we have revealed a role for another core SWI/SNF member, BAF155, in cell cycle checkpoint control and likely tumor suppression, via its leucine-zipper domain. We show expression of exongenous BAF155 in carcinoma cells lacking BAF155

consistently produces replicative senescence. This effect was also seen with low levels of BAF155 expression in the stable clones two weeks post-transfection. This low level of exogenous BAF155 was sufficient to cause substantial inhibition of colony forming ability as well as BrdU incorporation, and leads to an accumulation of cells in G0/G1 as determined by flow cytometry. These data indicate a vital (direct or indirect) role for BAF155 in the control of carcinoma cells SKOV3 and SNUC2B. The leucine zipper or proline/glutamine rich domain of BAF155 would seem to control this effect, as transfection with the Δ2 mutant lacking these domains inactivated its ability to suppress growth in the two carcinoma cell lines.

BAF155 is the third core member of the SWI/SNF complex to demonstrate tumor suppressive capabilities related to cell cycle control. Table 3-1 summarizes the tumor suppressive capabilities of the core members of the SWI/SNF complex. All core members are required for the basic task of remodeling nucleosomes, but the necessity for their presence in specific tumor suppressive functions of the complex varies. Rb-mediated cell cycle arrest requires only the catalytic core subunits BRG1/BRM, the specific functions served by SNF5 and BAF155 are dispensable for this particular tumor suppressive activity of the SWI/SNF complex. However, reexpression of any one core member above does induce growth arrest via senescence, a key characteristic of most tumor suppressors. This implies SWI/SNF complex may participate in more than one pathway to halt cell cycle progression. Additional mutational or inactivation studies are required to determine the pathways SNF5 and BAF155 operate through to halt tumor cell progression.

Table 3-1: Summary of the tumor suppressor capabilities of SWI/SNF core members. Data compiled from this and previous studies as discussed in the text.

SWI/SNF core subunit	Required for Rb-mediated arrest?	Can cause growth arrest?	Induces senescence?	Involved in Apoptotic signaling?
BRG1/BRM	YES	YES	YES	NO
SNF5	NO	YES	YES	NO
BAF155	NO	YES	YES	NO

Table 3-1

The lack of protein expression in SNUC2B without a mutation in the primary sequence or apparent degradation by the proteasome can be reconciled a few ways. The region of chromosome 3 where BAF155 is found is home to a cluster of suspected tumor suppressor genes. It is of note that it only rarely (only 5 of >200 primary human cancers) that somatic mutations have been found in 3p21 genes (Burbee, 2001; Dammann, 2003) although these genes are inactivated in the many cancers (Table 3-2). Further, the non-expressed but non-mutated 3p21 genes are found inactivated by other epigenetic mechanisms, such as chromosome instability, aneuploidy, promoter methylation, haploinsufficiency, altered RNA splicing, or defects in transcriptional, translational, or post-translational processes. Some are inactivated by rather exotic epigenetic means. For example, the FUS1 gene has detectable mRNA but no primary sequence mutation detectable, nor any protein detectable either, by western blot, or IHC, or microtissue array; similar to BAF155 in SNUC2B. Also, like so many 3p21 genes, FUS1 is a likely tumor suppressor gene, shown to be lost in >50% of lung cancers cell lines tested. It was eventually discovered that wild type FUS1 could be found as an N-myristoylated protein. Significant loss of expression or a myristoylation defect of the FUS1 protein was found in deficient lung cancer cell lines (Uno, 2004).

A more extensive proteasome-degradation inhibitor may be necessary ensure that degradation is not the cause of BAF155 protein absence in SNUC2B. however there may be another reason.

Table 3-2: Summary of multiple primary tumors and cancer cell lines displaying genetic abnormality in the 3p21 area of chromosome 3.

Adapted from Ji et al, (2005)

Primary Tumor Site	% Showing Genetic Abnormality in 3p21	References
Lung	65-95	Lerman, 2000; Zabarovsky, 2002; Minna, 2002; Wistuba, 2001
Breast	30-87	Miller, 2003; Maitra, 2001; Yang, 2002
Head and Neck	30-69	Chakraborty, 2003; Glavac, 2003; Riazimand, 2002
Ovary	>50	Manderson, 2002; Simsir, 2001
Cervix/ Uterus	40-90	Acevedo, 2002; Dellas, 1999; Herzog, 2001; Senchenko, 2003; Tzai, 2003
Colon/ rectum	14-33	Goel, 2003; Yashiro, 2001
Pancreas	35-45	Hessman, 1999; Iacobuzio-Donahue, 2004
Esophagus	13-55	Hu, 2000; Kuroki, 2003; Mueller, 2000; Shimada, 2000; Wei, 2002
Renal cell	45-87	Velickovic, 2001; Chino, 1999
Bladder	25-60	Li, 1996; Reznikoff, 1996
Nasopharyngeal	79-90	Liu, 2003; Lo, 2002; Lo, 2000
Follicular and papillary thyroid	17-67	Rodreigues-Serpa, 2003
Liver	35-46	Finkelstein, 2003
Skin	>15	Hussein, 2002
Gall bladder	35-65	Wistuba, 2001, 2002

Table 3-2

Another possibility is a mutation in the large non-coding exon accounting for the last 1500 bp of the BAF155 mRNA. Mutations within this region could result in significant changes in the tertiary structure of the mRNA leading to inefficient protein translation. We are currently determining the sequence of this region to assess this prospect.

The importance of SKOV3's lost leucine-zipper domain in BAF155 may be its ability to bind with other SWI/SNF complex members. It is known that leucine zipper domains in general allow the proteins possessing them to dimerize, with themselves (homodimerize) or with other proteins possessing a leucine zipper domain (heterodimerize). This has been suggested for BAF155 and BAF170. Chen and Archer recently demonstrated another function for BAF155's leucine zipper domain. This domain was shown to be necessary for BAF155's interaction with BAF57, stabilizing BAF57 in the cell. In fact, similar functions have been shown for several of BAF155's domains. The SANT domain of BAF155 has been shown to be necessary for BRG1 binding and stabilization (Sohn, 2007) and BRM binding (Crosby, 1999). The SWIRM domain of BAF155 was required for BAF60a and SNF5 binding and stabilization (Sohn, 2007). It has been proposed BAF155 is the scaffolding protein for the SWI/SNF complex, linking BRG1, and the therefore the remodeling capabilities of the complex, to the other subunits which interact with transcription factors or other targeting molecules. SNF5 interacts with c-Myc to recruit SWI/SNF, GR with BAF60a, the HMG domain of BAF57 binds DNA, and BAF155 binds them all together with BRG1 to remodel chromatin. To further the theory, reduced amounts of BRG1, SNF5, and BAF57 coincide with reduction, loss, or mutation of BAF155 (Chen, 2005; Sohn, 2007, DeCristofaro, 2001). Our data would seem to refute this, as we show the presence of the other SWI/SNF members in cells with no detectable expression of BAF155 protein (Figure

3-1A). The core complex also appeared to form in these cells lacking BAF155 (Figure 3-5A). If BAF155 was absolutely required as a scaffold for BRG1 and SNF5, as their only link to one another and the rest of the complex, we would not have been able to pull them down together in cells lacking BAF155 protein.

The apparent discrepancy may be explained by the following. A truncated protein may not be detected in these cells due to the lack of the antibody binding site we used to detect BAF155 expression. We have tested a recently released monoclonal antibody against BAF155 with a recognition site more central in the protein and still detect no protein in either cell line (data not shown) reference antibody. This furthers the suspicion that no BAF155 protein exists in these cell lines, although still not solving the conundrum of the complex forming in cells without a scaffold protein, if this is function of BAF155. Another explanation may be that the job of scaffold protein is not solely that of BAF155. There is significant sequence and domain homology among BAF155 and BAF170. BAF170 may take over as scaffold in these cells, allowing the complex to form, although other more specific functions attributed to BAF155 are lost leading to the cancerous phenotype of these cells that we see reversed upon exongenous BAF155 expression (Figure 3-3C, D). It is known that particular SWI/SNF members are vital to development (BRG1, SNF5, and BAF155) and others are dispensable (BRM) as demonstrated by the embryonic lethality of mice null for those subunits (Bultman,2000; Roberts, 2000; Kim, 2001). Many of the studies carried out to support the scaffold theory were done in immature cells, MEFs, developing thymocytes, or actively growing, transformed, immortalized cancer cell lines (Sohn, 2007, Chen, 2005). It is possible BAF155 serves the purpose of scaffolding during development but may share this function with BAF170 upon cellular differentiation. Further

characterization of BAF155 and BAF170 expression in developing or differentiating cells (such as the MEFs or developing thymocytes previously tested) would address these issues.

In conclusion, we submit an additional SWI/SNF core subunit, BAF155/SMARCC1 as a likely tumor suppressor gene due to its loss in two carcinoma cancer cell lines including a hemizygous mutation, its genomic location in the tumor suppressor cluster region of chromosome 3p21 and because of its ability to cause a G0/G1 growth arrest in deficient cancer cell lines upon reexpression. As mentioned earlier, BAF155 may be targeted as part of a tumor suppressor gene cluster on 3p21 to be epigenetically silenced, thereby affecting all SWI/SNF regulated genes, which are often cell cycle related. The next step in the determination of potential effectors of BAF155 loss in cancer cells is exploration of epigenetic silencing mechanisms such as promoter hypermethylation, aberrant acetylation patterns, and myristoylation in normal developing cell lines such as MEFs or developing thymocytes known to have highly regulated BAF155, as well as in differentiated cells, cancer cells, and ‘normal’ cells such as normal human fibroblasts.

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CHAPTER 4

Inactivation of Tumor Suppressor of SNF5 Increases Loss of p53 and Rhabdoid Tumor Formation in *Snf5*$^{+/-}$;*p53*$^{+/-}$ Mice

A. Abstract

Malignant rhabdoid tumors (MRTs), are highly undifferentiated pediatric cancers of unknown origin that arise in various anatomical locations with a very poor outcome. The vast majority of these malignancies are caused by loss of the SNF5/INI1 component of the SWI/SNF complex. However, the mechanism of tumor suppression associated with SNF5 loss remains unclear. SNF5's loss demonstrated effects on the regulation of the activity of, cyclin D1, $p16^{INK4A}$ and pRb suggests a likely function through the SWI/SNF complex to affect transcription of several factors involved in cell cycle control. However, previous studies in genetically engineered mouse (GEM) models have shown that loss of SNF5 on a p53 null background leads to 100% penetrance and significantly shorter latency periods of tumor development. Here, we use established GEM models to further define the relationship between the SNF5 and p53 tumor suppressor pathways. Combined haploinsufficiency in these mice also leads to decreased latency as well as increased penetrance of MRT formation, although MRTs do arise in alternate anatomical locations on the p53 heterozygous background. Our studies suggest that loss of SNF5 activity changes the differentiation status of the p53 spectrum of tumors to those of MRTs. They also raise the question whether SNF5 loss accelerates p53 tumorigenesis as opposed to the opposite paradigm.

B. Introduction

Although only representing a small percentage of pediatric neoplasms rhabdoid tumors are particularly devastating, killing the majority of affected children before their second birthday. These very aggressive tumors have been identified as rhabdoid or malignant rhabdoid tumors (MRTs) due to the morphological resemblance to rhabdomyoblasts (Haas, 1981). Historically considered a tumor of the kidney, extrarenal rhabdoid tumors are now recognized as typical MRTs as well, in as diverse locations as the liver, chest wall, and CNS (Gonzalez-Crussi, 1982; Lunch, 1983; Tsuneyoshi, 1985).

MRTs have always presented a diagnostic challenge given the largely undifferentiated although heterogeneous appearance, polyphenotypic immunoprofiles, and similarities to other tumor types. The designation of rhabdoid historically relied on the histological appearance of large epithelioid cells with eccentric eosinophilic cytoplasm, vesicular nuclei, prominent nucleoli, and whorled bundles of intermediate filaments. These characteristic rhabdoid cells, were not the typical cell in a malignant rhabdoid tumor, even rare in some tumors. Therefore, false diagnosis was frequent, leading to unsuccessful treatments. The discovery of deletions and mutations at 22q11.2 involving *SNF5/INI1* has since facilitated diagnosis and treatment choices (Versteege, 1998; Biegel, 1999; Sevenet 1999a&b; Rousseau-Merck, 1999). SNF5 function is now recognized as being lost in almost 100% of MRTs (Biegel, 2002). The genetic mutation in MRTs is usually specific to SNF5, not chromosome- or genome-wide losses or instability, implying it is the specific loss of SNF5 that allows these tumors to form (Biegel, 1999).

An array of mouse studies have followed the human analyses verifying SNF5's specific role in tumor suppression. *SNF5* nullizygotes are embryonic lethal, dying at peri-implantation stage, and conditional *SNF5* null mutants lead to death within 3 weeks of SNF5 loss due to hematopoietic failure. A reversible conditional mutant that causes only partial penetrance of SNF5 loss was able to avoid the bone marrow failure and death experienced with the fully penetrant conditional mutation. However, it showed a fully penetrant phenotype, developing CD8+ T cell lymphomas or MRTs by 11 weeks (Roberts, 2002). The 100% penetrance observed by 11 weeks of SNF5 loss brings SNF5 to the top of the list of deadly genetic mutations in cancer, as studied in mice. Loss of either tumor suppressor gene p53, or $p16^{INK4A}$ leads to tumor development at median ages of 20 weeks and 38 weeks, respectively (Sharpless, 2001; Donehower, 1992).

Attention has turned to how SNF5 suppresses tumor formation, including its effects on known oncogenic signaling pathways. Two groups showed that cyclin D1 absence allows SNF5+/- mice to survive tumor free, that primary mouse and human MRTs express cyclin D1 positive cells and that in some cases, several primary human rhabdoid tumors, but tumors lacking SNF5 inactivation, overexpressed cyclin D1 (Fujisawa, 2005; Tskitis, 2005). These findings, as well as that of SNF5 protein's interaction with c-Myc, Cyclin D1, and p21, suggest SNF5 tumor suppression abilities may function through manipulation of cell cycle pathways. Guidi et al showed in 2006 that loss of the Rb pathway is epistatic to loss of SNF5 in tumor formation. More recently, a similar cross of mice haploinsufficient for *SNF5* as well as losing the Rb family via cross with TgT_{121} mice (a truncated SV40 large T antigen that inactivates pRb and family members 107, and p130) have revealed a cooperation between SNF5 and the Rb family in tumor suppression as loss of both significantly

accelerates MRT formation (Chai, 2007). Although every step involved in SNF5's path to tumor suppression is not known, it seems fairly certain the Rb pathway is manipulated.

Aberrant proliferation can lead to Rb inactivation and subsequent apoptosis. Several studies have demonstrated the p53 dependence of this process (Debbas, 1993; Lowe, 1994; Mecleod, 1996; Morgenbesser, 1994; Howes, 1994; Sabbatini, 1995; Wagner, 1994). It seems apparent there are selective pressures to lose p53 for tumor survival and proliferation, demonstrated by Symonds et al 1994 where epithelial cell tumorigenesis occurs in response to pRb pathway inactivation, where normally Rb inactivation of the choroid plexus epithelium would result in p53-dependent apoptosis. In fact, it is well documented that most human tumors harbor inactivation of Rb and p53 (Weinberg, 1995). When considering the above evidence it becomes increasingly likely that loss of both p53 and Rb occur in many human neoplasms. It was this suspicion that has lead Yaniv and colleagues to investigate the interaction of *Snf5* and *p53* tumor suppressors in rhabdoid tumorigenesis. They demonstrated p53 nullizygosity indeed accelerated rhabdoid tumor formation in SNF5 heterozygous mice – with complete penetrance within 19 weeks (from 28 weeks on wild type p53 background). Neither the tumor spectrum, nor anatomical locations of the resultant rhabdoid tumors were altered (Klochendler-Yeivin, 2006). This implies a true cooperation, not simply a combined effect, as all tumors were MRTs (SNF5-type tumors) in the same locations, with a much reduced latency and 100% penetrance, opposed to the no more than 30% penetrance in $Snf5^{+/-}$ mice on a wild type *p53* background (Roberts, 2000).

Although the above mentioned study, like the conditional *Snf5* null mouse studies, are less likely to represent a true physiologic state, they do further the observation that combined *p53* and *Snf5* inactivation allows the propagation of increased numbers of MRTs. Therefore,

to address this question in a more physiologically relevant genetically engineered mouse (GEM) model, we characterized tumor development in *p53*$^{+/-}$, *Snf5*$^{+/-}$ and *p53*$^{+/-}$,*Snf5*$^{+/-}$ mice. Our results reveal a cooperation between these two important tumor suppressor pathways may exist. The cooperative effects of SNF5 and the p53 tumor suppressor pathways may extend beyond increasing MRT formation in general, with the p53 halploinsufficient background allowing MRT formation in alternate locations. Alternatively, SNF5 loss may alter the differentiation status of existing p53-deficient tumors changing them into MRTs.

C. Results

1. SNF5 heterozygosity leads to increased penetrance and decreased latency of tumor formation on p53+/- background.

Previous studies have shown combined nullizygosity of SNF5 and p53 leads to increased tumor formation, but this occurs at a rate no longer compatible with life, with 100% penetrance within 3 weeks (Roberts, 2005). We crossed *Snf5*$^{+/-}$ females with *p53*$^{+/-}$ or *p53*$^{+/-}$ males to yield the *Snf5*$^{+/-}$;*p53*$^{+/-}$ double heterozygous mice, as well as control genotypes: *Snf5*$^{+/-}$;*p53*$^{+/+}$, *Snf5*$^{+/+}$;*p53*$^{+/+}$, and *Snf5*$^{+/+}$,*p53*$^{+/-}$ (see Materials and Methods for background strain and origin of mice). The overall survival of these genotypes can be seen in Figure 4-1. In the *p53* heterozygote mice, we observe the expected >50% penetrance and 14.5 month tumor onset similar to that observed in previous *p53*$^{+/-}$ mouse studies (Donehower, 1992; Jacks,1994) . Approximately 40% of *SNF5*$^{+/-}$ mice form rhabdoid tumors at a median age of 15 months. While this penetrance is slightly higher than that reported in the original *Snf5* mouse studies published by Roberts in 2000, additional malignancies and pathologies were acknowledged after publication (personal

communication) that would rise to the level of penetrance we see here. The mice heterozygous for both *p53* and *Snf5* however, have a median age of tumor formation of only 9.5 months, and a total penetrance of over 85%. This increase in tumor penetrance in the double heterozygous mice appears to be more than an additive effect. Figure 4-1B displays only rhabdoid tumor formation in $Snf5^{+/-}$ $p53^{+/+}$ mice and $Snf5^{+/-}$ $p53^{+/-}$ mice, which reveals a decrease in latency of rhabdoid tumors in the double heterozygotes. The reduction in latency of the double heterozygote rhabdoid tumors is largely responsible for the overall reduction in latency of rhabdoid tumors in all mouse groups.

2. $Snf5^{+/-};p53^{+/-}$ mice develop MRTs, lymphomas, and osteosarcomas

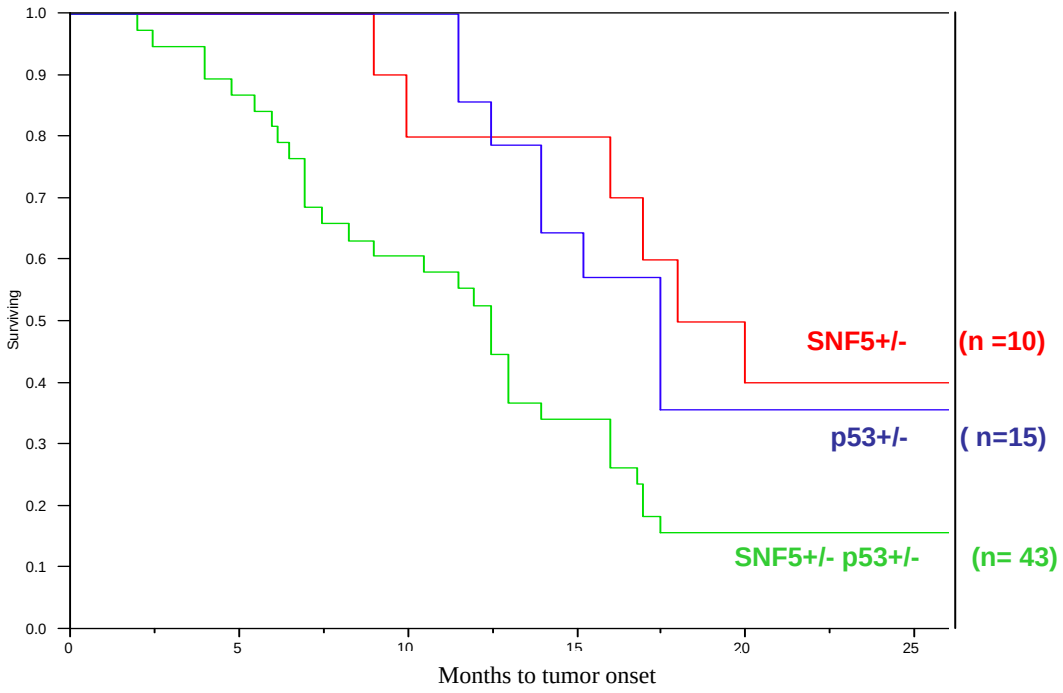
As shown by Donehower in 1992 and Harvey in 1993, *p53* heterozygous mice are predisposed to lymphomas (~30%), osteosarcomas(~60%), and some soft-tissue sarcomas and carcinomas as well, at a rate of 50% by 18 months. Just under half of $Snf5^{+/-}$ mice, form malignant rhabdoid tumors of the soft tissue of the head and neck, as well as CNS (Roberts, 2000 and personal communication). *Snf5* null conditional knockout mice formed lymphomas exclusively within 3 weeks (Roberts, 2002). We observed an >80% penetrance of rhabdoids, osteosarcomas, and lymphomas in the $Snf5^{+/-};p53^{+/-}$ mice. It is of note, that the penetrance of facial and CNS MRTs usually observed in $Snf5^{+/-}$ on a wild type *p53* background was not increased in these $Snf5^{+/-}$ mice on a $p53^{+/-}$ background. A small percentage of the total rhabdoid tumors from the double heterozygous group were histologically rhabdoid but presented at alternate locations to the expected soft tissues of the face. No tumors completely novel to either genotype were observed.

Figure 4-1: Survival of eouble heterozygous mice

(A) Overall survival of *Snf5*$^{+/-}$, *p53*$^{+/-}$, *and Snf5*$^{+/-}$*; p53*$^{+/-}$ mice in the study

(B) Rhabdoid incidence only in double heterozygote mice and *Snf5*$^{+/-}$ mice. Mice were sacrificed when an obvious tumor or paralysis was noticed. Healthy mice at 25 months were sacrificed and recorded as non-events. N values are noted on the figure. Wild type littermates experienced 100% survival at 25 months are not included on this survival curve.

A


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Surviving
0
5
10
15
20
25
Months to tumor onset
SNF5+/-
(n =10)
p53+/-
(n=15)
SNF5+/- p53+/-
(n= 43)

B

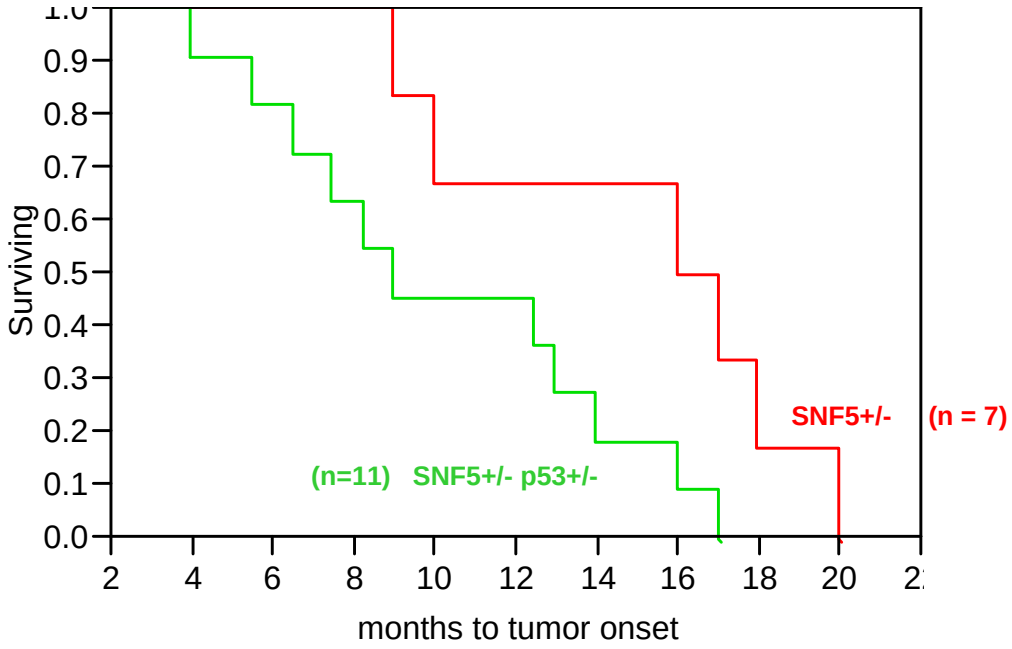

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Surviving
2
4
6
8
10
12
14
16
18
20
months to tumor onset
SNF5+/-
(n = 7)
(n=11)
SNF5+/- p53+/-

Figure 4-1

3. LOH of SNF5 and p53 occurs in osteosarcomas and variably in lymphomas of double heterozygous mice, but without an increase in penetrance.

As shown in Roberts et al 2005, *Snf5* nullizygosity can lead to early lymphomas. Lymphomas are common as well in *p53* $^{+/-}$ mice, displaying up to 50% *p53* LOH in those tumors. It would not have been surprising to observe an increase in lymphomas here. This is not what we observed. While the overall survival and tumor formation was affected, it was not due to increased lymphoma or osteosarcoma formation. The previously observed LOH rates are maintained here as well, with the majority lymphomas and osteosarcomas losing *p53* heterozygosity. Interestingly, no osteosarcomas formed in the double heterozygote mice that displayed p53 expression by IHC (sample staining in Figure 4-2C) and this was the only tumor type that 100% of the type lost expression of both *Snf5* (by PCR) and *p53* (by IHC). The age of onset, of these osteosarcomas was not extreme nor out of the normal latency range (table 4-1).

4. Immunoprofile of mouse rhabdoid tumors recapitulates that of human rhabdoid tumors.

One question about the validity of GEM models for human cancers is how accurately they embody the features of their representative human malignancy. The haploinsufficient *Snf5* mouse has been well characterized as producing MRTs with histologies similar to those observed in humans. We also examined a limited number of immunohistochemical markers to determine whether the MRTs in the *Snf5*$^{+/-}$ mice showed a similar pattern to their human counterparts. We also looked at the immunoprofile in a representative set of MRTs from the *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice to assess whether loss of p53

Table 4-1: Summary of *Snf5* and *p53* loss of heterozygosity in the tumors observed in the double heterozygotes as well as the two control genotypes.

Tumors were removed at necropsy and flash-frozen at necropsy for subsequent DNA extraction. LOH was determined by PCR. Not all tumors were able to be tested, percentages given are of the tumors able to be genotyped by PCR.

Mouse Genotype & tumor type		p53 LOH	Snf5 LOH
Snf5+/- p53+/+	Rhabdoids	Not tested	3 of 4 (75%)
Snf5+/+ p53+/-	Lymphomas + Osteosarcomas	4 of 4 (100%)	not tested
Snf5+/- p53+/-	Rhabdoids	1 of 5 (20%)	7 of 8 (88%)
Snf5+/- p53+/-	Non-Rhabdoids	2 of 3 (67%)	3 of 4 (75%)

Table 4-1

Figure 4-2: LOH determination of rhabdoid tumors formed in double heterozygote mice

(A) Representative loss of heterozygosity determination by PCR. Tumor samples were flash-frozen immediately at necropsy for subsequent DNA extraction and LOH determination.

(B) Representative image of SNF5 western blot used to determine SNF5 and p53 LOH. Protein was extracted from tumor tissue flash-frozen at necropsy. 30ug of protein was loaded into each well.

(C) Representative average signal strengths assigned to tumors immunohistochemically stained for p53. These average signal strengths were multiplied by the percentage of positive tumor cells in the core for a single number score for each core scored. These numbers were averaged and are summarized on table 4-2

A

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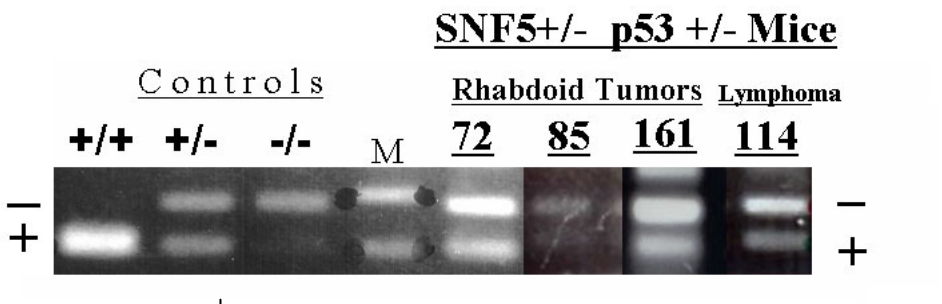
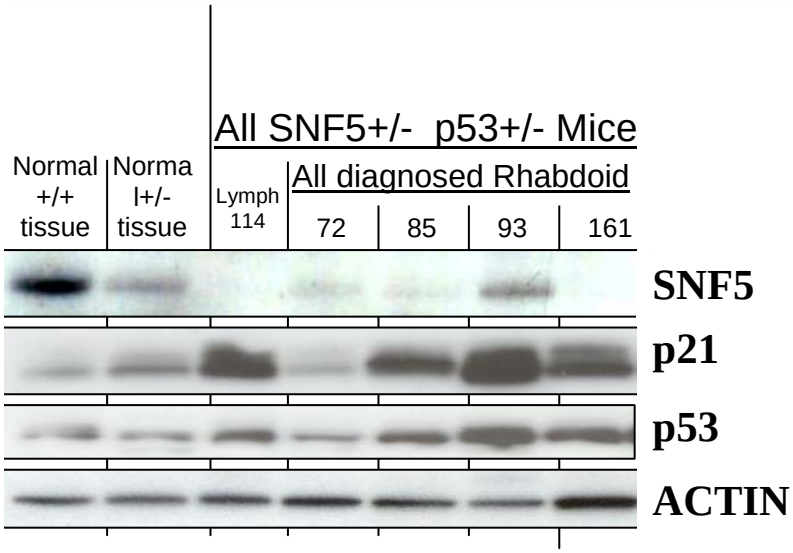


B



C

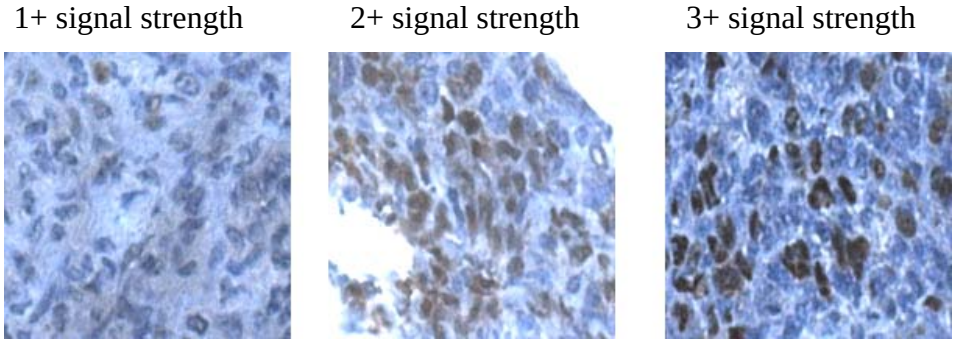


FIGURE 4-2

function affected the phenotype of these tumors. Finally, we determined the origin of the lymphomas in the *p53*$^{+/-}$ and *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice to ascertain if the loss of both tumor suppressor genes affected the origin of these hematopoietic tumors.

Approximately 90% of the MRTs, from either genotype, were positive for S100ß expression, similar to our previous report (Chai, 2007) (Table 4-2, figure 4-3). We also observed expression of vimentin, an intermediate filament found in cells of mesenchymal origin, in all MRTs (Table 4-2, Figure 4-3). The staining of lymphomas from the *Snf5*$^{+/-}$ mice as well as the double heterozygotes are suspected to be mature T-cell lymphomas, as they did not express the B-cell marker B220. Staining for the T-cell marker CD3 will verify these are mature T-cell lymphomas. While the majority of the lymphomas in the *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice are likely of T-cell origin, we found one B-cell lymphoma.

D. Discussion

A few decades have passed since the formal recognition and diagnosis of MRT came to exist (Beckwith, 1978; Haas, 1981; Rorke, 1996). While we have determined the underlying genetic causal relationship of *Snf5* loss with MRT formation, it has yet to change the prognosis of infants diagnosed with this devastating aggressive neoplasm.

Given the difficulty in studying the molecular mechanisms of human cancers, the genetically engineered mouse model has been invaluable for providing needed insights into this important problem. However, it has been difficult to find a GEM for MRT because *Snf5* loss

Table 4-2: Results of immunohistochemistry scoring of p53, S100β, Vimentin, and B220 (for lymphomas). Results are grouped according to genotype of the mouse

(A) $p53^{+/-}$ $Snf5^{+/+}$ mice

(B) $Snf5^{+/-}$ $p53^{+/+}$ mice

(C) wild type controls

(D) $Snf5^{+/-}$ $p53^{+/-}$ mice

Each core, once stained, was scored for average signal strength of positive tumor cells and for percentage of tumor cells demonstrating some positivity. These two values were multiplied for each score and averaged for the total number of scores. There were three cores of most samples on each array and two array slides stained, so the majority are an average of 6 scores. *Ncp = no core present to score. n/a = core was not scored due to lack of a core present on the array or the stain was not relevant to that tumor (B220 only relevant for lymphomas)*

A

Mouse #	Age in months at tumor onset	Histologic diagnosis	average p53	average S100β	average Vimentin	average B220
130	11.5	Lymphoma	1	120	144	0
113	12.5	Lymphoma	0	2	73	19
103	14	Lymphoma	4	3	100	80
144	14	Lymphoma	95	11	13	1
133	11.5	Osteosarcoma	0	125	180	N/A
B19	15.25	Osteosarcoma	0	52	153	N/A
1401	17.5	Osteosarcoma	0	63	188	N/A

B

mouse #	age at tumor onset (mo)	Histologic diagnosis	average p53	average S100β	average Vimentin	average B220
B47	9	Rhabdoid	0	2	100	N/A
B73	10	Rhabdoid	53	92	65	N/A
B29	16	Rhabdoid	30	127	93	N/A
B56	17	Rhabdoid	1	5	15	N/A
B27	20	Rhabdoid	50	122	180	N/A

C

mouse #	age at tumor onset (mo)	Histologic diagnosis	average p53	average S100β	average Vimentin	average B220
wt 1		normal thymus	n/a	n/a	115	n/a
wt 1		normal spleen	n/a	7	77	40

Table 4-2 (continued on next page)

D

mouse #	age at tumor onset (mo)	Histologic diagnosis	average p53	average S100β	average Vimentin	average B220
114	2	Lymphoma	90	ncp	ncp	ncp
1407	6.2	Lymphoma	0	2	95	62
106	13	Lymphoma	0	133	50	2
146	16	Lymphoma	2	10	163	N/A
111	12.5	Osteosarcoma	0	63	112	N/A
146	16	Osteosarcoma	0	10	148	N/A
72	4	Rhabdoid	0	110	193	N/A
141	5.5	Rhabdoid	0	10	180	N/A
1507	6.5	Rhabdoid	0	30	160	N/A
151	7.5	Rhabdoid	0	100	140	N/A
86	8.3	Rhabdoid	3	73	118	N/A
161	9	Rhabdoid	0	40	192	N/A
B34	12.5	Rhabdoid	20	80	70	N/A
85	13	Rhabdoid	0	78	121	N/A
93	13	Rhabdoid	0	78	112	N/A
81	14	Rhabdoid	60	80	149	N/A
143	16	Rhabdoid	1	38	186	N/A
1400	17	Rhabdoid	13	116	187	N/A
B42	17	Rhabdoid	0	145	138	N/A

Table 4-2

Figure 4-3: Representative immunohistochemical staining of mouse tumor tissue microarray assigned scores of low (1+) and high (2+ or 3+) average signal strengths Results are summarized on Table 4-2

B220 (A, B)

S100β (C, D)

Vimentin (E, F)

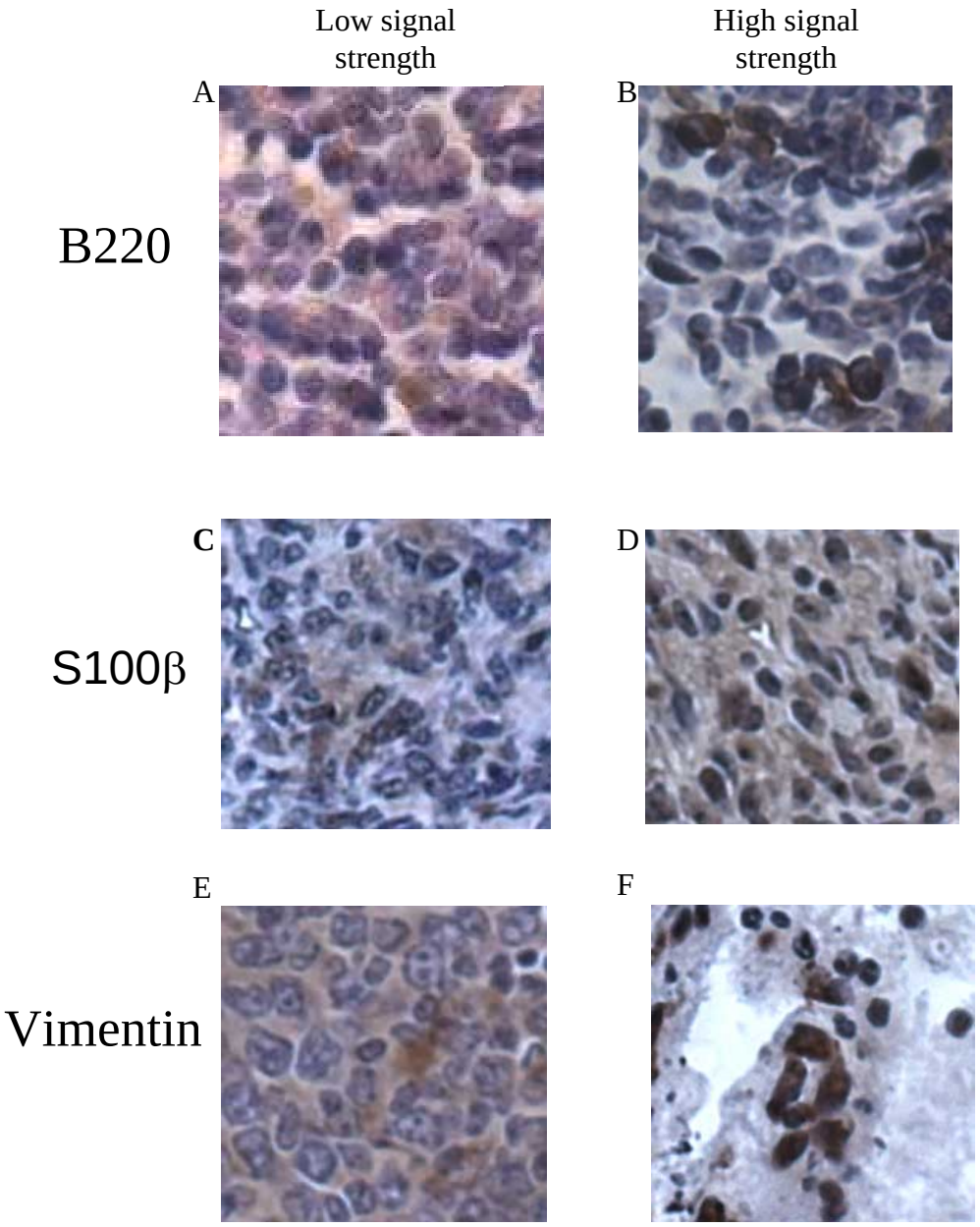

Low signal strength
High signal strength
A
B
B220
C
D
S100β
E
F
Vimentin

FIGURE 4-3

results in embryonic lethality and *Snf5* heterozygosity leads to histologically indistinguishable MRT but at a low penetrance with an extended latency. Complicating matters for conditional deletion studies, the cell of origin for MRTs remains unknown.

Despite these problems, we have gained invaluable information from GEM involving *Snf5* deletion. Multiple mouse studies have revealed cooperation between SNF5 and other tumor suppressors in the prevention of MRTs. Guidi et al, (2006) has demonstrated Snf5 loss may be epistatic to Rb loss, but a more recent study demonstrates loss of the entire Rb family accelerates MRT development (Chai, 2007). This would imply that SNF5 tumor suppression may involve the Rb pathway but clues to SNF5 mechanism of tumor suppression does not end with Rb. These data seem to fit with the etiology of human disease where MRTs do not appear to lose Rb or $p16^{INK4A}$ function.

Loss of SNF5 function on a *p53* null background in GEM led to 100% tumor penetrance within 19 weeks (Klochendler-Yeivin, 2006). This group also showed an accumulation of p53 in SNF5 deficient cells in culture displaying growth defects followed by apoptosis. In contrast, the SNF5 deficient cells on the p53-null background showed a reduced level of apoptosis. Therefore, one could attribute the increased tumor incidence in the $Snf5^{+/-}$;$p53^{+/-}$ mice to a reduced rate of apoptosis associated with the *p53* null background.

In the current mouse model, cells could lose either one or both of these tumor suppressor pathways in order to progress to tumorigenicity. In the case of the facial MRTs that presumably correspond to those found in the $Snf5^{+/-}$ mice, we did not observe an increase in penetrance. However, an apparent decrease in latency for the appearance of these tumors compared to the *Snf5* heterozygous mice on a *p53* wild type background (Figure 4-1). A low frequency of LOH for p53 in these tumors suggesting that p53 haploinsufficiency alone

could accelerate SNF5 tumorigenesis in these mice. We did observe an increase in the penetrance of the spinal cord tumors in the *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice reminiscent of what we found in the *TgT121*; *Snf5*$^{+/-}$ mice (Chai 2007). However, sufficient material was not obtained to carry out LOH analyses on these tumors. Therefore, we do not know if increase in the frequency of these MRTs was due to *p53* LOH or for other reasons. It is of note, that not only was *p53* loss of expression observed by IHC in the osteosarcomas formed in the double heterozygotes, but *Snf5* LOH was selected for as well, but without an observed change in latency, penetrance, or histologic appearance. This may mean that loss of SNF5 was incidental and perhaps late in the progression of the tumor so the Snf5 characteristic rhabdoid cell phenotype did not manifest.

In contrast, we found a high frequency of LOH for both *Snf5* and *p53* in the non-MRTs from the *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice (Table 4-1). The frequency of *p53* LOH was remarkably similar to that observed in the *p53*$^{+/-}$ mice alone. Again, a decrease in latency was observed for the development of these tumors when compared to the *p53* heterozygous mice. The fact that *p53* LOH occurred in both mouse models implies that the loss of remaining wild-type *Snf5* allele led to the acceleration of tumor formation in these mice.

Several possibilities may explain the observed increase in tumor formation with the combined haploinsufficiency of *p53* and *Snf5*. First, Vries et al demonstrated in their 2005 study that 10% of MRT cell lines are near tetraploid and over half of the MRT cell lines bear chromosomal aberrations. They went on to show these aberrations can be corrected in the cell population by addition of SNF5 which leads the genetically unstable cells to senesce. Therefore, SNF5 haploinsufficient mice may experience substantial genomic instability leading to secondary genetic events such as loss of p53 activity. This model could account

for the acceleration of development of non-MRTs in *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice. However, molecular and cytogenetic studies of human MRTs suggest that genomic instability occurs infrequently, with chromosomal aberrations localized mainly to region surrounding the *SNF5* gene (Biegel, 1999). Thus, this may not provide the most likely mechanism.

A second explanation for the increase in tumor formation in the double heterozygous mice is that p53 null cells allow genetic instablity, increasing the frequency of *Snf5* LOH. This scenario also appears unlikely since previous studies in GEM models suggest that loss of p53 function does not lead directly to global chromosomal instability (Lu, 2001). Furthermore, loss of p53 is not been found in the primary MRTs of children presenting with this disease (Dr. Jaclyn Biegel, personal communication). In fact, these tumors respond well to chemotherapy and radiation and are frequently ablated. Unfortunately, MRTs can return months to years later in a chemotherapy-resistant form in which the p53 status has not been established.

We favor a third explanation for observations in human MRT development as well as the increase in MRTs in the double heterozygous mice. *Snf5* LOH is likely occurring in the *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice at an equivalent rate and locations as it does in the *p53*$^{+/+}$ mice. However, on a *p53* wild type background, these *Snf5* null cells are arrested or undergo apoptosis via induction of p21 and subsequent halting cell cycle progression via Rb. On the *p53*$^{+/-}$ background, most cells possess enough functional *p53* still exists to eliminate themselves if they lose SNF5 function. However, a few cells are able to escape apoptosis due to low p53 levels causing a slight increase in tumor formation. Since tumors developing from *Snf5* loss have a shorter latency than *p53* (as determined in previous mouse studies), you would expect to see more of an increase in SNF5-type tumors (MRTs) and little or no increase in tumor

types associated with p53 LOH (lymphomas and osteosarcomas). This is precisely what we observed in this study. This has also been posed by Yaniv and colleagues, that loss of *p53* could be accelerating the LOH of *Snf5* in certain populations and then may be allowing or even enhancing the survival of these $Snf5^{-/-}$ cells to form MRTs (Klonchendler-Yeivin, 2006).

In this study the combined loss of *Snf5* and *p53* revealed a cooperation between the two tumor suppressors yielding reduced latency and increased penetrance of rhabdoid tumors. The increase in rhabdoids observed were not simply an increase in expected MRTs, but there were a subset of $p53^{-/-}$ tumors with a histologic rhabdoid appearance. We believe it is the loss of *Snf5* that made the neoplasm histologically rhabdoid (verified by the distinctive rhabdoid features seen by H&E and the IHC profile matching that of human MRTs (Figure 4-3) even though progression of the tumor may have been afforded by p53 inactivation. It seems that given the *Snf5* haploinsufficient status of the mice, haploinsufficiency of p53 was sufficient to allow a larger number of rhabdoid tumors to progress to frank, solid tumors.

Given the extensive loss of p53 in human tumors and the chemotherapy resistance of secondary rhabdoid tumors in humans, this study may lead to a deeper understanding of the mechanism of SNF5 tumor suppression and MRT behavior, specifically CNS MRTs, now considered typical MRTs, may be due to *p53* inactivation as well, noting the role of *p53* in survival and death checkpoints in the central nervous system neurons (Aloyz, 1998; Xiang, 1996). Further insight into the relationship of essential tumor suppressors like SNF5 will inevitably allow for more successful treatment avenues for devastating childhood neoplasms like MRT.

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CHAPTER 5

SUMMARY AND PERSPECTIVES

A. Summary

The status of the SWI/SNF chromatin remodeling complex's role in effecting the transcription of only 5% of the genome may underestimate the overall effects of its activity. It is likely to affect far more than 5% of the genome considering the profiles of the genes that fall into that 5%, their associations, and their effects on cell cycle. Solid links have been established between SWI/SNF subunits, cell cycle control, and often tumorigenesis, via specific complex members. SNF5 is one such member, a bone fide tumor suppressor gene, it is also vital to development. The mechanism in which SNF5 operates to affect cell cycle and tumorigenesis, and any associations necessary for this pathway have not been fully defined. This is also true of core member BAF155, which is minimally characterized at this point. Although known to be essential to development like other SWI/SNF core members, BAF155's role in tumor suppression is largely unknown. Given it's role as a necessary core member in a complex whose role involves the transcriptional regulation of genes important to cell cycle control, it is also likely BAF155 has tumor suppressive effects.

In addressing these questions, I sought to further characterize the tumor suppressive capabilities of two of SWI/SNF's core members, SNF5 and BAF155, in two aims. The first aim tests BAF155's potential tumor suppressive capabilities with specific standard tests of growth suppressive effects in cell culture. Specifically, exploring any growth control effects

exerted upon expression of BAF155 in tumor cell lines lacking endogenous expression. The second aim sought to further define the mechanism of SNF5's tumor suppression. Specifically, I sought to determine any cooperativity that may exist between the SNF5 and the established cell cycle control pathway, p53. The results of these studies lead me to conclude the following:

- Loss of BAF155 occurs in carcinoma cell lines. Complete loss of protein expression was observed in two carcinoma cell lines, SKOV3 (ovarian carcinoma) and SNUC2B, colorectal carcinoma). This loss was limited to the expression of protein because northern blots revealed full length BAF155 message in both cell lines. Loss may be due, at least in SKOV3, to a truncating mutation and an 855AA protein lacking its leucine-zipper domain.
- Loss of BAF155 is not due to protein degradation. Loss of this subunit is not due to aberrant degradation by the ubiquitin-proteasome pathway because when this pathway was interrupted, no accumulation of BAF155 was detected in these cells.
- BAF155 leads to reduction in growth due to induced senescence upon re-expression in cells lacking endogenous BAF155 expression. The reduction in colony forming ability observed upon re-expression of full length BAF155 did not occur upon transfection with a truncation mutant of BAF155, suggesting the need for full length BAF155 for this effect.
- BAF155's growth suppressive capabilities are dependent on its leucine-zipper and /or proline-glutamine rich domains. The truncation mutant that was unable to induce a growth arrest lacked the c-terminal leucine zipper and proline-glutamine rich domains.
- BAF155 is not required for RB-mediated cell cycle arrest. Upon expression of $p16^{INK4A}$, to induce a Rb-mediated cell cycle arrest, the BAF155 null cells arrested in G0.

BrdU assays showed a lack of cells entering S phase, and flow cytometry verified a G0/G1 arrest.

- Cooperativitiy exists between the tumor suppressive pathways of SNF5 and p53. The *Snf5*$^{+/-}$;*p53*$^{+/-}$ mice developed tumors at median age of 9.5 months, as opposed to 15 and 17 months in their single mutant *Snf5*$^{+/-}$ or *p53*$^{+/-}$ control genotype littermates, respectively.
- *Snf5* and *p53* cooperate to suppress MRT formation in the mouse. The increase in penetrance of tumor formation in the double heterozygotes can be attributed to an increased numbers of malignant rhabdoid tumors. There were a subset of MRTs with a loss of heterozygosity of p53, accounting for a subset of the additional rhabdoids seen in the double heterozygotes . An increase in penetrance of lymphomas or osteosarcomas was not seen, nor were any novel tumor types observed.
- The immunoprofile of mouse rhabdoid tumors recapitulates that of human MRTs. Immunohistochemical staining of several rhabdoids for vimentin and S100ß revealed the same staining patterns seen in human MRTs. The mouse MRTs express vimentin invariably and S100ß to varying degrees.

B. Perspectives

The field of molecular cancer genetics is currently at the doorway of a new way of looking at tumor causation, with the onslaught of information describing epigenetic gene silencing in cancer flooding the literature. The perfect example is that of the RB /E2F pathway, specifically p16^{INK4A} and its involvement in cell cycle control. p16^{INK4A} is the poster-protein for epigenetic silencing, as one of the more important genes to be well characterized as being silenced due to hypermethylation. Increasingly more cancer

phenotypes are attributed to some epigenetic cause, much more so than simple somatic genetic mutations. Large regions of the human genome may contain a cluster of tumor suppressor genes, such as chromosome 3p21, where few somatic mutations are found but many genes related to cell cycle control, differentiation, and proliferation, are epigenetically silenced (see discussion in chapter 3 and Ji, 2005). These new discoveries continue to add to the complexity of a disease already appearing infinitely complex.

The SWI/SNF complex is the epitome of the intricacy of cancer causation, as it is involved in gene transcription directly, albeit by independent targeting. As we find that the loss of more specific subunits correlate with specific cancer phenotypes, we are also realizing the mechanism involved appears to be more epigenetic than genetic.

Despite the compounding complexity of cancer, I believe the simplest answer to questions like the causation of rhabdoid tumorigenesis is still the best. Knudsen's two hit hypothesis is generally connoted with the accumulation of genetic lesions. It seems more precise now to think of the two required hits as being hits to mechanisms, or pathways more than single coding region mutations or losses. For example, the inability to successfully treat recurrent rhabdoids in children successfully treated with chemotherapy of the original rhabdoid and the appearance of non-renal, non-CNS rhabdoid tumor formation in adults may be due to loss of an additional tumor suppressing pathway. Loss of p53 or some other mechanism or pathway, the second hit required, may be what is causing the alternate location rhabdoids in humans and mice, as well as the chemotherapy -resistance of the recurrent rhabdoids in children. It would follow that the first, apparently successful round of chemotherapy or radiation selected for the tumor cells that could by-pass this second pathway. This second pathway, perhaps p53, may be what allows the development of SNF5

null cells to malignant rhabdoid tumors in the alternate location and that this usually occurs later in life. Preliminary indications are that p53 loss of heterozygosity is found only in the alternate location MRTs in the mice in this study.

A loss of Snf5 heterozygosity along with p53 loss of expression was observed in both of the only 2 osteosarcomas developed in the double heterozygotes. This was the only tumor type observed to lose Snf5 heterozygosity and not appear histologically as a rhabdoid or a lymphoma. An opposing mechanism may have lead to the development or progression of the osteosarcomas than was observed in the rhabdoids developed in the double heterozygotes. Or perhaps, as with the rhabdoids, loss of p53 occurred as usual in these tumors and, given the speed and aggressive nature of these tumors, it may be likely some genetic instability occurred leading indirectly to Snf5 loss. Therefore *Snf5* loss was incidental, and perhaps late in the progression of the osteosarcoma, as no other sign of Snf5 LOH was observed, such as the histologic rhabdoid cell type.

The other core member that was the focus of this study is also likely to involve several pathways in its proper functioning. Several functions have been proposed for nearly every domain of BAF155 from transcriptional control to protein-protein interactions to self-dimerization. It has been proposed BAF155 is the scaffolding protein for the SWI/SNF complex, as it has been shown to bind specifically to several other SWI/SNF subunits and this binding is necessary and sufficient for the stabilization and nuclear localization of those other subunits. This directly implicates BAF155 in the regulator of the steady state levels of the other SWI/SNF subunits and arguably the entire complex. BAF155 does not seem to be affected by the proteasomal degradation that befalls other SWI/SNF subunits not properly bound to BAF155. It begs the question, what mechanism controls the steady state levels of

BAF155? The recent studies would imply that he who controls BAF155, controls the SWI/SNF complex entire. I find this conclusion questionable given the presence and associations of SWI/SNF core members in our cell lines lacking BAF155 expression. Either this is not the case, that BAF155 is a scaffold for SWI/SNF, or the mechanism is overridden in these cells, perhaps part of a larger loss of mechanism leading to their cancerous phenotype. However, we show re-expression of full-length wild type BAF155 is sufficient to cause a G0/G1 growth arrest due to senescence, and this response is specific to full length wild type BAF155, as a truncation mutant BAF155 does not accomplish arrest.

Previous studies have demonstrated LOH and chromosomal abnormalities in numerous cancer types (shown in Table 3-1) and as a marker of poor prognosis in the metastasis of neuroblastoma in the specific area of chromosome 3 where the BAF155 gene resides, 3p21 (Busson, 2004; Kuroki T, 2005; Mitelman, 1997; Todd, 1996; Killary, 1992; Cheng, 1998; Braga, 1999). Therefore, evidence of a role for BAF155 in the regulation of the cell cycle control makes it another candidate tumor suppressor gene from this region. It has been proposed that this region of chromosome 3, 3p21, houses a tumor suppressor gene cluster of several identified and unidentified tumor suppressor genes important in the suppression of carcinomas specifically (Ji, 2005).

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