p16^{INK4a} Is Required for hSNF5 Chromatin Remodeler-induced Cellular Senescence in Malignant Rhabdoid Tumor Cells*

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The hSNF5 chromatin-remodeling factor is a tumor suppressor that is inactivated in malignant rhabdoid tumors (MRTs). A number of studies have shown that hSNF5 re-expression blocks MRT cell proliferation. However, the pathway through which hSNF5 acts remains unknown. To address this question, we generated MRT-derived cell lines in which restoration of hSNF5 expression leads to an accumulation in G_0/G_1 induces cellular senescence and increased apoptosis. Following hSNF5 expression, we observed transcriptional activation of the tumor suppressor p16^{INK4a} but not of p14^{ARF}, repression of several cyclins and CD44, a cell surface glycoprotein implicated in metastasis. Chromatin immunoprecipitations indicated that hSNF5 activates p16^{INK4a} transcription and CD44 down-regulation by mediating recruitment of the SWI/SNF complex. Thus, hSNF5 acts as a dualistic co-regulator that, depending on the promoter context, can either modulate transcription or repression. Three lines of evidence established that p16^{INK4a} is an essential effecter of hSNF5-induced cell cycle arrest. 1) Overexpression of p16^{INK4a} mimics the effect of hSNF5 induction and leads to cellular senescence. 2) Expression of a p16^{INK4a}-insensitive form of CDK4 obstructs hSNF5-induced cell cycle arrest. 3) Inhibition of p16^{INK4a} activation by siRNA blocks hSNF5-mediated cellular senescence. Collectively, these results indicate that in human MRT cells, the p16^{INK4a}/pRb, rather than the p14^{ARF}/p53 pathway, mediates hSNF5-induced cellular senescence.

The genesis of most human cancers requires disabling of the p53 or retinoblastoma protein (pRb) tumor suppressor pathways, which form the primary cell-autonomous defense against unrestrained proliferation (1). The activities of these two pathways are modulated by p14^{ARF} and p16^{INK4a}, two structurally unrelated proteins encoded by a single locus (2–4). These two tumor suppressors act through distinct mechanisms. Whereas p16^{INK4a} modulates pRb activity, p14^{ARF} regulates p53 function. pRb is a corepressor that binds several E2F family transcription factors to silence a host of genes required for cell cycle advancement from G_1 to S-phase. In the presence of growth-promoting signals, the cyclin D1-CDK4 complex phosphorylates pRb, causing its dissociation from E2F. This in turn allows activation of E2F responsive genes, driving cell cycle progression into S-phase. p16^{INK4a} regulates pRb through inhibition of the cyclin D1-CDK4 kinase activity, thus keeping pRb in its active antiproliferative state. The second tumor suppressor encoded by the INK4a/ARF locus, p14^{ARF}, binds HDM2 to prevent p53 degradation. Stabilization of the p53 transcription factor activates gene expression programs that can lead to cell cycle arrest or apoptosis.

ATP-dependent chromatin-remodeling factors (remodelers) play a crucial role in the regulation of gene expression during cellular differentiation and development (5, 6). Consequently, aberrant gene control due to mutations in chromatin-regulating enzymes can lead to tumor formation (7). The multiprotein SWI/SNF complex is the prototypical remodeler, present in all eukaryotes (8–11). SWI/SNF utilizes the energy derived from ATP-hydrolysis by its SWI2/SNF2-related ATPase subunit to alter nucleosome structure. Human SWI/SNF complexes (hSWI/SNF or BRM/BRG1-associated factor (BAF) complex) contain either BRM or BRG1, the two SWI2/SNF2p homologues present in human cells.

Several studies have implicated misregulation of SWI/SNF in the development of human cancers (7). The central BRG1/BRM ATPases are mutated in multiple human tumor cell lines and their loss correlates with poor prognosis of non-small cell lung cancers (12, 13). A role for SWI/SNF in tumorigenesis is further supported by its association with proteins with a well-established role in human cancers including pRb, BRCA1, MLL, and β-catenin (7, 14–21). Most compellingly, the human SNF5 gene (hSNF5, a.k.a. Ini1, Baf47, or SmarcB1), encoding a core hSWI/SNF component, was found to be deleted or mutated in virtually all atypical teratoid and malignant rhabdoid tumors (ATRT and MRT) (22, 23). These rare cancers of early childhood have a grim prognosis and very high lethality. hSNF5 mutations were also found associated with a number of other neoplasms, including chronic myeloid leukemia, choroid plexus carcinoma, medulloblastoma, and central primitive neuroectodermal tumors (24, 25). In addition to somatic mutations, germ-line mutations have been identified. Carriers are predisposed to various cancers, including MRTs and tumors of the central nervous system (23, 26, 27). In a large proportion of these tumors, the wild-type allele is either lost or deleted, consistent with a classic tumor suppressor phenotype.

SNF5 is a universal SWI/SNF component, conserved from yeast to man. SNF5 is a direct target for several distinct DNA-
binding regulators (28–30), stimulates the in vitro remodeling activity of BRG1 (31), and is required during postrecruitment remodeling in vivo (32). Gene inactivation studies in mice revealed that, while homozygous SNF5-null mice die very early during embryogenesis, heterozygous mice survive but are prone to soft tissue tumors (33–35). The loss of the remaining SNF5 allele in these tumors strongly suggests that SNF5 functions as a tumor suppressor. Likewise, mice with mono-allelic BRG1 expression are predisposed to tumors, reinforcing the role of SWI/SNF in neoplasia (36). Utilizing a reversibly inactivating conditional allele, Orkin and co-workers (37) showed that loss of SNF5 function resulted in a highly penetrant and extremely short latency development of cancers, in particular lymphomas or rhabdoid tumors. Their elegant approach, which circumvents the critical requirement for SNF5 during development, highlighted its essential tumor suppression function. This study, however, also uncovered the requirement of SNF5 for survival of most normal, nonmalignant cells. However, when particular additional mutations are present, loss of SNF5 may trigger tumor progression.

The human pediatric cancers and the mouse gene inactivation studies have firmly established SNF5 as a novel tumor suppressor. Moreover, during the course of the present study, a number of reports showed that hSNF5 re-expression blocks MRT cell proliferation (38–41). These studies revealed a G1 cell cycle arrest upon re-expression of hSNF5 in MRT cells. These distinct studies emphasized different targets. Factors reported to be affected by hSNF5 expression include several cyclins, p16INK4a, and pRb. However, the central question, which of these factors is a critical mediator of hSNF5-induced cell cycle arrest, remained unsolved.

To address this issue, we have generated MRT-derived cell lines, which inductively express hSNF5. We found that hSNF5 induction leads to a G1 cell cycle arrest and cellular senescence. We demonstrate that the transcriptional activation of the tumor suppressor p16INK4a, but not of p14ARF, plays a pivotal role in this hSNF5-induced proliferative block. Chromatin immunoprecipitation indicated that the p16INK4a promoter is an hSNF5-dependent, direct target of the SWI/SNF chromatin-remodeling complex. We directly tested the importance of p16INK4a activity for the hSNF5-induced cell cycle arrest and cellular senescence. Three different approaches, involving p16INK4a overexpression, the use of a p16INK4a–/– knockout, and CDK4 and p16INK4a small interfering RNA duplex (siRNA) knockdown, revealed that p16INK4a is both necessary and sufficient to mediate hSNF5-dependent cellular senescence. We conclude that in human MRT cells, the p16INK4a cyclin D1/CDK4-pRb pathway, rather than the p14ARF-HDM2-p53 pathway, is indispensable for hSNF5 tumor suppressor function.

EXPERIMENTAL PROCEDURES

Cell Procedures—Stable cell lines were generated using the LacZ-\(\beta\)-galactosidase reporter system (Stratagene). First pCMVLacI followed by either pOSVST or pOSVST-hSNF5-FLAG, generated by PCR-based strategy, encoding hSNF5 with a C-terminal FLAG epitope, were stably transfected into G401 MRT cells. FuGENE 6 (Roche Applied Science) was used to transfect the cells. Selection with G418 (500 μg/ml), G418 (105 cells/ml), and G418 (105 Lac-hSNF5 cells per well) were seeded in 10-cm-diameter dishes and counted. For colony formation, cells were seeded at 105 cells/ml in 35 mm dishes. After 7–10 days, colonies were stained with methanol/acetic acid and counted. hSNF5 expression was controlled by standard indirect immunofluorescence. Apoptotic cells were identified by DAPI staining. To determine proliferation curves, 1.5 × 106 cells were plated in triplicate into 5 cm dishes. At the indicated time points, cells were harvested and counted. For colony formation, cells were seeded at 105 cells/ml in 35 mm dishes. After 7–10 days, colonies were stained with methanol/acetic acid and counted with Giemsa. BrdU incorporation was measured according to the manufacturers instructions (BrdUrd labeling and detection kit I, Roche Applied Science). Flow cytometric analyses were typically performed after 3–5 days according to standard procedures (42). Briefly, cells were washed with ice-cold PBS. Pelleted cells were resuspended in 0.5 ml of ice-cold phosphate-buffered paraformaldehyde (pH 7.2) containing 100 μg/ml t-lysophosphatidylcholine (Lysolecithin, Sigma) was added dropwise under constant swirling. The cell suspension was kept on ice for 5 min after which the reaction was stopped by the addition of 2 ml phosphate-buffered saline/30% formamide. Cells were pelleted and resuspended in 1 ml of phosphate-buffered saline/1% bovine serum albumin. Cells were then pelleted and resuspended in 0.5 ml of p16INK4a reporter assay mix, and analyzed by FACSort flow cytometer (BD Biosciences). Typically, at least 10,000 events per sample were acquired, and data were analyzed using CELLQuest software (BD Biosciences, Immunochemistry Systems, San José, CA). Senescence-associated β-galactosidase staining was performed as described (43).

 Lentivirus Production and Transduction—Details of the lentiviral transfer vectors used in this study will be published elsewhere. Briefly, pMRLeptptgkmcspreSin (44) was adapted by insertion of an internal ribosome entry site (IRES) for entry site (IRES) for expression of eGFP. Lentiviral particles were produced in 293T cells (44). Briefly, the expression vectors for HIV Gag/Pol, HIV Rev, and VSVG and the viral transfer vector were co-transfected by the calcium phosphate method. Next day, the medium was changed and the supernatants were collected 48 h after transfection. Filtered through a 0.45-μm pore size filter and stored at −80°C. The viral titer was estimated by determining the number of foci formed by HIV-1 p24 antigen ELISA (ZeptoMetrix Corporation, NY). For transfections, 5 × 104 Lac-hSNF5 cells were seeded in 10-cm-diameter dishes and incubated overnight with 50 ng of virus in the presence of 8 μg/ml polybrene. Next day, the medium was changed, and 2 days after transduction, the cells were harvested, seeded, and induced with IPTG for growth curves, colony-forming assays or FACs analysis. FACs sorting for eGFP expression revealed a 70–90% transduction efficiency.

 siRNA Knockdown of p16INK4a—The siRNA duplex oligonucleotides (sense: 5′-CGCAGCGGAAGUAGUACGUGTT-3′; antisense: 5′-ACCGU- ACAAUACUGCGUGCGTTT-3′) corresponding to positions 93–111 bp downstream of the p16INK4a AGT initiation codon to which 2 additional bases were added to enhance siRNA processing were prepared in 10 mM Tris, pH 7.5, 20 mM NaCl. The solution was incubated for 2 min at 95°C and then allowed to slowly cool to room temperature. For transfection, 3 × 104 Lac-hSNF5 cells were seeded in 6-well plates containing 1 ml of media. 1-h prior transfection, Lac-hSNF5 cells were induced by addition of IPTG. For each well, 10 μl of oligonucleotide stock (50 μM) was diluted into 175 μl of Opti-MEM serum-free media (Invitrogen). Next, 4 μl of Oligofectamine reagent (Invitrogen) was added to serum-free Opti-MEM media by gentle mixing and following a 10 min incubation added to the oligonucleotide solution, mixed gently, and incubated at room temperature for 15–20 min. Next, 200 μl of the solution was added to each well. Cells were incubated 4 h at 37°C prior to addition of media either lacZ or Lac-hSNF5. The transfection was repeated every 72–96 h for a maximum of three consecutive transfections. At the indicated time points, cells were collected for each assay performed as described above.

 Protein and mRNA Expression Analysis—Cell extracts were prepared in radioimmunoassay precipitation assay buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Nonidet P-40, 1% NaDOC, 0.1% SDS, 5 mM MgCl2, 50 μM DTT, 2 μg/ml Aprotinin) for protein concentration, and 15 μg (45 μg for p14ARF blot) of extract was resolved by SDS-PAGE, and analyzed by immunoblotting. Primary antibodies: 5D4, Upstate Biotechnology for cyclin D1, C-19 (p21WAF1/CIP1), M20 (cyclin E), H432 (cyclin A), C22 (cdk4), DO-1 (p53), SMP14 (HDM2), H88 (BRG1), H-116 (BAP170), H-76 (BAP155) all from Santa Cruz Biotechnology, G-25 (CDK4), Phospho-hSNF5 (1D12, Sigma), Hermes-1, and H4C4 (DSHB, University of Iowa) for CD44, anti-p53 (TM311, T-2780, Sigma), Hermes-1, and H4C4 (DSHB, University of Iowa) for CD44, anti-p53 (TM311, T-2780, Sigma); 4C6 (p14ARF) and Ref. 46; JC8 (p16INK4a, Ref. 47). The anti-hSNF5 antibodies were raised by immunization of rabbits with bacterially expressed full-length hSNF5.
protein using standard procedures. Enhanced chemiluminescence (Amersham Biosciences) was used for detection of proteins. For immunoprecipitations, cell extracts were prepared 48 h after IPTG addition in HEMG buffer (25 mM Hepes-KOH pH 7.6, 0.1 mM EDTA pH 8.0, 12.5 mM MgCl2, 10% glycerol, 0.1% Nonioid P-40, and 150 or 400 mM NaCl). Protein lysates were incubated with antibodies followed by addition of either protein A- or G-Sepharose beads (Amersham Biosciences). Bound proteins were washed extensively and resolved by SDS-PAGE followed by immunoblotting. For RT-PCR analysis of mRNA levels, cells were harvested 48 h after IPTG addition, total RNA was extracted with RNAzol (Campro Scientific), and cDNA was prepared using random hexamers and Superscript™ II Reverse Transcriptase (Invitrogen, Life Technologies, Inc.). PCR amplification was performed using the Advantage® PCR Kit (Clonetech) and the following primers: p166′/6′, ATGGAGGCACAGGAGGGGAGC and TCAATCGGGGATG-TCTGA; p144′/4′, GTTTTCGTGGTCTACATCCC and ACCAGCCTGTC- CAGGAAG; p213′/3′, ATGCTACAACCCGCTGGGGAT and TTAGGCG- TTCTCTTGGAGAA; cycl. A, AGACCTTCATTTGGGTTGGAA and ACAAACTCTGCTACTTCTTG; cycl. D1, CCAGCCATCCTGGGATTG and CCAGCCATCCTGGGATTG; cycl. G1, CCAGACCAATAGT and TGTGCTTCTGGGATGACC; cycl. HDH, AATCCACATCACCATCTCTC and AGTCTTGCTCCAGCAT- ACC; CDC4, CAGACACGCAAATGGGATGGGACCC and CCAAAGCCAAAAGCCAGGAGGATGCC.

Promoter Assays and Chromatin Immunoprecipitations—Cells were plated and transfected with the appropriate reporter plasmid and pCDNA-lacZ control plasmid using FuGENE 6 (Roche Applied Science). 12 h later, IPTG was added and after 24 or 48 h, cells were harvested and assayed for luciferase and β-galactosidase activity as described (48). The following reporter plasmids were used: p166′/6′, p144′/4′, p213′/3′, p169′-luc (49), p14-800-luc (50) and p21-luc. ChIP assays were performed essentially as published (51). Cross-linked chromatin was prepared from cells harvested 48 h after IPTG addition and sheared to an average length of 0.6 kb. After immunoprecipitation with antibodies against Ac-H3 (Upstate Biotechnology 06–599), BRG1, hSNF5 or FLAG-M2 (Sigma). The recovered DNA was analyzed by PCR with primers corresponding to the p169′-luc, p14-800-luc (49), and p21-luc promoters. DNA was visualized by ethidium bromide staining.

RESULTS

Expression of hSNF5 in MRT Cells Blocks Cell Proliferation and Induces Senescence—The aim of our study was to elucidate the pathway controlled by hSNF5 whose disruption causes tumorigenic transformation of MRT cells. Therefore, we reintroduced the hSNF5 gene under control of the Lac repressor operator system. We established "Lac-hSNF5" cells derived from G401 MRT parental cells. These cells lack the hSNF5 gene but express wild-type p53 and pRb. In Lac-hSNF5 cells, the expression of hSNF5, tagged with a C-terminal FLAG epitope, is under control of the lac operator and can be induced by addition of IPTG. Immunofluorescence experiments demonstrated that, while there is no detectable hSNF5 in the absence of IPTG, in its presence, hSNF5 is induced and localizes to the nucleus (Fig. 1A). The level of hSNF5 in Lac-hSNF5 cells upon induction with IPTG is comparable or even less than that observed in other cell lines, as established by Western immunoblotting (Fig. 1B). Because hSNF5 functions as a component of the SWI/SNF complex, we tested its association with BAF155, BAF170, and BRG1 (Fig. 1C). BRG1 is the only human SWI2/SNF2p homologue expressed in G401 cells (data not shown). In extracts prepared from IPTG-induced cells, hSNF5 was found associated with immunoprecipitated BRG1 and, conversely, BRG1, BAF155, and BAF170 were present in hSNF5-FLAG immunoprecipitates (Fig. 1C). The anti-FLAG beads did not immunoprecipitate BRG1, BAF155 or BAF170 in the absence of IPTG or in extracts prepared from the Lac-empty control cell line. In summary, we have generated an MRT-derived cell line in which hSNF5 expression can be tightly controlled. The induced hSNF5 appropriately localizes in the nucleus and associates with a BRG1-containing SWI/SNF complex.
induced Lac-hSNF5 cells can proliferate in the absence of serum (data not shown). We found that hSNF5 induction dramatically impaired cell proliferation, even when cultured in the presence of 10% serum (Fig. 2A). In contrast, addition of IPTG had no effect on Lac-empty control cells lacking the hSNF5 gene. Similar results were obtained with independently isolated Lac-hSNF5 cell lines (data not shown). We also assessed the effect of hSNF5 on the colony-forming capacity of Lac-hSNF5 cells, and found that hSNF5 expression dramatically reduced the number of colonies formed, after seeding these cells at a very low density (Fig. 2B).

We next determined which stage of the cell cycle is affected by hSNF5. As shown in Fig. 2C, expression of hSNF5 leads to impaired DNA synthesis, indicated by the strongly reduced incorporation of BrdUrd. FACS analysis revealed that the hSNF5-expressing cells accumulate in G1, suggesting that the

Fig. 2. hSNF5 expression induces cell cycle arrest and senescence in MRT cells. A, proliferation curves for Lac-hSNF5 cells (circles) and Lac-Empty control cells (triangles) in the presence or absence of IPTG. Cells were grown in media containing 10% serum. All S.D. were less than 10%. B, Lac-hSNF5 cells were seeded at a very low density and allowed to form colonies either in the absence of presence of IPTG. C, effect of hSNF5 induction on the relative S-phase cell numbers as determined by BrdUrd incorporation in asynchronous growing Lac-hSNF5 cells. S.D. were less than 10%. D, cell cycle distribution of Lac-hSNF5 cells grown in either the presence or absence of IPTG, as determined by FACS analysis. E, representation of the difference in percentage of gated cells at a given stage of the cell cycle upon hSNF5 induction, as determined in D (induced minus non-induced). F, senescence-associated β-galactosidase activity in Lac-hSNF5 and Lac-Empty cells grown either in the presence or absence of IPTG. G, apoptotic cells were identified by indirect immunofluorescence using antibodies against cytochrome c to monitor its release from the mitochondria and antibodies specific for cleaved PARP. DNA was visualized by DAPI staining. Cell counting revealed that the proportion of apoptotic cells increased from less than 1% to over 5%, following hSNF5 expression.
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...cell cycle block affects entry into S-phase (Fig. 2D). The difference in percentage of gated cells at a given stage of the cell cycle in cells expressing hSNF5 versus uninduced cells is shown in Fig. 2E. Finally, we found that a large proportion of the hSNF5-expressing cells displayed the characteristic features of cellular senescence, including an enlarged, flat cell morphology and the presence of activated acidic β-galactosidase (Fig. 2F). We also examined the effect of hSNF5 induction on apoptosis. There was a clear increase in the number of apoptotic cells, from less than 1% to more than 5%, following hSNF5 expression, as determined by the release of cytochrome c from mitochondria, the presence of the caspase 3 substrate cleaved PARP and a DAPI staining characteristic for apoptotic cells (Fig. 2G). Because, at least in these cells, the induction of cell cycle arrest appears to be the major cause of the proliferative block, we decided to focus on the role of hSNF5 in cell cycle control rather than apoptosis. In conclusion, our results, and those of others (38–41), demonstrated that hSNF5 re-expression in MRT cells blocks cell proliferation and activates the cellular senescence program. However, the mechanism through which hSNF exerts its antiproliferative function remained unknown.

**hSNF5 Activates p16\textsuperscript{INK4a}, but Not p14\textsuperscript{ARF} Expression**—To delineate the pathway by which hSNF5 expression can reverse a tumor cell phenotype, we sought to identify effectors of cell cycle arrest and senescence that are activated by hSNF5. Since the INK4a/ARF locus is believed to play a prominent role in these processes, we first analyzed the expression of p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} in Lac-hSNF5 cells (Fig. 3A). Western immunoblotting revealed a strong up-regulation of p16\textsuperscript{INK4a} protein levels following hSNF5 expression, whereas no increase or even a small reduction of p14\textsuperscript{ARF} was observed. In common with other cells containing wild-type p53, p14\textsuperscript{ARF} levels are very low in Lac-hSNF5 cells. For comparison, we have loaded 3-fold less HeLa cell extract in which p14\textsuperscript{ARF} was readily detected. The extracts shown in Fig. 3A were harvested 4 days after induction of hSNF5. It should be noted that the onset of p16\textsuperscript{INK4a} induction varied between different experiments and has been detected as early as day 1 or as late as day 5.

Because p16\textsuperscript{INK4a} is a specific inhibitor of the Rb-regulating cyclin D1-CDK4 complex, we determined the phosphorylation status of Rb in Lac-hSNF5 cells. In support of a role of hSNF5 in activating the expression of p16\textsuperscript{INK4a}, Rb became hypophosphorylated following hSNF5 induction. As might be expected for cells arrested in G1, the levels of cyclins A, D, and E were reduced in hSNF5-expressing cells whereas we observed no major changes in CDK4. The levels of HDM2, p53, BAX, and p21\textsuperscript{WAF1} remained largely unchanged, indicating that, at least in these cells, hSNF5 has no major effects upon the p53 pathway. Finally, it is of interest to note that the CD44 gene, encoding a family of cell surface glycoproteins whose overexpression is often associated with tumor metastasis (52), is down-regulated after hSNF5 induction.

We next tested whether the increase of 16\textsuperscript{INK4a} protein resulted from enhanced mRNA levels, using RT-PCR. As shown in Fig. 3B, p16\textsuperscript{INK4a} mRNA expression is clearly induced in hSNF5-expressing cells. In contrast, we consistently observed a robust reduction in CD44, cyclin A, D1, and E mRNA levels, a modest reduction of p14\textsuperscript{ARF} mRNA, while expression of p21\textsuperscript{WAF1} and GAPDH was not influenced by hSNF5. Taken together, these results indicate that the p16\textsuperscript{INK4a}, but not the p14\textsuperscript{ARF} gene expression is activated by hSNF5.

**hSNF5 Is Required for Recruitment of BRG1 to the p16\textsuperscript{INK4a} Promoter**—How does hSNF5 mediate activation of the p16\textsuperscript{INK4a} gene? The specific up-regulation of p16\textsuperscript{INK4a}, but not p14\textsuperscript{ARF}, by hSNF5 suggests that it acts in a promoter-selective manner. To explore this idea further, we used transient transfection assays to compare the effects of hSNF5 expression on a number of distinct promoters (Fig. 4A). While hSNF5 induction activated the p16\textsuperscript{INK4a} promoter, the p14\textsuperscript{ARF} and p21\textsuperscript{WAF1} promoters were not significantly affected. These results are in good agreement with the effects of hSNF5 expression on the corresponding endogenous genes, and establish that hSNF5 acts as a promoter-specific transcription factor.

Since hSNF5 is in a complex with BRG1 and activates the p16\textsuperscript{INK4a} promoter, we tested whether hSNF5 plays a role in promoter targeting of the hSWI/SNF complex. We utilized formaldehyde cross-linking and chromatin immunoprecipitation (ChIP) to study the occupancy of the p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} promoters in living cells. Using either control beads or beads coupled with antibodies against hyperacetylated histone H3, FLAG, or BRG1, we immunoprecipitated chromatin isolated from Lac-hSNF5 cells (Fig. 4B). Hyperacetylated histone H3 was present at the p16\textsuperscript{INK4a} promoter, irrespective of whether it is active (hSNF5 expressed), or not (hSNF5 silent). In contrast, hyperacetylated histone H3 was not associated with the p14\textsuperscript{ARF} promoter. After induction, hSNF5-FLAG bound to the...
p16\textsuperscript{INK4a} promoter. Strikingly, only in hSNF5-expressing cells is BRG1 found associated with the p16\textsuperscript{INK4a} promoter. It should be noted that hSNF5 expression does not affect BRG1 levels (see Fig. 1C). Neither hSNF5 nor BRG1 was detected at the p14\textsuperscript{ARF} promoter. These results suggest that the SWI/SNF complex is recruited to the p16\textsuperscript{INK4a} promoter to stimulate transcription, and that its tethering is hSNF5-dependent.

Next, we performed ChIP experiments on the CD44 promoter to determine the role of hSNF5 at a promoter that is repressed upon its induction. In cells lacking hSNF5, when CD44 is highly expressed, BRG1 was not detectable at the promoter(Fig. 4C, top panel). In agreement with its high activity, histone H3 at the CD44 promoter appears to be hyperacetylated. Following its induction, hSNF5 but also BRG1 are bound to the CD44 promoter (Fig. 4C, bottom panel). Interestingly, histone H3 at the promoter became hypo-acetylated. Thus, concomitant with hSNF5-dependent repression of the CD44 promoter, the SWI/SNF complex was recruited and histones were de-acetylated. Taken together, our results suggest that hSNF5 is a dualistic transcriptional co-regulator, which can mediate gene activation as well as repression.

The molecular mechanism of hSNF5 function at both the activated p16\textsuperscript{INK4a} promoter and the repressed CD44 promoter involves BRG1 recruitment.

hSNF5 Induces Cell Cycle Arrest and Cellular Senescence through p16\textsuperscript{INK4a}—Having established that the p16\textsuperscript{INK4a} gene is a major target for activation by hSNF5, we investigated the consequences of its activation in MRT cells. We first tested whether activation of p16\textsuperscript{INK4a} alone would be sufficient to drive cell cycle arrest and induction of senescence. To this end, we overexpressed p16\textsuperscript{INK4a} in the absence of hSNF5 induction. Lac-empty cells transfected with a vector expressing p16\textsuperscript{INK4a} displayed a clear senescence phenotype, which was not observed after transfection with an empty vector (Fig. 5). Thus, our results demonstrate that hSNF5 up-regulates p16\textsuperscript{INK4a}, and that stimulation of p16\textsuperscript{INK4a} expression suffices to activate the cellular senescence program in MRT-derived cells. However, they do not exclude alternative hSNF5-dependent growth control pathways or unambiguously establish the critical requirement of p16\textsuperscript{INK4a} for cellular senescence in these cells.

p16\textsuperscript{INK4a} exerts its effect by inhibiting the activity of CDK4, thereby maintaining pRB in its active, antiproliferative state. We therefore asked whether expression of a tumor-associated mutant form of CDK4, CDK4\textsuperscript{R24C} (45), which is insensitive to kinase inhibition by p16\textsuperscript{INK4a}, could override the hSNF5-induced arrest. We utilized recombinant lentiviruses to express either CDK4 or CDK4\textsuperscript{R24C} in Lac-hSNF5 cells. As shown in Fig. 6A, expression of the p16\textsuperscript{INK4a}-insensitive mutant CDK4\textsuperscript{R24C}, but not of wild type CDK4, counteracts the hSNF5-mediated block of cell proliferation. Using Western immunoblotting, we confirmed that the p16\textsuperscript{INK4a} induction by hSNF5 was not affected in these cells (Fig. 6B). The obstruction of hSNF5-induced cell cycle arrest by CDK4\textsuperscript{R24C} was also observed in colony-formation assays (Fig. 6C). Finally, FACS analysis revealed that CDK4\textsuperscript{R24C} expression led to a strong reduction of
FIG. 6. 
p16<sup>INK4a</sup>-insensitive CDK4<sup>R24C</sup> overrules the hSNF5-induced block to cell proliferation. Recombinant lentiviruses were used to transduce Lac-hSNF5 cells to express GFP and either no additional protein (LV-empty), wild-type CDK4 (LV-CDK4), or CDK4<sup>R24C</sup> (LV-CDK4<sup>R24C</sup>), in addition to. A, proliferation curves for lentivirus infected Lac-hSNF5 cells grown in either the presence (closed circles) or absence of IPTG (open circles). All S.D. were less than 10%. B, extracts were prepared from these cells and equal amounts of protein were separated by SDS-PAGE followed by Western immunoblotting using antibodies directed against the indicated proteins. Tropomyosin (TM) serves as a loading control. C, effect of CDK4<sup>R24C</sup> expression on the hSNF5-dependent proliferative block was tested by colony-formation assays. D, FACS analysis of the effect of CDK4<sup>R24C</sup> expression on the cell cycle distribution of Lac-hSNF5 cells grown in either the presence or absence of IPTG, as determined by FACS analysis. Results were represented as described in Fig. 1E, showing the difference in percentage of gated cells at a given stage of the cell cycle of induced versus uninduced cells.
the accumulation of cells in the G1 phase of the cell cycle upon hSNF5 induction, while wild-type CDK4 or transduction with a control virus did not significantly influence the G1 arrest (Fig. 6D). These results establish that p16<sup>INK4a</sup> inhibition of CDK4 represents a critical control point of MRT cell proliferation.

Finally, we tested the essential role of p16<sup>INK4a</sup> in hSNF5-dependent cell cycle arrest by siRNA knockdown experiments. Lac-hSNF5 cells were treated with p16<sup>INK4a</sup>-specific siRNA or, as controls, with sense or antisense oligonucleotides. As revealed by Western immunoblotting analysis, the p16<sup>INK4a</sup> controls, with sense or antisense oligonucleotides. As revealed by Western immunoblotting analysis, the p16<sup>INK4a</sup> silencing efficiency efficiently reduces the level of p16<sup>INK4a</sup>-protein even following hSNF5 induction (Fig. 7A). Strikingly, in cells that lack p16<sup>INK4a</sup> expression no longer leads to cell cycle arrest and cellular senescence (Fig. 7, B–E).

Collectively, the results from p16<sup>INK4a</sup> overexpression, the experiments with a p16<sup>INK4a</sup>-insensitive CDK4 and p16<sup>INK4a</sup> siRNA knockdown assays show that p16<sup>INK4a</sup> induction is both necessary and sufficient for the hSNF5-mediated proliferative block in these MRT cells.

**DISCUSSION**

p16<sup>INK4a</sup> and p14<sup>ARF</sup> form part of a central tumor suppressor network whose disruption appears to be mandatory for the transformation of normal cells into tumor cells. Much recent attention has focused on the relative contributions of p16<sup>INK4a</sup> versus p14<sup>ARF</sup> inactivation during tumorigenesis (reviewed in Ref. 4). The aim of this study was to identify the pathway through which the chromatin remodeling factor hSNF5 acts as a critical tumor suppressor protein in MRT cells. The main conclusion from our work is that in these cells the p16<sup>INK4a</sup>-cyclin-D1/CDK4-pRb, rather than the p14<sup>ARF</sup>-HDM2-p53 tumor suppressor pathway, is critical for the hSNF5-implemented proliferative barrier. hSNF5 acts as a transcriptional coactivator, which is required for the recruitment of the BRG1 containing SWI/SNF chromatin remodeling complex to the p16<sup>INK4a</sup> promoter. The increased p16<sup>INK4a</sup> levels result in inhibition of the cyclin D1/CDK4 complex, thus retarding pRb in its hypophosphorylated antiproliferative state.

Consistent with a critical role for pRb downstream of hSNF5 signaling, it has been shown that ilicit over-expression of the viral pRb-binding oncoproteins SV40 T, adenovirus E1A or HPV-16 E7 counteracts the hSNF5-induced arrest (38, 41). Previously, a number of reports have implicated a role for BRG1 and hBRM, the core enzymes of the SWI/SNF complexes, downstream of pRb (17–21). Our results now clearly establish that hSNF5 mainly acts upstream of the p14<sup>ARF</sup>/p16<sup>INK4a</sup> pRb pathway. In agreement with this notion, two independent recent studies also suggested that hSNF5 ablation is not equivalent to BRG1/BRM loss and that hSNF5 acts upstream of pRb (38, 41). Clearly, these results do not exclude alternative anti-proliferative roles for hSNF5 such as repression of cyclin D1 transcription (40). However, the results from our experiments with the p16<sup>INK4a</sup>-insensitive CDK4<sup>R24C</sup> and siRNA knockdown of p16<sup>INK4a</sup> reveal a strict requirement for p16<sup>INK4a</sup> during hSNF5-mediated cell cycle arrest. These findings suggest that, at least in these cells, this requirement cannot be bypassed through alternate pathways. Indeed, we found that over-expression of cyclin D1 does not overcome the hSNF5-induced proliferative block (data not shown). A very high rate of metastasis is a critical clinically characteristic of MRTs. Therefore it is of interest to note that our gene expression profiling experiments uncovered an array of potential hSNF5 targets involved in cell mobility and adhesion, including CD44.2

An important step toward understanding the development of cancer is to determine the relative importance of the p16<sup>INK4a</sup> versus p14<sup>ARF</sup> (p19<sup>ARF</sup>) in mice) proteins in the control of cell cycle progression, cell immortalization, and cellular senescence. Results from different, but complementary, experimental approaches to this problem suggested that the proportional contribution of ARF versus INK4a to restricting cell proliferation might be cell-type- or even species-dependent (4, 53–56). Thus, in some settings p14<sup>ARF</sup>, rather than p16<sup>INK4a</sup>, appears to be vital for a proliferative block whereas in others p16<sup>INK4a</sup> is critical. Our results provide an example of a clear distinction between the roles of p16<sup>INK4a</sup> and p14<sup>ARF</sup> in regulation of cell proliferation. In hSNF5 MRT cells, hSNF5 activates transcription of the p16<sup>INK4a</sup> gene, but not of p14<sup>ARF</sup>. Consequently, the activity of the cyclin D1/CDK4 complex is inhibited by p16<sup>INK4a</sup> resulting in the accumulation of antiproliferative, hypophosphorylated pRb. Consistent with the absence of p14<sup>ARF</sup> induction, which acts mainly through the HDM2/p53 pathway, the levels of HDM2, p53, and its targets BAX and p21<sup>CIP2</sup>, remained unchanged following hSNF5 expression. The critical importance of the p16<sup>INK4a</sup> induction for cell cycle arrest was established by inactivation of p16<sup>INK4a</sup> function by siRNA and by expression of mutant CDK4<sup>R24C</sup>. We conclude that in human MRT cells, it is the p16<sup>INK4a</sup>-cyclin D1/CDK4-pRb pathway, rather than the p14<sup>ARF</sup>-HDM2-p53 pathway, which implements hSNF5-dependent cellular senescence.

It should be stressed that hSNF5 expression does not always induce cell cycle arrest or senescence. Perhaps paradoxically, while hSNF5 re-expression blocks proliferation of MRT cells, mouse studies utilizing a reversibly inactivating conditional allele revealed the hSNF5 requirement for survival of most normal cells (37). This suggests that only when additional mutations are present, can loss of SNF5 trigger tumor progression. In many cell lines the establishment through transformation with a viral oncogene will inactivate pRb and bypass the antiproliferative function of hSNF5. Importantly, the MRT cells used in this, and related studies, were tumor cell lines, which have not been transformed with a viral oncprotein. Therefore, it appears that in these cells, re-introduction of the hSNF5 tumor suppressor restores the limited life span duration, typical for normal diploid cells, and triggers the senescence program. Moreover, it has been established that some cells require BRG1 function for cell growth or differentiation rather than cell cycle arrest (34, 36, 57). Overexpression of a dominant negative BRG1 in NIH3T3 cells interferes with muscle cell differentiation, but does not block MyoD induced cell cycle arrest (58). Thus, the SWI/SNF complex(es) act in a cell type-specific manner and, whereas some cell types cannot survive without its activity, in others it activates the senescence program.

Genetic studies in *Drosophila* have shown that SWI/SNF belongs to the trithorax group of activators, which counteracts the Polycomb group protein BMI1 as a repressor of the INK4a/ARF locus (59). Therefore, it appears that SWI/SNF and Pcg proteins act antagonistically, not only during regulation of developmental pathways, but also during control of proliferative lifespan. However, while BMI1 regulates the whole INK4a/ARF locus, hSNF5 selectively activates only p16<sup>INK4a</sup>.

Our results suggest that, at least in these MRT cells, hSNF5 is critical for the recruitment of the SWI/SNF complex to the activated p16<sup>INK4a</sup> promoter as well as the repressed CD44 promoter. This provides an attractive molecular explanation for the co-regulator function of hSNF5. Presumably depending on promoter context, chromatin restructuring by the SWI/SNF
**Fig. 7.** *p16^{INK4a}* is essential for hSNF5-dependent cell cycle arrest, as revealed by siRNA knockdown experiments. *A*, extracts were prepared from induced or uninduced Lac-hSNF5 cells treated with either *p16^{INK4a}* siRNA or with control sense or antisense oligonucleotides. Next, equal protein amounts were separated by SDS-PAGE followed by Western immunoblotting using antibodies directed against *p16^{INK4a}* or the FLAG epitope or the tropomyosin (TM) loading control. *B*, proliferation curves for Lac-hSNF5 cells grown in either the presence (closed circles) or absence of IPTG (open circles) and treated with either *p16^{INK4a}* siRNA or with corresponding sense or antisense control oligonucleotides. All S.D. were less than 10%. *C*, effect of hSNF5 on the colony-forming capacity of Lac-hSNF5 cells treated with *p16^{INK4a}* siRNA, with control sense or antisense oligonucleotides. *D*, FACS analysis of the effect of the loss of *p16^{INK4a}* on the cell cycle distribution of Lac-hSNF5 cells grown in either the presence or absence of IPTG. Results were represented as described in Fig. 1*E*, senescence-associated β-galactosidase activity in Lac-hSNF5 cells, treated with *p16^{INK4a}* siRNA, control sense or antisense oligonucleotides.
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complex will result in either mediating activation or repression of transcription. Recent genome wide expression profiling experiments have emphasized the dualistic effects of hSNF5 in gene activation as well as repression.\(^\text{1}\) Two unsolved problems remains why the cell type-specific effect of hSNF5 in tumorigenesis. One possible explanation might be that distinct activators recruit SWI/SNF through binding to distinct subunits.\(^\text{2}\) p16INK4a expression seems to be subject to multiple control pathways (2, 3, 47, 59, 60). Thus, cell-type-specific differences in the requirement for transcriptional activators may determine whether p16\(^{INK4a}\) expression is dependent on hSNF5 or not. It is of interest to identify the DNA binding regulators that activate p16\(^{INK4a}\) through association with the hSNF5 chromatin-remodeling factor. To this end, we have performed an extensive analysis of the regulatory sequences of the p16\(^{INK4a}\) gene. Progressive deletions of the upstream regions and the promoter only reveal a gradually diminished activity concomitant with a gradually diminished effect of hSNF5 on p16\(^{INK4a}\) transcription (data not shown). Our results suggest that hSNF5-dependent regulation of this complex natural promoter is the result of accumulative small contributions by different activators, and is not consistent with the existence of a single key regulatory element or activator. In conclusion, our results provide a direct link between mutations in a chromatin-remodeling factor associated with certain human cancers and inactiva-
tion of the p16\(^{INK4a}\) tumor suppressor pathway.

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