ELUCIDATIG SNF5 RREGULATED GENE EXPRESSION IN MALIGNANT RHABDOID TUMOR DEVELOPEMNT

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ABSTRACT

Darmood Wei: Elucidating SNF5 Regulated Gene Expression in Malignant Rhabdoid Tumor Development
(Under the direction of Bernard E. Weissman)

Malignant Rhabdoid Tumors (MRTs), a pediatric renal cancer, lack SNF5, a subunit of the SWI/SNF chromatin remodeling complex which regulates nucleosome positioning and gene expression. MRTs offer a unique model for an epigenetically driven cancer because, unlike other cancers, they often have no other detectable mutations. Recent data on SWI/SNF mutations in cancer reveal mutations of 20 SWI/SNF subunit genes across 18 different cancers. Combined, the mutation rate of SWI/SNF complex members occurs at a frequency of 19% comparable to the 26% mutation frequency of TP53. Therefore understanding the SWI/SNF complex is highly relevant in our understanding of the mechanisms of not only tumorigenesis but also to the contribution of non-genotoxic carcinogens to this process. In these dissertation studies, we focused on the role of SNF5 inactivation in the development of MRTs. We hypothesize that SNF5 loss compromises the SWI/SNF complex resulting in aberrant targeting of the SWI/SNF complex, altered gene expression and tumorigenesis. To test this notion, we re-expressed SNF5 in MRT cell lines and examined the subsequent effects in SWI/SNF complex composition and gene expression. Our results indicated that SNF5 mediates the composition of the SWI/SNF complex, and its loss potentially disrupts SWI/SNF complex variants required for differentiation. SWI/SNF subunits are post-transcriptionally regulated in an interdependent fashion for stability. The changes in

SWI/SNF complex composition also alter targeting of the complex with subsequent changes gene expression. We validated our results using two known targets of SNF5, p21 and p16, and also through the identification of 2 two novel SNF5 targets, NOXA and CCNG2. These genes are upregulated after the reconstitution of the SWI/SNF complexes with SNF5 and may play critical roles in MRT development. These data demonstrate the intricacies of chromatin regulation and our incomplete understanding of this process in tumorigenesis. Together, this body of work serves as another milestone on our path to gain a better understanding of the relationships between chromatin structure and regulation, cancer biology, and toxicology.

This dissertation is dedicated in loving memory of my father, Han-liang Wei. It is your example of hard work I try to emulate in all that I do. Thank you.

PREFACE

This Doctoral Dissertation contains the results of research conducted by the author, Darmood Wei, at the Curriculum in Toxicology at the University of North Carolina. The goal of this dissertation seeks to elucidate the role of SNF5 in the SWI/SNF complex using malignant rhabdoid tumors as a model system. These data are published or are in the process of being published.

A version of Chapter 1 was published at eLS. I was the lead author responsible for the majority of the manuscript and Weissman BE was the supervisory author.

A version of Chapter 2 has submitted for publication at Molecular Cancer Research. I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as the majority of manuscript composition. Song S and Sakellariou-Thompson D assisted in the characterization of inducible SNF5 cell lines. Kuwahara Y completed the chromatin immunoprecipitation experiments. Cannon C and Emannuelle M provided the compound, MG132, and the expertise in ubiquitin dependent degradation. Goldfarb D and Major MB provided the mass spectrometry data and analysis. Weissman BE was the supervisory author.

A version of Chapter 3 was published at Molecular Cancer Research. Kuwahara Y was the lead investigator, Responsible for all major areas of concept formation, data collection and analysis, as well as the majority of manuscript composition. I was second author and assisted in data collection and analysis, as well as the majority of manuscript

composition. Durand J also assisted in the data collection and analysis and manuscript composition. Weissman BE was the supervisory author.

A version of Chapter 4 will be submitted for publication. I was the lead investigator, responsible for all major areas of data collection and analysis, as well as manuscript composition. Charboneau A, Davies B, was involved in the early stages of concept formation, data collection, and contributed to the manuscript, and Sakellariou-Thompson D contributed to the data collection. Weissman BE was the supervisory author on this project and was involved throughout the project in concept formation and manuscript composition.

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LIST OF ABBREVIATIONS

Ad - Adenoviral Vector

AT/RT – Atypical Teratoid/Rhabdoid Tumor

ATP – Adenosine Triphosphate

BAF0 – BRG1 (or BRM) associated factor

BRG1 – Brahma-Related Gene 1

BRM – Brahma

CCNG2 – Cyclin G2

CCNH – Cyclin H

cDNA – Complementary Deoxyribonucleic acid

ChIP – Chromatin Immunoprecipitation

CMV – Cytomegalovirus Promoter

DNA – Deoxyribonucleic Acid

GFP - Green Fluorescent Protein

HA – Hemagglutinin Tag

H3K4m3 – Histone H3 Lysine 4 Trimethylation

MOI – Multiplicity of Infection

MRT – Malignant Rhabdoid Tumor

PBS – Phosphate Buffered Saline

PCA – Principal Components Analysis

PCR – Polymerase Chain Reaction

Rb – Retinoblastoma Protein

RTK – Rhabdoid tumor of the kidney

RT-PCR – Real Time Polymerase Chain Reaction

shRNA – short hairpin Ribonucleic Acid

siRNA – Small Interfering Ribonucleic Acid

SMARC - SWI/SNF related, matrix associated, actin dependent regulator of chromatin

SWI/SNF – Switch/Sucrose Non-Fermenting

QPCR – Quantitative Polymerase Chain Reaction

Chapter 1: Genetics and Genomics of Malignant Rhabdoid Tumors[1]

1.1 Summary

Malignant rhabdoid tumors (MRTs) represent an aggressive pediatric cancer with limited treatment options. While MRTs mainly arise in the kidney and brain of patients under the age of 8 years, they may appear in other major organs as well as the soft tissues. Remarkably, these tumors possess few mutations other than ones that inactivate the SNF5/INI1 gene, the smallest member of the SWI/SNF chromatin-remodeling complex. In addition, these cancers lack other hallmarks of adult malignancies including genomic instability, aberrant karyotypes and abnormal regulation of cellular stress response pathways. Their major defect appears to arise from epigenetic instability, presumably through changes in nucleosome positioning due to defective chromatin remodeling activity. Recent reports have shown that SNF5 loss affects key signaling pathways such as cell cycle regulation, DNA damage repair and gene transcription. Thus, MRTs offer a unique model for studying the role of epigenetics in driving tumorigenesis and for the development of novel treatment approaches.

1.2 Introduction

The National Wilms Tumor Study first described malignant Rhabdoid Tumor (MRT), a highly aggressive pediatric cancer, in 1978 as a rhabdomyosarcomatoid variant of a Wilms tumor (Tomlinson et al., 2005a). Rhabdomyosarcomatoid, later abridged to rhabdoid,

[1] This chapter appeared as an article in the journal eLS. Orginial Citation is as follows Wei D and Weissman BE, Genetics and Genomics of Maligant Rhabdoid Tumors, eLS , 2014

referred to absence of muscular differentiation in these tumors despite their microscopic appearance (Tomlinson et al., 2005a). MRTs were later determined to be a distinct cancer from Wilms tumors and rhabdomyosarcomas. While MRTs were first reported in kidney, they have been found in various locations in the body, including the brain, liver, lung, skin, and heart (Parham et al., 1994; Rorke et al., 1996). MRTs occur with the greatest frequency in the kidneys, designated as rhabdoid tumors of the kidney (RTK), and in the brain, atypical teratoid/rhabdoid tumors (AT/RT).

At first the diagnosis of MRT relied upon the expertise of a limited number of pathologists, based upon the histology of the tumor and the appearance of the diagnostic rhabdoid cell (Figure 1.1). However, MRTs are now identified by the bi-allelic loss of the SMARCB1/INI1/SNF5/BAF47 gene, hereafter referred to as SNF5. SNF5 is a highly conserved subunit of the SWI/SNF chromatin-remodeling complex. Intriguingly, SNF5 loss does not affect genetic stability in MRT, but causes epigenetic instability(McKenna et al., 2008a). Next generation sequencing of these tumors revealed few mutations in MRTs beyond those found in *SNF5* (Lee et al., 2012). These results pose the question of how loss of a singular protein can profoundly affect tumorigenesis. Thus, these tumors offer a true epigenetically driven model for human cancer development. Further insights into the etiology of MRTs will promote our understanding of the role of epigenetic changes during cancer development as well as lead to new translational studies.

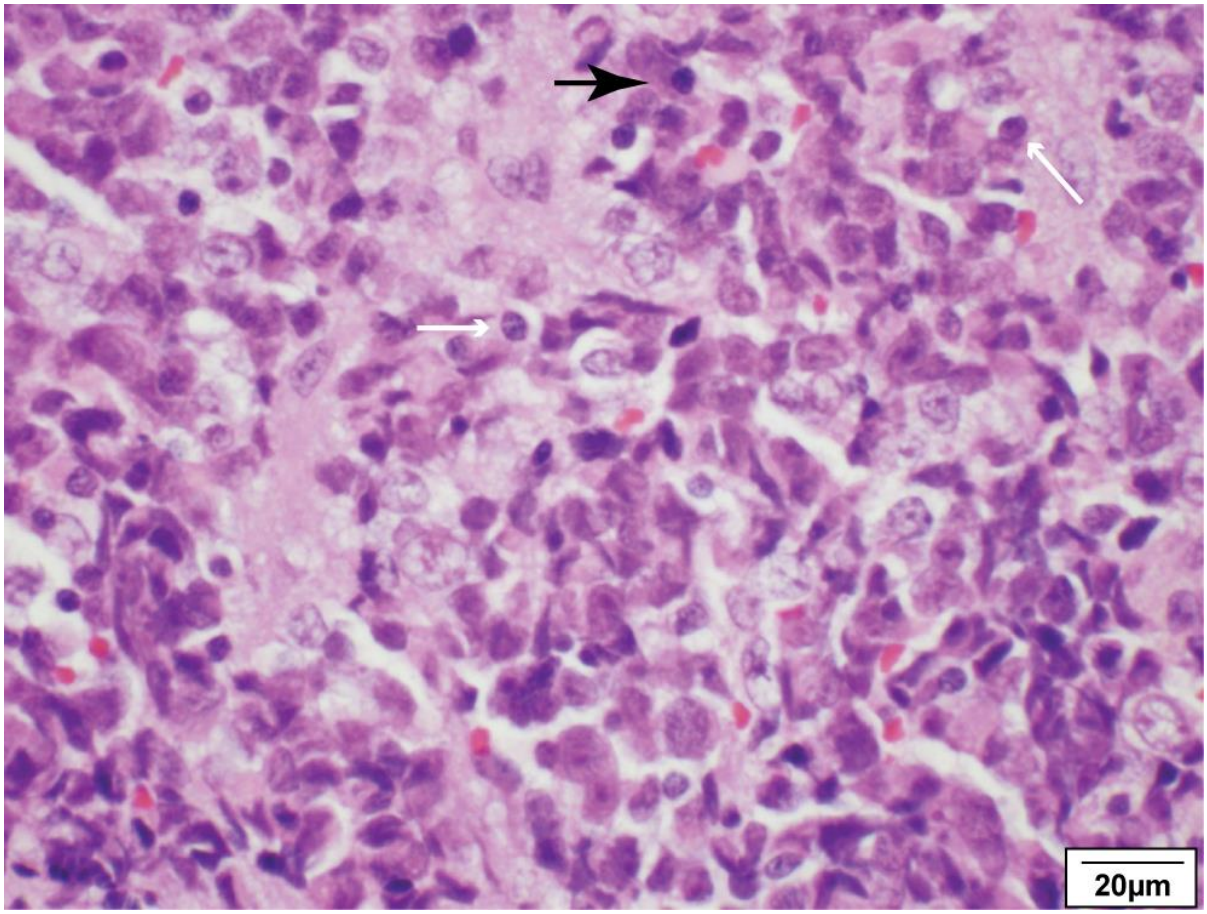


Figure 1.1 – Histopathology of MRT. Hematoxylin and eosin staining of a brain malignant rhabdoid tumor (AT/RT) from a young pediatric patient. The black arrow denotes a rhabdoid cell with significant accumulation of intermediate filaments with the cytoplasm. The white arrows demonstrate the prominent single nucleolus often observed in MRTs. Photograph courtesy of Dr. Dmitri Trembath, University of North Carolina, Chapel Hill, NC

1.3 Pathology

MRTs are extremely aggressive- approximately, 80-90% of children die from the disease within a year of diagnosis (Tomlinson et al., 2005a). For patients with MRT, the age at diagnosis appears to be a prognostic factor. Infants diagnosed between 0 and 5 months of age have the worst 4-year survival rate- 8.8% compared to 41.1% in infants 24 months or older (Tomlinson et al., 2005a). Additionally, high-stage (stage III/IV) disease at diagnosis is also correlated with an adverse outcome. The median appearance of tumors in children, 11 months, suggests that younger patients may likely exhibit germ line mutations resulting in a more aggressive disease (Tomlinson et al., 2005a). Furthermore, 70% of patients present with stage III or IV disease (Tomlinson et al., 2005a). The existing protocols for treatment include tumor resection, followed by adjuvant chemotherapy and/or radiation (Biswas et al., 2009). This current protocol suffers from several inadequacies, including the difficulty of resection due to the tumor size and the contraindication of radiation in young patients, especially in the developing brain (Biswas et al., 2009). It is unknown whether the aggressiveness of the disease is the result of underlying germ line mutations or the presence of multiple tumors (Roberts and Biegel, 2009a). While malignant rhabdoid tumors are generally considered pediatric malignancies, there have been rare occurrences in adults. Approximately 31 cases have been reported between 1992 and 2011, with the mean age of diagnosis at 30 years of age (Samaras et al., 2009; Shonka et al., 2011). MRTs in adults are no less aggressive with mean survival times of slightly less than 2 years (Samaras et al., 2009).

Classic malignant rhabdoid tumors contain diagnostic cells with a distinctive phenotype. The hallmarks of a rhabdoid cell include large acentric nucleus, and single

prominent nucleoli. The acentric nucleus is the result of swirls of intermediate filaments pushing the nucleus to the side (Schmidt et al., 1982). Additionally, the loss of SNF5 can be easily distinguished through immunohistochemistry (IHC) staining. Histology and IHC staining for SNF5 now comprise the diagnostic features of malignant rhabdoid tumors (Roberts and Biegel, 2009a).

1.4 SNF5 loss in MRT patients

SNF5 loss in MRTs occurs through a combination of frame shifts, nonsense mutations, and deletions, with the latter alteration appearing most commonly (Versteege et al., 1998c). *SNF5* mutations can either arise de novo or be inherited from a parent. An estimated 35% of MRT patients carry a germ line mutation of SNF5 (Eaton et al., 2011). Eaton et al. undertook a study to examine the SNF5 status in 100 MRT cases. The majority of parents of MRT patients with germ line mutations appears healthy and has a normal *SNF5* sequence (Eaton et al., 2011). This finding suggests a de novo origin for most of the *SNF5* mutations in these patients, with the mutations occurring pre-zygotically, during gametogenesis, or post-zygotically, during the early stages of embryogenesis. Eaton et al. observed only 9 cases where the mutated *SNF5* gene came from a parent. In 8 of the 9 cases, family members have been diagnosed with malignant rhabdoid tumors or schwannomas (Eaton et al., 2011). The parents who were carriers of the *SNF5* mutation often developed schwannomas. In cases of asymptomatic parents, their siblings were affected by the *SNF5* mutations, indicative of gonadal mosaicism. Presently, no data suggest a parental bias for the origin of *SNF5* mutations (Eaton et al., 2011). In rare cases, mutations in the *BRG1/SMARCA4* gene, coding for the ATPase of the complex, are found in MRTs in lieu of SNF5 mutations (Hasselblatt et al., 2011; Schneppenheim et al., 2010). The similar

pathology of these tumors to those with SNF5 loss further emphasizes the key role of loss of SWI/SNF complex activity in the development of this cancer.

1.5 RTK vs. AT/RT

There has been significant controversy in the field regarding the distinction between RTKs and AT/RTs. While these rhabdoid tumors arise in different anatomical sites, they are linked by one commonality, SNF5 loss. One must therefore ask whether both RTKs and AT/RTs should be reclassified as a single group of MRTs. A recent study found compared mRNA to microRNA (miRNA) expression in RTKs and AT/RTs (Grupenmacher et al., 2013b). Intriguingly, they found that while gene expression varied between these two classes, microRNA expression appeared very similar (Grupenmacher et al., 2013b). miRNAs are small non-coding RNA molecules that have been implicated in both positive and negative gene regulation. Mammalian miRNA has partial complementarity to its mRNA targets allowing it to target multiple mRNA targets. Additionally, as demonstrated by Grupenmacher and colleagues, a given target may be targeted by several distinct miRNAs (Grupenmacher et al., 2013b). Given the complex nature of miRNA regulation of gene expression, it seems surprising that the differences in mRNA expression patterns between these tumor subtypes are not reflected in divergent miRNA patterns. One possible explanation put forth by Grupenmacher and colleagues suggests that the mRNA differences between RTK and AT/RT reflect the divergent microenvironments in their tissues of origin (Grupenmacher et al., 2013b). In contrast, similar miRNA patterns may indicate a common cell of origin for MRTs in both tissues.

Because MRTs are found in different tissues, the cellular origin remains unclear. However, previous studies have suggested that different MRT subtypes arise from the same

the cell of origin, potentially a neuronal stem cell. For example, Okuno et al. found that MRTs express neuronal stem cell markers including CD133, nestin, and Musashi-1 (Msi-1) (Okuno et al., 2010). More intriguingly, MRT cell lines can be induced to differentiate by N-(4-hydroxylphenyl) retinamid (4-HPR) with concomitant downregulation of CD133, nestin, and Msi-1 (Okuno et al., 2010). The possible origin of MRTs from neuronal stem cells could support the observation that families with history of SNF5 loss often have members that develop schwannomas (Eaton et al., 2011). SNF5 and NF2 mutations, frequently observed in schwannomas are located in close proximity on chromosome 22 (Boyd et al., 2008). Thus, a single genetic event could result in loss of both genes (Boyd et al., 2008). The identification of the cell(s) of origin for MRTs will allow provide clearer insights into their development.

1.6 SNF5 is a member of the SWI/SNF complex

SNF5 is a core member of the SWI/SNF chromatin-remodeling complex. The SWI/SNF complex has multiple variants and is classified by their mutually exclusive ATPase subunits, BRM and BRG1. However, in the majority of MRTs, BRM is believed to undergo epigenetically silencing leaving only the BRG1 containing complexes (Glaros et al., 2007). BRG1 containing SWI/SNF complexes can be subdivided into two mutually exclusive groups BAF180/PBRM1 and BAF250A/ARID1A containing complexes (Wilson and Roberts, 2011). While SNF5 appears in all the SWI/SNF complexes, little is known about the effects of SNF5 loss upon SWI/SNF activity (Figure 2). One previous study by Doan et al. showed that the SWI/SNF complex appeared intact in MRT cell lines despite the loss of SNF5 (Doan et al., 2004). They also demonstrated that BRG1-dependent genes did not show altered expression in MRT cell lines (Doan et al., 2004). However, in genetically engineered mouse models, T-cell lymphoma development depends upon the retention of BRG1-

containing SWI/SNF complexes, suggesting that its loss fundamentally alters the complex activity (Wang et al., 2009). In general, previous reports have suggested two distinct roles for SNF5 loss during MRT development: as a classical tumor suppressor and as a critical element for cell survival (Isakoff et al., 2005; Roberts et al., 2000). Interestingly, MRTs seem to have overcome the requirement for SNF5 for survival.

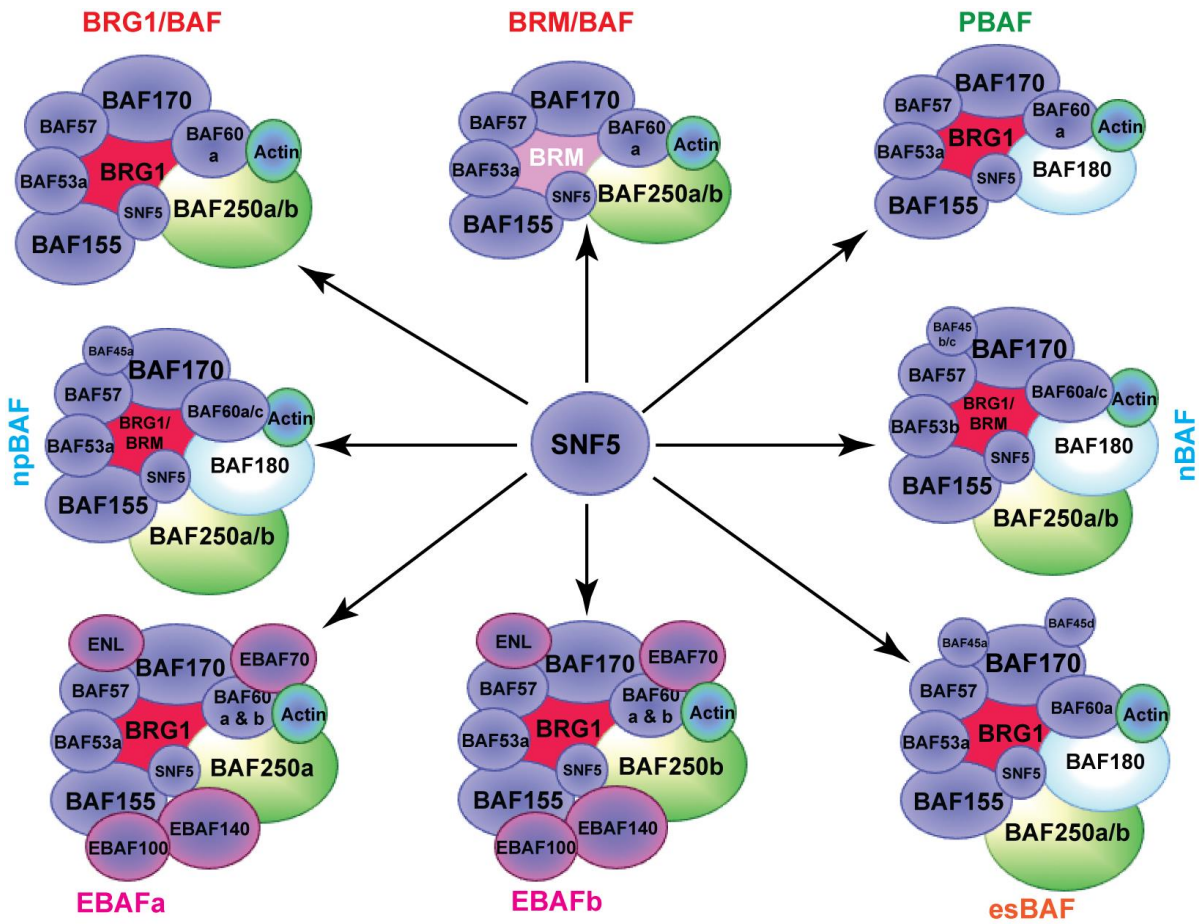


Figure 1.2 – SNF5 comprises all SWI/SNF complexes. Each SWI/SNF complex identified by previous studies varies in their subunit composition. However, all SWI/SNF complexes contain the SNF5 protein suggesting that its loss would affect all SWI/SNF complex activities.

1.7 Mechanisms of SNF5 Tumor Suppression

To understand the contribution of SNF5 loss to the etiology of MRT, considerable studies have focused upon identifying the underlying mechanisms. As shown in Table 1.1, inactivation of SNF5 functions impacts a broad range of cellular functions including cell cycle control, DNA damage response and cellular differentiation. We have not included all pathways associated with SWI/SNF complex activities, only those directly linked to SNF5 loss. Below, we discuss in more detail some of the most intriguing possibilities for how SNF5 loss drives MRT development.

Table 1.1 – Cellular Pathways Affected by SNF5 Inactivation		
Pathway	Effect Due to SNF5 loss	Reference
WNT	Aberrant activation of beta-catenin target genes	(Mora-Blanco et al., 2013)
c-MYC	Decreased c-MYC transactivation function, downregulation of BIN1	(Cheng et al., 1999a; McKenna et al., 2012)
GLI1	Hyperactivation of Heddgehog-GLI1 pathway	(Jagani et al., 2010)
Cell differentiation	Inhibits stem cell development and adipocyte, hepatic, neural & smooth muscle differentiation	(Albanese et al., 2006; Caramel et al., 2008a; Gresh et al., 2005; Ho et al., 2009; Kato et al., 2003; Kidder et al., 2009; Wang et al., 2006; You et al., 2013)
Cell cycle regulation	Decreased p16, p21 and p57 expression, Down-regulation of Rb pathway and CCND1 expression, increased c-FOS expression	(Algar et al., 2009; Betz et al., 2002; Chai et al., 2005; Kuwahara et al., 2010; Oruetxebarria et al., 2004; Pan et al., 2005; Zhang et al., 2002)
TP53	Decreased p53 dependent transcription of p21 and MDM2	(Xu et al., 2010)
DNA damage repair	Increased sensitivity to doxorubicin and UV damage, inhibits nucleotide excision repair	(Gong et al., 2006; Klochendler-Yeivin et al., 2006; Ray et al., 2009; Rosson et al., 2002a; Rosson et al., 2002b; Wu et al., 2002)
FGF	Increased expression of FGF receptors	(Wohrle et al., 2013)
EGF	Increased expression of EGF receptors	(Charboneau et al., 2006; Kuwahara et al., 2004b; Satomi et al., 2013)
AKT	Increased pAKT levels; AKT phosphorylation of SNF5	(Charboneau et al., 2006; Darr et al., 2013; Foster et al., 2006)
Polycomb silencing	Increased EZH2 levels and Polycomb complex activity	(Kuwahara et al., 2010; Oruetxebarria et al., 2004; Wilson et al., 2010)
RNA splicing	Altered elongation by RNA Polymerase II and completion of pre-mRNA transcripts	(Zraly and Dingwall, 2012)
Interferon	Increased PLK1 expression	(Morozov et al., 2007)
Mitotic checkpoint	Increased AURKA expression, inhibits polyploidy	(Lee et al., 2011; Vries et al., 2005)
Cell migration	Increased RHOA expression, promotes disorganized cytoskeleton	(Caramel et al., 2008b; Medjkane et al., 2004)
Nuclear receptors	Decreased T3, GR & AR-dependent transcriptional activation	(Choi et al., 2007; Lee et al., 2009)

1.7.1 Cell Cycle Regulation

A consistent property of MRT cell lines is that upon SNF5 re-expression, they undergo a G1 cell cycle arrest (Betz et al., 2002; Versteege et al., 2002). SNF5 re-expression results in increased levels of 2 cyclin-dependent kinases, $p21^{WAF1/CIP1}$ and $p16^{INK4A}$, two well-established tumor suppressors (Kamb et al., 1994; Yang et al., 1995). Of note, induction of p21 and p16 occurs through two distinct mechanisms (Betz et al., 2002; Kia et al., 2008c; Kuwahara et al., 2010).

The SWI/SNF complex forms independently of SNF5 (Doan et al., 2004), and ChIP data suggest that the constant presence of BRG1 containing complexes missing SNF5 along the p21 promoter (Kuwahara et al., 2010). It is believed that these "incomplete" complexes initiate transcription but cannot displace the nucleosomes at the promoter. These obstacles prevent the elongation of RNA polymerase II resulting in promoter pausing concomitant with decreased p21 expression (Brown et al., 1996; Neely et al., 1999). During SNF5 re-expression, SNF5 stabilizes and recruits BAF180 to the SWI/SNF complex thus changing the complex's stoichiometry (unpublished observations). The reconstituted SWI/SNF complex binds to the TSS of p21 promoter, as evidenced by the similar binding profile of SNF5 and BAF180 (Wei, Weissman and Kuwahara- unpublished observation). The "complete" SWI/SNF complex is able to effect chromatin remodeling and to allow robust p21 transcription. Alternatively, these events could indicate a model where BAF180 can be recruited to the p21 promoter and initiate expression independent of SNF5. However, levels of p21 remain low due to degradation of BAF180 in the absence of SNF5 (Wei, Weissman and Kuwahara-unpublished data). SNF5 re-expression stabilizes BAF180 within the

complex leading to increased p21 expression. Interestingly, this p21-dependent mechanism can function in either a p53-independent or p53-dependent manner (Kuwahara et al., 2010).

In contrast to p21 expression, p16 expression is silenced epigenetically by binding of the polycomb complex to its promoter (Jacobs et al., 1999). Kia and colleagues demonstrated in MRT cell lines that SNF5 recruits BRG1 and by extension the SWI/SNF complex to the p16 promoter resulting in polycomb eviction and p16 re-expression (Kia et al., 2008b). Additionally, they showed that p16 expression is BRG1 dependent (Kia et al., 2008b). The silencing of p16 by the polycomb complex in MRTs probably occurs normally in its cell of origin i.e. a stem cell, rather than an active process initiated by SNF5 loss. Thus, this mechanism of gene expression differs from the mechanism at the p21 promoter where the SWI/SNF complex remains in the absence of SNF5 (Kuwahara et al., 2010). However, on closer examination, the expression of p21 occurs prior to p16 suggesting that SNF5 induced cell cycle arrest is initially driven by a p21-dependent mechanism.

1.7.2 DNA Damage Repair

The SWI/SNF complex has been implicated in DNA damage repair, including nucleotide excision repair. In general, nucleosomes can obstruct protein complexes from binding to DNA, including DNA repair enzymes. Thus, the presence of SWI/SNF complex affects chromatin remodeling to allow access by DNA repair enzymes and increase the rate of DNA repair (Gaillard et al., 2003). Of note, MRT cell lines retain intact p53 pathways (Chai et al., 2005). Complementary studies conducted in MEFs found that reduction of SNF5 leads to accumulation of p53 (Klochendler-Yeivin et al., 2006). Klochnendler-Yeivin et al. demonstrated, in SNF5-null MEFs, that DNA damage could activate p53 (Klochendler-Yeivin et al., 2006). The induction of p53 results in 2 to 3 fold increases in expression of p53

dependent genes MDM2 and PUMA, yet had no effect on other p53 targets such as p21 and BAX (Klochendler-Yeivin et al., 2006). Additionally, they observed that SNF5 loss in MEFs increased susceptibility to UV irradiation (single strand breaks) and doxorubicin (double strand breaks) (Klochendler-Yeivin et al., 2006). We have observed similar results in human MRT cell lines where either PUMA or NOXA expression increases after SNF5 re-expression (Kuwahara et al., 2013a). These data imply that SNF5 plays an important role in a cell's ability to survive DNA damage. These observations appear consistent with the aggressive malignancies caused by SNF5 loss, but not with their lack of additional mutations, i.e. no genetic instability. The paradoxical nature of SNF5 further underscores the complexity of this disease.

1.7.3 Dysregulation of transcription factor activity

SNF5 has been demonstrated to interact with a wide range of transcription factors. Consequently, SNF5 loss can lead to dysregulation of the pathways regulated by each transcription factor. For example, SNF5 has been demonstrated to interact with glioma-associated oncogene family zinc finger-1 (GLI1) as well as c-Myc. SNF5 loss results in aberrant activation of GLI1, an effector protein for the Hedgehog signaling (Jagani et al., 2010). Jagani et al. demonstrated that that SNF5 not only interacts with GLI1 but also localizes to GLI1-regulated promoters. Intriguingly, loss of SNF5 results in hyperactivation of GLI1 and PTCH1, while SNF5 re-expression MRT cell lines results in their down-regulation (Jagani et al., 2010). These findings suggest that SNF5 plays repressive role in relation to the Hedgehog-GLI pathway, an oncogenic pathway associated with many tumors including MRT (Jagani et al., 2010).

A second example is c-MYC, a sequence specific transactivator that interacts with SNF5 resulting in the recruitment of the SWI/SNF complex (Cheng et al., 1999a). It is further believed that this interaction results in the transcription of a small subset of c-MYC target genes including those that are involved in apoptosis (Cheng et al., 1999a). Many human tumors overexpress the C-MYC oncogene including a large number of pediatric cancers. It can also act as an oncogene in many animal models of cancer (Albihn et al., 2010). Thus, the normal suppression of their transcriptional activities may play a role in SNF5's tumor suppressor functions (Morton and Sansom, 2013).

1.7.4 Altered Nucleosome Positioning

SNF5 has been found to be important for chromatin remodeling during differentiation. You et al. demonstrated that SNF5 is recruited to both OCT4 activated and repressed genes as indicated by H3 methylation patterns. The OCT4 transcription factor plays a key role in maintaining pluripotency (Young, 2011). Yet, SNF5 levels appears to be inversely correlated with OCT4 expression levels which seems to suggest that levels of SNF5 may change as a cell differentiates (You et al., 2013). During differentiation, SNF5 is recruited to genes activated by OCT4, resulting in nucleosome enrichment, and to genes repressed by OCT4, leading to nucleosome depletion (You et al., 2013). This interaction supports that notion that SNF5 and the SWI/SNF complex play critical roles in differentiation. Furthermore, the kinetics of the activity suggest that given a differentiation cue, SNF5 condenses chromatin regions of active transcription and then opens the chromatin regions of Oct4 repressed genes in a two-step manner. Given this scenario, it is not surprising that SNF5 loss results in a stem cell like state and over-expression of SNF5 biases the cell towards differentiation. Therefore, SNF5 may maintain the balance between

embryonic and differentiated cellular states. Loss of SNF5 can disrupt the timing of gene expression during differentiation, further underscoring the impact of disruption of nucleosome positioning. Paradoxically, there is evidence that the SWI/SNF complex plays an intimate role in maintain pluripotency. Over-expression of BRG1 and BAF155, other SWI/SNF complex members, increases re-programming efficiency in iPS cells (Singhal et al., 2010). Also BAF155 and BRG1 have been shown to be critical for self-renewal in ES cells (Ho et al., 2009). These observations appear consistent with enrichment of the stem cell-associated signatures as well as their associated proliferation deficient signatures found in SNF5-deficient MEFs (Wilson et al., 2010).

1.7.5 SWI/SNF Complex Disruption

Previous reports have shown the effects of loss of individual SWI/SNF complex members upon the organization and stability of the remaining complex members. For example, studies by Chen and Archer posited that BAF155 serves as a scaffolding element required for stability of other SWI/SNF complex components including BRG1 (Sohn et al., 2007) and BAF57 (Chen and Archer, 2005). Another report demonstrated that ARID2/BAF200 regulated BAF180 expression at the level of mRNA expression (Yan et al., 2005). Other reports have shown increased expression of BRM upon loss of BRG1 expression. Recent evidence has also shown decreased BAF57 and SNF5 levels in BAF155 deficient cells lines (DelBove et al., 2011). In drosophila, loss of the SNF5 ortholog, SNR1, leads to SWI/SNF complex instability (Zraly and Dingwall, 2012).

Only one previous study addressed the effects of SNF5 loss in mammalian cells upon SWI/SNF complex assembly and function. Doan et al. showed assembly of the complex in the absence of SNF5 and no effect on expression of BRG1-dependent genes (Doan et al.,

2004). However, loss of SNF5 protein has profound impact on another SWI/SNF complex member, BAF180. Our lab has demonstrated that BAF180 is regulated post-transcriptionally (unpublished observations). BAF180 protein is stabilized by the presence of SNF5; however, in the absence of SNF5, BAF180 is degraded via an ubiquitin-ligase dependent manner. This reconstituted complex has been shown to target to the p21 promoter (Kuwahara et al., 2013a). This demonstrates that the stoichiometry of the complex has a broad impact on gene expression, consistent with previous evidence demonstrating that other SWI/SNF complex members are interdependent in their role to maintain complex integrity. Future studies into understanding the mechanism of BAF180 instability could prove instrumental in treating not only MRTs but also in cancers have demonstrated SNF5 loss.

1.8 Environment As Etiological Factor in MRTs

Little else is known regarding the etiology of this cancer. The low incidence of MRTs has made it difficult to conduct epidemiological studies to better understand this unique cancer. However, a variety of environmental agents are known to modulate epigenetic machinery in living organisms, including ATP-dependent chromatin remodeling. Some reports have suggested MRTs may arise from a shared parental environment implicating environmental factors in mutations and/or loss of SNF5 (Swinney et al., 2006b). The most compelling case for environmental factors as the etiological agent was published in 2006 where doctors observed 3 cases in which unrelated children developed rhabdoid tumors, lived in the same geographic area, and had parents that worked at the same job site (Swinney et al., 2006b). These investigators examined the relevant parent of two of these patients: a 4-month old Caucasian female with an AT/RT and a 4-month old Asian American male with an MRT. The father of the first patient worked at the jobsite prior to conception

through infancy. Subsequent genetic analysis revealed that she had a germ line SNF5 mutation. Genetic analysis revealed found no germ line mutation of SNF5 in either the second patient or the parents. The mother of the second patient also worked at the same job site prior to conception to through infancy. No genetic analysis was conducted on the third case.

In the majority of patients the SNF5 mutations have been found to be de novo in origin with the parents unaffected (Eaton et al., 2011). This would suggest the majority of the cases are a result of a mutation arising either pre-zygotically (gametogenesis) or post-zygotically (embryogenesis). This time course is consistent with possibility of environmental exposure as an etiological agent. However, due to the low frequency of these tumors, conducting epidemiological studies has been challenging.

1.9 Future Directions

Currently, the protocols for MRT treatment include tumor resection, followed by adjuvant chemotherapy with vinicristine/actinomycin D/cyclophosphamide (VAC) regimen (Biswas et al., 2009). These treatment options suffer from several inadequacies including the difficulty of resecting large tumors, sometimes in sensitive sites such as the brain and spinal cord, as well as the contraindication of chemotherapy in young patients (Biswas et al., 2009). Therefore, a great need exists for treatment approaches that will improve patient outcome in these pediatric patients. Unfortunately, direct targeting of the SWI/SNF complex does not appear feasible because of the potential cross-reactivity with a wide range of other ATP-dependent enzymes (Keppler and Archer, 2008). Therefore, a better understanding of how SNF5 loss contributes to MRT development should provide new targets for novel drugs or therapeutic interventions.

SNF5 is a multifaceted protein with involvement in a wide array of cellular process, consistent with its contribution to SWI/SNF complex activities. Identifying the pathways activated during SNF5 re-expression should reveal potential targets for specific therapeutic interventions. Furthermore, it has been conjectured that the cell of origin may be a neuronal progenitor cell. Confirmation of the cell of origin would provide information not only on the critical development period when these tumors arise but also biomarkers for identifying exposures. This research will further our understanding of chromatin remodeling, and provide knowledge for translation into the clinic in the context of improving the outcomes of patients with MRTs. Furthermore, this knowledge will extend beyond the scope of MRT patients to the treatment of other cancers with mutations in other members of the SWI/SNF complex. Finally, dissecting the molecular mechanisms driving MRT development will provide new insights into the general role of epigenetic changes during human tumor development.

Chapter2: SNF5/INI1 Deficiency Destabilizes the SWI/SNF Complex During Malignant Rhabdoid Tumor Development[2]

2.1 Summary

Malignant Rhabdoid Tumors (MRTs), a pediatric cancer that frequently arises in the kidney, lack SNF5, a subunit of the SWI/SNF chromatin-remodeling complex. Recent studies have established that multiple SWI/SNF complexes exist due to the presence or absence of different complex members. Therefore, we investigated the effect of SNF5 loss upon SWI/SNF complex formation in human MRT cell lines. We initially observed that MRT cell lines and primary tumors exhibited reduced levels of many complex proteins. Furthermore, reexpression of SNF5 in MRT cell lines increased SWI/SNF complex protein levels without concomitant increases in mRNA. Mass spectrometry analysis of a MRT cell line before and after SNF5 reexpression indicated the recruitment of different components into the complex along with the expulsion of others. IP-Western blotting confirmed these results and demonstrated similar changes in other MRT cell lines. Finally, we found that reduced expression of SNF5 in normal human fibroblasts led to altered levels of these same complex members. Our data establish that SNF5 loss during MRT development alters the repertoire of available SWI/SNF complexes, generally disrupting those associated with cellular differentiation. Our findings support the model that SNF5 inactivation blocks the conversion of growth promoting SWI/SNF complexes to differentiation inducing ones.

[2] A version of this manuscript has been submitted to Molecular Cancer Research and is currently in revision.

Therefore, restoration of these complexes in tumors cells may provide an attractive approach for the treatment of these tumors.

2.2 Introduction

Malignant Rhabdoid Tumors (MRT) are a highly aggressive pediatric cancer commonly found in the kidneys (RTK) and brain (AT/RT). These tumors have a median onset of 11 months and 80-90% of children die from the disease within a year of diagnosis (Roberts and Orkin, 2004). MRTs are characterized by the loss of SNF5/BAF47/INI1/SMARCB1, a core member of the SWI/SNF complex, hereafter referred to as SNF5 (Biswas et al., 2009). Interestingly, SNF5 loss does not affect genetic stability in MRT, but causes epigenetic instability (McKenna et al., 2008a). Thus, this consistent and specific molecular change, SNF5 inactivation, provides a unique model for understanding the role of epigenetics in human tumor development. Numerous studies have shown that SNF5 loss affects multiple signaling pathways (Wei and Weissman, 2014). However, little else is known regarding the etiology of this cancer.

The SWI/SNF complex has multiple variants and is classified by their mutually exclusive ATPase subunits, BRM and BRG1. BRG1 containing SWI/SNF complexes can be subdivided into two mutually exclusive groups- BAF180/PBRM1 and BAF250A/ARID1A containing complexes. While SNF5 appears in all the SWI/SNF complexes, little is known about the effects of SNF5 loss upon SWI/SNF activity. One previous study by Doan et al. showed that the SWI/SNF complex appeared intact in MRT cell lines despite the loss of SNF5 (Doan et al., 2004). They also demonstrated that BRG1-dependent genes did not show altered expression in MRT cell lines (Doan et al., 2004). However, they did not address the stoichiometry of the complex components in MRT cell lines or the interaction of the complex

with chromatin. Other studies have indicated that loss of a SWI/SNF complex member could affect the stability of other complex members in mammalian cells and in flies(Chen and Archer, 2005; Curtis et al., 2011; Decristofaro et al., 2001; DelBove et al., 2001; Jung et al., 2012; Moshkin et al., 2007; Zraly et al., 2006). Furthermore, a growing number of studies have identified multiple SWI/SNF complexes defined by the presence or absence of different components (Eaton et al., 2011; Lessard et al., 2007; Tonelli and Wachholtz, 2014; Wade and Chang, 2014). Importantly, these different complexes can promote either growth or differentiation depending upon their composition (Ho et al., 2009; Kadoch and Crabtree, 2013; Lessard et al., 2007; Wei and Weissman, 2014).

In order to address whether SNF5 loss affects SWI/SNF complex composition during MRT development, we examined the effects of SNF5 expression in MRT cell lines. Our studies demonstrate that re-expression of SNF5 in MRT cell lines altered total protein levels of many components while knock down of SNF5 in normal human fibroblasts showed the opposite effect without altering mRNA expression. After SNF5 reexpression, many components were recruited into SWI/SNF complexes that are generally associated with induction of differentiation. Therefore, SNF5 loss may promote MRT development by preventing a switch from SWI/SNF complexes that promote proliferation to those associated with differentiation. These studies emphasize the need to resolve the scope and composition of SWI/SNF complexes among different tissues and may account for the low incidence of these deadly childhood cancers.

2.3 Materials and Methods

2.3.1 Cell lines.

Our laboratory previously described the A204.1, D98OR, and G401.6 cell lines (Chai et al., 2005a; Weissman, 1987; Weissman and Stanbridge, 1980). The MCF-7, A673, Jurkat, DAOY and RD cell lines were obtained from the American Type Tissue Collection (ATCC). The TTC642 and TTC549 cell lines were kindly provided from Dr. Timothy Triche, Children's Hospital of Los Angeles. Dr. Peter Houghton, St. Jude's Children's Hospital kindly provided the BT-12 AT/RT cell line. 293FT cells were kindly provided by Dr. Inder Verma, Salk Institute. All cell lines were cultured in RPMI 1640 plus 10% fetal bovine serum (Gibco, Grand Island, NY) and were used within 30 passages of their initial arrival to minimize chances of cross-contamination.

2.3.2 Adenovirus infection.

The Ad/pAdEasyGFPINI-SV+ adenoviral vectors expressing hSNF5 and coexpressing green fluorescent protein (GFP; designated Ad-hSNF5) and the Ad/pAdEasyGFP expressing GFP (designated Ad-GFP) were previously published (Reincke et al., 2003). To generate adenovirus expressing HA-tagged SNF5, we used the pAdEasy system (He et al., 1998). We first subcloned the insert from the pMDK225 lentivirus (Kaeser et al., 2008) expressing SNF5 with a c-terminal triple-HA tag into the pShuttle vector. The UNC Vector Core generated Ad-SNF5-HA following the pAdEasy system protocol. The SNF5-HA was sequenced to verify the wild-type gene and the triple HA tag at the c-terminus. To achieve infection of over 90% of cells, we infected at a multiplicity of infection (MOI) of 20 for the A204.1 cell line, 10 for the G401 cell lines, 10 for the TTC549 cell line, and 200 for the TTC642 cell line. Infection of cell lines were carried out as previously described (Kuwahara et al., 2010).

2.3.3 Protein extracts and Western blotting.

Isolation of nuclear protein and Western blotting was carried out as described previously (Chai et al., 2005; DeCristofaro et al., 1999). Western blot analyses for protein expression were carried out using anti-ß-ACTIN (A2066; Sigma), anti-BAF57 (A300-810A; Bethyl), anti-BRG1 (A300-813A; Bethyl or G-7; Santa Cruz), anti-BRD7 (A302-304A;Bethyl), anti-BAF170 (A301-038A; Bethyl), anti-BAF200 (A302-229A; Bethyl), anti-BAF250A (A301-040A; Bethyl), anti-BAF60A (A301-594A; Bethyl), anti-BAF60B (A301-596A; Bethyl), anti-HA (F-7; Santa Cruz), anti-hSNF5 (A301-087A; Bethyl or 612111; BD Transduction Laboratories), anti-p21CIP1/WAF1 (AB1; Calbiochem), anti-BAF180 (A2218; a kind gift from Dr. Ramon Parsons, Columbia University), anti-BAF155 (DXD7; Santa Cruz), anti-KU70/80 (a kind gift from Dr. Dale Ramsden, University of North Carolina), anti-Ran (sc-271376; Santa Cruz), anti-c-FOS (H-125; Santa Cruz), anti-H3 (Cat#39163; Active Motif) and horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (GE Healthcare). Densitometry was carried out using the BioRad Imagelab 4.1 software.

2.3.4 RNA extraction and quantitative real-time reverse transcription–PCR analysis.

RNA was extracted using the RNeasy mini kit (Qiagen), and 1 μg was used for cDNA synthesis primed with random primers (Invitrogen). cDNA was analyzed using TaqMan (Applied Biosystems) quantitative real-time reverse transcription–PCR (qPCR) analysis, with β-ACTIN as the reference gene in each reaction. Reactions were performed on an ABI 7900 HT sequence detection system (Applied Biosystems), and relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method (Chai et al., 2005a). The following TaqMan gene expression assay primer/probe sets (Applied Biosystems) were used: p21CIP1/WAF1-Hs00355782_m1, BAF180/PBRM1-Hs00217778_m1, SNF5/SMARCB1-Hs00268260_m1, BAF250/ARID1A-Hs00195664_m1, BRM/SMARCA2-Hs00268234_m1, BRD7-

Hs04186169_g1, BAF200/ARID2-Hs00326029_m1, BAF170/SMARCC2-Hs00161961_m1, BAF155/SMARCC1-Hs00268265_m1, BAF57/SMARCE1-Hs00705034_s1, BRG1/SMARCA4-Hs00946396_m1, BAF60A/SMARCD1-Hs00161980_m1, β-ACTIN-Hs99999903_m1 (Applied Biosystems).

2.3.5 Immunoprecipitation.

Cells were infected with adenovirus expressing either HA tagged SNF5 (Ad-SNF5-HA) or empty vector (Ad-CMV). Proteins were isolated from the infected cells using IP buffer (50mM Tris, 400 mM NaCl, 2mM EDTA, 10% Glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1 mM PMSF, 4 mM sodium fluoride, 40 nM sodium orthovanadate, 1x complete Mini protease inhibitor cocktail (Roche Diagnostics)). The isolated protein was quantified and adjusted to 500ug/ml. 500 ug of protein from each sample was incubated with BRG-1 (A303-877A ; Bethyl), or normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) rotating overnight at 4oC with 30 µl of a 50% slurry of protein A/G Sepharose beads. The beads were then washed 3 times with IP wash buffer (1x PBS, 10% glycerol, 1% Triton) and then suspended in 1x Nupage LDS loading buffer supplemented with 125mM DTT and boiled for 5 minutes. The supernatants were then run on 4-12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with anti-SNF5, anti-BRG1, anti-BAF155, anti-BAF180, anti-BAF200, anti-BAF250A, anti-BAF170, anti-BAF60A, or anti-BAF57 (referenced above).

2.3.6 Mass Spectrometry

The determination of SWI/SNF complex components by mass spectrometry was carried out on immunoprecipitated samples prepared as in the immunoprecipitation protocol above following our previously published protocols (Hast et al., 2013).

2.3.7 Lentiviral procedures and small hairpin RNA

Lentivirus was generated using 293FT cells following the protocol of Kafri and colleagues (Xu et al., 2001). Either pLKO.1, a nontargeting small hairpin RNA (shRNA) control vector (SHC002; Sigma), or SNF5 shRNA lentiviral transduction particles (TRCN 39585 and 39587) were cotransfected with the packing construct ΔNRF (from Dr. Tal Kafri, University of North Carolina; (Xu et al., 2001)) and the VSV-G envelope expression plasmid (pMDK64; from Dr. Matthias Kaeser, Salk Institute) into 293FT cells using calcium phosphate transfection. pLKO.1 is a negative control containing an inert sequence that does not target any human or mouse gene but will activate the RNAi pathway. For infection, cells were incubated with lentiviral particles and polybrene and then selected with puromycin.

2.3.8 Inducible SNF5 Cell lines

Flag tagged SNF5 was amplified from an existing plasmid, pcDNA3-fSNF5, by PCR (T7 promoter F primer, hSNF5 ORF R primer – TTA CCA GGC CGG CGT GTT) (Chai et al., 2005a). The resulting PCR product was TOPO-cloned into a Gateway vector using the pCR®8/GW/TOPO® TA cloning kit (45-0642, Invitrogen). The fSNF5 was transferred into the pINDUCER20 vector that provided a c-terminal HA tag using Gateway® LR Clonase ™ II Plus Enzyme Mix (12538-120, Invitrogen) . The identity of the fSNF5HA insert was confirmed by DNA sequencing.

2.3.9 ß-galactosidase staining.

To identify ß-galactosidase positive cells, we used the Senescence β-Galactosidase Staining kit (Cell Signaling #9860). Cells were prepared and stained following the kit protocol.

2.3.10 Protein degradation inhibition.

To inactivate the proteasome, cells were treated with 10μM MG132 (1748, Boston Biochem) or DMSO vehicle control for 6 hours. Cells were harvested and prepared lysates were analyzed by immunoblotting, as described above.

2.4 Results

2.4.1 SWI/SNF Complex Components Are Post-Transcriptionally Regulated in MRT Cell Lines.

We first examined whether component protein levels differed between SNF5-deficient MRT and AT/RT cell lines and SNF5-positive non-MRT cell lines including pediatric (A673 Ewing sarcoma, DAOY medulloblastoma, and RD rhabdomyosarcoma) and adult (D98OR Hela derivative, Jurkat acute T cell lymphoma and MCF-7 breast cancer) tumor cell lines. As expected, we found a clear loss of SNF5 protein in the four MRT cell lines and one AT/RT cell line in comparison to non-MRT cancers (Figure 2.1A) (DeCristofaro et al., 1999). We also found no expression of BRM/SMARCA2 protein or mRNA, consistent with previous reports (Figure 2.1A & B) (Jagani et al., 2010; Yamamichi et al., 2005) while levels of the other ATPase, BRG1/SMARCA4, appeared similar among all cell lines. Surprisingly, the other component proteins showed generally lower levels in MRTs compared to other pediatric and adult tumor cell lines ranging from nearly complete absence (ARID1A, SMARCC2 and SMARCD2) to moderate reduction (ARID2) (Figure 2.1A).

We next asked whether the reduced protein levels reflected changes in mRNA expression. Thus, we assessed component mRNA levels using quantitative PCR (qPCR) normalized to the HeLa-derived D98OR cell line, a cell line often used for the purification of

SWI/SNF complex members (Wang et al., 2006; Wang et al., 1996). Consistent with the western blot data, SNF5 mRNA expression was undetectable in MRT cell lines, with the exception of TTC642, a cell line that contains a nonsense mutation in the SNF5 gene, (DeCristofaro et al., 1999). In contrast, we could not observe consistent changes in the mRNAs of other complex components in MRT cell lines that could account for their lower protein levels (Figure 2.1B)

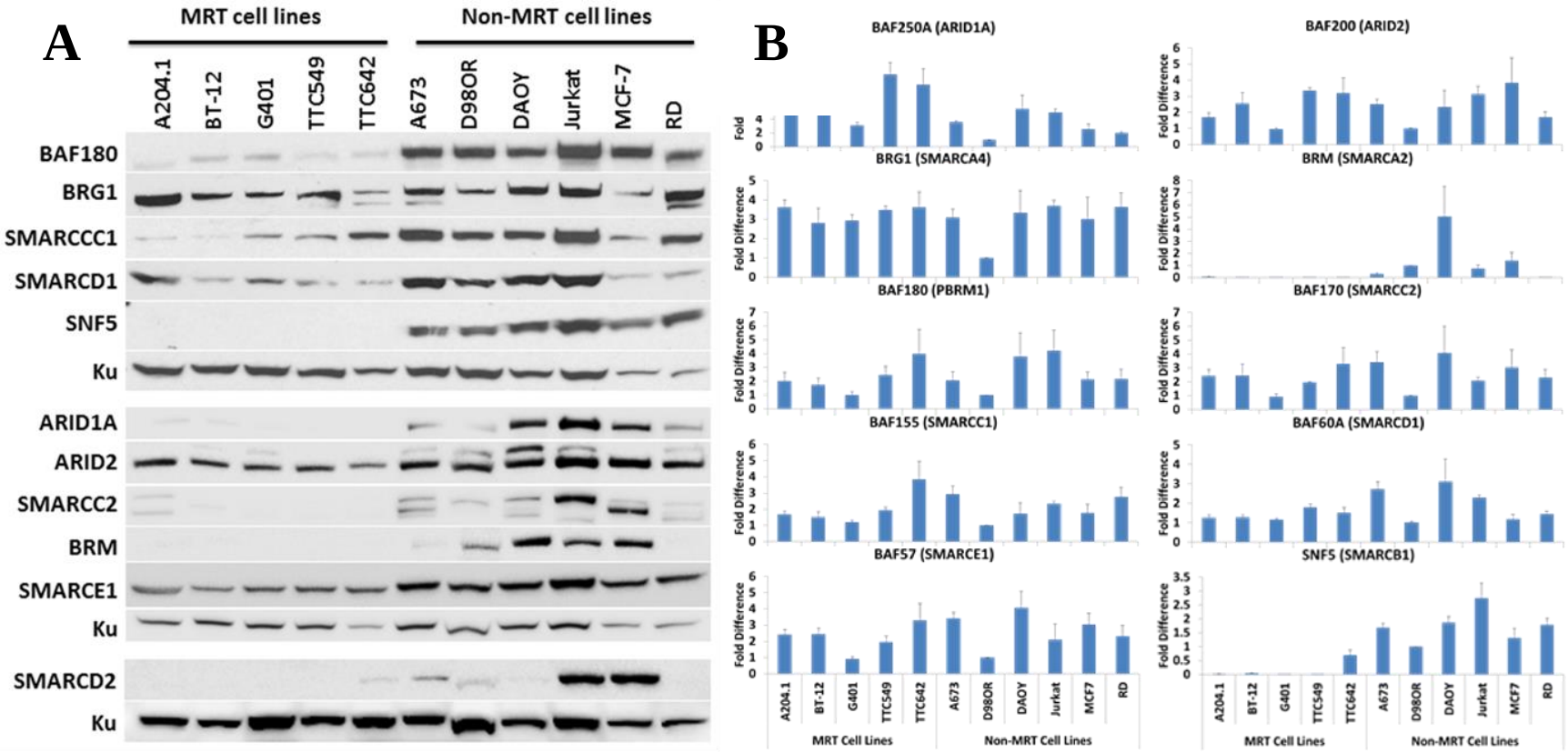


Figure 2.1 – Decreased SWI/SNF complex protein but not mRNA in MRT cell lines. (A) Total cellular proteins (30 μg) isolated from MRT and non-MRT cell lines were separated on a 4% to 12% SDS-polyacrylamide gel, transferred to PVDF membranes and probed with antiserum against SWI/SNF complex members. We used Ku70/80 as the loading control. (B) RNA was extracted from the indicated cell lines. The mRNA levels were measured for each gene by qPCR and normalized to D98OR β-actin expression. Columns – mean of four independent experiments; Error bars – standard error

To extend these findings to primary MRTs, we examined protein expression in nuclear extracts from a series of primary tumors derived from MRTs as well as other prototypical childhood cancers, including Wilm's tumors (WT), and rhabdomyosarcomas (RMS) (DeCristofaro et al., 1999). Due to limited amount of starting material, we could only assess a subset of SWI/SNF components. Protein levels in these nuclear extracts were normalized to histone H3 protein levels and aggregated by MRT vs non-MRT tumor type. Loss of SNF5 protein again was apparent in the MRTs compared to the other tumor types (Figure 2.2A). The average ratio of BAF180 and BAF57 to H3 in MRTs was significantly lower than in non-MRT samples.while the BRG1 levels remained the similar (Figure 2.2B). Thus, the primary tumor data for MRTs recapitulate the findings derived from cell lines.

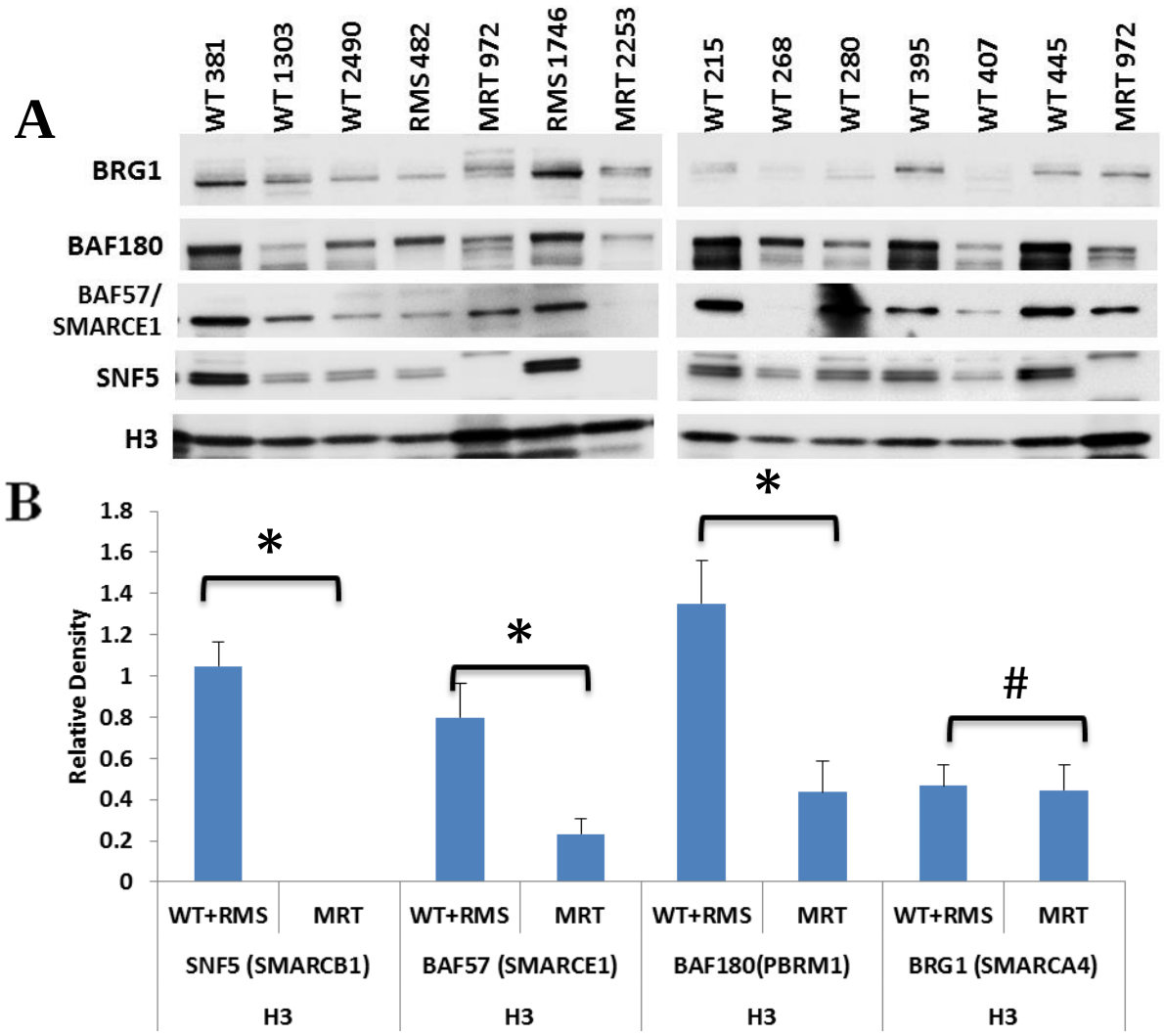


Figure 2.2 – Decreased SWI/SNF complex proteins in primary tumor samples. (A) Nuclear protein (3 µg) isolated from primary tumors were separated on a 4 to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with anti-BRG1, anti-BAF57, anti-SNF5, anti-BAF180, or anti-H3. WT = Wilms' tumor; RMS = Rhabdomyosarcoma; MRT = Malignant Rhabdoid Tumor. (B) Densitometry was carried out using Bio-Rad Imagelab 4.1. All values expressed as a ratio to H3. The values were then aggregated by tumor types and averaged. Columns, mean of ratio of each tumor type; Error bars – standard error *,$P < 0.05$ relative to MRT; #, $P > 0.05$ relative to MRT using Student's T-test .

2.4.2 hSNF5 Re-expression in MRT Cell Lines Increases SWI/SNF Complex Component Levels.

We next examined the effects of SNF5 re-expression on SWI/SNF complex members in MRT cell lines. To carry out these experiments, we utilized adenovirus to re-express SNF5 in 3 representative MRT cell lines. We examined SWI/SNF complex component protein expression by Western blotting and mRNA levels by qPCR before and after 24 hours of doxycycline treatment. We focused on the MRT cell lines, G401, TTC549 and TTC642 and omitted the soft tissue derived A204 to maintain our focus on renal-derived MRTs. As shown in Figure 2.3A, we observed a consistent increase in the protein levels of several components as a result of SNF5 re-expression in all MRT cell lines including BAF180 and BAF155. In contrast, BRG1 and BAF200 protein levels did not change significantly after SNF5 reexpression. Other complex members showed differential changes with BAF155 increasing in the G401 and TTC642 cell line and BAF250A showing an increase in the TTC549 cell line. These findings appear consistent with the observations in primary tumors (Figure 2.1C) in which SNF5 negative MRTs demonstrated decreased BAF180 and BAF57 levels but not BRG1 in comparison to WT and RMS. Similar to the results in Figure 2.1 A & B, changes in protein levels did not result from altered mRNA expression (Figure 2.3B), consistent with post-transcriptional regulation.

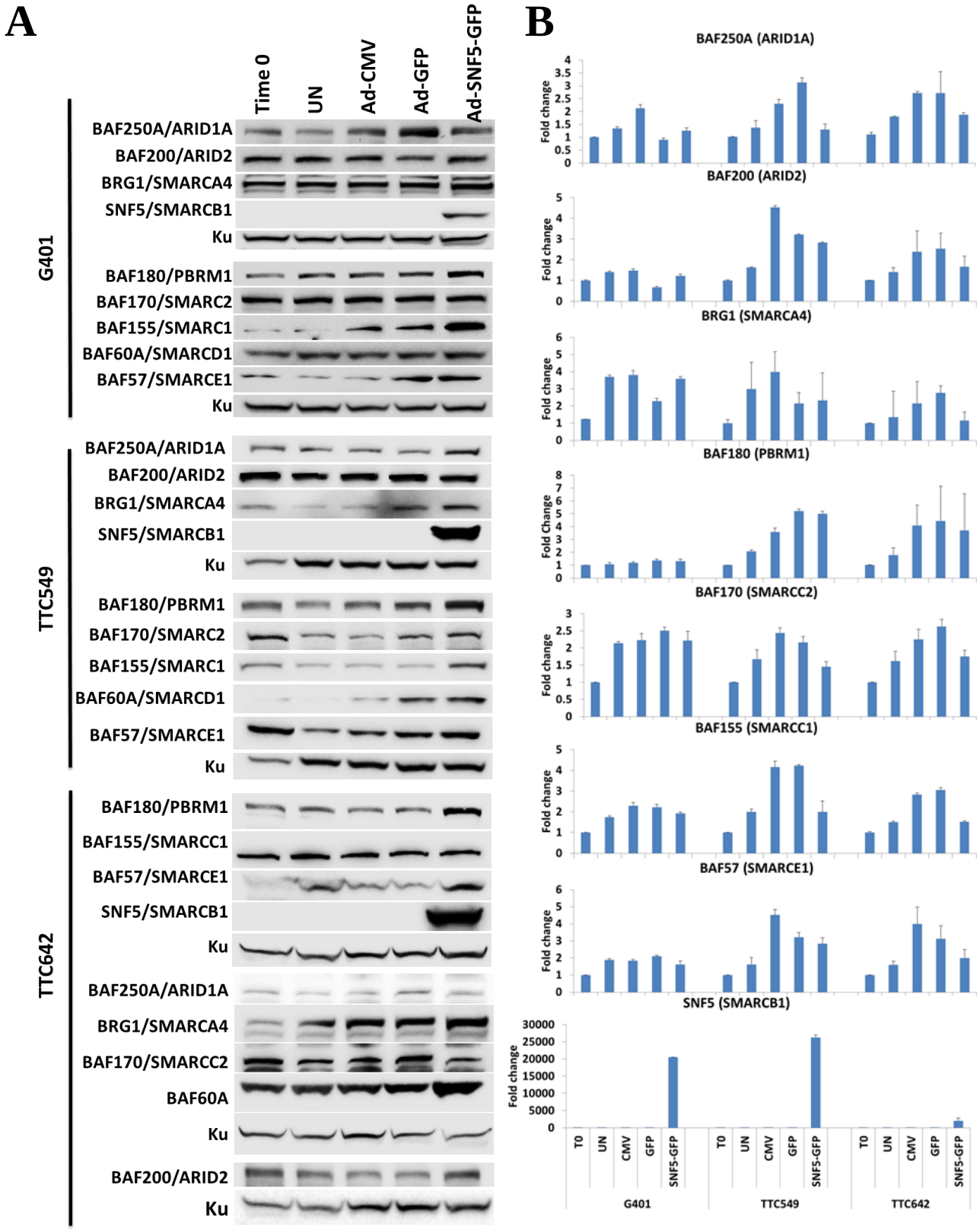

A
Time 0
UN
Ad-CMV
Ad-GFP
Ad-SNF5-GFP
G401
BAF250A/ARID1A
BAF200/ARID2
BRG1/SMARCA4
SNF5/SMARCB1
Ku
BAF180/PBRM1
BAF170/SMARC2
BAF155/SMARC1
BAF60A/SMARCD1
BAF57/SMARCE1
Ku
TTC549
BAF250A/ARID1A
BAF200/ARID2
BRG1/SMARCA4
SNF5/SMARCB1
Ku
BAF180/PBRM1
BAF170/SMARC2
BAF155/SMARC1
BAF60A/SMARCD1
BAF57/SMARCE1
Ku
TTC642
BAF180/PBRM1
BAF155/SMARCC1
BAF57/SMARCE1
SNF5/SMARCB1
Ku
BAF250A/ARID1A
BRG1/SMARCA4
BAF170/SMARCC2
BAF60A
Ku
BAF200/ARID2
Ku
B
BAF250A (ARID1A)
BAF200 (ARID2)
BRG1 (SMARCA4)
BAF180 (PBRM1)
BAF170 (SMARCC2)
BAF155 (SMARCC1)
BAF57 (SMARCE1)
SNF5 (SMARCB1)
Fold change
T0
UN
CMV
GFP
SNF5-GFP
G401
TTC549
TTC642

Figure 2.3 – hSNF5 Re-expression in MRT Cell Lines Increases SWI/SNF Complex Component Levels. (A) Cells were harvested at time 0 and 24 hours after infection with Ad-CMV, Ad-GFP or Ad-SNF5-GFP. Total cellular proteins (30 μg) were separated on a 4% to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with indicated antibody . UN, uninfected control. (B) Cells were harvested at time 0 and 24 hours after infection with Ad-CMV, Ad-GFP or Ad-SNF5-GFP and RNA extracted from the indicated cell lines. The mRNA levels were measured for each gene by qPCR and normalized to β-ACTIN expression at time 0. Columns, mean of four independent experiments; Error bars-standard error.

2.4.3 hSNF5 Expression Increases the Complexity of SWI/SNF Complexes.

Our data thus far demonstrated that global SWI/SNF complex component levels increase following SNF5 over-expression. Next, we determined if this global change is mirrored within the SWI/SNF complex. To carry out these experiments, we developed a tet-inducible SNF5 vector using the pINDUCER system (Meerbrey et al., 2011b) to develop MRT cell lines with inducible SNF5 expression (Figure 2.4). To maximize hSNF5 expression, we first determined the temporal course of induction in the A204.1pIND20-fSNF5-HA cell line as well as a dose response for doxycycline concentration (Supplementary Figure 2.1). Based upon these results, we used 1.0 μg/ml doxycycline for maximum induction of SNF5 for analyses. As shown in Figure 2.4, the tet-inducible cell lines validated the results from the adenovirus infections studies in the previous section.

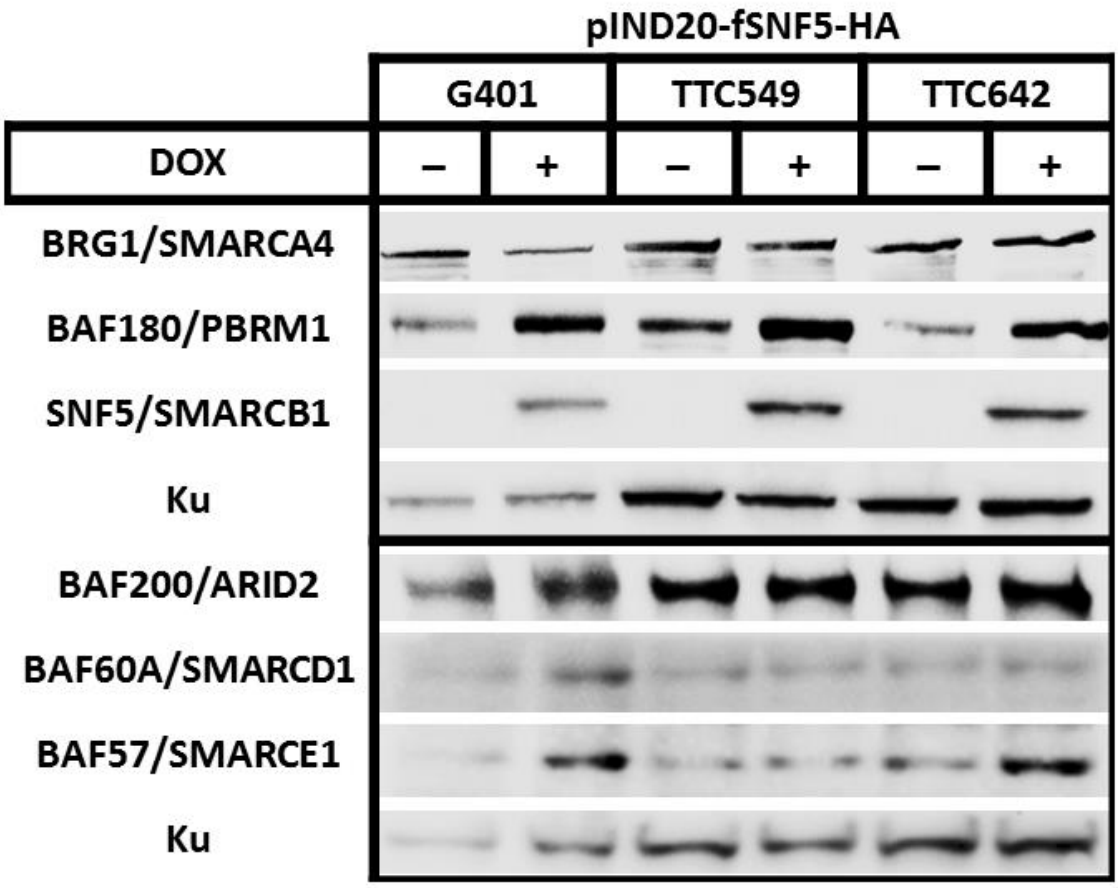


Figure 2.4 – Inducible MRT cell lines. Cells were induced with doxycycline (DOX) at Time 0 and samples were harvest at 24 hours after induction. Total cellular proteins (30 μg) were separated on a 4% to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with indicated antibody. UN, untreated control

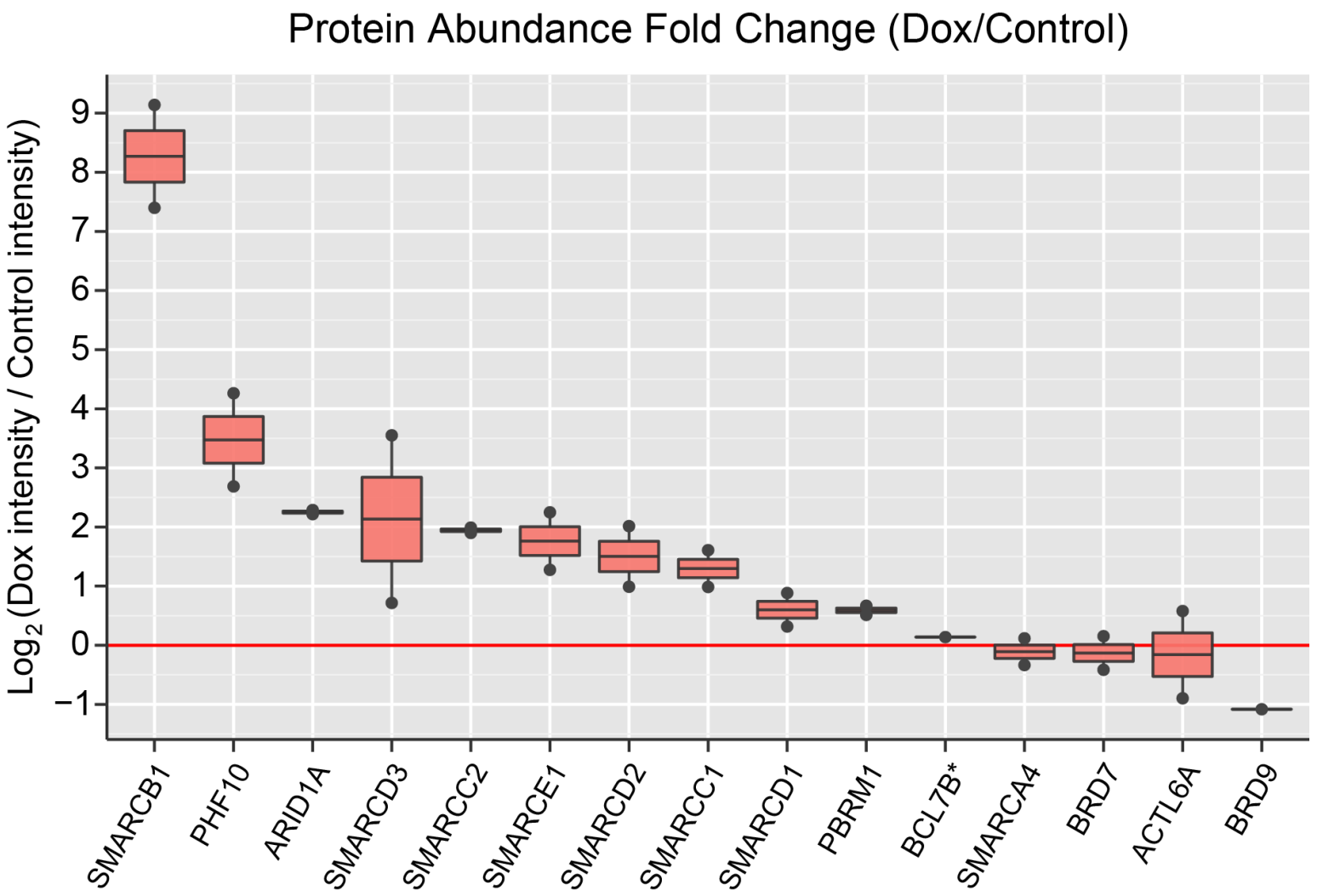
To determine the composition of the SWI/SNF complex in the presence and absence of SNF5, we initially carried out mass spectrometry on the TTC642 cell line. We isolated SWI/SNF complexes from this cell line +/- SNF5 from whole cell lysates by immunoprecipitation with an antibody against BRG1. Because the MRT cell lines cell lines used in this study do not express the mutually exclusive BRM ATPase (Figure 2.1A), functional complexes must possess the BRG1 ATPase. As shown in Figure 2.5A, reexpression of SNF5 resulted in the incorporation of the other components with increased overall expression.

In order to confirm the semi-quantitative mass spectrometry results, we assessed individual component protein levels by Western blotting after immunoprecipitation with our BRG1 antibody. Because BRG1/SMARCA4 levels did not change or are decreased after SNF5 reexpression (Figures 2.3 and 2.4), we used the ratio of complex subunits to BRG1 to measure changes in component levels within the complex. Whole cell protein extracts from MRT cell lines, G401.6, TTC549 and TTC642 cells, either untreated or induced with doxycycline for 24 hours, were harvested, immunoprecipitated with α-IgG or α-BRG1 antibodies and Western blotted to determine the protein levels of BRG1 and other complex members.

While the overall levels of most component proteins increased, BRG1 levels either remained the same or decreased after SNF5 reexpression (compare Input lanes in each cell line in Figure 2.5B). Furthermore, without the confounding issue of viral infection, we found increased expression of BAF57 and BAF170 in all cell lines. As shown in Figure 2.5B, the ratio of most component proteins to BRG1 increased in all MRT cell lines after SNF5 reexpression (Figure 2.5B). Surprisingly, several components, such as BAF250A/ARID1A

and BAF170/SMARCC2 were absent from the complex in the absence of SNF5. Other components showed a differential pattern among the cell lines. For example, the complexes in the TTC549 and TTC642 cell lines contain BAF200/ARID2 after SNF5 reexpression while it is absent in complexes from the G401 cell line. Therefore, the increased component protein levels observed in MRT cell lines after SNF5 reexpression coincides with increased association with SWI/SNF complex members.

A


Protein Abundance Fold Change (Dox/Control)
Log2 (Dox intensity / Control intensity)
9
8
7
6
5
4
3
2
1
0
−1
SMARCB1
PHF10
ARID1A
SMARCD3
SMARCC2
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BRD7
ACTL6A
BRD9

B

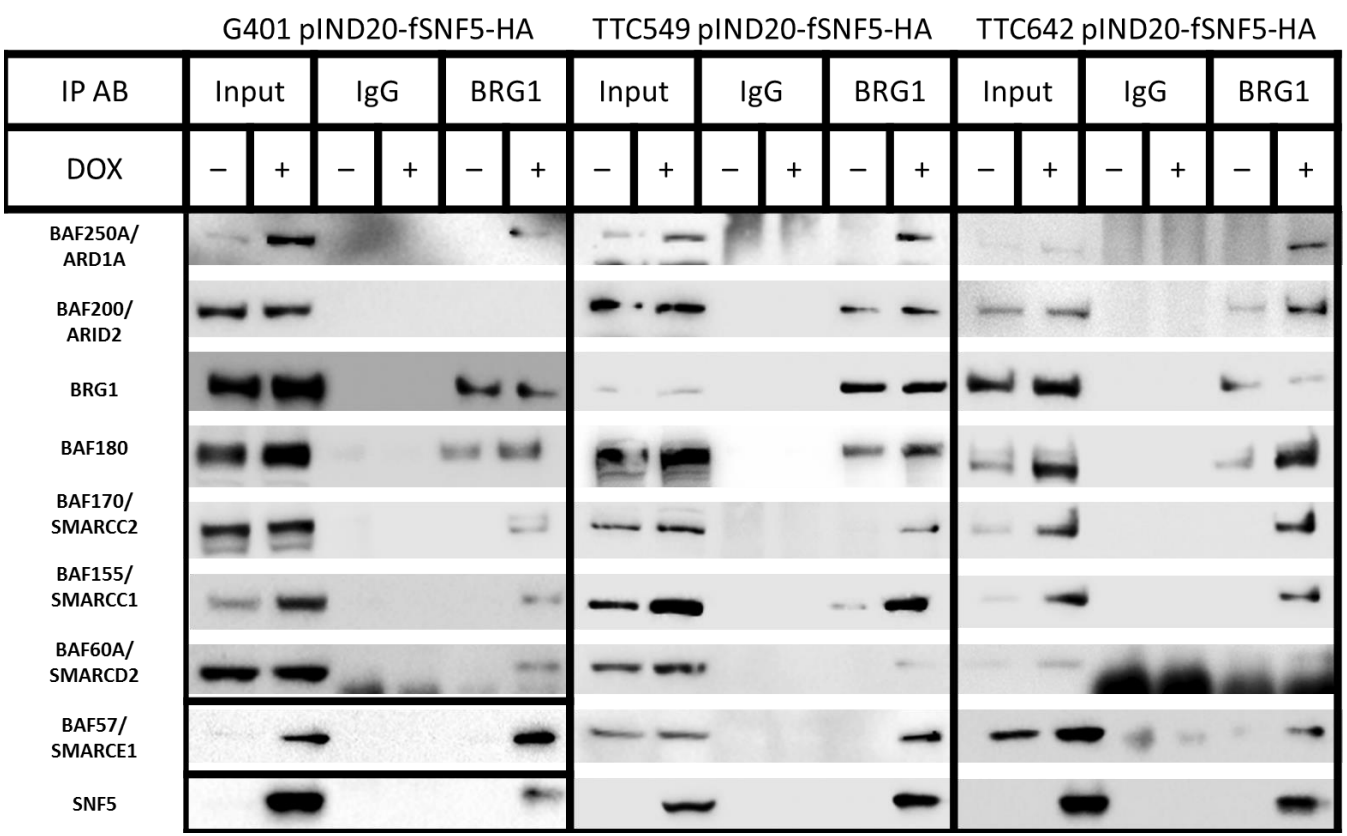

Figure 2.5 –SWI/SNF complex composition changes after SNF5 re-expression. (A) The relative abundance of SWI/SNF complex components in the absence (untreated) and presence (Dox) of SNF5 in the TTC642 cell line was determined by mass spectrometry. Each data point is the average of 2 technical replicates of 2 biological replicates. (B) The indicated cell lines (A-C) were induced with doxycycline, and the samples were immunoprecipitated with either rabbit IgG or anti-BRG1. IP samples were then separated on a 4% to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with the indicated antibodies.

2.3.5 Inhibition of SNF5 Expression in Normal Human Fibroblasts Causes Reduced Complex Component Expression.

While our studies indicated that restoration of SNF5 in MRT cell lines increased expression of SWI/SNF complex components, they did not address whether SNF5 loss in normal cells would lead to decreased protein levels. To address this issue, we infected normal human fibroblasts (NHF-1) with 2 different shRNAs against SNF5 and assessed component mRNA and protein levels. Because the cell of origin for MRTs remains unknown, we used NHFs as a well-characterized normal human cell line model. As shown in Figure 2.6A, we achieved an approximately 80% decrease in SNF5 expression 48 hours after lentiviral infection with 2 different shRNAs. We observed a similar decrease in BAF180 protein levels only after expression of the shRNA against SNF5. The non-targeting shRNA control, pLKO.1, did not show any effect. However, similar to our results with the MRT cell lines, the change in component protein levels did not mirror its mRNA expression (Figure 2.6B). We also observed an increase in p21 protein levels with either the non-targeting control or shSNF5 expression suggesting a stress response after lentiviral infection. However, using β-galactosidase staining, we observed senescence only in the cells with SNF5 knockdown (Figure 2.6C).

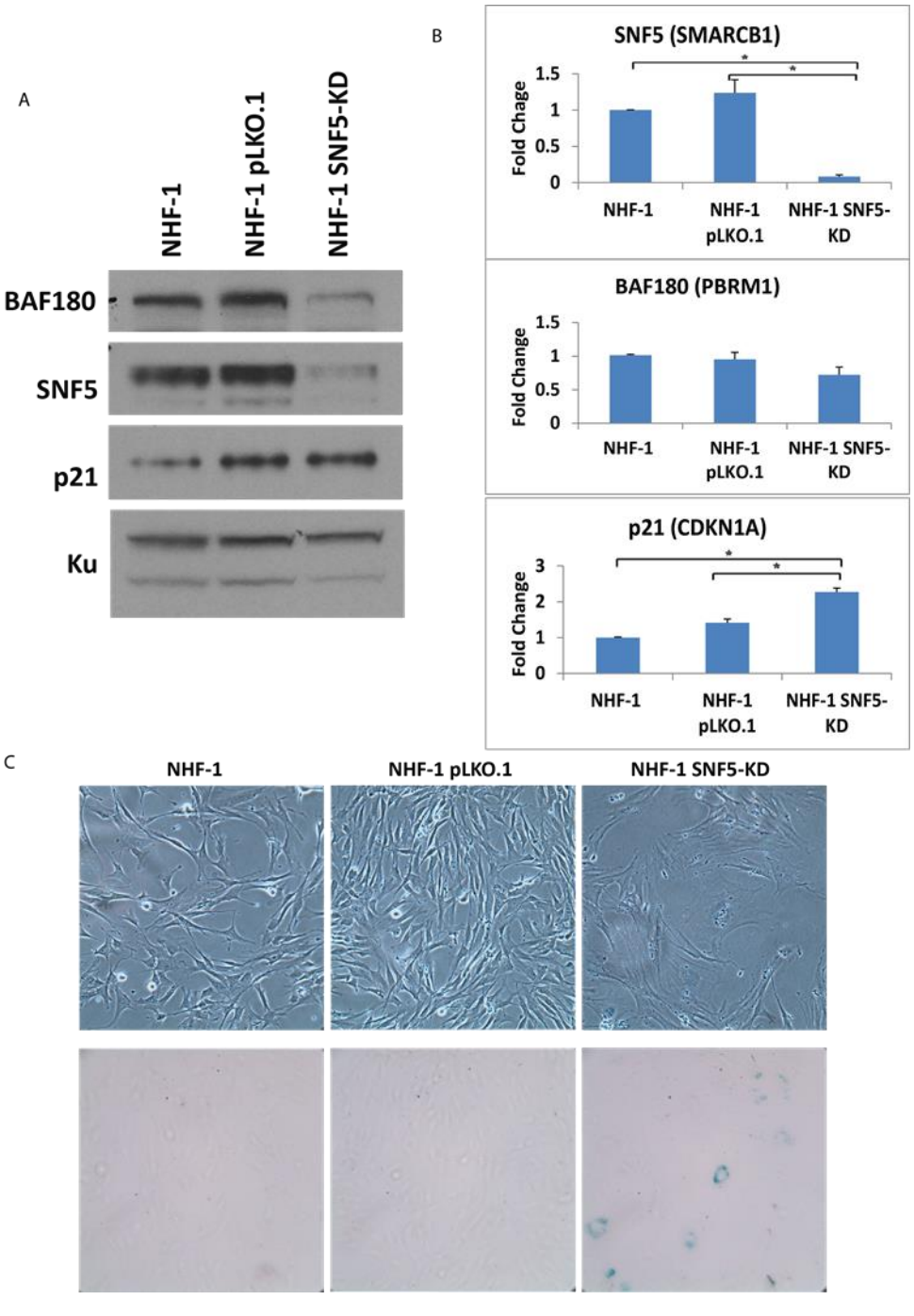
A
NHF-1
NHF-1 pLKO.1
NHF-1 SNF5-KD
BAF180
SNF5
p21
Ku
B
SNF5 (SMARCB1)
Fold Chage
1.5
1
0.5
0
NHF-1
NHF-1 pLKO.1
NHF-1 SNF5-KD
BAF180 (PBRM1)
Fold Change
p21 (CDKN1A)
3
2
C
NHF-1
NHF-1 pLKO.1
NHF-1 SNF5-KD

Figure 2.6 – Reduction of BAF180 protein levels after inhibition of SNF5 in normal human fibroblasts. (A)SNF5 knockdown cells were generated by infecting normal human fibroblasts (NHF-1) with lentivirus expressing 2 different shRNAs against SNF5 (TRCN 39585 and TRCN 39587) or a non-targeting shRNA control (pLKO.1) and immediate selection with puromycin. After 72 hrs. on selective medium, total cellular protein (30 μg), separated on a 4% to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with anti-SNF5, anti-BAF180, or anti-KU70/80 antibodies.(B) RNA was extracted from the SNF5 knockdown cells generated as in part A. The mRNA levels were measured for each gene by qPCR and normalized to β-ACTIN expression at time 0. Columns, mean of four independent experiments; Error bars – standard error. (C) Knockdown of SNF5 in NHF-1 cells results in senescence. SNF5 knockdown cells were generated by infecting normal human fibroblasts (NHF-1) with lentivirus expressing 2 different shRNAs against SNF5 (TRCN 39585 and TRCN 39587) and immediate selection with puromycin. After 72 hrs. on selective medium, the cells were stained using a senescence β-Galactosidase cell staining protocol (Cell Signaling).

2.3.6 SWI/SNF complex components are degraded in a proteasome-independent mechanism

The levels of many cellular proteins are regulated through ubiquitination and subsequent degradation via the proteasome machinery (Asher et al., 2006). To confirm a posttranscriptional change in component levels in the MRT cell lines, we examined the effects of MG132, a potent proteasome inhibitor, on protein expression in TTC642 cell lines (Lee and Goldberg, 1998).

We treated the cell lines with DMSO or 10uM MG132. After 6 hrs., we assessed representative SWI/SNF complex members as well as c-FOS (positive control) and RAN (negative control/loading control) protein levels by Western blotting. As shown in Figure 2.7A, MG132 treatment either increased expression (BAF180) or augmented the increase after SNF5 re-expression (BAF250A and SNF5).

We then asked whether MG132 treatment would restore BAF250A and BAf180 expression after SNF5 knockdown in NHF-1 cells. Figure 2.7B shows that exposure to MG132 increased BAF180 levels after SNF5 knockdown by 2 different shRNAs. However, BAF250A levels were unaffected. The pLKO.1 lentivirus served as a negative control. These results implicate proteasome-dependent mechanism in lowering some component protein levels in the obscene of SNF5 in MRTs..

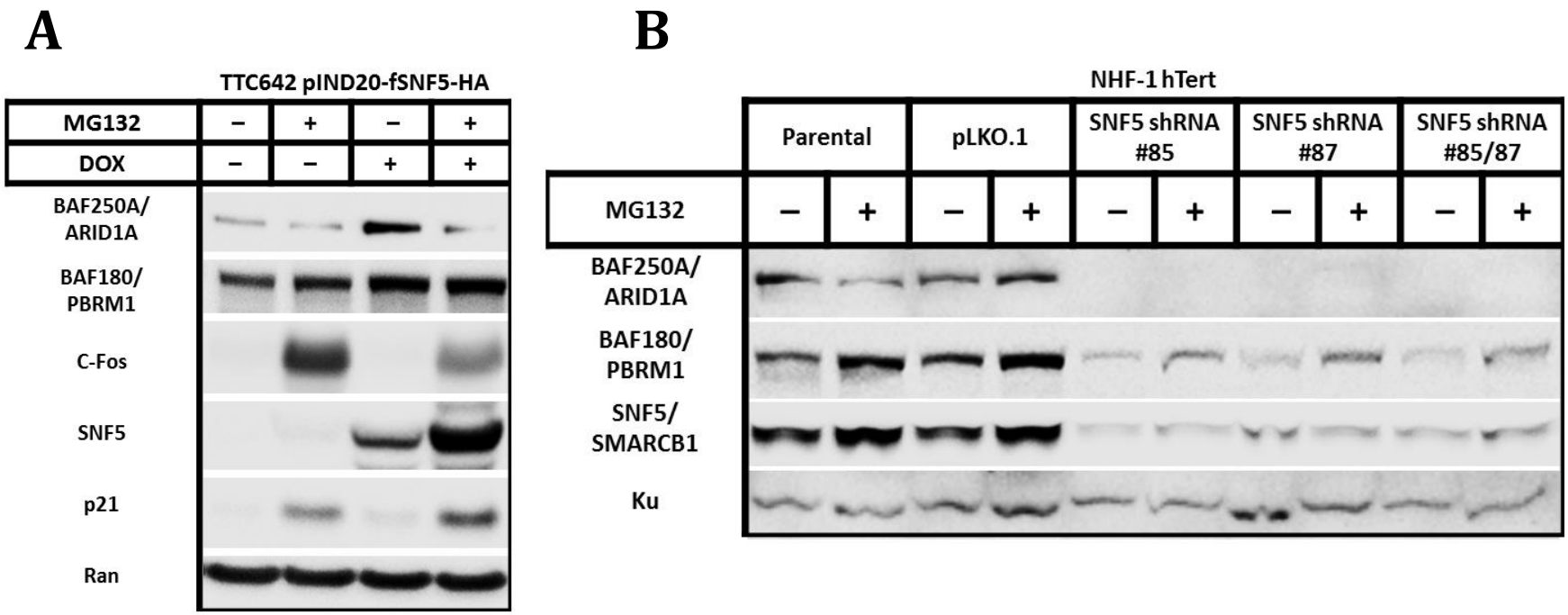

Figure 2.7 – SWI/SNF complex components are degraded in a proteasome-independent mechanism. (A) The TTC642 pINDUCER20-fSNF5-HA cell line, +/- SNF5 expression, were treated with DMSO or 10uM MG132 for 6 hrs. After protein isolation, protein levels for BAF180/PBRM1, BAf250A/ARID1A, c-ROS and RAN were detected by SDS-PAGE and Western blotting as previously described (Chai et al., 2005a). c-FOS served as a positive control for inhibition while Ran, unaffected by MG132 treatment, served as a loading control (B) NHF-1 hTert cells were infected with indicated lentivirus and placed in selective media for 3 days. Then the samples were treated with MG132 for 6 hours before harvesting. After protein isolation, protein levels for BAF180/PBRM1, BAF250/ARID1A, SNF5/SMARCB1 and Ku were detected by SDS-PAGE and Western blotting.

2.4 Discussion

Previous reports have shown the effects of loss of individual SWI/SNF complex members upon the organization and stability of the remaining complex components. For example, studies by Chen and Archer posited that BAF155 serves as a scaffolding element required for stability of other SWI/SNF complex including BRG1 (Sohn et al., 2007) and BAF57 (Chen and Archer, 2005). Another report demonstrated that ARID2/BAF200 regulated BAF180 expression at the level of mRNA expression (Yan et al., 2005). Other reports have shown increased expression of BRM upon loss of BRG1 expression (Cohen et al., 2010; Reyes et al., 1998; Wang et al., 2009). Recent evidence has also shown decreased BAF57 and SNF5 levels in BAF155 deficient cells lines (DelBove et al., 2011). Only a few studies have addressed the effects on complex formation in the absence of different complex members (Doan et al., 2004). Doan et al,. previously showed assembly of the complex in the absence of SNF5 with no effect on expression of BRG1-dependent genes (Doan et al., 2004). Our data demonstrate increases in SWI/SNF complex members binding to the complex after SNF5 ectopic expression, implying that SNF5 plays a role in the stability of the complex members but not the corresponding mRNA. This role does not contradict prior research but further expands the functions of SNF5 within the SWI/SNF complex.

We have previously shown that SNF5 reexpression in MRT cell lines causes increased $p21^{WAF1/CIP1}$ expression associated with preferential recruitment of SNF5 to the p21 TSS (Kuwahara et al., 2013a). This information, in the context of our data, suggests that the constant presence of BRG1 containing complexes lacking SNF5 along the p21 promoter maintains transcription but cannot remodel nucleosomes at the TSS. This obstacle could

prevent the elongation of RNA polymerase II resulting in promoter pausing and minimal p21 expression (Brown et al., 1996; Neely et al., 1999). Similar effects have been observed for the regulation of developmental genes in *Drosophila* (Zraly and Dingwall, 2012; Zraly et al., 2006). SNF5 reexpression could stabilize the SWI/SNF complex resulting in targeting to TSSs and increased gene transcription. Alternatively, our results could support a model where SNF5-deficient complexes are already recruited to TSSs and maintain a basal level of gene expression. However, gene expression levels remain low due to the short half-life of most complex members in the absence of SNF5 or the type of SWI/SNF complex present at the promoter. SNF5 expression would stabilize the complex or recruit a different type of SWI/SNF complex causing increased gene expression.

Our finding that SWI/SNF complex components are degraded in a proteasome-dependent manner appears consistent with previous reports indicating their instability in the absence of other complex members (Chen and Archer, 2005; Sohn et al., 2007). However, SNF5 loss appears to induce degradation of many complex members, in contrast to the limited numbers affected by loss of other complex components (Chen and Archer, 2005; Sohn et al., 2007; Yan, 2005). The identity of the proteasome pathway responsible for the degradation of complex components remains unknown. However, our observations appear consistent with previous results that cells maintain tight control over the protein levels of SWI/SNF complex members (Bourgo et al., 2009; Cohen et al., 2010; Guidi et al., 2006).

The mechanisms by SNF5 loss initiates MRT development remain unresolved. Recent reports have identified at least 9 different forms of the SWI/SNF complex, based upon protein composition, that promote diverse biological functions including growth and differentiation (Lessard et al., 2007; Wei and Weissman, 2014). Our current study implicates

changes in SWI/SNF complex composition after SNF5 inactivation as a mechanism for MRT development. This makes an attractive model because it accounts for several hallmarks of this cancer. Presumably, the transition from a growth promoter configuration of the SWI/SNF complex to a differentiating inducing one occurs within a narrow window of development. Therefore, SNF5 loss would only exert an effect if it happened within this time frame. This strict requirement for timing could account for the relative paucity of these tumors. Second, if MRTs arise from retention of growth promoting complexes, affecting gene expression, one would expect little genomic instability in these tumors. In agreement with this notion, a recent report from Lee et al. demonstrates a lack of significant changes in MRTs(Lee et al., 2012).

The changes in gene expression observed after SNF5 re-expression in MRT cell lines or its inactivation in normal cells may arise from differential binding of the SWI/SNF complexes present under each condition (Isakoff et al., 2005; Morozov et al., 2007). Therefore, future ChIP-seq experiments should identify additional unique and mutual binding sites for SWI/SNF complex members with and without SNF5. These data, in conjunction with gene expression analyses, will allow for further insights into the role of SWI/SNF complex activities after SNF5 inactivation in MRT development. It will also be important to investigate the activities of SWI/SNF complexes that do form in the absence of SNF5. These complexes may account for the observation of Roberts and colleagues that lymphoma development after SNF5 inactivation requires BRG1 expression (Wang et al., 2009).

The existing protocols for treatment of MRT include tumor resection, followed by adjuvant chemotherapy and/or radiation (Biswas et al., 2009). These current protocols suffer from several inadequacies, including the difficulty of resection due to the tumor size and the

contraindication of radiation in young patients (Biswas et al., 2009). Understanding the molecular mechanisms behind MRT development and growth will allow for improved patient treatment and survival rate. For example, the posttranscriptional regulation of SWI/SNF complex components suggests that they can be potential targets for therapeutic intervention. Further screening of inhibitors of the 20S proteasome could prove fruitful in finding a drug candidate to stabilize the complex in the absence of SNF5. Additionally, identifying the pathways regulated by the SWI/SNF complexes present in the absence of SNF5 should reveal additional therapeutic targets. Furthermore, our results, showing that BAF180 and BAF250 stability inextricably depends upon SNF5's presence, suggests a potential mechanistic link between SNF5 loss in MRT and BAF180 and BAF250A losses in renal cell carcinoma and ovarian carcinomas, respectively. Future studies will further our understanding of chromatin remodeling functions and provide knowledge for translation into the clinic for improving the outcomes of patients with MRTs and other cancers with SWI/SNF complex mutations.

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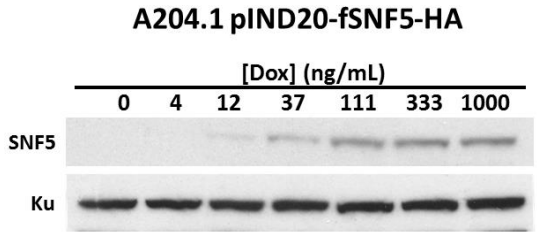
2.5 Supplementary Figures



Supplementary Figure 2.1 – A204.1 pIND20-fSNF5-HA Time Course. The A204.1 pINDUCER20-fSNF5-HA- with the indicated amount of doxycycline for 24 hrs. After protein isolation, protein levels for SNF5 and Ku were detected by SDS-PAGE and Western blotting as previously described (Chai et al., 2005a).

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Chapter 3: hSNF5 reexpression in malignant rhabdoid tumors regulates the transcription of a subset of p53 target genes by recruitment of SWI/SNF complexes and RNAPII to their promoters.[3]

3.1 Summary

Malignant rhabdoid tumor (MRT), a highly aggressive cancer of young children, displays inactivation or loss of the *hSNF5/INI1/SMARCB1* gene, a core subunit of the SWI/SNF chromatin-remodeling complex, in primary tumors and cell lines. We have previously reported that reexpression of hSNF5 in some MRT cell lines causes a G_1 arrest via $p21^{CIP1/WAF1}$ (*p21*) mRNA induction in a p53-independent manner. However, the mechanism(s) by which hSNF5 reexpression activates gene transcription remains unclear. We initially searched for other hSNF5 target genes by asking whether hSNF5 loss altered regulation of other consensus p53 target genes. Our studies show that hSNF5 regulates only a subset of p53 target genes, including *p21* and *NOXA*, in MRT cell lines. We also show that hSNF5 reexpression modulates SWI/SNF complex levels at the transcription start site (TSS) at both loci and leads to activation of transcription initiation through recruitment of RNA polymerase II (RNAPII) accompanied by H3K4 and H3K36 modifications. Furthermore, our results show lower NOXA expression in MRT cell lines compared with other human tumor cell lines, suggesting that hSNF5 loss may alter the expression of this important apoptotic

[3] This chapter appeared as an article in the journal Molecular Cancer Research. The citation is as follows: Kuwahara Y, Wei D, Durand J, Weissman BE., SNF5 reexpression in malignant rhabdoid tumors regulates transcription of target genes by recruitment of SWI/SNF complexes and RNAPII to the transcription start site of their promoters. Mol Cancer Res. 2013 Jan 30

gene. Thus, one mechanism for MRT development after hSNF5 loss may rely on reduced chromatin-remodeling activity of the SWI/SNF complex at the TSS of critical gene promoters. Furthermore, because we observe growth inhibition after NOXA expression in MRT cells, the NOXA pathway may provide a novel target with clinical relevancy for treatment of this aggressive disease.

3.2 Introduction

Malignant rhabdoid tumor is a rare and extremely aggressive childhood cancer, originally described as an unfavorable histological variant of the pediatric renal Wilms' tumor (Beckwith and Palmer, 1978). The most common locations are in the kidney and central nervous system, although MRTs may arise in almost any site (Biegel et al., 2002b; Hoot et al., 2004). Despite significant advances in treatment, for MRTs diagnosed before the age of 6 months, patient survival at 4 years drops to ~8.8% (Tomlinson et al., 2005b). Therefore, improved patient outcome requires a better understanding of malignant rhabdoid tumorigenesis and the development of novel therapeutic strategies. The common genetic alteration in MRTs is the inactivation of *hSNF5*, located in chromosome band 22q11.2 (Versteege et al., 1998b), that implicates the loss of hSNF5 function as the primary cause of these tumors (Biegel et al., 2002a). Therefore, the elucidation of hSNF5 function should lead to the identification of the key molecular steps necessary for MRT tumorigenesis.

hSNF5 is a component of the SWI/SNF chromatin remodeling complex. SWI/SNF complexes are ATP-dependent chromatin remodeling complexes that regulate gene transcription by causing conformational changes in chromatin structure (Wilson and Roberts). SWI/SNF subunits can be subclassified into three categories: (1) ATPase subunit (either BRG1 or BRM), (2) core subunits (hSNF5, BAF155 and BAF170), (3) accessory

subunits (BAF53, BAF57, BAF180 and so on) (Weissman and Knudsen, 2009). How loss of one of the core subunits leads to the development of a rare pediatric cancer remains one of the most challenging questions in the cancer epigenetic field.

Our previous study demonstrated that while hSNF5 reexpression in MRT cells increases both $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ expression during the induction of G_1 cell cycle arrest, $p21^{CIP1/WAF1}$ up-regulation precedes $p16^{INK4A}$. Furthermore, we demonstrated that $p21^{CIP1/WAF1}$ transcription shows both p53 dependent and independent mechanisms of induction after hSNF5 reexpression. hSNF5 was confirmed to bind to the $p21^{CIP1/WAF1}$ promoter by ChIP (chromatin immunoprecipitation) analysis (Kuwahara et al., 2010). However, little is known about whether hSNF5 generally regulates p53 target genes or how hSNF5 activates transcription at its target promoters. This question seems especially important because mutations that inactivate the p53 pathway in MRTs rarely appear (Rosson et al., 1998; Xu et al., 2012).

Therefore, in this study, we assessed whether hSNF5 regulates the transcription of representative p53 target genes by Q-RT-PCR and Western blotting. We then clarified how hSNF5 regulates its target genes by performing ChIP assays for hSNF5, histone modifications and SWI/SNF complexes. Our results show that hSNF5 can regulate a subset of p53 target genes either through its modulation of SWI/SNF complex activity or recruitment of complementary transcriptional factors. We also establish the NOXA/PMAIP1 gene as a downstream target of hSNF5 that may provide a new therapeutic target for treatment of MRTs.

3.3 Materials and Methods

3.3.1 Cell culture and adenovirus infection.

MCF7, A204.1, G401.6, TTC642, TM87-16 and TTC549 cells were cultured in RPMI 1640 containing 10% fetal bovine serum. The MCF7, A204 and G401 cell lines came from the American Type Tissue Collection and were used within 6 months of receipt or from frozen stocks within a similar timeframe. The remaining 3 cell lines came directly from their originator, Dr. Timothy Triche of the Children's Hospital of Los Angeles. Similar time limitations were also used to maintain the identities of these cell lines. The Ad/pAdEasyGFPINI-SV+ adenoviral vectors expressing hSNF5 and co-expressing the green fluorescent protein (GFP) (designated Ad-hSNF5) and the Ad/pAdEasyGFP expressing GFP (designated Ad-GFP) were previously published (Chai et al., 2005; Kuwahara et al., 2010; Reincke et al., 2003). In order to achieve infection of over 90% cells, we infected at a multiplicity of infection (M.O.I.) of 10 for the G401.6TG cell line, 20 for the A204.1 and TTC549 cell line, 80 for the TM87-16 cell line and 200 for the TTC642 cell line.

3.3.2 Protein extracts and Western blotting.

Western blotting was carried out as described previously (Chai et al., 2005). Western analyses of proteins were carried out by using anti-NOXA (OP180 ; Calbiochem), anti-hSNF5 (612110, BD-Transduction Laboratories) anti-actin (A2066; Sigma) and horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (GE Healthcare).

3.3.3 RNA extraction and Quantitative real-time reverse transcription-PCR analysis.

RNA was extracted using the RNeasy mini kit (Qiagen), and 1 μg was used for cDNA synthesis primed with Random Primers (Invitrogen). cDNA was analyzed using TaqMan

(Applied Biosystems) quantitative real-time reverse transcription-PCR (QT-PCR) analysis with beta-actin as the reference gene in each reaction. Reactions were performed on an ABI 7900 HT sequence detection system (Applied Biosystems) and relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method (Donner et al., 2007; Livak and Schmittgen, 2001b). The primers used The TaqMan gene expression assay primer/probe set used in this study is described in Table. 3.1.

Table 3.1 – TaqMan gene expression primer/probes

Gene symbol	**Gene name**	**Assay ID**
ACTB	Actin, β	Hs99999903_m1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Hs00355782_m1
TP53	Tumor protein p53	Hs01034249_m1
BBC3	BCL2-binding component 3 (PUMA)	Hs00248075_m1
BAX	BCL2-associated X protein	Hs00180269_m1
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1(NOXA)	Hs00560402_m1
SFN	Stratifin (14-3-3σ)	Hs00968567_s1
MDM2	Mdm2 p53-binding protein homolog	Hs01066930_m1
GADD45B	Growth arrest and DNA-damage-inducible, β	Hs00169587_m1

3.3.4 Chromatin immunoprecipitation.

Chromatin immunoprecipitation (ChIP) was carried out as described by Donner et al (Donner et al., 2007). Immunoprecipitation was performed with an antibody specific to hSNF (Dr. Tony Imbalzano), histone H3 trimethylation of lysine 4 (H3K4 me3) (39159 ; Active Motif), BRG-1 (J1; Dr. Weidong Wang), BAF155 (sc-10756 ; Santa Cruz), RNA polymerase II (8WG16 ; Covance), histone H3 trimethylation of lysine 4 (H3K36 me3) (ab9050 ; abcam), normal Rabbit IgG (sc-2027; Santa Cruz Biotechnology), normal mouse IgG (sc-2025; Santa Cruz Biotechnology), histone H3 C-terminal (39163 ; Active Motif) or p53 (DO-1; Calbiochem). DNA present in each IP was quantified by QT-PCR using gene-specific primers on an ABI 7000 sequence detection system. All expression values were normalized against input DNA or histone H3. PCR primer sequences are shown in Table 3.2(Donner et al., 2007).

Table 3.2 – PCR primer sequences for ChIP assays

Gene	Site	Forward primer	Reverse primer
NOXA	−4,578 bp	GGT TGG TGT GAT TGC TTG GCC G	AGG GCT GCC TGG GAG AGC AA
	−769 bp	ACT CAT GGC CTC GCC AAA CAT T	AGG GCT GAG CTA CCT GGG AAC G
	−158 bp	GCG GGT CGG GAG CGT GTC	AGA CGG CGT TAT GGG AGC GGA
	43 bp	CGG GCC GGG CGT CTA GTT TC	CGC GCC AGA GAC CAC GCT TT
	104 bp	CCC TGC CTG CAG GAC TGT TCG	CCC GGG AAC CTC AGC CTC CA
	874 bp	AGT TTT CAG GCC AGC GCC CC	GGC CCA CAC AGA CTT CGG GC
	2,573 bp	AGA GCT GGA AGT CGA GTG TGC T	TGC CGG AAG TTC AGT TTG TCT CCA
**p21*$^{CIP1/WAF1}$	−3,000 bp	CCGGCCAGTATATATTTTTAATTGAGA	AGTGGTTAGTAATTTTCAGTTTGCTCAT
	−2,283 bp	AGCAGGCTGTGGCTCTGATT	CAAAATAGCCACCAGCCTCTTCT
	−1,391 bp	CTGTCCTCCCCGAGGTCA	ACATCTCAGGCTGCTCAGAGTCT
	−20 bp	TATATCAGGGCCGCGCTG	GGCTCCACAAGGAACTGACTTC
	182 bp	CGTGTTCGCGGGTGTGT	CATTCACCTGCCGCAGAAA
	507 bp	CCAGGAAGGGCGAGGAAA	GGGACCGATCCTAGACGAACTT
	2,786 bp	GCACCATCCTGGACTCAAGTAGT	CGGTTACTTGGGAGGCTGAA
	4,001 bp	AGTCACTCAGCCCTGGAGTCAA	GGAGAGTGAGTTTGCCCATGA

Note: *p21 primers were based on Donner AJ, Szostek S, Hoover JM, Espinosa JM. CDK8 is a stimulus-specific positive coregulator of p53 target genes. Mol Cell 2007;27:121

3.4 Results

3.4.1 The effects of reexpression of hSNF5 on p53 target genes in MRT cell lines.

To determine the role of hSNF5 in expression of p53 target genes, we analyzed the change of mRNA levels using Q-RT-PCR in 3 MRT cell lines, A204.1, TTC642 and TTC549, at 24 hrs after infection with Ad-hSNF5 and Ad-GFP (negative control) adenoviruses (Kuwahara et al, 2010). Reexpression of hSNF5 differentially modified expression of subsets of p53 target genes among the MRT cell lines (Figure 3.1). All 3 cell lines showed modest to robust induction of $p21^{CIP1/WAF1}$ expression and decreased expression of 14-3-3σ. However, expression of pro-apoptotic genes varied. While the A204.1 cell line showed induction of both PUMA and BAX expression, the other 2 cell lines demonstrated only NOXA expression (Figure 3.1). Expression of other p53 regulated genes also diverged among the lines with increased MDM2 expression in A204.1 and higher GADD45b expression in TTC642. These results suggest that hSNF5does not regulate the consensus repertoire of p53 target genes in MRT cell lines.

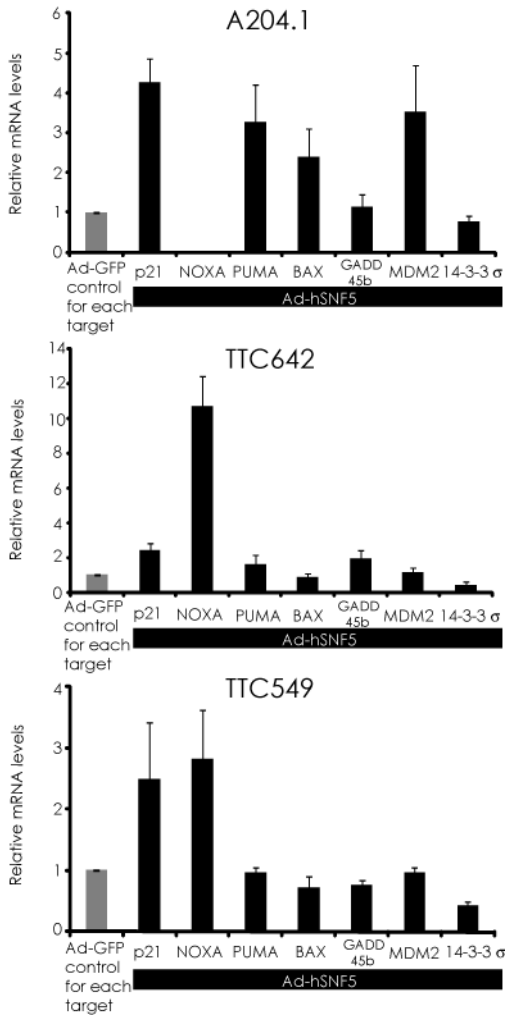


Figure 3.1 – hSNF5-induced p53 target genes' expression. hSNF5-induced p53 target genes' expression. RNA was extracted at 24 hours after infection with Ad-hSNF5 and Ad-GFP. The mRNA levels were measured for each gene by real-time qRT-PCR and normalized for β-actin expression and relative to the Ad-GFP for each genes. Values are the mean of 3 independent experiments; bars, ±SD.

3.4.2 hSNF5-induced NOXA mRNA and protein expression in MRT cell lines.

Intriguingly, our Q-RT-PCR analysis showed that hSNF5 reexpression caused an increase in NOXA mRNA in the TTC642 and TTC549 cell lines. This result suggested that NOXA might be a common downstream target of the hSNF5 protein in MRT cell lines. To test this notion, we examined NOXA mRNA levels by Q-RT-PCR in 2 additional MRT cell lines (TM87-16 and G401.6TG) after hSNF5 reexpression. We found the level of *NOXA* mRNA increased 24 hours after Ad-hSNF5 infection in comparison with Ad-GFP infection in the TM87-16 MRT cell line followed by a marked increase at 48 hours after infection. In the G401.6TG cell line, although basal expression on NOXA mRNA is not detected, reexpression of hSNF5 induced NOXA mRNA within 24 hrs (Figure 3.2A). We next evaluated the basal NOXA mRNA expression level in all MRT cell lines by Q-RT-PCR. We used six MRT cell lines as well as the MCF7 human breast carcinoma cell line. We found significantly lower levels of NOXA mRNA in the MRT cell lines compared to MCF7 except for the TM87-16 cell line with no NOXA mRNA expression in the A204.1 and G401.6TG cell lines (Figure 3.2B). Furthermore, we tested whether NOXA protein levels increased after Ad-hSNF5 infection in comparison with Ad-GFP infection. We observed that the level of NOXA protein increased at 24 hours after Ad-hSNF5 infection in comparison with Ad-GFP infection in two MRT cell lines (TTC642 and TTC549), followed by a marked increase at 48 hours after infection (Figure 3.3).

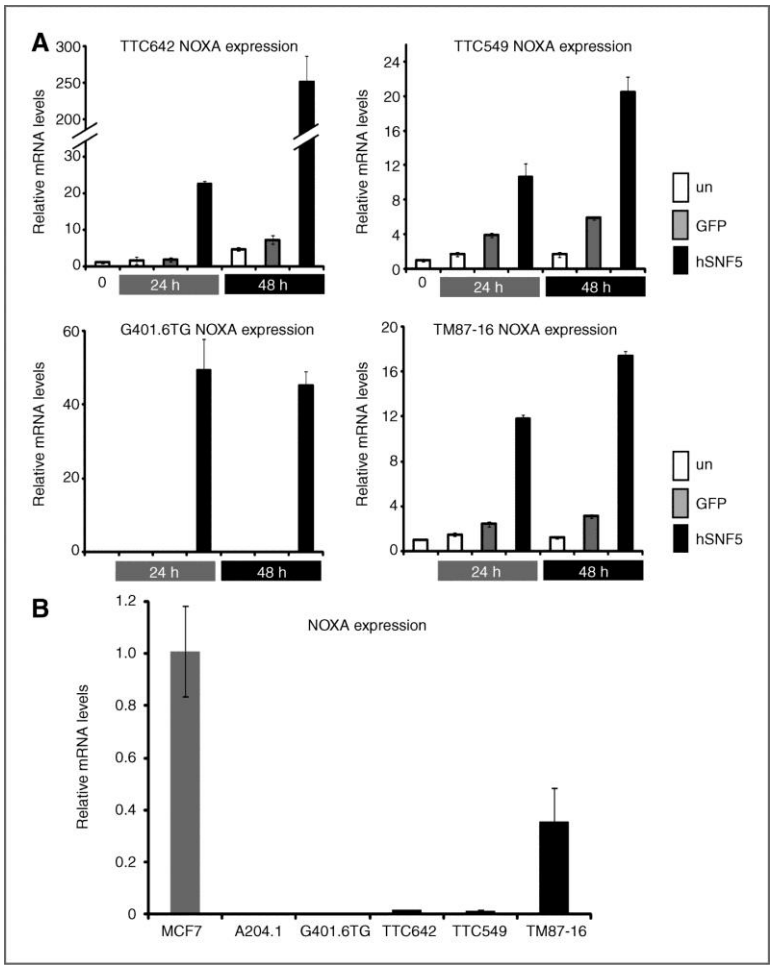


Figure 3.2 – hSNF5-induced NOXA mRNA expression.(A) RNA was extracted at the indicated times after infection with Ad-hSNF5 and Ad-GFP. The mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Values are the mean of three independent experiments; bars, ± SD. un; uninfected control.(B) *NOXA* mRNA expression in 6 MRT cell lines (A204.1, TTC642, G401.6TG, TTC549, TM87-16, TTC549) by Q-RT-PCR. The MCF7 cell line was used as a control. The NOXA mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Values are the mean of three independent experiments; bars, ± SD.

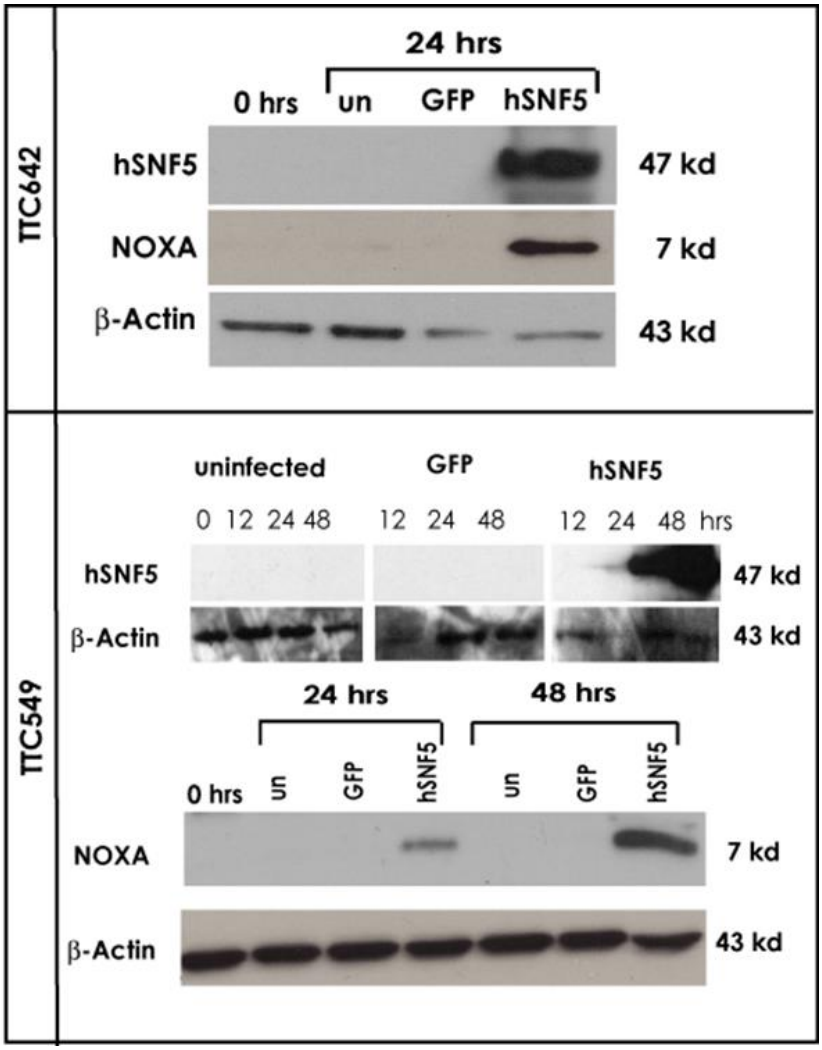


Figure 3.3 – hSNF5-induced NOXA protein expression. Cells were harvested at the indicated times after infection with Ad-hSNF5 and Ad-GFP. Total cell protein (30 μg) were separated on a 4-20% SDS-polyacrylamide gel and probed with either anti-SNF5, anti-β-actin or anti-NOXA. un; uninfected control.

3.4.3 Recruitment of hSNF5 on NOXA locus correlates with NOXA transcription in MRT cell lines.

We analyzed the chromatin status at *NOXA* promoter in both TTC642 and TTC549 cell, at 24 hours after Ad-hSNF5 infection to clarify the mechanism of *NOXA* activation by hSNF5. To analyze the recruitment of hSNF5 and other factors associated with gene transcription, we made sets of primers for the NOXA promoter regions from - 4758 to +2573 (Figure 3.4A). Our ChIP data confirmed that hSNF5 bound within 1 kb of the transcription start site (TSS), with maximal enrichment at the TSS in both cell lines (Figure 3.4B). Furthermore, a modest increase of BRG1 and BAF155 (~2X) also appeared across the promoter region after hSNF5 induction of NOXA expression in the TTC642 cell line (Figure 3.4c). We next determined the effect of hSNF5 reexpression on the H3K4me3, a chromatin mark associated with gene activation. H3K4me3 increased after hSNF5 reexpression at TSS, with the maximal peak near the TSS in both the TTC642 (Figure 3.4C). These results showed that lower levels of NOXA mRNA expression in MRT cell lines correlates with the absence of hSNF5 expression. This finding suggests that loss of hSNF5 expression might lead to an epigenetic modification at the NOXA promoter altering transcriptional activity.

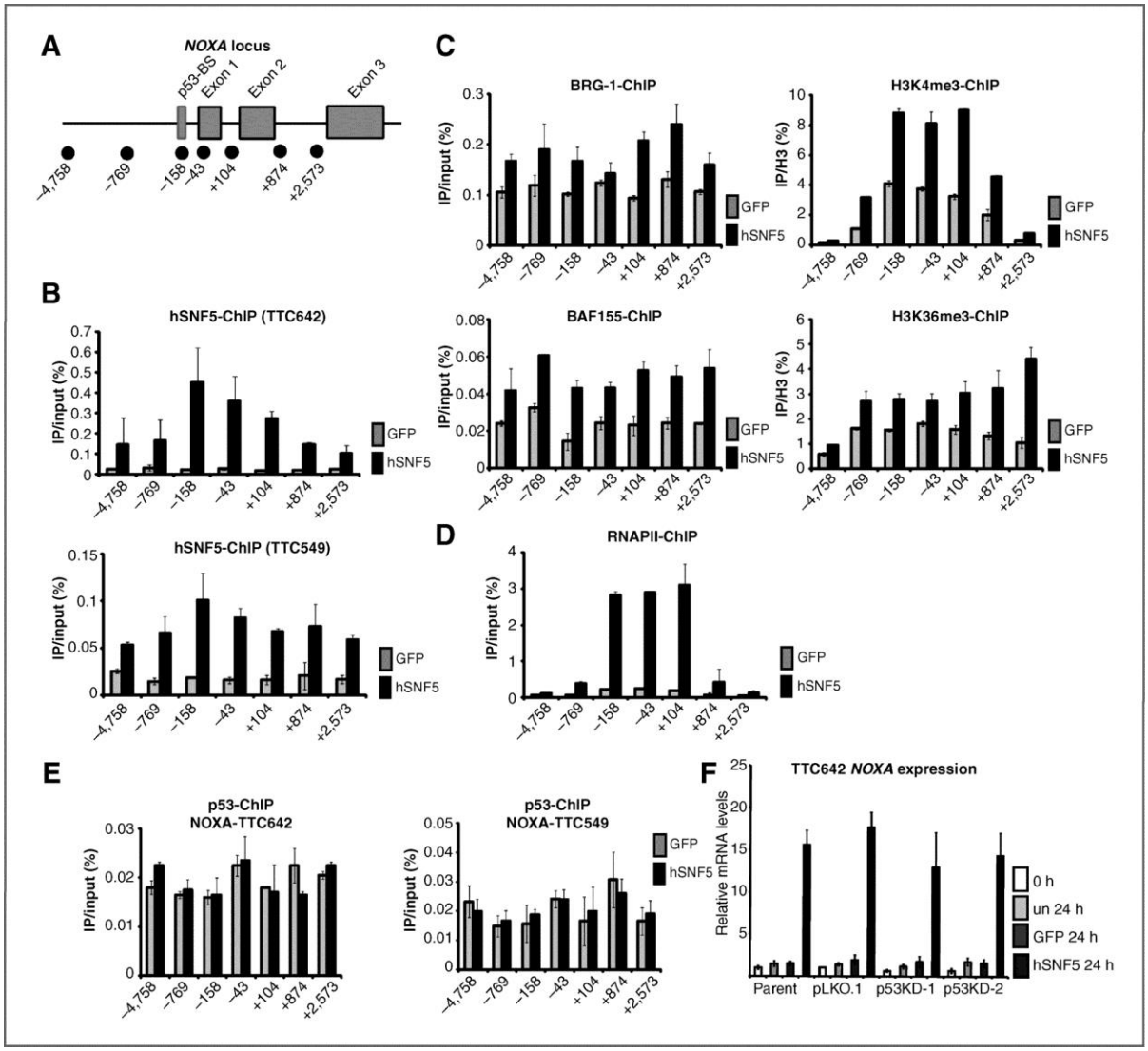


Figure 3.4 - Recruitment of hSNF5, SWI/SNF complexes and RNAPII to the NOXA locus and histone modification on NOXA locus after hSNF5 reexpression. (A) Schematic of the NOXA locus indicating the one p53 binding sites and overall gene structure. Primers used in QRT-PCR of ChIP-enriched DNA are named according to their relative distance (bp) to the transcription start site (TSS). (B, C, D, E) At 24 hours after infection with Ad-hSNF5 and Ad-GFP, protein was extracted for ChIPs assays. ChIPs assays were performed using antibodies directed against hSNF5(b), BRG-1(c), BAF155(c), H3K4me3(c), H3K36me3(c), RNAPII(d) and p53 (e) on indicated site of NOXA promoter. Values are the mean of

duplicate or triplicate; bars, ± SD. (f) RNA was extracted at 24 hours after infection with Ad-hSNF5 and Ad-GFP. The mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Values are the mean of three independent experiments; bars, ± SD. un; uninfected control.

3.4.4 Reexpression of hSNF5 induces p21CIP1/WAF1 and NOXA transcription accompanied with SWI/SNF complex recruitment.

In our previous report, we demonstrated that hSNF5 directly controled $p21^{CIP1/WAF1}$ transcription activity. Here, we confirmed hSNF5 mediated $p21^{CIP1/WAF1}$ and NOXA gene activation. However, the mechanism of $p21^{CIP1/WAF1}$ and NOXA transcription activity induced by hSNF5 has not yet been clarified. To investigate the occupancy of reexpressed hSNF5 on the $p21^{CIP1/WAF1}$ locus, we first performed a ChIP assay for hSNF5, BAF155 and BRG-1 at 24 hrs after infection of Ad-hSNF5 compared with Ad-GFP infection. We made sets of primers for the $p21^{CIP1/WAF1}$ promoter regions from - 3000 to +4001 as previously described (Donner et al., 2007) (Figure 3.5A). We found that reexpressed hSNF5 binds within 1 kb of TSS with maximal enrichment site at TSS in both A204.1 and TTC642 cell lines (Figure 3.5B). In contrast, BRG-1 and BAF155 levels increased on $p21^{CIP1/WAF1}$ locus throughout the whole region (Figure 3.5B). These results obtained from $p21^{CIP1/WAF1}$ locus were congruent with those obtained from the NOXA locus (Figure 3.4). These observations suggest that hSNF5 might bind near the TSS and either recruit other SWI/SNF complex contents such as BRG-1 and BAF155 to the target gene locus or re-activate an existing SWI/SNF complex to initiate gene transcription.

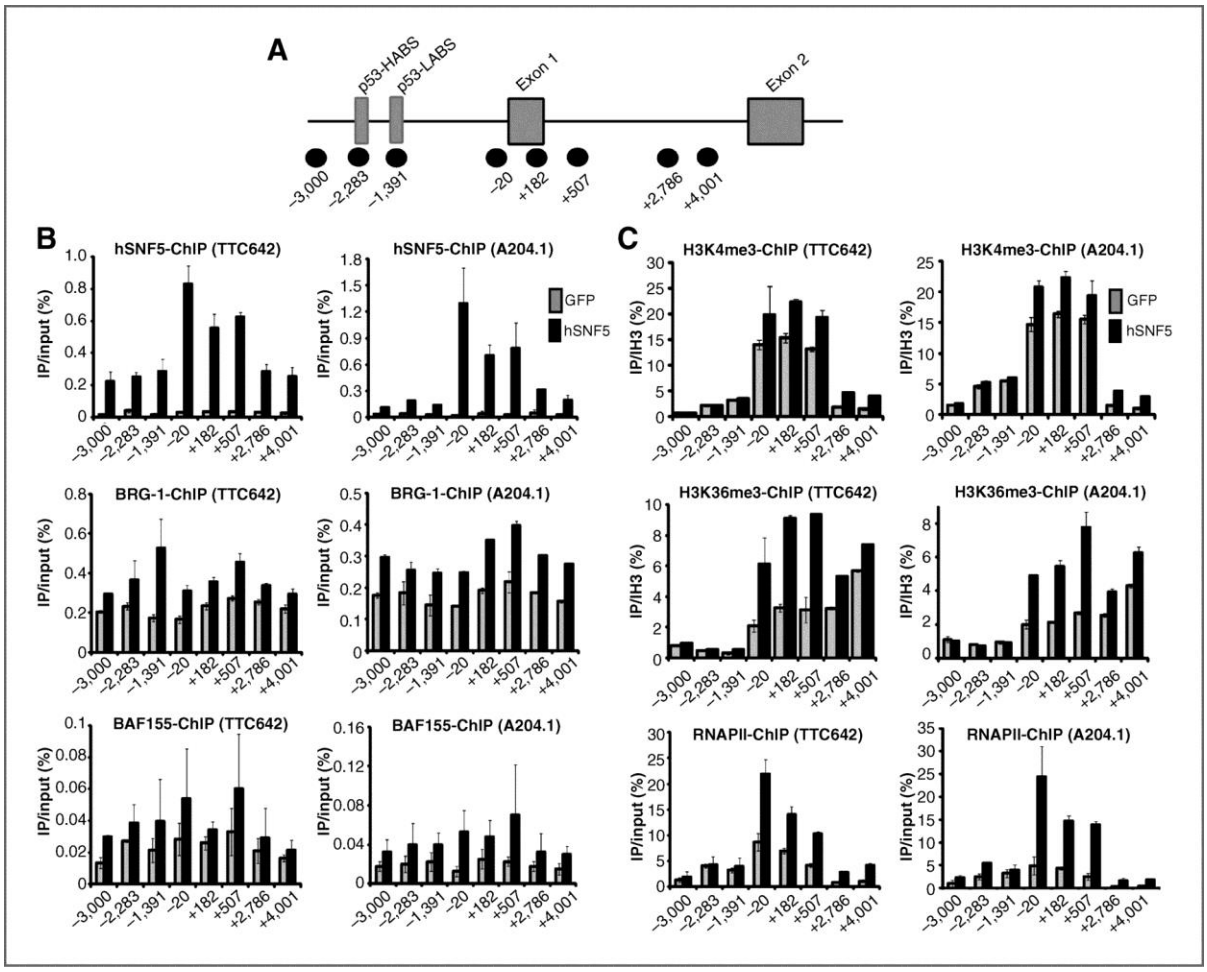


Figure 3.5 – Recruitment of hSNF5, SWI/SNF complexes, RNAPII, and p53 to the *NOXA* locus and histone modification on NOXA locus after hSNF5 reexpression. (A), schematic of the *p21* locus indicating the 2 p53-binding sites (p53-HABS: high-affinity p53-binding site and p53-LABS: low-affinity p53-binding site) and overall gene structure. Primers used in real-time qRT-PCR of ChIP-enriched DNA are named according to their relative distance (bp) to the TSS.(B) and (C), at 24 hours after infection with Ad-hSNF5 and Ad-GFP, protein was extracted for ChIP assays. ChIP assays were conducted using antibodies directed against hSNF5 (B), BRG-1 (B), BAF155 (B), H3K4me3 (C), H3K36me3 (C), and RNAPII (C) on indicated site of *p21* promoter. Values are the mean of duplicate or triplicate; bars, ±SD

3.4.5 Reexpression of hSNF5 induces p21CIP1/WAF1 and NOXA transcription accompanied with RNAPII recruitment and histone modification.

To assess the effects of reexpressed hSNF5 on the known steps of transcriptional activation, we performed ChIP assays for RNAPII, H3K4me3 and H3K36me3 on the $p21^{CIP1/WAF1}$ and NOXA loci at 24 hrs after infection of Ad-hSNF5 compared with Ad-GFP infection. Our ChIP results for RNAPII showed that RNAPII increased at TSS on both $p21^{CIP1/WAF1}$ and NOXA locus after hSNF5 reexpression with maximal enrichment site at TSS (Figure 4D, Figure 5C). The RNAPII occupancy pattern was similar to the hSNF5 occupancy pattern. Moreover, the H3K4me3 binding pattern on $p21^{CIP1/WAF1}$ locus demonstrated that H3K4me3 increased after hSNF5 reexpression at TSS, with a maximal peak near the TSS in both the A204.1 and TT642 cell lines (Figure 5C). These results support the H3K4me3 ChIP results of obtained from NOXA experiments (Figure 4C). Because H3K4me3 correlate with promoter activation (Santos-Rosa et al., 2002), our results strongly suggest that hSNF5 reexpression leads to the transcriptional activation by inducing the initiation step with recruitment of RNAPII. Furthermore, H3K36me3 levels also increased downstream of the TSS in the $p21^{CIP1/WAF1}$ and NOXA promoters (Figure 4C, Figure 5C). These results imply that reexpression of hSNF5 presumably precedes the transcription elongation step (Strahl et al., 2002).

3.4.6 p53 is not required for hSNF5-induced transcriptional activity on the NOXA and p21CIP1/WAF1 promoters.

We next determined whether hSNF5 recruitment to the NOXA promoter affected p53 binding. We performed a ChIP assay for p53 binding at these promoters in the TTC642 and TTC549 cell lines. We assessed p53 binding site on the NOXA promoter at -158bp, the consensus p53 binding site (Nikiforov et al., 2007), and did not observe binding after hSNF5 reexpression (Figure 4E). In our previous study, we determined that up-regulation of

$p21^{CIP1/WAF1}$ transcription by hSNF5 operated through a p53-dependent mechanism in A204.1 cells, but through a p53-independent mechanism in TTC642 cells (Kuwahara et al., 2010). To determine p53 dependency for NOXA transcription, we used 2 previously characterized p53 stable knock-down MRT cell lines from TTC642 cells and a negative control cell line (pLKO.1) (Kuwahara et al., 2010). Infection of the pLKO.1 and p53KD cells with Ad-hSNF5 or Ad-GFP resulted in increased levels of *NOXA* mRNA at 24 hours after Ad-hSNF5 infection in pLKO.1 cells as in the parental cell lines. We found the increase of *NOXA* mRNA by hSNF5 reexpression was not significantly different among TTC642, TTC642 pLKO.1 and all TTC642 p53KD cells (Fig. 4f). Those observations suggest that hSNF5 binding to could initiate transcription on the $p21^{CIP1/WAF1}$ and NOXA promoters in the TTC642 cell line or that recruitment of another transcription factor(s) was involved.

3.5 Discussion

The objective of this study was to determine the role of hSNF5 in the regulation of transcription of p53 target genes in MRT cells and to assess the impact of hSNF5 on their promoters as a regulator of their transcriptional activity. Our results provide three major insights into the control of hSNF5 target genes and how hSNF5 and the SWI/SNF complex functions in the regulation of their transcription.

First, our results indicate that hSNF5 may regulate a subset of p53 target genes such as $p21^{CIP1/WAF1}$ and NOXA in MRT cell lines but generally through a p53-independent mechanism. Reexpression of hSNF5 increased the transcription of p53 target genes within 24 hours. However, the robustness of the effects and the target genes affected varied among MRT cell lines. These results indicate that hSNF5 may operate with either p53 or other transcriptional factors but not in the same pattern or with the same genes in all MRT cell

lines. Intriguingly, we observed an increase in NOXA mRNA and protein levels after reexpression of hSNF5 in most MRT cell lines. Our ChIP assays also showed that reexpression of hSNF5 increased NOXA transcriptional activity through the recruitment of BRG1 and RNAPII to the NOXA promoter. This is the first report that hSNF5 regulates NOXA transcriptional activity and establishes NOXA as a clinically relevant hSNF5 target gene.

NOXA was initially identified as a phorbol ester-responsive gene (Ploner et al., 2008). The NOXA protein, containing a Bcl2 homology domain3 (BH-3), has been previously implicated in apoptosis associated with DNA damage, hypoxia or exposure to inhibitors of the proteasome (Oda et al., 2000). Apoptosis –associated NOXA activation is primarily achieved through transcriptional up-regulation, and a number of transcription factors including p53 and Myc has been shown to regulate NOXA expression (Ploner et al., 2008). NOXA binds to the antiapototic Bcl-2 family members Mcl-1 with high-affinity binding, and this BH3-only protein appears to be a mediator of apoptosis in cells showing a dependency on Mcl-1 expression (Alves et al., 2006; Chonghaile and Letai, 2008). Furthermore, some reports showed that NOXA and Mcl-1 are related to sensitivity to some chemotherapeutics agents such as Taxol, vincristine and platinum-based drugs(Sheridan et al., 2010

; Wertz et al., 2011)

MRTs have a chemo-resistance character so that chemotherapy does not prove an effective treatment for most patients(Rosson et al., 2002a). To resolve the chemo-resistance

character of MRTs, some candidate drugs such as EGFR kinase inhibitors, HER2 inhibitors, fenretinide, HDAC inhibitors and flavopilidol have been described (Das et al., 2008; Katsumi et al., 2008; Kuwahara et al., 2004a, b; Smith et al., 2008; Smith et al.; Watanabe et al., 2009). However, a promising effective therapy has not yet been established. On the other hand, Nocentini et al. reported that perturbation of the p53 pathway and a reduced apoptotic response in MRT cell lines might contribute to the resistance of MRT to chemotherapies (Nocentini, 2003). They suggested that the lack of positive correlation between an increase in the Bax/Bcl2 ratio and cell death contributes their abnormalities in the control of apoptosis. In this study, our results suggest that loss of hSNF5 results in a failure to regulate NOXA expression in the case of DNA damage by some chemotherapy agents such as doxorubicin. Although further experiments are needed to clarify the mechanisms of chemo-resistance in MRT cells, our results indicate that one key mediator could be NOXA.

Second, our results demonstrated the function of hSNF5 in the context of SWI/SNF complex. Some researchers have reported that reexpressed hSNF5 appeared at one or multiple sites within the promoters of several genes (Chai et al., 2005; Kia et al., 2008a; Zhang et al., 2002). In our previous report, we also demonstrated hSNF5 binds at a point close to the TSS on the $p21^{CIP1/WAF1}$ and p16 promoters by ChIP assay (Kuwahara et al., 2010). However, the pattern of hSNF5 occupancy of target gene promoters has remained unclear. Our results showed hSNF5 appeared on the promoter region with maximal enrichment site at the TSS. Kia and colleagues reported that on p16 promoter, reexpressed hSNF5 binds more at -0.3kb and +85 bp site than other upstream and downsteam sites (Chai et al., 2005; Kia et al., 2008a; Zhang et al., 2002). Our results with the $p21^{CIP1/WAF1}$ and NOXA promoters provide strong evidence that hSNF5 is generally associated with the

transcriptional activity at TSS on its target genes. In contrast, BRG-1 and BAF155 occupancy increased equally from downstream to upstream on $p21^{CIP1/WAF1}$ and NOXA locus after reexpression of hSNF5. Therefore, our results indicate a discrepancy between hSNF5 occupancy and BRG-1 occupancy after hSNF5 reexpression. A more detailed analysis using ChIP-seq to identify the binding sites of the hSNF5 and the other SWI/SNF complex members will resolve this question.

Does this recruitment of the SWI/SNF complex lead to a change in the histone modification in their target genes? In our previous study, we found that H3K4me3 decreased at the p53 binding sites in the $p21^{CIP1/WAF1}$ promoter (−2,283 kb and −1,391 kb) after hSNF5 reexpression, although $p21^{CIP1/WAF1}$ transcription increased (Kuwahara et al., 2010). Previous studies have concluded that nucleosomes with H3K4me3 are associated with actively transcribed genes in various eukaryotes (Bernstein et al., 2002; Pokholok et al., 2005; Schneider et al., 2004). Guenther and colleagues also found H3K4me3 enriched within 1 kb of known TSS, with maximal enrichment downstream of the TSS (Guenther et al., 2007). Our results showed H3K4me3 increased at or near the TSS in the $p21^{CIP1/WAF1}$ and NOXA promoters, similar to the results from Guenther's report. Lee and colleagues indicated that the C-terminal SET domain of H3K4 methyltransferase (MLL3 and MLL4) directly interacts with hSNF5 (Lee et al., 2009). The fact that H3K4me3 occupancy is likely linked to hSNF5 occupancy suggests that reexpressed hSNF5 potentially interacted with a H3K4 methyltransferase. In contrast, our H3K36me3 ChIP data showed that H3K36me3 occupancy was detected in transcribed regions of the $p21^{CIP1/WAF1}$ and NOXA genes, peaking toward the 3' end (Bernstein et al., 2002; Pokholok et al., 2005; Schneider et al., 2004; Vastenhouw et al.). Our results concur with several earlier reports demonstrating (Bernstein

et al., 2002; Pokholok et al., 2005; Schneider et al., 2004; Vastenhouw et al.) that H3K36me3 occupancy tracks within the body of transcriptionally active genes and associates with transcriptional elongation activity. These histone modifications after hSNF5 reexpression indicate that hSNF5 can regulate the transcription initiation followed by elongation activity in MRT cells.

We also showed that RNAPII occupancy also increased after hSNF5 reexpression on $p21^{CIP1/WAF1}$ and NOXA promoter. Guenther and colleagues found that most promoters (98%) occupied by RNAPII were also occupied by H3K4me3, whereas RNAPII occupied few genes (2%) that lack H3K4me3 (Guenther et al., 2007; Hughes et al., 2004). Other researchers have shown that components of H3K4 methyltransferase complexes interact with the Ser5-phosphorylated form of RNAPII, indicating that transcription initiation coincides with H3K4me3 deposition (Hughes et al., 2004). Similar studies showed that H3K4me3 modification occurs subsequent to RNAPII recruitment and Ser5 phosphorylation of RNAPII C-terminal domain (Ng et al., 2003; Pokholok et al., 2005; Santos-Rosa et al., 2002). Indeed, we also showed that the occupancy of RNAPII follows a similar pattern for H3K4me3 occupancy. The increase of H3K4me3 occupancy in the $p21^{CIP1/WAF1}$ and NOXA promoters may result from recruitment of H3K4 methyltransferase and RNAPII by hSNF5 reexpression.

However, H3K4me3 was still detected at the inactive NOXA promoter in the absence of hSNF5 with a low occupancy of RNAPII. Then, after reexpression of hSNF5, both RNAPII and H3K4me3 immediately increased on or near the TSS. Vastenhouw and colleagues indicated that many non-expressed genes in ES cells also carry only H3K4me3 marks (Vastenhouw et al.). They also demonstrated most of these H3K4me3 domains are not

associated with detectable levels of RNAPII, and H3K4me3 marks might be established in the absence of sequence-specific activators and without the stable association of RNAPII. These H3K4me3 domains might be paused genes for activation by creating a platform for the transcriptional machinery (Reincke et al., 2003). Our data suggest that the inactive NOXA gene in MRT cells might be paused by loss of hSNF5 and reexpression of hSNF5 can release the pausing followed by recruitment of RNAPII. On the other hand, because transcriptional activity appears at the $p21^{CIP1/WAF1}$ promoter, it has both H3K4me3 and RNAPII occupancy before reexpression of hSNF5. Taken together, hSNF5 can activate a transcription initiation step by recruitment of RNAPII accompanied with H3K4me3 nucleosome modification.

In conclusion, our results show that hSNF5 reexpression in MRT cells increases both $p21^{CIP1/WAF1}$ and NOXA expression. hSNF5 reexpression leads to activation of transcription initiation by either recruitment of SWI/SNF complexes or activation of existing ones. Increased RNAPII binding at the TSS accompanied with H3K4 and H3K36 modifications follows. Because MRT cells display repressed NOXA transcription activity due to loss of hSNF5, targeting reexpressing of the NOXA pathway might be a promising new paradigm to treat MRT in near future.

Chapter 4: Cyclin G2, a Novel Target of the SNF5/BAF47 Tumor Suppressor Gene[4]

4.1 Summary

Malignant Rhabdoid Tumors (MRTs) are a rare and aggressive form of pediatric cancer. Previous studies have shown that reintroducing SNF5 into MRT cell lines results in an inhibition of growth through induction of $p21^{WAF1/CIP1}$, $p16^{INK4A}$ and $p57^{KIP2}$ expression. However, the mechanisms behind this growth arrest remain incompletely characterized. In this current report, we used a RT-PCR Cell Cycle SuperArray to identify six candidate genes that showed increased expression after SNF5 reexpression but not after expression of a constitutively active RB gene. One of these genes, Cyclin G2 (CCNG2), a member of the non-canonical Cyclin G family, is thought to act as a negative regulator of the cell cycle. Chromatin immunoprecipitation analyses (ChIP) verified that SNF5 binds to the CCNG2 promoter with peak binding at the transcription start site (TSS) after its reexpression in a MRT cell line. Importantly, primary MRT samples display reduced CCGN2 expression when compared to normal brain tissue or other types of pediatric brain cancers. Using a panel of MRT and AT/RT cell lines, we confirmed that SNF5 reexpression leads to induction of CCGN2 in many cell lines. Therefore, CCNG2 represents a new SNF5 target gene whose downregulation may play a role during MRT development.

[4] This manuscript is expected to be submitted to journal, Cell Cycle.

4.2 Introduction

Malignant Rhabdoid Tumors (MRTs) are a rare and aggressive form of pediatric cancer. Because MRTs are refractory to treatment by both traditional chemotherapeutics and radiotherapy, patients with MRT have an overall four year survival rate of less than 25%(Tomlinson et al., 2005c). Therefore, most MRT studies has concentrated on understanding the molecular genetics of the disease to identify critical targets for targeted treatment approaches. These efforts have shown that the majority of MRTs display inactivation of SNF5/BAF47/INI1/SMARCB1, a core subunit of the SWI/SNF ATP-dependent chromatin remodeling complex(Versteege et al., 1998a).

To uncover the mechanisms of SNF5-associated oncogenesis, previous studies have shown that reintroducing SNF5 into MRT cell lines results in an inhibition of growth(Wilson and Roberts, 2011). These studies have established $p21^{WAF1/CIP1}$, $p16^{INK4A}$, $p57^{KIP2}$ and BIN1 as important downstream regulators of SNF5-induced growth inhibition(Algar et al., 2009; Betz et al., 2002; Kuwahara et al., 2010; McKenna et al., 2012). However, whether these genes represent the only downstream targets that contribute to the growth arrest remains unresolved. Previous studies from our laboratories have shown that neither p16 nor p21 alone regulated the cell cycle after SNF5 reexpression(Chai et al., 2005; Kuwahara et al., 2010). Other studies have also identified multiple signaling pathways that undergo regulation via SNF5 expression(Wei and Weissman, 2014).

In an effort to further elucidate other downstream targets of SNF5 involved in growth regulation, we looked for genes whose expression significantly changes after SNF5

reintroduction into the A204 MRT cell line. We used a RT-PCR Cell Cycle SuperArray to identify six candidate genes that showed increased expression after SNF5 reexpression but not after expression of a constitutively active RB gene. Two of these genes, *p21*$^{CIP1/WAF1}$ and *p16*^{INK4A}, have been previously confirmed as SNF5 targets(Chai et al., 2005; Kuwahara et al., 2010). Potentially new target genes identified in this analysis include *CCNG2* (Cyclin G2), *CCNH* (Cyclin H), *CDK8*, and *HERC5*.

Using qPCR, we validated the increased expression of these 4 genes in the A204 cell line after SNF5 reexpression. We then focused on CCGN2 because of its known tumor suppressor activity and its restricted expression pattern in normal tissues(Horne et al., 1996; Ito et al., 2003; Kim et al., 2004). Chromatin immunoprecipitation analyses (ChIP) verified that SNF5 binds to the CCNG2 promoter with peak binding at the transcription start site (TSS) after its reexpression in a MRT cell line, similar to our results at the p21 and NOXA promoters(Kuwahara et al., 2013b). Furthermore, primary MRT samples display reduced CCGN2 expression when compared to normal brain tissue or other types of pediatric brain cancers. Although a panel of additional MRT cell lines showed CCGN2 protein induction after SNF5 reexpression, we did not observe an increase in mRNA levels. We also did not find CCNG2 protein within the SWI/SNF complex. Therefore, CCNG2 represents a new SNF5 target gene whose reduced expression may contribute to MRT development.

4.3 Materials and Methods

4.3.1 Cell Culture*:*

A204.1 (American Type Culture Colleciton, ATCC), G401 (ATCC), TTC642, TTC549, TTC709 (Dr. Timothy Triche, Children's Hospital of Los Angeles), and BT-12 (Dr.

Peter Houghton, Ohio State University) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The adenoviral vectors Ad/pAdEasyGFPINI-SV^{+} (Ad-SNF5-GFP) and Ad/pAdEasyGFP (Ad-GFP) have been described previously.(Reincke et al., 2003) The adenoviral vector Ad/pAdEasySNF5-HA (Ad-SNF-HA) was produced by the UNC Viral Vector Core Facility. Adenoviral infections were performed at a multiplicity of infection (MOI) to infect at least 90% of cells. The MOI used for A204.1 was 20, G401 and TTC549 were 10, TTC642 was 200, TTC709 was 40, and BT-12 was 25.

4.3.2 Western Blotting*:*

Protein extractions were performed as described previously (Chai et al., 2005). 30 μg of total proteins were separated on either 4-20% SDS-polyacrylamide or 4-12% Bis-Tris gels by electrophoresis. Proteins were transferred onto Immobilon-FL PVDF membranes (Millipore). Analysis of proteins was performed by using anti-actin (A2066; Sigma), anti-KU70/80 (a kind gift from Dr. Dale Ramsden, University of North Carolina), anti-BAF180 (A2218; a kind gift from Dr. Ramon Parsons, Columbia University), anti-CCNG2 (sc7266; Santa Cruz), anti-SNF5 (612110; BD Transduction), anti-p21$^{CIP1/WAF1}$ (sc-297; Santa Cruz) and horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (GE Healthcare). Antibodies were visualized using IRDye 800CW or IRDye 680 and visualized using the LI-COR Odyssey Western Blot Detection System or using ECL reagents (GE Healthcare) followed by exposure to X-ray film.

4.3.3 Chromatin Immunoprecipitation*:*

Chromatin immunoprecipitation (ChIP) was performed as described previously (Gomes et al., 2006) with the following modifications: protein extracts were precleared with 40 μl of 50% protein A/protein G slurry. Immunocomplexes were washed once with RIPA

buffer, three times with Szak IP wash buffer, once with RIPA buffer, then twice with 1x TE. Immunocomplexes were eluted by the addition of 200 μl of 1.5x Talianidis elution buffer and incubation at 65°C for 10 minutes. 2 ug of the following antibodies were used for immunoprecipitation: HA (ab9110; Abcam), Histone H3 (39163, Active Motif), Histone H3 Lysine 4 trimethylation (H3K4m3, 39159, Active Motif) and RNA Polymerase II (MMS-126R, Covance). Normal rabbit IgG (sc2027, Santa Cruz) was used as a negative control. DNA recovered was quantified by an ABI 7900 HT sequence detection system using QT-PCR. Values were normalized to input DNA for each cell line. The primers used for each promoter site can be found in Table4. 4.

4.3.4 RNA extraction and RT-PCR*:*

RNA was extracted using the RNeasy Plus mini kit (Qiagen). cDNA was synthesized using 1 ug RNA primed with random primers (Invitrogen). cDNA was analyzed by RT-PCR using TaqMan (Applied Biosystems). *β-actin* was used as a reference gene for each reaction. The ABI 7900 HT sequence detection system was used, and data were analyzed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001a). The primer/probe sets (Applied Biosystems) used for each gene are listed in Table 4.1.

Table 4.1: Primer/Probes for genes analyzed by RT-PCR.	
beta-Actin	Hs99999903_m1
CCNG2	Hs00171119_m1
CCNH	Hs00236923_m1
CDK8	Hs00176209_m1
Herc5	Hs00180943_m1
$p21^{CIP1/WAF1}$	Hs00355782_m1

4.3.5 Immunoprecipitation:

Cells were treated with 1mg/ml doxycycline for 24 hours. Proteins were then isolated from treated and untreated cells using IP buffer (50mM Tris, 400 mM NaCl, 2mM EDTA, 10% Glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1 mM PMSF, 4 mM sodium fluoride, 40 nM sodium orthovanadate, 1x complete Mini protease inhibitor cocktail (Roche Diagnostics)). The isolated protein was quantified and adjusted to 500ug/ml. 500 ug of protein from each sample was incubated with BRG-1 (A303-877A ; Bethyl), or normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) rotating overnight at 4^{o}C with 30 µl of a 50% slurry of protein A/G Sepharose beads. The beads were then washed 3 times with IP wash buffer (1x PBS, 10% glycerol, 1% Triton) and then suspended in 1x Nupage LDS loading buffer supplemented with 125mM DTT and boiled for 5 minutes. The supernatants were then run on 4-12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with anti-SNF5 (612110; BD Transduction), anti-ARID2 (A302-229A; Bethyl), anti-SMARCC2 (A301-038A; Bethyl), anti-SMARCC1 (DXD7; Santa Cruz), anti-SMARCE1 (A300-810A; Bethyl) or anti-CCNG2 (sc7266; Santa Cruz).

4.3.6 Cell Cycle SuperArray

Briefly, we plated 5 x 10^5 A204.1 cells per 100mm tissue culture dish. After 48 hours, we infected with Ad-GFP, Ad-SNF5 or Ad-RbΔcdk at an m.o.i. of 20. Although we observed SNF5 protein expression as early as 12 hours after infection, for these initial experiments, we harvested cells 24 hours after infection. We extracted total RNA and measured expression of the 84 cell cycle genes using the RT^2 Profiler™ plate following the manufacturer's instructions. We analyzed the resulting data by the $\Delta\Delta C_t$ method.

4.3.7 Primary MRT Gene Expression Analyses

The expression of CCND1, CCND2 and SNF5 in primary AT/RTs, medulloblastomas and in normal brain was determined as previously described (McKenna et al., 2008a).

4.4 Results

4.4.1 Identification of novel SNF5 target genes-

To identify changes in gene expression that occur after reexpression of SNF5 in the A204.1 cell line, we carried out an analysis of Ad-SNF5 infected A204.1 cells using the SuperArray RT^{2}Profiler Cell Cycle PCR Array. We used the Human Cell Cycle RT2 Profiler that contains 84 key cell cycle regulatory genes including *CCNs, CDCs, CDKIs* and others. By using a focused pathway approach, we hoped to rapidly obtain insight into the mechanisms of growth arrest induced by reexpression of SNF5. As a control for genes whose expression change with any growth arrest in the A204.1 cell line, we also infected with an adenovirus expressing RBΔCDK, a constitutively active form of the RB protein, that also causes a growth arrest in this cell line(Braden et al., 2006; Kuwahara et al., 2010). Therefore, if SNF5 reexpression functioned strictly through an induction of the RB pathway, we should observe similar changes in gene expression after infection with Ad-RBΔCDK.

We have carried out 3 independent experiments to analyze gene expression after Ad-RbΔCDK infection and 4 independent experiments for Ad-SNF5 infection. The average of the results from these experiments are summarized in Tables 4.2 and 4.3. As expected from previous studies, RBΔCDK expression in the A204.1 cells resulted in decreased expression of many E2F regulated genes (Table 4.2)(Markey et al., 2002). The only gene that showed increased expression was *RB,* confirming the successful infection of the cells by adenovirus. When we examined the expression of these same genes after SNF5 expression in these cells, we also found a similar decrease in gene expression (Table 4.2). This result appears consistent with the reduction in hyperphosphorylated RB observed in MRT cells following

SNF5 reexpression(Betz et al., 2002; Kuwahara et al., 2010). The magnitude of the reduction in the Ad-SNF5 infected cells was generally less than in the Ad-RbΔCDK infected cells probably reflecting the large amount of RBΔCDK protein produced by the adenovirus. Similar changes for *CDC2* and the *MCM2-5* genes were reported by Vries et al. after SNF5 reexpression in the G401 MRT cell line(Vries et al., 2005).

Table 4.2 – Summary of gene expression common to Ad-RbΔcdk and Ad-SNF5 infection of A204.1 cells

Gene	Avg. Change (Range)	
	RbΔcdk	**SNF5**
Birc5/survivin	-4.33 (-2.03 to -7.94)	-1.60 (-1.26 to -1.90)
CCNE1/cyclinE1	-1.63 (-1.39 to -1.80)	-1.89 (-1.73 to -2.06)
CDC2	-2.64 (-2.10 to -2.92)	-1.48 (-1.34 to -1.86)
CDK2	-2.64(-2.54 to -3.11)	-1.49 (-1.34 to -1.63)
DDX11	-5.42 (-4.77 to -6.19)	-2.76 (-1.82 to -3.73)
MCM3	-1.64 (-1.39 to -1.99)	-2.25 (-2.01 to -2.65)
MCM4	-3.21 (-1.90 to -4.39)	-2.54 (-2.22 to -2.89)
MCM5	-2.99 (-2.12 to -4.39)	-1.72 (-1.54 to -2.01)
RAD51	-3.15 (-2.15 to -5.01)	-1.77 (-1.11 to -2.88)
RB1	16.62 (7.54 to 25.12)	1.13 (1.10 to 1.15)

Table 4.3 – Summary of gene expression changes correlated to SNF5 infection of A204.1 cells		
Gene	**Ave. Change (Range)**	
	RbΔcdk	**SNF5**
CCNG2/CyclinG2	1.39 (1.32 to 1.47)	3.06 (2.27 to 4.06)
CCNH/CyclinH	-0.38 (-1.28 to 1.14)	5.74 (3.63 to 8.25)
CDK8	-1.18 (-1.35 to -1.00)	2.49 (1.71 to 2.90)
CDKN1A/p21	0.45 (-1.11 to 1.32)	2.17 (1.82 to 2.54)
CDKN2A/p16	-1.10 (-1.26 to -1.01)	2.27 (1.50 to 2.99)
HERC5	0.46 (-1.24 to 1.43)	3.40 (1.74 to 6.21)

While genes that showed reduced expression after SNF5 expression generally overlapped those with RBΔCDK expression, a unique set of genes displayed increased expression only after SNF5 expression (Table 4.3). As shown earlier, the cyclin-dependent kinase inhibitors p16 and p21 were both induced by SNF5 while RBΔCDK apparently inhibited their expression (Table 4.3). Expression of two genes involved in the regulation of RNA Polymerase II activity, *cyclin H (CCNH)* and *CDK8*, were also increased by SNF5 expression(Kobor and Greenblatt, 2002). *HERC5* is a E3 ubiquitin ligase involved in interferon signaling(Wong et al., 2006). Intriguingly, a recent report has linked SNF5 loss to a block in interferon signaling in MRT cells(Morozov et al., 2007). However, we were particularly excited by the induction of *cyclin G2* (*CCNG2*). CCNG2 is a non-canonical cyclin that acts as a growth suppressor rather than promoting growth(Horne et al., 1997a). It also does not appear to partner with a classical cyclin-dependent kinase but can interact with PP2A, another potential tumor suppressor gene(Bennin et al., 2002a). It also plays a role in early development as a negative regulator of the cell cycle during cellular differentiation including B cells, heart, muscle and nervous system(Horne et al., 1997a; Houldsworth et al., 2002; Yue et al., 2005).

4.4.2 Validation of gene expression in A204.1 cells by qPCR-

We next confirmed the expression change results for the 4 novel targets genes in the A204.1 cell line by qPCR, a more stringent measure of mRNA expression. In order to reduce the level of transgene expression induced by the Ad-SNF5 infection, we used a new adenovirus expressing a HA-tagged *Snf5* gene, Ad-HA-SNF5, derived by Kaeser et al(Kaeser et al., 2008). We observe significantly lower SNF5 protein levels in cells than levels produced by the Adenovirus-SNF5-GFP (Supplemental Figure 4.1). The use of this virus

also eliminated the caveat of potential alterations in gene expression induced by the co-expression of GFP. As a control for Ad-HA-SNF5 expression, we used a replication-deficient adenovirus, Ad-CMV, which did not contain a transgene.

As shown in Figure 4.1, reexpression of HA-SNF5 increased p21 expression in the A204.1 as previously reported(Kuwahara et al., 2010). We also observed increased expression after Ad-HA-SNF5 infection for the 4 target genes in A204.1 compared to the Ad-CMV control (Figure 4.1). Although these genes showed increased expression, the fold change did not correlate with the results found using the SuperArray (Table 4.3). This may reflect differences in the levels of SNF5 expression, RT-PCR vs. qPCR assays and/or the lack of GFP expression after Ad-HA-SNF5 infection. Nonetheless, we could reproduce the induction of the 4 novel target genes after Ad-HA-SNF5 infection.

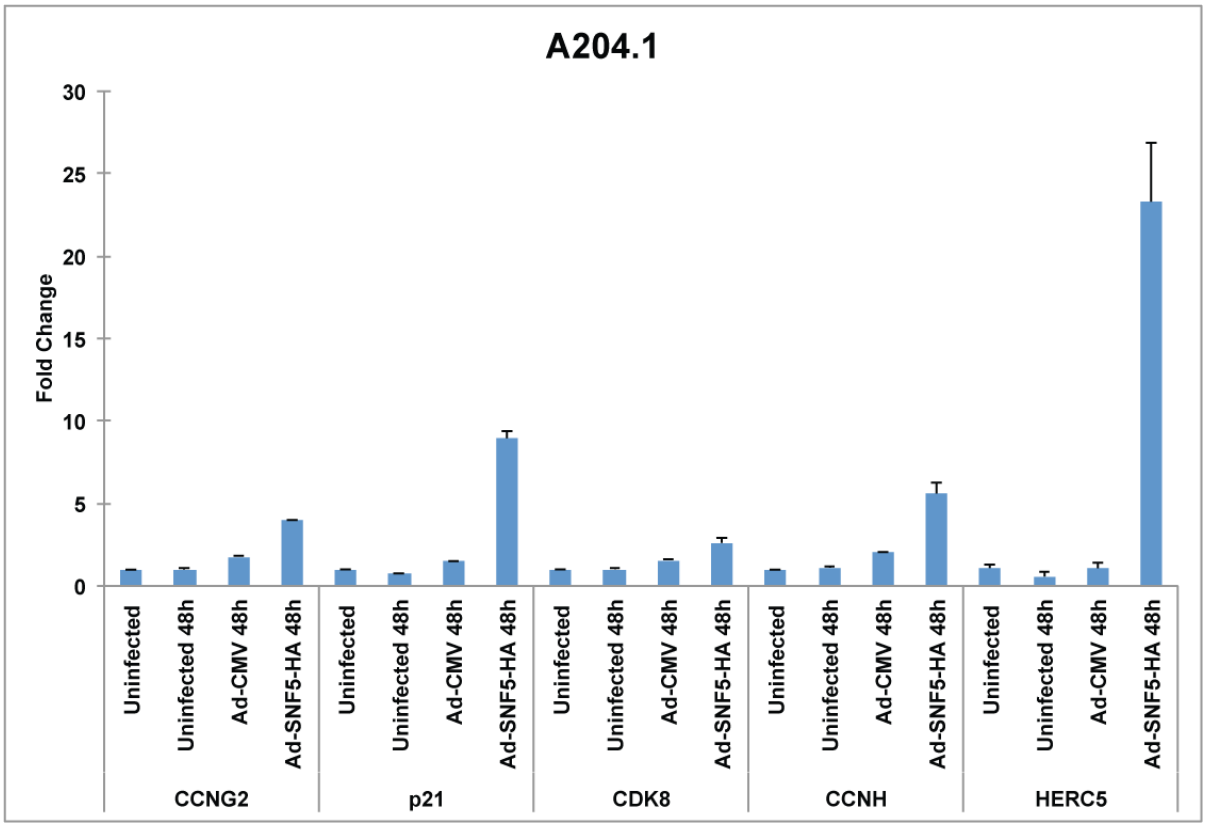


Figure 4.1- Effect on target gene expression after SNF5 reexpression in A204.1 cells. RNA was extracted from the A204.1 cell line immediately before infection or 48 hours after infection. The mRNA levels for HERC5, CCNH, CCNG2, CDK8 and p21 were assessed by qPCR. All values are normalized to actin, with relative expression shown. Each gene was examined in duplicate from 2 biological experiments, with error bars indicating standard deviation among the replicates.

4.4.3 Chromatin Immunoprecipitation (ChIP) analysis

Although we validated all 4 genes by qPCR, we decided to focus upon the CCNG2 gene for further analyses. Previous studies have established CCNG2 as a tumor suppressor gene for oral, colorectal, esophageal and thyroid papillary carcinomas among others (Ito et al., 2003; Kim et al., 2004; Sun et al., 2014a; Sun et al., 2014b). Furthermore, CCNG2 expression is needed for optimal DNA damage-induced G(2)/M checkpoint response(Zimmermann et al., 2012). Therefore, we checked whether SNF5 directly regulates CCNG2 expression by recruitment to its promoter as seen for other target genes such as p21, BIN1 and NOXA (Kuwahara et al., 2010; Kuwahara et al., 2013b; McKenna et al., 2012). We conducted ChIP analysis on the CCNG2 promoter in A204.1 to determine the pattern of SNF5 binding and epigenetic changes across the promoters. We designed 4 primers in the promoter region of CCNG2, with two upstream of the transcription start site (TSS), one near the TTS, and one downstream site. The sequences of the primers and locations are given in Table 4.4. The +1013 site was designed based off work performed by Stossi et al(Stossi et al., 2006). We used the Ebox promoter as a negative control, as it is known to be a site where SNF5 does not bind(Kuwahara et al., 2010; Kuwahara et al., 2013b).

Table 4.4: Primer sequences used for ChIP QT-PCR.		
CCNG2 Site	Forward	Reverse
-3kb	CACACTTGTCAGGAGTCAGGGATT	TAATAAGCAGGGAGTGCCCACACA
-660	AAACTCTCCCGTGGCTGAAA	GCGCTTCTCCTAACAGCTAACCTT
-55	GGAAGTGCAGGATCCCTCCG	TTTGTTAAGAGTTTCGACGCCC
+1013	TCAGGTGGGGCAGACCGAGG	GTTTCACAAACAGGAAACTGTCCGC

A representative ChIP analysis of the CCNG2 promoter region +/- SNF5 is shown in Figure 4.2. As we have observed with the p21 and NOXA promoters, reexpression of SNF5 leads to recruitment across the promoter region in the Ad-HA-SNF5 infected sample, at significantly higher recovery than Ad-CMV infected cells(Kuwahara et al., 2013b). We also observed more binding upstream and at the TSS than at the downstream site. We also found a clear increase in RNA Polymerase II binding at the TSS and the downstream site, correlating with increased transcription. Surprisingly, we did not observe an increased H3K4 trimethylation as we have observed at other SNF5 target gene promoters(Kuwahara et al., 2010; Kuwahara et al., 2013b). All positive signals were significantly above the background binding found by normal Rabbit IgG immunoprecipitation. Therefore, reexpression of SNF in the A204.1 cell line leads to significant binding at the CCNG2 promoter.

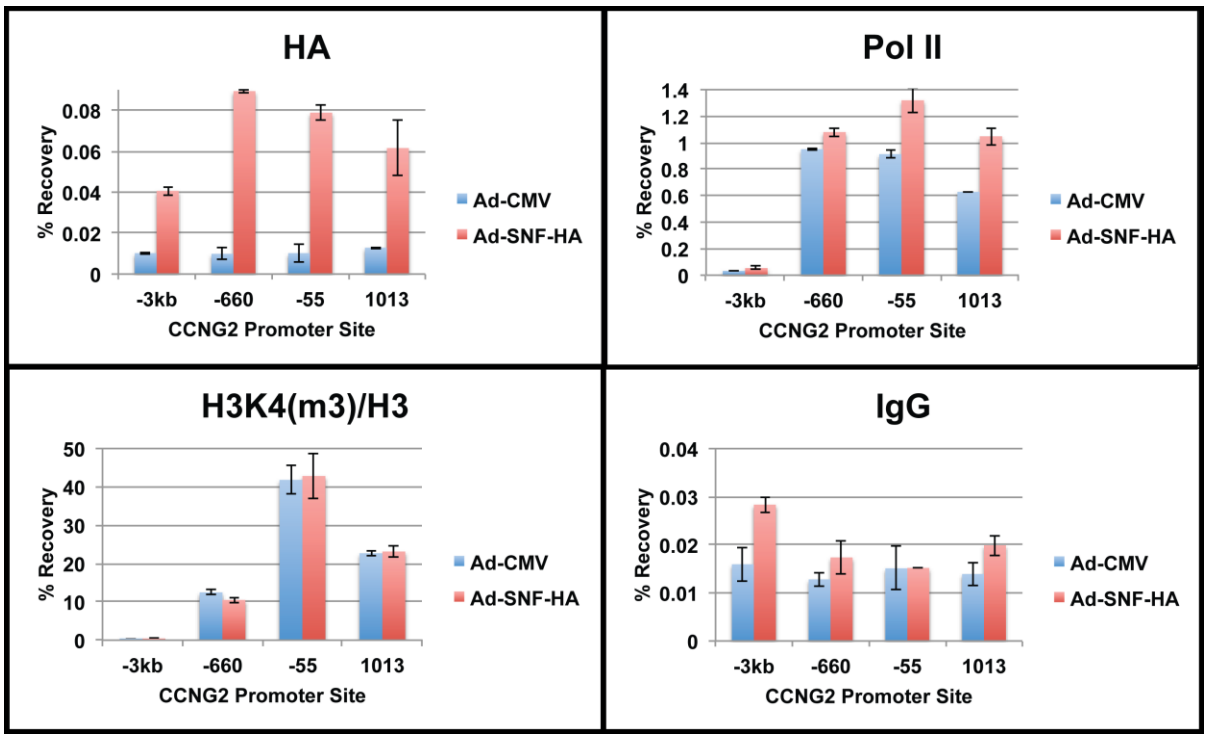


Figure 4.2- CCNG2 ChIP Analysis. ChIP analysis of the CCNG2 promoter region in A204.1 cells 24 hours after SNF5 introduction. Ad-CMV infections are shown in blue while Ad-HA-SNF5 infections are shown in red. Error bars indicate standard deviation between replicates. Two replicates for each promoter site were completed. Antibodies used include RNA Polymerase II CTD, total Histone H3, Histone H3 Lysine 4 trimethylation, SNF5 (Imbalzano), and normal rabbit IgG. Data are normalized to input DNA or Histone H3 (Histone H3K4m3 only).

4.4.4 Downregulation of CCNG2 expression in primary MRTs

While we observed a strong correlation between SNF5 reexpression and increased CCNG2 expression in MRT cell lines cultures, we wanted to determine whether this relationship exists in primary MRTs. To answer this question, we took advantage of our previous gene expression profiling comparing MRTs, medulloblastomas, and normal cerebellum(McKenna et al., 2008a). As shown in Figure 4.3A, we observed downregulation of CCNG2 in almost all MRTs compared to normal cerebellum. This contrasts with our previously reported increase in CCND1 expression in MRTs(McKenna et al., 2008a). Furthermore, CCNG2 downregulation appeared specific to MRTs because we did not observe changes in expression in another pediatric brain tumor, medulloblastoma (Figure 4.3A). Therefore, our results in the MRT cell line correlate well with changes in CCNG2 expression observed in vivo. These results also raise questions about a potential association between CCNG2 inactivation and increased CCND1 activity.

We next asked whether SNF5 reexpression induced CCNG2 in additional MRT cell lines. In order to overcome the caveat of unregulated levels of expression induced by Ad-HA-SNF5 infection as well as the apparent effects on gene expression induced by adenovirus infection, we repeated these experiments using an inducible SNF5 expression vector controlled by tetracycline (Meerbrey et al., 2011b). As shown in Figure 4.3B, SNF5 reexpression in the G401.6 and TTC642 MRT cell lines as well as the BT-12 AT/RT cell line resulted in increased CCNG2 protein expression. However, in contrast to SNF5 reexpression in the A204.1 cell line, we did not observe an increase in CCNG2 mRNA levels (Figure 4.3C).

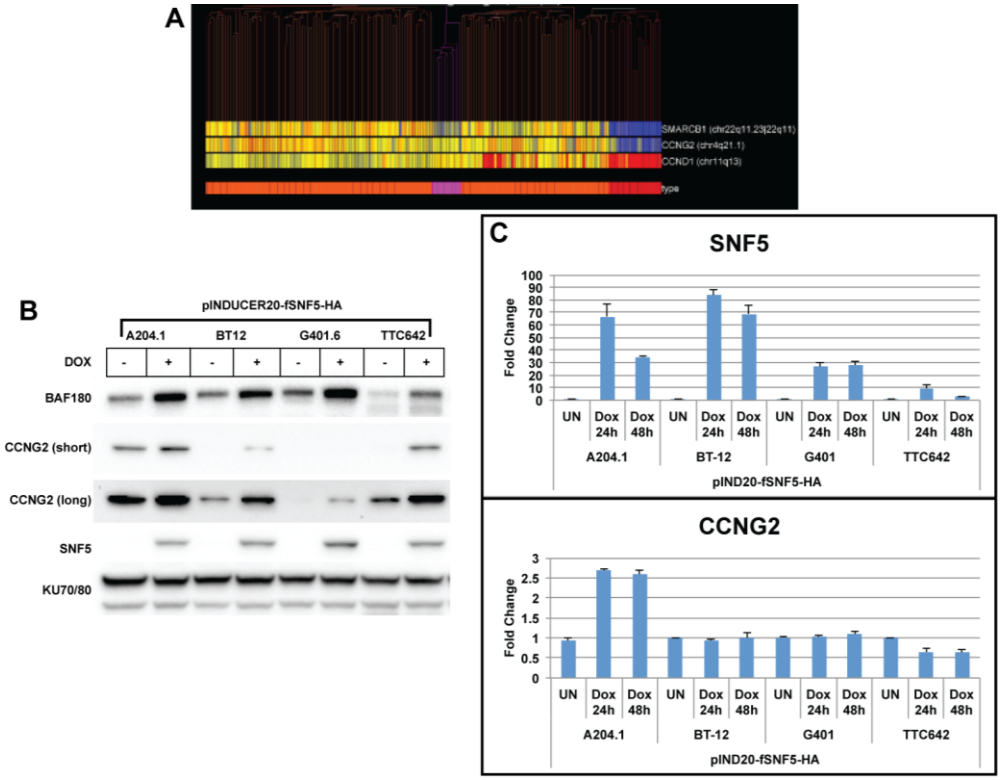


Figure 4.3- SNF5 expression correlates with CCNG2 expression in MRT cell lines and primary tumors. (A) Cyclin G2 expression was measured using Affymetrix U133A2 microarray data from a panel of medulloblastomas, normal cerebellum, and MRTs. Data was visualized using GeneSpring GX 7.3.1 software(McKenna et al., 2008b). (B) Cells were harvested at the indicated times after induction by 1 µg/ml doxycycline. Total cellular proteins (30 µg) were separated on a 4% to 12% Bis-Tris polyacrylamide gel and probed with anti-SNF5, anti-CCNG2, anti-BAF180, or anti-KU70/80 antibodies. UN, untreated; Dox, induced with 1 µg/ml doxycycline for 24 hours. (C) RNA was extracted from the 4 MRT cell lines at the indicated times after induction by 1 µg/ml doxycycline. The mRNA levels for SNF5 and CCNG2 were assessed by qPCR. All values are normalized to actin, with relative expression shown. Each gene was examined in duplicate from 2 biological experiments, with error bars indicating standard deviation among the replicates

4.4.5 CCNG2 does not associate with the SWI/SNF complex

The increase in CCNG2 protein in the absence of a corresponding rise in its mRNA levels suggested that SNF5 reexpression might affect protein stability. Therefore, a potential mechanism for increased CCNG2 protein after SNF5 reexpression is an interaction with the SWI/SNF complex as observed for other proteins such TP53, AKT and CMYC(Cheng et al., 1999b; Foster et al., 2006; Lee et al., 2002). To test this notion, we induced SNF5 expression in the G401pIND20-fSNF5-HA MRT cell line and the BT-12pIND20-fSNF5-HA AT/RT cell lines. We then immunoprecipitated the SWI/SNF complex via binding to a BRG1 antibody and examined its components before and after SNF5 expression. As shown in Figure 4.4, we found known components that either appeared or increased in the complex after SNF5 reexpression. However, we did not observe CCNG2 protein associated with the complex under either condition. Therefore, another mechanism must account for the increased CCNG2 protein levels observed in TTC642, G401.6 and BT-12 cell lines.

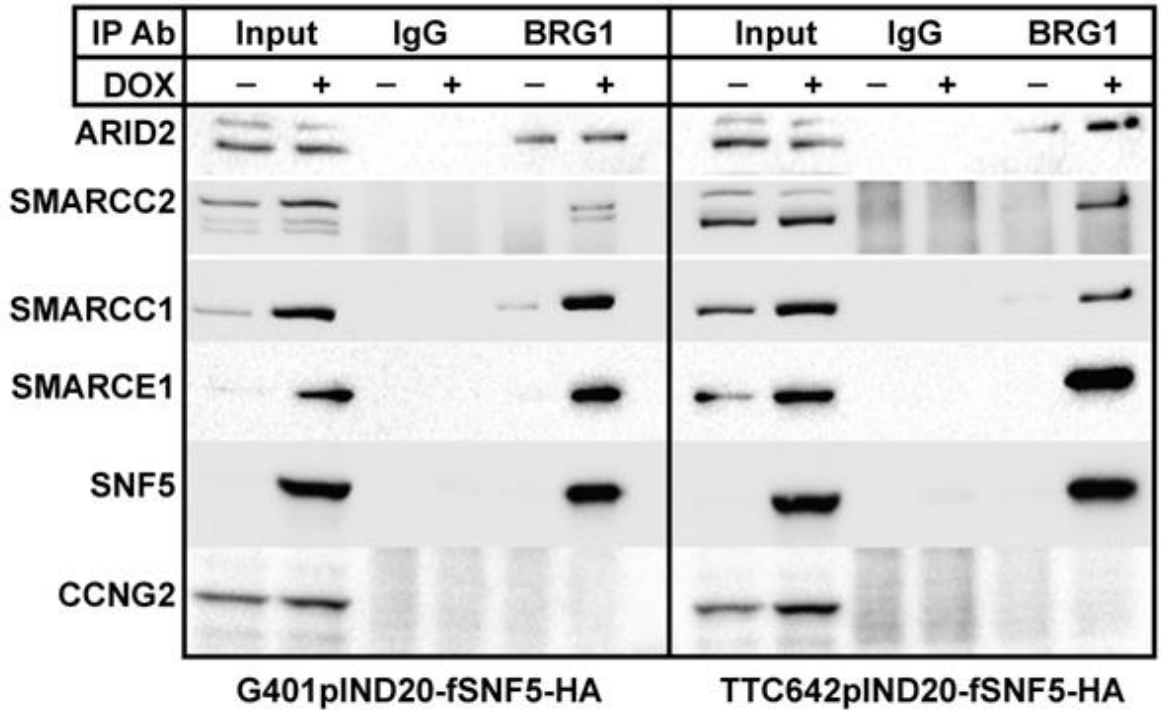


Figure 4.4. CCNG2 does not associate with the SWI/SNF complex. The G401pIND20-fSNF5-HA and TTC642 G401pIND20-fSNF5-HA cell lines were treated with 1 μg/ml doxycycline for 24 hours. Treated and untreated samples were then immunoprecipitated with rabbit IgG, or anti-BRG1. IP samples were then separated on a 4% to 12% Bis-Tris polyacrylamide gel, transferred to PVDF membranes and probed with anti-SNF5, anti-ARID2, anti-SMARCC2, anti-SMARCC1, anti-SMARCE1, or anti-CCNG2. After probing with the appropriate secondary antibodies, protein bands were visualized using the LI-COR Odyssey Western Blot Detection System or using ECL reagents (GE Healthcare) followed by exposure to X-ray film

4.5 Discussion

Although the discovery that SNF5 inactivation occur in virtually all MRTs and AT/RTs, the exact mechanisms that drive tumorigenesis remain unclear(Roberts and Biegel, 2009b; Versteege et al., 1998c). Previous studies have shown implicated SNF5 loss as altering cell cycle control through multiple signaling pathways including RB, p53 and BIN1(Roberts and Biegel, 2009b; Wei and Weissman, 2014). However, disruption of any of these pathways could not completely block SNF5 induced growth arrest after reexpression in MRT cell lines(Chai et al., 2005; Kuwahara et al., 2010; McKenna et al., 2012). Our current study identifies 4 additional genes, CCNG2, HERC5, CCNH and CDK8, whose increased expression after SNF5 reexpression may prove important to the subsequent growth arrest and induction of replicative senescence. Importantly, these genes are not associated with growth arrest alone because they do not change after inhibition of proliferation induced by activation of the RB pathway (Table 4.2).

Previous studies have implicated each gene as a potential regulator of the cell cycle. Cyclin G2 (CCNG2), CDK8, a member of the Mediator complex, is recruited to promoters undergoing active transcription by RNA Polymerase II (Donner et al., 2007). Interestingly, CDK8 is recruited to the *p21* promoter, a known SNF5 binding target, during activation by p53 signaling (Donner et al., 2007). Decreased levels of CDK8 can reduce activation of p53 downstream targets, while overexpression of CDK8 can promote cell growth in colon cancer(Donner et al., 2010). HERC5 is a Hect3-type E3 ligase involved in addition of ISG15 to cellular proteins during an active innate immune response(Dastur et al., 2006). CCNH is a member of the cdk-activating complex (CAK), which is involved in cell cycle control(Rossignol et al., 1997). CAK can associate with TFIIH and modulate RNA

polymerase II activity(Rossignol et al., 1997). Some polymorphisms in the *CCNH* gene have been linked to an increased risk of glioma development(Rajaraman et al., 2007).

In the current study, we primarily focused on the role of Cyclin G2 (CCNG2) in SNF5 driven growth arrest in MRT and AT/RT cell lines. CCNG2, a member of the non-canonical Cyclin G family, is thought to act as a negative regulator of the cell cycle(Horne et al., 1997b). CCNG2 is located primarily in the cytoplasm, yet it also can enter the nucleus. Additionally, overexpression can create unusual nuclear structures(Bennin et al., 2002b). The evidence that reduced levels of protein expression have been found in multiple human cancers as well as its ability to induce growth suppression in cultured tumor cells implicate it as a classic tumor suppressor(Ito et al., 2003; Kim et al., 2004; Sun et al., 2014a; Sun et al., 2014b). Furthermore, it has been implicated as an inducer of apoptosis during early development(Yue et al., 2005). Therefore, its reduced expression upon SNF5 inactivation could block terminal differentiation consistent with the paradigm that MRTs arise from arrested differentiation of multipotent progenitor cells(Biegel, 2006).

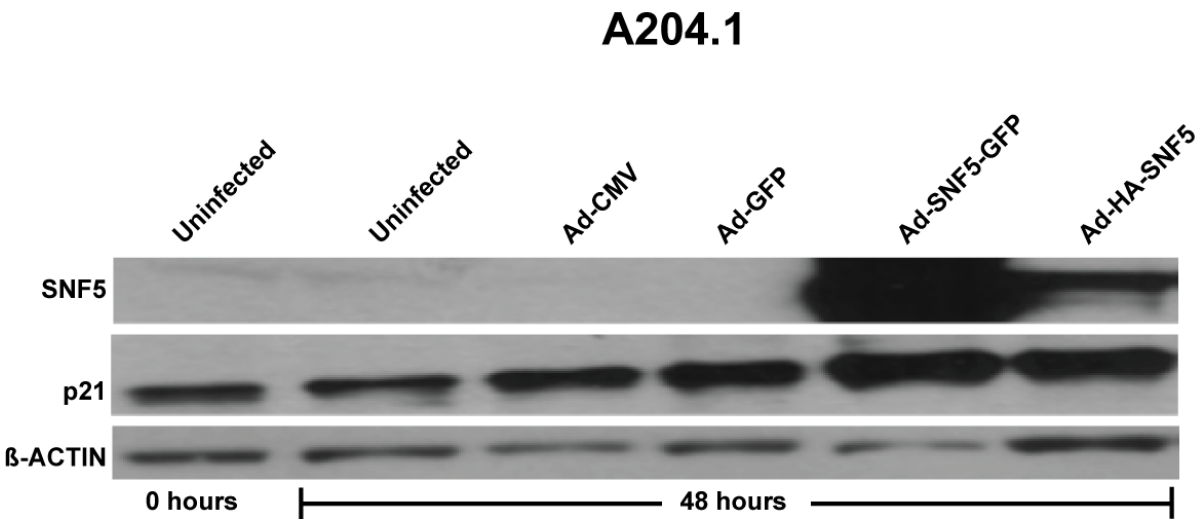
Induction of gene expression via restoration of functional SNF5 in MRTs may be due to restoration of proper SWI/SNF maintenance of nucleosome positioning and epigenetic marking in their promoter regions(Tolstorukov et al., 2013). This concept appears consistent with our ChIP analysis where SNF5 binds to the transcription start site of CCNG2 (Figure 4.2). We have observed a similar pattern of SNF5 binding after its reexpression in the TTC642 cell line for other genes(Kuwahara et al., 2013a). The modest increase in RNA Polymerase II binding at the CCNG2 promoter after SNF5 reexpression may reflect the limited increase in mRNA expression. A more extensive ChIP analysis of the promoter region including additional epigenetic marks, such as histone acetylation and methylation and

polycomb proteins may shed light on other changes that contribute to altered gene expression.

We also attempted to knockdown CCNG2 expression in MRT cell lines to assess its role in SNF5-induced growth arrest. Using several different shRNAs against CCNG2, we could not isolate viable colonies from A204.1 cell line. While we did isolate colonies after CCNG2 knockdown in the TTC642 cell line, they showed only modest reductions in its protein levels (<50%). These results may indicate that CCNG2 plays a biologically significant role in the normal growth of these MRT cell lines. We are currently developing vectors for inducible expression of CCNG2 shRNAs to better address this question.

The difference in mRNA expression of CCNG2 in A204.1 versus the other cell lines after SNF5 reexpression suggests a considerable level of variability among them. We also observe different expression patterns for the other 3 putative target genes among the cell lines after SNF5 reexpression (data not shown). As the cell type of origin for MRTs remains unknown, these differential responses among the cell lines could indicate different cells of origin. These differences could result in a varying basal gene expression patterns before and after transformation. The fact that both primary MRTs and the A204.1 cell line show reduced expression of CCNG2 mRNA suggests it may play a role in MRT oncogenesis; however, further characterization is warranted to solidify this hypothesis. Additional insights may also provide new avenues for the development of safer and more effective treatments of MRTs. Further analyses of the additional target genes may valuable insights into the molecular mechanisms underlying the etiology of this deadly pediatric cancer.

4.6 Supplementary Figures



Supplementary Figure 4.1 - Comparison of SNF5 expression after Ad-SNF-GFP vs. Ad-HA-SNF5 infection. Cells were harvested before and 48 hours after infection by Ad-CMV, Ad-GFP, Ad-SNF5-GFP or Ad-HA-SNF5. Total cellular proteins (30 µg) were separated on a 4% to 12% Bis-Tris polyacrylamide gel and probed with anti-SNF5, anti-p21 or anti-ß-actin antibodies. Antibodies were visualized by ECL-Plus (GE Amersham) and X-ray film exposure.

Chapter 5: Conclusions and Perspectives

5.1 Summary of Findings

In the past decade, epigenetics has become an area of intense research; however, only recently has the spotlight fallen on the SWI/SNF complex. Recent advances in high throughput technologies have made whole genome sequencing readily accessible and increased the coverage and depth of the sequencing data. This increase in capability has enhanced the scientific community's ability to examine tumor genomes comprehensively. Using this approach, scientists have identified genes consistently mutated but at low frequencies that had been previously overlooked. The fruit of this labor has been the identification of mutations in SWI/SNF complex members in cancer. Each SWI/SNF subunit has a relatively low mutation rate. Consequently, they lacked the notoriety of TP53, PTEN, and BRCA1 mutations. However, when one aggregates the data of SWI/SNF mutations in cancer, we find mutations in 20 SWI/SNF subunit genes across 18 different cancers (Shain and Pollack, 2013). Combined, the mutation rate of SWI/SNF complex members occurs at a frequency of 19% comparable to the 26% mutation frequency of TP53(Shain and Pollack, 2013).

The identification of mutations in subunits of the SWI/SNF chromatin remodeling complex in cancer should not be surprising because of its multi-faceted roles in cells. The SWI/SNF complex repositions nucleosomes to regulate transcription of target genes, and it has also been implicated in other cellular processes including DNA synthesis, viral integration and expression, and mitotic gene regulation (Roberts and Orkin, 2004). However,

the effects of SWI/SNF subunit mutations on these cellular processes have yet to be fully characterized. Furthermore, whether mutations in different subunits contribute to cancer development through distinct and/or overlapping mechanisms remains unclear. Thus, understanding the roles of the SWI/SNF complex in the regulation of these processes must be a high priority in cancer research.

In these dissertation studies, we focused on the role of SNF5 inactivation in the development of MRTs. Our results have indicated that SNF5 mediates the composition of the SWI/SNF complex, and its loss potentially disrupts SWI/SNF complex variants that are required for differentiation. While the SWI/SNF complex still formed in MRTs in the absence of SNF5, its re-expression vastly changed the stoichiometry of the complex. Specifically, ectopic expression of SNF5 in MRTs led to its incorporation into the complex followed by changes in other subunits in the complex. Intriguingly, the mRNA levels of these complex members do not change after SNF5 re-expression. Therefore, in MRT, SWI/SNF subunits are post-transcriptionally regulated in an interdependent fashion for stability. The changes in the SWI/SNF complex also alters gene expression and results in a p21-dependent growth arrest (Kuwahara et al., 2010). However this pathway does not completely account for the growth arrest, as knocking out p21 does not completely abrogate SNF5 induced growth arrest (Kuwahara et al., 2010). Therefore, we also investigated the changes in SNF5-dependent gene expression. In this project we examined several known targets of SNF5, p21 and p16. Additionally, we also examined 2 novel targets of SNF5, NOXA and CCNG2. Identification of NOXA came about during efforts to identify the subset of p53 dependent genes that are also SNF5 dependent. CCNG2 was identified through the use of microarrays.

Both NOXA and CCNG2 levels increased after SNF5 re-expression and their expression are known to be growth inhibitory, thus they may play a role in SNF5-induced growth arrest.

Furthermore, the loss of SNF5 results in down-regulation of various SWI/SNF complex member proteins that have also been implicated as tumor suppressors. Ultimately, the loss of SNF5 does not constitute a loss of a single tumor suppressor; it affects the activities of multiple tumor suppressors that regulate targeting of the SWI/SNF complex resulting in aberrant transcription. These findings also emphasize the notion that mutations of SWI/SNF subunits must be evaluated in a biological context. Thus, the magnitude of the effects of SNF5 loss on cellular processes has been overlooked due to limitations of a sequencing-centric approach. The value of the sequencing studies is greatly improved when complemented with other approaches including high throughput proteomics, gene expression analyses, ChIP-seq and MNase-seq. These global approaches require substantial investment in human capital, expertise, and equipment that is difficult to achieve at any one institution. Pursuit of this goal compels an evolution from the current research model to a network-centric research model that coordinates research efforts to allow for better integration of scientific data.

5.2 Challenges encountered and observations

Current studies of the SWI/SNF complex have often been centered on the re-expression of subunits in mutant cell lines; the study of SNF5 is no exception. There are 2 major stumbling blocks in this approach. Firstly, SNF5 and other SWI/SNF subunits have been implicated as tumor suppressors. SNF5 re-expression in the SNF5-null MRT cell lines

results in growth arrest. Secondly, the methods to re-expresses SNF5 are limited and have their shortcomings. The most common methods include DNA transfection and viral transduction. DNA transfection using liposomal transfection reagents have been implicated in eliciting stress response and altering gene expression (Fiszer-Kierzkowska et al., 2011). Furthermore, our laboratory has found that the transfection rate for most MRT cell lines is poor, 10% or less. Our laboratory generally utilized adenovirus transduction as the primary method to re-express SNF5. Adenoviruses are double strand DNA viruses that can introduce DNA into the nucleus of cell without incorporation into the genome. Adenoviral vectors are considered a valuable tool for gene therapy for several reasons. Adenoviruses are capable of infecting a broad range of human cells, including non-proliferating cells, and they are believed to have low pathogenicity. Additionally, adenoviral vectors can accommodate large DNA fragments and have a high DNA transfer efficiency. Lastly, this method is considered safe because the transduced DNA is not integrated into the host genome, therefore it will not be replicated. However, we observed a stress response in the form of p21 induction following adenoviral infection. This finding is problematic because p21 is a known mediator of growth arrest and its induction may confound the pathways of growth arrest activated by SNF5. Furthermore, adenovirus infection causes considerable overexpression of the transgene raising questions of physiological relevance (Wei et al., 2008).

In order to address these challenges caused by the use of adenoviral vectors, we sought to make an inducible SNF5 expression system. Our laboratory has previously made several abortive attempts to produce such a system. The main barrier for an inducible SNF5 MRT cell line is the “tightness” of expression. Residual expression of SNF5 is sufficient to prevent the propagation of the cell line. We were able to successfully generate a tetracycline-

inducible SNF5 vector using the pINDUCER system designated pINDUCER20-flag-SNF5-HA (pIND20-fSNF5-HA). The pINDUCER system is a novel system with minimal basal expression of the gene of interest, high inducibility, and antibiotic resistance gene for selection of infected cells (Meerbrey et al., 2011a). We had previously examined the effect of SNF5 re-expression on SWI/SNF complex composition using adenovirus. We sought to replicate our findings using pIND20-fSNF5-HA. There was striking difference in the α-BRG1 IP of the Ad-CMV infected sample and untreated pIND20-fSNF5-HA sample. We observed higher levels SWI/SNF subunits pulled down in Ad-CMV treated samples compared to untreated pIND20-fSNF5-HA BRG1 pull down. This demonstrates that use of adenovirus has a reproducible effect on the SWI/SNF complex and potentially confounds previous data. Thus, the use of pIND20-fSNF5-HA proved critical and highly relevant to the understanding of the SWI/SNF complex.

5.3 Relevance to toxicology

These studies were initiated in the context of assessing the potential contribution of environmental toxicants on the development of this rare but deadly pediatric cancer. While, the etiology of MRTs remain unclear, discussion of environmental exposures is relevant because there is evidence to suggest that parental environmental exposure may lead to MRTs in their offspring (Swinney et al., 2006a). An epidemiological study conducted in California found that rhabdoid tumors were associated with low birth weight, preterm labor, and twin pregnancies (Heck et al., 2013). Maternal exposure to environmental toxicants, such as cigarette smoke and sulfur dioxide, have been shown to influence such factors such as low birth weight and preterm labor, respectively(Chomitz et al., 1995). Also, a US Navy study found paternal exposures to pesticides have also been associated with pre-term labor

(Hourani and Hilton, 2000).This phenomenon of paternal exposures as a contributing factor is not a novel concept. As early as 1974, a father's occupational exposure to hydrocarbons was associated with the increased risk of his offspring developing malignancies(Fabia and Thuy, 1974). Additionally, paternal age or geographic location was also determined not to be contributing factors (Fabia and Thuy, 1974). This information underscores the relevancy of environmental exposures in a discussion regarding pediatric cancers.

Environmental exposures have been shown to impact the epigenome. These changes to the epigenetics machinery result in gene expression changes which can ultimately alter an organism's sensitivity to toxic exposures and disease(Szyf, 2011). Chromatin plays a major role in the response to these changes and recently we have begun examining epigenetic changes as an endpoint. Alterations of chromatin by toxicant exposure can result from changes in DNA methylation, histone posttranslational modifications, and ATP-dependent chromatin remodeling. DNA methylation and histone modification are common endpoints utilized when studying epigenetics in the context of toxiciology. For example, arsenic exposure results in aberrant methylation patterns (Reichard and Puga, 2010). Additionally, neurotoxic pesticides have been implicated in the histone hyper-acetylation by inhibiting the degradation of a histone acetyltransferase (HAT) (Song et al., 2010). There is evidence some epigenetic changes in response to environmental stress facilitate alterations in chromatin structure and gene activity to generate a proper cellular response (Clayton and Mahadevan, 2003).

A more relevant toxicological endpoint to this dissertation is chromatin remodeling. This can occur as a result of nuclear receptor (NR) activation, DNA damage response, and changes to histone tail modifications. The activated nuclear receptors are able to recruit

other co-factors and effect changes in the chromatin landscape to facilitate gene transcription (Moggs and Orphanides, 2004; Morris et al., 2014). The role of the SWI/SNF complex in nuclear receptor regulated gene expression was first described in the mechanism of glucocorticoid receptors (Trotter and Archer, 2007). Since then, the SWI/SNF complex has been implicated in other nuclear receptor dependent gene expression. The mechanism of action of most chemical carcinogens is direct damage of DNA. For example, chromium exposure causes DNA adducts that can be repaired by nucleotide excision repair. In order to repair such damage, the chromatin landscape needs to be altered to allow access to the damage sites by DNA repair enzymes (Green and Almouzni, 2002). Additionally, a neurotoxic pesticide, dieldrin, prevents the degradation of Creb-binding protein (CBP) which contains a HAT domain, and this increase of CBP results in hyper-acetylation of histones (Song et al., 2010). Acetylated histone H3 is known to recruit the SWI/SNF complex although their exact interaction is unclear. However, it may play a role as an additional layer of regulation of cellular processes (Chatterjee et al., 2011). Thus, disruption of the SWI/SNF complex will dramatically alter response to insult from environmental agents.

In the context of MRTs, our observations suggest that environmental stress, specifically adenoviral infection, can have a direct effect on the SWI/SNF complex. SNF5 is critical for the transcription of interferon inducible genes (Cui et al., 2004). This suggests that MRT cell lines are unable to mount an effective antiviral response. Intriguingly, we observed that the act of infecting MRT cell lines was sufficient to increase the basal levels subunits within the SWI/SNF complex. One possible explanation is that adenoviral infections induce SNF5-independent expression of co-factors that then bind to their respective SWI/SNF complex components to affect an immune response. The binding of co-factors

helps reduce the innate instability resulting in low levels observed. Based on these suppositions, a provocative hypothesis is that MRTs arise from the confluence of 2 low probability events- SNF5 loss and a rare environmental exposure. SNF5 loss is known to be a lethal mutation; however, there may be critical window of opportunity during which an environmental stress (viral, chemical, etc.) stabilizes the complex in such a way that it disrupts the senescence programming resulting in malignancy. Taken all the data together, it is evident that an understanding of chromatin is highly relevant to toxicology.

5.4 MRT cell of origin

The cell of origin of MRT has remained a mystery thus far. MRTs have been found primarily in the kidneys (RTK) and the central nervous system (AT/RT); however, MRTs also arise from a variety of other soft tissues. The primary method for the diagnosis of MRTs has been 2–fold: (1) histological staining and identification of hallmarks of MRT cells such as acentric nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm and (2) the absence of SNF5. There has been significant controversy in the field regarding the distinction between RTKs, AT/RTs and MRTs found in other organs. These rhabdoid tumors arise in different sites but are linked by one commonality, SNF5 loss. Therefore, whether these tumors are the same or represent cancers with a common genetic lesion must be examined.

A recent study demonstrated that while gene expression varies, microRNA (miRNA) expression remains fairly similar in RTKs and AT/RTs (Grupenmacher et al., 2013a). miRNAs are small non-coding RNA molecules that have been implicated in both positive and negative gene regulation. There is evidence to suggest that miRNA expression and function is highly regulated in a cell type dependent manner (Kuppusamy et al., 2013). This similarity suggests that RTKs and AT/RTs are derived from the same progenitor cell type.

Based on our current understanding, the most likely candidate is a neuronal stem cell. This observation creates the opportunity to test a new methodology to diagnose MRTs utilizing miRNA profiling. This disconnect between miRNA expression and gene expression is not unprecedented. Intriguingly, a similar pattern exists for colon cancer and ulcerative colitis patients, where miRNA profiles are sufficiently similar to be used diagnostically while gene expression profiles are less consistent (Ahmed et al., 2009; Birkenkamp-Demtroder et al., 2002). Pilot studies conducted in melanoma, colon cancer, and ulcerative colitis patients have demonstrated that diagnosis by this approach may prove non-invasive, accurate and fast (Ahmed et al., 2009; Leidinger et al., 2010).

Additionally, the issue of composite rhabdoid tumors (CRTs) must also be addressed. CRTs are similar to MRTs in that they share many of the same histopathological feature of rhabdoid tumors. However there are several distinctions. While MRTs are typically pediatric cancers, CRTs are mostly adult tumors. As the name suggests CRTs contain a component of rhabdoid cells, but surprisingly these rhabdoid cells retain SNF5 expression (Perry et al., 2005). Therefore, miRNA profiling maybe provide a molecular basis to determine if there is a distinction between MRTs and CRTs. This information would shed more light and enhance our understanding

5.5 Therapeutic strategies for cancers with SWI/SNF mutations

There are currently no effective treatments for patients with MRTs (Ahmed et al., 2007). The existing protocols for treatment of MRT include tumor resection, followed by adjuvant chemotherapy and/or radiation (Biswas et al., 2009). These current protocols suffer from several inadequacies, including the difficulty of resection due to the tumor size and the contraindication of radiation in young patients (Biswas et al., 2009). Improvements in

patient treatment and survival warrants further study to understand the molecular mechanisms behind MRT tumorigenesis. Three potentials treatment strategies standout based on currently understating of the SWI/SNF complex: synthetic lethality, stabilization of SWI/SNF subunits, and short term tumor suppressor expression.

5.5.1 Synthetic lethality

Synthetic lethality refers to concurrent loss of 2 or more genes that result in cellular death, where loss of either gene alone would have little to no effect. This strategy can be utilized in targeted therapy by knocking out a gene that is synthetic lethal with a cancer gene mutation. This method, in theory, would result in the death of cancer cells while normal tissue would remain unaffected. This is an appealing strategy because many SWI/SNF complex members share a high degree of homology. Thus, while cells can survive loss of either subunit, the absence of both homologous proteins leads to growth inhibition or cell death. One example is normal cells tolerating either BAF250A or BAF250B loss, but not both. Therefore, knocking down of BAF250B in cancers with pre-existing inactivating mutations of BAF250A results in growth inhibition while cells with wild-type BAF250A continue to grow (Helming et al., 2014). Another potential target is BRM in BRG1-null cancers. BRM and BRG1 are mutually exclusive core ATPase units of SWI/SNF and loss of BRG1 results in greater incorporation of BRM into the complex (Hoffman et al., 2014; Wilson et al., 2014). Depleting BRM in BRG1-null cancers results in cell cycle arrest and senescence but has little effect on BRG1 wild-type cells(Hoffman et al., 2014). Additionally, recent reports have suggested that BRM-containing complexes in cancers lacking BRG1 may acquire oncogenic functions (Oike et al., 2013; Wilson et al., 2014). However, one of the challenges with this approach for homologous SWI/SNF complex members is the high risk

of off target effects. While this approach is viable for some pairs of SWI/SNF complex members, it does seem applicable to SNF5, a unique component of the complex. The fact that SNF5 loss is poorly tolerated in fibroblasts supports this notion(Wang et al., 2009). However, one report showed that BRG1 loss abrogated tumor development in a genetically engineered mouse model based upon SNF5 inactivation (Wang et al., 2009). This may occur due to the lack of BRM expression in most MRTs.

5.5.2 Stabilization of SWI/SNF subunits

The results of these dissertation studies have shown that SNF5 is critical for the stability of other complex members. Based on preliminary MNase-seq data, it seems SNF5 re-expression in MRTs does not have a profound effect on the global nucleosome positioning, implicating the changes in complex composition as a major oncogenic mechanism. Little research has focused on the effects of stabilizing SWI/SNF complex proteins in the absence of SNF5. Therefore, determining the effect of stabilizing the complex sans SNF5 for tumor suppressor activity is of probative value. There are two distinct approaches to understand the interactions of SNF5 and SWI/SNF subunits.

The first strategy is to mutate the domains of SNF5 to identify specific residues interacting with each SWI/SNF subunit. Using this knowledge, one could then generate peptides that might mimic the effects of the whole protein by blocking certain ubiquitinylation sites and prevent degradation of SWI/SNF subunits. Literature evidence has shown p53 interacts with the SWI/SNF complex through BAF60A which is stabilized by SNF5(Oh et al., 2008). If small peptides can stabilize proteins such as BAF60A in SNF5 mutant cancers then one might expect increased binding of p53 to the SWI/SNF complex. As a result, the tumor may become sensitized to DNA damage as p53 targets the complex to

DNA damage response genes, such as p21. Alternatively, this approach can be used to develop probes that bind complex members, obstruct their subunit interactions, and disrupt SWI/SNF complex entirely. In the absence of SNF5, the SWI/SNF complex still forms despite the instability of the member subunits. Complete disruption of the complex could result in decreased cell viability if not cell death. Using either approach has value as an investigational chemical probe by overriding SNF5-dependency. Limitations of this approach for tumor therapy include difficulties in drug delivery and in targeting a small peptide into the nucleus where the SWI/SNF complex is found.

A second approach would take advantage of the degradation of SWI/SNF proteins in an ubiquitin-ligase dependent manner. Treatment with MG132, a broad spectrum ubiquitin-ligase inhibitor, seems to stabilize SWI/SNF subunits including BAF180 and SNF5. A key issue is whether increasing the global level of these proteins will result in increased incorporation into the complex. Compounds like MG132 offer several advantages including its cell permeability and the potential for oral administration. MG132 treatment induces in apoptosis in cancer cells; however, there is a report showing that MG132 can also induce death of human pulmonary fibroblast cells(Guo and Peng, 2013; You and Park, 2011). Thus, a screening approach is needed to identify selective inhibitors to increase the potential of this strategy as a therapy. In addition, targeted inhibition of specific ubiquitin ligases may help us to further understand SWI/SNF complex subunit action in the absence of SNF5. Much about the process of SWI/SNF complex assembly remains poorly understood.

5.5.3 Short-term expression of tumor suppressor

The least understood treatment strategy is perhaps the most obvious- re-expression of the tumor suppressor. Using an MRT cell line with a doxycycline-inducible SNF5 expression

vector, one has unprecedented control over SNF5 re-expression. One unexpected observation is that induction of SNF5 expression for 24 hours is sufficient for irreversible growth inhibition in vitro (Figure 5.1). While no experiments have been conducted to examine the effects of SNF5 over-expression in wild type cells, it would seem unlikely to have a significant effect. SNF5 protein seems to be highly regulated as evidenced by an increase in SNF5 following MG132 treatment in fibroblasts. It would appear the re-expression of SNF5 results in a critical irreversible epigenetic change, perhaps $p16^{INK4A}$ expression, that commits the cells to growth arrest after 24 hours. Localized treatment with an inducible SNF5 vector maybe an effective treatment approach and minimize off target effects.

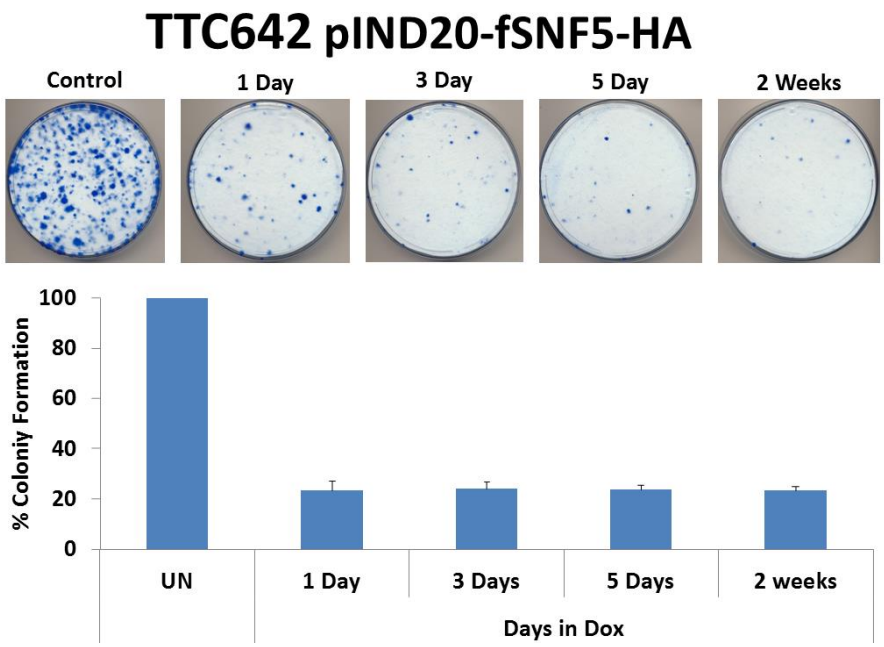


Figure 5.1 – 24hrs of SNF5 induction sufficient for reduction in colony formation. To determine the effects of *SNF5* re-expression on individual cell growth colony formation assays were carried out. Cells were treated with doxycycline for the duration indicated, and then washed. Colonies were then fixed and stained with Coomasie blue after 2 weeks. Colonies containing at least 1000 were inspected visually. Error bars – S.E. of 3 biological replicates .

5.6 Future Directions

The study of the SWI/SNF complex has a reached a watershed moment in our understanding of the biological world. There is clear evidence supporting the importance of understanding chromatin regulation, and by extension the SWI/SNF complex, in not only cancer biology but also toxicology. Given these relationships, there are many potential avenues of further studies that query SNF5's role in toxicology. Three objectives are perhaps most intriguing. Firstly, effort must be made to investigate the effects of stabilizing SWI/SNF members through various means (e.g. small peptides, proteasome inhibitors) in order to identify potential therapeutic strategies. Secondly, identifying the mechanism of how short-term tumor suppressor re-expression leads to senescence. This surprising phenomenon suggests that one mechanism of senescence occurs epigenetically and is irreversible. Lastly, in depth miRNA profiling may provide an additional level of understanding of the processes that underlie MRT tumorigenesis. Together these findings will better inform attempts at understanding the underlying pathology and identify targets of therapeutic value.

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