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

Yasumichi Kuwahara1,2, Darmood Wei1,3, Joel Durand1,4 and Bernard E. Weissman1,3,4,5

1Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA, 2Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan, 3Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA, 4Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA, and 5Department of Pathology and Laboratory Medicine

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Corresponding Author- Bernard E. Weissman, Lineberger Comprehensive Cancer Center, Room 32-048, University of North Carolina, 450 West Drive, Chapel Hill, NC 27599-7295; Office (919) 966-7533; FAX (919) 966-3015; weissman@med.unc.edu

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Abstract

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 G1 arrest via p21CIP1/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 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 demonstrate lower NOXA expression in MRT cell lines compared to other human tumor cell lines suggesting that hSNF5 loss may alter the expression of this important apoptotic 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.
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 (1). The most common locations are in the kidney and central nervous system, although MRTs may arise in almost any site (2, 3). Despite significant advances in treatment, for MRTs diagnosed before the age of 6 months, patient survival at 4 years drops to ~8.8% (4). 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 inactivation of hSNF5 (also known as SMARCB1, INI1, and BAF47), located in chromosome band 22q11.2 (5), that implicates the loss of hSNF5 function as the primary cause of these tumors (6). 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 (7). SWI/SNF subunits can be sub classified into three categories: (1) ATPase subunit (either BRG1 or BRM), (2) core subunits (hSNF5, BAF155 and BAF170), (3) accessory subunits (BAF53, BAF57, BAF180 and others) (8). How loss of the core SNF5 subunit 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 (9). However, little is known about how hSNF5 activates transcription at its target promoters.

Therefore, in this study, we initially sought additional HSNF5 regulated genes to characterize the effects of HSNF5 reexpression of its target genes. We focused on HSNF5 regulation of other p53 target genes because mutations that inactivate the p53 pathway rarely appear in MRTs (10, 11). 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 its 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.
Materials and Methods

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 (9, 12, 13). 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.

Colony Forming Assay. Approximately 1 x 10^6 TTC642 cells were then transfected with 6μg plasmid DNA (pcDNA3, pcDNA3-fSNF5, pcDNA-wt-hNOXA) using Fugene 6 Reagent (Promega). At 24h post transfection, each 10cm plate was split into 8x 10cm plates and placed on selective media containing 300μg/ml Neomycin (Gibco, Grand Island, NY). Two weeks after transfection, the plates were fixed with 95% ethanol and stained with Coomassie blue (50% Methanol, 10% Glacial Acetic Acid, 1g/L Coomasie Blue). Colonies with greater than 1000 cells were counted by visual inspection.

Protein extracts and Western blotting. Western blotting was carried out as described previously (13). 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).
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 $2^{-\Delta\Delta C_t}$ method (14, 15). The primers used in this study is described in Table 1.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was carried out as described by Donner et al (15). 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 2 (15).
Results

The effects of reexpression of hSNF5 on p53 target genes in MRT cell lines. To determine whether hSNF5 regulates additional 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 1). All 3 cell lines showed modest to robust induction of p21\textsuperscript{CIP1/WAF1} expression and decreased expression of 14-3-3\textgreek{sigma}. 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 1). The expression of the remainder of the genes also diverged among the lines with increased MDM2 expression in A204.1 and higher GADD45b expression in TTC642. These results suggest that inactivation of hSNF5 does not recapitulate p53 loss in MRT cell lines.

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 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 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 3A). Finally, we asked whether NOXA expression might contribute to the potent growth arrest induced by HSNF5. Using a colony forming assay, we found that both HSNF5 and NOXA expression in the TTC642 cell line gave similar levels of growth inhibition (Figure 3B).

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 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 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 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 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.
Reexpression of hSNF5 induces p21CIP1/WAF1 and NOXA transcription accompanied with SWI/SNF complex recruitment. In our previous report, we demonstrated that hSNF5 directly controlled p21CIP1/WAF1 transcription activity. However, the mechanism of p21CIP1/WAF1 and NOXA transcription activity induced by hSNF5 has not yet been clarified. To investigate the occupancy of reexpressed hSNF5 on the p21CIP1/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 p21CIP1/WAF1 promoter regions from -3000 to +4001 as previously described (15) (Figure 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 5b). In contrast, BRG-1 and BAF155 levels increased on p21CIP1/WAF1 locus throughout the whole region (Figure 5b). These results obtained from p21CIP1/WAF1 locus were congruent with those obtained from the NOXA locus (Figure 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.

Reexpression of hSNF5 induces p21CIP1/WAF1 and NOXA transcription accompanied with RNAPII recruitment and histone modification. To assess the effects of reexpressed hSNF5 on known steps of transcriptional activation, we performed ChIP assays for RNAPII, H3K4me3 and H3K36me3 on the p21CIP1/WAF1 and NOXA loci at 24 hrs. after infection of Ad-hSNF5 compared with Ad-GFP infection. Our ChIP results for RNAPII showed an increase at the TSS on both p21CIP1/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 p21CIP1/WAF1 locus after hSNF5 reexpression demonstrated an increase in binding at the TSS, with a maximal peak near the TSS (Figure 5c). These results support the
H3K4me3 ChIP results of obtained from the NOXA experiments (Figure 4c). Because increased H3K4me3 binding correlate with promoter activation (16), 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 p21CIP1/WAF1 and NOXA promoters (Figure 4c, Figure 5c). These results imply that reexpression of hSNF5 presumably precedes the transcription elongation step (17).

**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 (18), and did not observe binding after hSNF5 reexpression (Figure 4e). In our previous study, we determined that up-regulation of p21CIP1/WAF1 transcription by hSNF5 operated through a p53-dependent mechanism in A204.1 cells, but through a p53-independent mechanism in TTC642 cells (9). 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) (9). Infection of the pLKO.1 and p53KD cells with Ad-GFP did not alter levels of NOXA mRNA 24 hours after infection compared to the uninfected parental cell lines (Fig. 4f). However, we found an increase of NOXA mRNA after hSNF5 reexpression that was not significantly different among the TTC642, TTC642 pLKO.1 and TTC642 p53KD cells (Fig. 4f). These observations suggest that hSNF5 binding could initiate transcription on the p21CIP1/WAF1 and NOXA promoters in the TTC642 cell line without additional p53 recruitment.
Discussion

The objective of this study was to identify additional genes, besides $p21^{\text{WAF/CIP1}}$, regulated by both hSNF5 and p53 in MRT cells and to determine the mechanism of hSNF5 regulation of their transcriptional activity. While HSNF5 could regulate expression of varying p53 target genes among the MRT cell lines, NOXA was the only common target gene in 5/6 cell lines. We then used ChIP analyses of the promoter regions of the $p21^{\text{WAF/CIP1}}$ and NOXA genes to understand how HSNF5 regulates their expression. Our results provide major insights into the control of hSNF5 target genes and how hSNF5 and the SWI/SNF complex functions in the regulation of their transcription.

Recently, Lee RS et al. demonstrated hSNF5 is the only gene recurrently mutated at a high frequency in MRTs (19). This finding implicates MRTs as true “epigenetically-driven” cancers and indicates that downstream target genes of hSNF5 must drive tumorigenesis for this cancer. Because the patterns of epigenetic changes, especially those associated with chromatin landscape, can vary from cell to cell, it does not seem surprising that we observed a slightly different pattern of gene expression after restoration of HSNF5 expression in each MRT cell line. Our results also indicate that hSNF5 may regulate a subset of p53 target genes such as $p21^{\text{CIP1/WAF1}}$ and NOXA in MRT cell lines but generally through a p53-independent mechanism.

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 (20). 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 (21). Apoptosis–associated NOXA activation is primarily achieved through transcriptional up-regulation through a number of transcription factors including p53 and Myc (20). Because NOXA binds with high affinity to the anti-apoptotic Bcl-2 family members Mcl-1, it appears to act as a mediator of apoptosis in cells showing a dependency on Mcl-1 expression (22, 23). Furthermore, some reports showed that NOXA and Mcl-1 control sensitivity to some chemotherapeutics agents such as Taxol, vincristine and platinum-based drugs (24, 25).

MRTs have a chemo-resistance character such that chemotherapy does not prove an effective treatment for most patients (26). 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 (27-32). However, a promising effective therapy has not yet been established. Nocentini et al. reported that perturbation of the p53 pathway and a reduced apoptotic response in MRT cell lines might contribute to their resistance to chemotherapies (33). They suggested that the lack of positive correlation between an increase in the Bax/Bcl2 ratio and cell death constitutes an abnormality in the control of apoptosis. In this study, we demonstrated that NOXA expression in MRT cell lines inhibited growth as efficiently as HSNF5 reexpression. Although several reports have shown NOXA as a positive growth regulator, taken together, these data implicate NOXA expression as a contributor to the strong growth inhibition induced by hSNF5 reexpression (34, 35). Alternately, hSNF5 loss may result in a failure to regulate NOXA expression in the case of DNA damage by some chemotherapy agents such as doxorubicin. Additional studies will help elucidate the function of NOXA in MRT development.

Our results also 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 (13, 36-38). In our previous report, we also demonstrated hSNF5 binds
at a point close to the TSS on the p21CIP1/WAF1 and p16 promoters by ChIP assay (9). 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 downstream sites (13, 36, 37). Our results with the p21CIP1/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 p21CIP1/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 p21CIP1/WAF1 promoter (~2,283 kb and ~1,391 kb) after hSNF5 reexpression, although p21CIP1/WAF1 transcription increased (9). Previous studies have concluded that nucleosomes with H3K4me3 are associated with actively transcribed genes in various eukaryotes (39-41). Guenther and colleagues also found H3K4me3 enriched within 1 kb of known TSS, with maximal enrichment downstream of the TSS (42). Our results showed H3K4me3 increased at or near the TSS in the p21CIP1/WAF1 and NOXA promoters, similar to the results from Guenther’s report. Since the transcriptional activity changes is reflected the occupancy of H3K4me3, it is unclear whether the methylation of H3K4 is primary or secondary effects of hSNF5 reexpression. However, Lee and colleagues indicated that the C-terminal SET domain of H3K4 methyltransferase (MLL3 and MLL4) directly interacts with hSNF5 (43). 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<sup>CIP1/WAF1</sup> and NOXA genes, peaking toward the 3′ end (39-41, 44). Our results concur with several earlier reports demonstrating (39-41, 44) 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 increased after hSNF5 reexpression on p21<sup>CIP1/WAF1</sup> 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 (42, 45). 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 (45). Similar studies showed that H3K4me3 modification occurs subsequent to RNAPII recruitment and Ser5 phosphorylation of RNAPII C-terminal domain (16, 40, 46). Indeed, we also showed that the occupancy of RNAPII follows a similar pattern for H3K4me3 occupancy. The increase of H3K4me3 occupancy in the p21<sup>CIP1/WAF1</sup> 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 (44). 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 (12). 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\textsuperscript{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\textsuperscript{CIP1/WAF1} and NOXA expression. Reexpression of hSNF5 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. These results raise the exciting possibility that one mechanism for epigenetic changes cause by HSNF5 inactivation involves changes in promoter pausing. Furthermore, because MRT cells display repressed NOXA transcription activity due to hSNF5 loss, reexpression of the NOXA pathway might prove a promising new paradigm to treat MRT in the near future.
References


Figure Legends

**Figure 1.** 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 QT-PCR and normalized for β-actin expression and relative to the Ad-GFP for each genes. Values are the mean of three independent experiments; bars, ± SD.

**Figure 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.** hSNF5-induced NOXA protein expression.

(A) 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. (B) Cells were transfected with the designated plasmids and selected in neomycin for 2 weeks. The results represent the colony numbers from 3 independent experiments; bars, ± SD.
Figure 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 ChIP assays. ChIP 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.

Figure 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 two 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 QRT-PCR of ChIP-enriched DNA are named according to their relative distance (bp) to the transcription start site (TSS). (b, c) At 24 hours after infection with Ad-hSNF5 and Ad-GFP, protein was extracted for ChIP assays. ChIP assays were performed 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.
Table 1 TaqMan gene expression assay primer/probes

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<td>phorbol-12-myristate-13-acetate-induced protein 1(NOXA)</td>
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<td>PMAIP1</td>
<td>Mdm2 p53 binding protein homolog</td>
<td>Hs01066930_m1</td>
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<td>SFN</td>
<td>stratifin (14-3-3 sigma)</td>
<td>Hs00968567_s1</td>
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<td>growth arrest and DNA-damage-inducible, beta</td>
<td>Hs00169587_m1</td>
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<td>GADD45B</td>
<td>growth arrest and DNA-damage-inducible, beta</td>
<td>Hs00169587_m1</td>
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Table. 2 PCR primer sequences for ChIP Assays

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<th>Gene</th>
<th>Site (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>AGG GCT GCC TGG GAG AGC AA</td>
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<tr>
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<td>ACT CAT GGC CTC GCC AAA CAT T</td>
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<td>AGA CCG CTG TAT GGG AGC GGA</td>
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<td>CGG GCC GGG CGT CTA GTT TC</td>
<td>CGC GCC AGA GAC CAC GCT TT</td>
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<td>104</td>
<td>CCC TGC CTG CAG GAC TGT TCG</td>
<td>CCC GGG AAC CTC AGC CTC CA</td>
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<tr>
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<td>874</td>
<td>AGT TTT CAG GCC AGC GCC CC</td>
<td>GGC CCA CAC AGA CTT CGG GC</td>
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<td>TGC CGG AAG TTC AGT TCG TCT CCA</td>
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<td>*p21CIP1/WAF1</td>
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<td>AGTCACATCGCCCGCTGAGTCAA</td>
<td>GGAGATGGATGGCCCATGA</td>
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Note: * p21 primers were based on (15).
Figure 3: Western Blot Analysis

(A) TTC642

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<tr>
<th>Time (hrs)</th>
<th>un</th>
<th>GFP</th>
<th>hSNF5</th>
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<tbody>
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<tr>
<td>43 kd</td>
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</table>

(B) TTC549

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<th>GFP</th>
<th>hSNF5</th>
</tr>
</thead>
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<tr>
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<tr>
<td>47 kd</td>
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</tr>
<tr>
<td>7 kd</td>
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<tr>
<td>43 kd</td>
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<td></td>
</tr>
</tbody>
</table>

B) TTC642

<table>
<thead>
<tr>
<th>Colony per 10 cm plate</th>
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</thead>
<tbody>
<tr>
<td>pdx mutant</td>
</tr>
<tr>
<td>pds mutant</td>
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<tr>
<td>pds WT</td>
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</tbody>
</table>

Kuwahara et al., Figure 3-Revised
Molecular Cancer Research

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

Yasumichi Kuwahara, Darmood Wei, Joel Durand, et al.

Mol Cancer Res  Published OnlineFirst January 30, 2013.

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