Inhibition of Expression of the Chromatin Remodeling Gene, SNF2L, Selectively Leads to DNA Damage, Growth Inhibition, and Cancer Cell Death

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Abstract
SNF2L, a chromatin remodeling gene expressed in diverse tissues, cancers, and derived cell lines, contributes to the chromatin remodeling complex that facilitates transcription. Because of this wide expression, it has not been exploited as a cancer therapeutic target. However, based on our present studies, we find that cancer cells, although expressing SNF2L at similar levels as their normal counterparts, are sensitive to its knockdown. This is not observed when its imitation SWI ortholog, SNF2H, is inhibited. SNF2L siRNA inhibition using two different siRNAs separately reduced SNF2L transcript levels and protein in both normal and cancer lines, but only the cancer lines showed significant growth inhibition, DNA damage, a DNA damage response, and phosphorylation of checkpoint proteins and marked apoptosis. DNA damage and the damage response preceded apoptosis rather than being consequences of it. The damage response consisted of increased phosphorylation of multiple substrates including ATR, BRCA1, CHK1, CHK2, and H2AX. Both the total and phosphorylated levels of p53 increased. The downstream targets of p53, p21, GADD45A, and 14-3-3σ, were also upregulated. The alterations in checkpoint proteins included increased phosphorylated cdc2 but not Rb, which resulted in a modest G2-M arrest. Although apoptosis may be mediated by Apaf-1/caspase 9, other caspases could be involved. Other members of the chromatin remodeling or SWI/SNF gene families exhibited overall reduced levels of expression in the cancer lines compared with the normal lines. This raised the hypothesis that cancers are sensitive to SNF2L knockdown because, unlike their normal counterparts, they lack sufficient compensation from other family members.


Introduction
The regulation of chromatin structure is of fundamental importance for many DNA-based processes in eukaryotes. Activation or repression of gene transcription or DNA replication depends on enzymes that can generate the appropriate chromatin environment. Several of these enzymes utilized the energy of ATP hydrolysis to alter nucleosome structure. Chromatin remodeling ATPases are found in all eukaryotic organisms. All known ATP-dependent chromatin-remodeling enzymes belong to the helicase superfamily 2 (SNF2) and the ATPase domain harbors seven motifs that are characteristic of helicases (1). In cancer development, chromatin remodeling is a key component to epigenetic silencing of key genes. Mutations in chromatin remodeling genes accelerate cell cycle progression and oncogenic transformation and inactivate the p21 and p16 pathways (2, 3). Recent data have also shown that histone modification, DNA methylation, and ATP-dependent chromatin remodeling play important roles in DNA damage responses and DNA repair (4-17). The imitation SWI (ISWI) chromatin remodeling ATPase was first identified in Drosophila due to its sequence homology to the yeast SWI2/SNF2 enzyme (18). In contrast to Drosophila, yeast and humans each have two ISWI proteins: ISW1 and ISW2 in yeast; hSNF2L (SMARCA1) and hSNF2H (SMARCA5) in humans (19-21). ISW1 members exist in all eukaryotes and constitute a prominent subgroup of the SNF2 ATPase superfamily (22). In Drosophila, ISWI is a component of three known chromatin remodeling complexes: nucleosome remodeling factor (NURF), ATP-utilizing chromatin assembly and remodeling factor (ACF), and chromatin accessibility complex (23-26).

In all three complexes, ISWI serves as the ATP-dependent motor that drives nucleosome assembly or changes in nucleosome structure. ISWI cooperates with other complex subunits to bring about the specific activities of NURF, ACF, and chromatin accessibility complex (27, 28). In Drosophila, NURF has four subunits—ISWI, NURF301, NURF55, and NURF38. In humans, the NURF complex (hNURF) is composed of three subunits—hSNF2L, BPTF, and RbAP46/RbAP48 (29-31). NURF regulates expression of homeotic genes, including ATR, BRCA1, CHK1, CHK2, and H2AX.

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genes, modulates Wnt-signaling, and affects higher order chromatin structure; in vitro NURF catalyzes formation of regularly spaced nucleosomal arrays and facilitates transcription activation (31).

Although SNF2L and its chromatin-remodeling complexes have been studied in development (9, 29, 32-36), little is known about the functional importance of this gene in cancer. In preliminary studies observing an inverse relationship between levels of E-cadherin and SNF2L expression in a series of human breast cancer cell lines (data not shown), we inhibited SNF2L expression with the expectations of increasing E-cadherin expression; but instead, we observed that inhibiting SNF2L expression had dramatic effects on the viability of a series of different cancer cell lines. We decided to investigate and extend this observation further in the present study.

Results

Expression of SNF2L in Normal and Tumoral Tissues and Cell Lines

The expression of human and mouse SNF2L, analyzed with species-specific primers, indicated that SNF2L is highly expressed in most normal human and select murine tissues. Totally, 20 different human tissues and 19 murine tissues were analyzed. Our results showed that murine SNF2L was highly expressed in brain, testis, and ovary (data not shown). Human SNF2L was expressed nearly ubiquitously (Fig. 1A). Like murine SNF2L, human SNF2L was also expressed at high levels in the ovary and testis (Fig. 1A). To confirm these reverse transcription-PCR (RT-PCR) findings, we searched a database from the National Center for Biotechnology Information Web site and obtained a SNF2L expression profile by analyzing the expressed sequence tags (EST) intensities in human and mouse. The database showed that SNF2L ESTs were present in most of the human tissues tested. A greater abundance of SNF2L EST were found in ovary, soft tissue, testis, and umbilical cord. SNF2L EST were also present in heart, kidney, and liver but were found in greater abundance in the embryo and fetus. In contrast, murine SNF2L EST were detected in fewer murine tissues with the greatest abundance in dorsal root ganglion, embryonic tissue, epididymis, ovary, pituitary gland, and uterus. The SNF2L EST data from the National Center for Biotechnology Information were consistent with our RT-PCR results.

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![Figure 1](mcr.aacrjournals.org)
SNF2L was also expressed in all human tumoral tissues examined (Fig. 1B). Again, the National Center for Biotechnology Information EST database showed that SNF2L EST were present in a wide variety of human tumors, with ovarian tumors showing the highest expression. SNF2L was also expressed nearly ubiquitously and at similar levels in different cell lines including highly malignant (HM), low-grade (LG) or benign, and normal untransformed (NU) human lines (Fig. 1C). When cancers of different types were directly compared with their normal tissue counterparts, there was no appreciable difference in levels of SNF2L expression (Fig. 1D).

**Inhibition of SNF2L Expression and Selective Growth Inhibition in HM Lines**

We applied siRNA transfection (SNF2L siRNA v12578) to inhibit the expression of SNF2L in a number of different cell lines including HM, LG, and NU lines (complete list of lines provided in Materials and Methods). In all but one of the HM lines were SNF2L transcripts detected. These SNF2L transcript levels were reduced by 80% to 90% in all cell lines. With this siRNA approach, protein levels of SNF2L were concomitantly reduced (Fig. 2A and B). We then examined the effect of the inhibition on the growth of these different cell lines. Our results showed that the growth of all the HM lines was dramatically inhibited (Fig. 3A). In the HM lines, not only was there growth inhibition but the cell numbers were reduced by day 3 below starting numbers, indicating induction of cell death. This was most striking in the MDA-MB-468 and HeLa cell lines (Fig. 3A). In the HM lines, not only was there growth inhibition but the cell numbers were reduced by day 3 below starting numbers, indicating induction of cell death. This was most striking in the MDA-MB-468 and HeLa cell lines (Fig. 3A).

Negative control siRNA (NCSI) had no effect. However, the use of the second SNF2L siRNA to some extent has achieved this purpose.

We similarly applied siRNA knockdown to inhibit the ISWI ortholog of SNF2L, SNF2H. Although similar knockdown was achieved, there was no effect on growth in either the HM, LG, or NU lines (Fig. 3D and E). We then investigated the effects of SNF2H overexpression on rescuing the HM cells from the effects of SNF2L knockdown. We attempted to overexpress SNF2H before, concurrently with, and following SNF2L knockdown. We could not overexpress SNF2H in cells having undergone SNF2L knockdown for the same reason we could not overexpress SNF2L in these cells. Cells having undergone

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**FIGURE 2.** Western blot and derived histograms following SNF2L siRNA indicates that the SNF2L protein was successfully knocked down in A, a representative HM line (MDA-MB-468), and B, a representative NU line (HLF). Use of the negative control (NCSI) had no effect.
SNF2L knockdown undergo apoptosis. Unlike the situation with SNF2L overexpression, which by itself caused apoptosis, we were able to overexpress SNF2H in the MDA-MB-468 cells before SNF2L knockdown and the cells survived and grew. We then carried out SNF2L knockdown in these SNF2H-transfected cells. The results showed a profound degree of growth arrest and apoptosis despite the SNF2H overexpression (Fig. 3F).

Inhibition of SNF2L Expression and Selective DNA Damage with a DNA Damage Response in HM Lines

The dramatic growth inhibition in the HM lines following inhibition of SNF2L expression with either of the two SNF2L siRNAs together with the fact that SNF2L is a component of the NURF chromatin remodeling complex suggested to us the possibility that SNF2L knockdown might cause DNA

FIGURE 3. The loss of SNF2L expression significantly inhibited the growth of HM lines but not LG or NU lines although SNF2L was similarly inhibited. A, Two representative HM lines, the MDA-MB-468 and HeLa, showed significant growth inhibition whereas B, two NU fibroblast cell lines, HDF and HLF, did not. All lines were treated with either SNF2L siRNA or with NCSI, the latter of which had no effect. All lines were analyzed for their growth rates and for the expression levels of SNF2L by RT-PCR. The derived growth curves represent three independent experiments. C, To rule out potential off-target effects of a single SNF2L siRNA, we used a second SNF2L siRNA (#142643) and compared it with the first SNF2L siRNA (#12578). MDA-MB-468 cells were transfected with either siRNA. The treated cells were analyzed for their growth rates and for the expression levels of SNF2L by RT-PCR. Both SNF2L siRNAs showed the same results—the dramatic growth inhibition in the HM lines following inhibition of SNF2L expression. D, Knockdown of SNF2H, in contrast to SNF2L knockdown, in the HM lines did not inhibit growth. E, Neither knockdown affected growth of the NU lines. In the comparative knockdowns of SNF2L versus SNF2H, the former siRNA served as an additional negative control of the latter and vice versa, F, Overexpression of SNF2H could be achieved in the MDA-MB-468 line but could not rescue the cells from apoptosis from subsequent SNF2L knockdown.
damage. We used the Comet assay method to detect this DNA damage. Forty-eight hours after treatment with SNF2L siRNA, the HM lines selectively exhibited DNA damage (Fig. 4A). The percentage of cells with comet tail DNA (DNA damage) was very high in the HM lines, lower in the LG lines, and lowest in the NU lines (Fig. 4B). Because significant DNA damage usually results in a DNA damage response involving the activation of H2AX, ATM, ATR, or other phosphoinositide-3-kinase–related kinases, which, in turn, can phosphorylate multiple substrates (37), we examined these molecules and their substrates: CHK1, CHK2, BRCA1, and p53. We examined both their expression levels as well as their phosphorylation status. There was no change in expression levels by both real-time PCR (data not shown) and Western blot of total ATM, ATR, H2AX, CHK1, CHK2, and BRCA1 protein (Fig. 4C). Western blotting revealed however an increase in levels of phosphorylated ATR, H2AX, BRCA1, CHK1 (8-fold), and CHK2 (13-fold) but could not detect any phosphorylated ATM in multiple experiments (Fig. 4C). This DNA damage response was much lower or absent in the LG and NU lines studied. Collectively, these findings suggested that the expression of SNF2L plays a very important role in maintaining the stability of chromatin structure and protecting against DNA damage and that this role is especially critical for cancer cell survival.

Inhibition of SNF2L Expression and Selective Upregulation and Phosphorylation of p53 in HM Lines

Because the tumor suppressor protein p53 plays a major role in cellular response to DNA damage (38), we investigated both the expression of p53 as well as its phosphorylation with SNF2L knockdown. Real-time PCR revealed an increase in the p53 mRNA levels in the HM lines up to 6-fold by 3 days (Fig. 5A). Real-time PCR also indicated an increase in the critical targets of p53: the Cdk inhibitor, p21, GADD45, and 14-3-3σ (Fig. 5B). Western blot confirmed an increase in total p53 protein but also showed an increase in phosphorylated p53 (Fig. 5C). These findings suggested then that SNF2L regulated p53 by two mechanisms: transcription and phosphorylation.
Knockdown of SNF2L led to alterations in cell cycle checkpoint proteins and upregulation of p53 and its downstream genes selectively in the HM lines. A. Various lines were transfected with SNF2L siRNA or NCSI. Forty-eight hours after transfection, relative p53 mRNA levels using real-time PCR were determined. B. MDA-MB-468 cells were similarly transfected and the relative mRNA levels of p53 and its target genes including p21, 14-3-3σ, and GADD45A were determined at various times. C. In the MDA-MB-468 cells, phosphorylated p53 protein, phosphorylated cdc2 (Tyr15) and phosphorylated Rb (Ser795) proteins as well as their respective total protein levels were also measured by Western blot. Whereas both p-p53 and total p53 increased following SNF2L knockdown, only the phosphorylated form of cdc2 increased. D. Also in the MDA-MB-468 cells, cell cycle analysis performed 12 to 24 h after SNF2L knockdown revealed a mild G2-M arrest (bottom). Untreated (top) and negative control (NCSI; middle) showed no effects on the cell cycle.
Inhibition of SNF2L Expression and Selective Alterations in Cell Cycle and Checkpoint Proteins in HM Lines

Because the cellular responses to DNA damage such as cell cycle arrest, DNA repair, chromatin remodeling, and apoptosis are well coordinated (39), we investigated whether SNF2L inhibition led to alterations in the cell cycle and in the cell cycle checkpoint proteins. Our results indicated that the HM lines exhibited a mild G2-M arrest with SNF2L knockdown (Fig. 5D), which was observed 12 to 24 hours after SNF2L siRNA knockdown. Our results also showed that the level of phosphorylated cdc2 was increased but not the level of phosphorylated Rb (Ser795; Fig. 5C). The respective levels of cdc2 and Rb total protein were unchanged (Fig. 5C). Because the cdc2/cyclin B kinase is thought to be pivotal in regulating the G2-M transition and Rb is thought to control progression through late G1-S (40), our findings indicated that the effects of SNF2L inhibition on the cell cycle were largely directed at checkpoints that regulate G2-M, a finding confirmed by the mild G2-M arrest noted on flow cytometric analysis. Because p53 can play an equally important role in triggering either a G1-S or G2-M arrest or both and our findings had indicated that total and phosphorylated p53 were both increased (Fig. 5C), we also investigated the critical targets of p53: the Cdk inhibitor, p21, GADD45A, and 14-3-3. We found that the mRNA levels of p21, GADD45A, and 14-3-3 were also significantly increased (Fig. 5B). Because GADD45A and 14-3-3 both target the cyclin B/cdc2 complex (41), this would appear as further decreased (Fig. 5B). These alterations in the cell cycle and in checkpoint proteins were less altered in the LG and NU lines with SNF2L knockdown.

Inhibition of SNF2L Expression and Selective Apoptosis in HM Lines

For the HM lines, the dramatic decrease in cell numbers below the starting numbers by 48 to 72 hours (Fig. 3A, C, D, and F) with SNF2L knockdown suggested that cell death was occurring. FITC-conjugated Annexin V and propidium iodide (PI) was used to identify subpopulations of cells exhibiting both early (membrane immuno-reactivity) and late apoptosis (membrane immuno-reactivity and nuclear staining). Marked apoptosis was observed in all the HM lines 48 to 72 hours after SNF2L siRNA transfection. Of the HM lines, the HeLa line showed the most apoptosis with 34% of the cells showing early apoptosis and 21% of the cells showing late apoptosis by 48 hours (Fig. 6A). The LG and NU lines, in contrast, showed only low levels of apoptosis. We then investigated, by real-time PCR, which apoptotic pathway might be activated. We found that Apaf-1 and caspase 9 were selectively increased among all the other apoptosis-mediating proteins (Fig. 6B). We postulated that inhibition of SNF2L expression might result in increased Apaf-1, which increased caspase 9, which then activated the rest of the caspase cascade.

DNA Damage and the DNA Damage Response Precede Apoptosis

A legitimate question to ask was whether the DNA damage and the DNA damage response preceded apoptosis or whether they could be the consequence of it. To investigate whether the DNA damage was primary or secondary, we applied caspase inhibitors to inhibit apoptosis and then analyzed the DNA damage. Our results showed that the general caspase inhibitor I had strong inhibitory effects on the apoptosis induced by SNF2L knockdown (Fig. 7A). In NCSI-treated cells, the percentage of early apoptosis was 5.3% (R2) and late apoptosis was 8.3% (R3). With SNF2L siRNA–treated cells, early apoptosis predictably increased to 11.7% (R2) and late apoptosis to 14.5% (R3; P = 0.05). The comet assay revealed however that DNA damage continued unabated even when apoptosis was inhibited (Fig. 7B). The percentage of cells with comet tail DNA (DNA damage) was 39.0% with SNF2L knockdown compared with only 3% with NCSI control knockdown (P = 0.01). When apoptosis was inhibited with the general caspase inhibitor I, the percentage of cells with comet tail DNA remained high at 35.0% (P = 0.5). Western blot indicated that a DNA damage response also occurred with SNF2L knockdown even when apoptosis was inhibited (Fig. 7C). Western blot showed that the cleaved poly ADP ribose polymerase (PARP) protein (a surrogate for apoptosis) was not detected in cells treated with NCSI control knockdown but appeared...
with SNF2L siRNA knockdown. Specific caspase inhibitors to caspase 3, caspase 8, and caspase 9 were only minimally effective at inhibiting cleaved PARP. A general caspase inhibitor strongly inhibited cleaved PARP (apoptosis) however. With SNF2L knockdown, increased p-H2AX was observed as a surrogate for the DNA damage response. With NCSI control knockdown, p-H2AX was not observed. However p-H2AX was still present with SNF2L knockdown when cleaved PARP (apoptosis) was inhibited. Our results indicated that the DNA damage and the DNA damage response preceded apoptosis.

Hierarchical Clustering of Chromatin Remodeling and SWI/SNF Genes in Normal and Tumoral Tissues and Cell Lines

To gain insight into the selective sensitivity of HM lines to SNF2L knockdown, we identified 24 other genes in the chromatin remodeling group, of which SNF2L is a member, and 20 other genes in the switching defective/sucrose nonfermenting (SWI/SNF) family, to which SNF2L also belongs. We examined these two groups of genes in seven cell lines, which included representative HM, NU, and LG lines, and three human breast cancer tissues and their corresponding normal

FIGURE 7. DNA damage, DNA damage response, and apoptosis. A. Flow cytometry of cells stained in Annexin-V and PI solution and treated with NCSI (left), SNF2L siRNA (center), and SNF2L siRNA with a general caspase inhibitor (right). Only with SNF2L knockdown alone was there an increase in both early (Annexin-V) and late (PI) apoptosis (top right). Inhibition of apoptosis abolished this effect (bottom) and the control negative siRNA (NCSI) produced no increased apoptosis (top left). B. Comet assay confirmed that DNA damage was induced by SNF2L knockdown (center) and was not observed when the negative control (NCSI) was used (left). Significantly, DNA damage still occurred even when apoptosis was inhibited with a general caspase inhibitor (right). C. Western blot indicated that DNA damage and the resultant DNA damage response, e.g., p-H2AX from SNF2L knockdown preceded and did not follow apoptosis. SNF2L siRNA, but not NCSI, increased cleaved PARP (a surrogate of apoptosis) and p-H2AX. Specific caspase inhibitors to caspase 3, caspase 8, and caspase 9 were only minimally effective at inhibiting cleaved PARP. The general caspase inhibitor I, on the other hand, showed significant inhibition of PARP cleavage (apoptosis), yet p-H2AX was still expressed.
tissue counterparts. In the chromatin remodeling group, expression of all 24 of the genes could be detected in at least one of the samples (Fig. 8A); in the SWI/SNF family, expression of all 20 genes could be detected in at least one of the samples (Fig. 8B). Hierarchical clustering revealed overall dramatic differences between the HM lines and the NU and LG lines, and equally dramatic differences between the human breast cancers and their normal tissue counterparts (Fig. 8A and B) for both groups of genes. In the NU and LG lines and the normal human tissues, most of the genes from either group were expressed at a detectable level (called “present”) or at a low level of expression. In contrast, in the HM lines and the human breast cancer tissues, most of the genes were either expressed at undetectable levels (called “absent”) or at a low level of expression. However, these differences did not apply to either SNF2L or SNF2H, which were expressed at similar levels among the different cell lines (Figs. 1, 8, and 9). To confirm the relatively low levels of expression of these families observed on microarray, we measured selected gene expression of these families by real-time PCR. In all genes studied, lower levels of expression were observed in the HM lines (Fig. 9).

Discussion

In this study, we have observed that inhibiting the expression of SNF2L, a gene that is nearly ubiquitously expressed in normal and neoplastic human tissues and cell lines, selectively leads to growth inhibition, DNA damage, a DNA damage response, upregulation of genes related to cell cycle checkpoint arrest, a mild G2-M arrest, and apoptosis in the HM lines. Although the LG and NU lines similarly expressed SNF2L, which could be as effectively inhibited with a siRNA approach, these lines showed fewer effects from this treatment. Operationally, we defined HM lines as those derived from aggressive malignant human tumors that exhibited relatively rapid growth and/or metastasis in immunodeficient mice; LG lines as those derived from benign or LG human tumors that were tumorigenic in immunodeficient mice but ones that grew slowly without metastasis; and NU lines as those derived from normal human cells that were not immortal nor exhibited tumorigenicity.

We chose to study SNF2L in the first place because this gene had surfaced in a microarray analysis of breast carcinoma cell lines that showed a range of E-cadherin expression. Our laboratory had been studying the role of E-cadherin in the generation of the tumor lymphovascular embolus in a model of inflammatory breast cancer (42, 43) and wanted to study its gene regulation. We had conducted a microarray analysis of gene expression levels in E-cadherin–positive and E-cadherin–negative cell lines and three genes showed a strong inverse correlations: snai1, snai2 (slug), and SNF2L. The first two genes were known repressors of E-cadherin transcription and their inverse correlation was not at all surprising. The third gene, SNF2L, had not previously been studied with respect to its regulation of E-cadherin expression, and so we attempted to inhibit SNF2L expression initially in E-cadherin–negative lines when we observed the dramatic effects of this knockdown on cell growth and viability. We decided to study this phenomenon further in the present study.

Because the SNF2L gene is a component of the NURF complex, the component that contains ATPase, the component able to harness the energy of ATP to directly drive nucleosome assembly or changes in nucleosome structure, and the component that also binds to histones to silence or activate genes, it is undoubtedly a very important upstream component that regulates the expression of many genes including those essential for cancer cell survival. Usually, ubiquitously expressed genes do not make good specific targets and it was not clear why the HM lines were so exquisitely sensitive to SNF2L inhibition. Interestingly, one of the HM lines, the inflammatory breast cancer line (MARY-X), did not express SNF2L when grown as tumoral spheroids in vitro (Fig. 1C). Clearly not every HM line is dependent on the expression of SNF2L for growth or survival.

The effects of SNF2L knockdown seemed fairly specific for SNF2L. However, to rule out the potential off-target effects of the single SNF2L siRNA used in the initial knockdown studies, we used a second SNF2L siRNA directed against a different exon of SNF2L. The treated cells were analyzed for their growth rates and for the expression levels of SNF2L by RT-PCR and showed the same results—the dramatic growth inhibition in the HM lines following inhibition of SNF2L expression.

To further rule out the potential off-target effects of the two SNF2L siRNAs, we attempted to reexpress SNF2L in siRNA-depleted HM cells to formally establish the specificity of the SNF2L siRNA knockdown. We attempted specifically to overexpress SNF2L before, simultaneously with, and following SNF2L knockdown to completely rule out off-target effects of the SNF2L siRNA. The results with all these approaches were disappointing but enlightening. In all these transfection strategies, cells underwent profound apoptosis. With SNF2L knockdown preceding or simultaneous with SNF2L overexpression, the findings of apoptosis were not unexpected. After all, SNF2L knockdown caused apoptosis in the HM lines, and once apoptosis was triggered, SNF2L overexpression could not rescue the cells. The somewhat interesting finding was that SNF2L overexpression done before SNF2L knockdown also caused apoptosis even before SNF2L knockdown could be carried out. Therefore, we could not even prime the cells with SNF2L. This finding, however, was not totally surprising. In most recent studies, we discovered an isoform of SNF2L (termed SNF2LT). This isoform represented an alternately spliced molecule with an intronic insertion containing a stop codon that truncated the protein. As it turns out, the ratio of the full-length SNF2L protein to the truncated SNF2LT protein appears critical to normal cell growth and/or the triggering of an apoptosis response. Knockdown of either the full-length or the truncated isoform separately results in profound apoptosis, but knockdown of both permits cell growth. Overexpression of SNF2L would increase the ratio of SNF2L to SNF2LT and would be the equivalent of selective SNF2LT knockdown and therefore would be expected to result in apoptosis. We do not yet understand the mechanism of this effect but this is beyond the scope of the present study. Suffice it to say, then we could not use SNF2L overexpression to exclude the off-target effects of SNF2L knockdown.

5 Unpublished observations.
Mammalian genomes encode two ISWI orthologs, SNF2H and SNF2L. These mammalian ISWI proteins have high sequence homology to each other with 86% identity. But our results showed that the knockdown of SNF2H did not inhibit cell growth. Given the high level of sequence similarity shared by SNF2L and SNF2H and their critical role in chromatin structure maintenance, it is striking that knockdown of SNF2H did not cause similar inhibitory effects on cell growth. Furthermore, SNF2H overexpression in the HM cells was not able to rescue the cells from SNF2L knockdown–induced apoptosis. These

FIGURE 8. Microarray data revealed striking differences in both the chromatin remodeling group A, as well as SWI/SNF family B, in overall levels of expression in the various samples. Hierarchical clustering revealed globally low or nonexpression in the HM lines (top right columns) and the human breast cancer cases (bottom right columns) compared with high levels of expression in the NU and LG lines (top left and middle columns) and the normal tissue counterparts (bottom left columns).
later results suggested that SNF2H in the HM cells does not compensate for the loss of SNF2L. These results should not be too surprising because sequence similarity alone does not guarantee structural or functional equivalence. For example although SNF2L and SNF2H exhibit a high level of sequence similarity, they are thought to bind to different complexes (31). SNF2L is part of the NURF complex that consists of SNF2L, BPTF, RbAP46, and RbAP48. NURF regulates expression of homeotic genes, modulates Wnt-signaling, and affects higher order chromatin structure; in vitro NURF catalyzes formation of regularly spaced nucleosomal arrays and facilitates transcription activation. SNF2H, on the other hand, is part of the ACF complex that consists of SNF2H and WCRF180/hACF1. This complex is involved in transcription, DNA replication through heterochromatin, and proper chromatin assembly. In vitro, ACF interacts with naked DNA and nucleosomal arrays independent of ATP, assembling nucleosomes into regularly spaced chromatin. Therefore it is not at all surprising that the effects of SNF2L versus SNF2H knockdown were quite different and that SNF2H, even when overexpressed in the HM cells, could not compensate for the loss of SNF2L.

SNF2L is only one component of the NURF complex and conceivably other components of this complex may regulate cell growth and apoptosis depending on whether they are knocked down or upregulated. Still SNF2L, being an ATPase, is likely a very important component of this complex and a potential cancer target.

To our knowledge, no one previously has investigated the role of the SNF2L gene in cancer but this gene has been studied in murine development. Lazzaro and Picketts (36) examined the spatial and temporal expression patterns of SNF2L during development and found SNF2L to be predominantly expressed in terminally differentiated neurons in the brain after birth. In adult mice, SNF2L has been found to be highly expressed in testis, ovary, uterus, and placenta. Unlike murine SNF2L, human SNF2L is expressed nearly ubiquitously and its levels have been found to be particularly high in ovary and testis. Because of this, it has been postulated that SNF2L exerts a critical role in the survival of the ovarian follicle and spermatogenesis (34). Because HM lines frequently exhibit properties shared by embryonic or germ cells, it would be anticipated that SNF2L would play a critical role in cancer cell survival, growth, and renewal as well.

We compared the levels of SNF2L in HM versus LG and NU lines as well as cancer tissues versus their normal counterparts by both RT-PCR and real-time PCR studies. No differences were observed, and therefore, the levels of SNF2L alone could not account for the selective sensitivity of the HM lines to SNF2L knockdown.

In Drosophila, it has been known that ISWI is a component of three chromatin remodeling complexes: NURF, ACF, and chromatin accessibility complex (23-26). The deletion or inhibition of the expression of this gene would lead to functional loss of all three known complexes containing ISWI. Because ISWI is directly involved in the nucleosome assembly, it follows that one direct consequence of ISWI loss would be changes in nucleosome structure that directly would lead to DNA damage. In this article, our results showed that the inhibition of expression of SNF2L led to DNA damage in cancer cells. This indicated that the reduction of SNF2L protein likely led to structural changes of chromatin and directly induced the DNA lesions. Although the presence of double-stranded DNA breaks can be assumed from the Comet assay, these double-stranded breaks did not trigger detectable ATM phosphorylation. Rather the DNA damage activated a complex and multifaceted DNA damage response that was characterized by phosphorylation of p53, CHK1, CHK2, BRCA1, and ATR. H2AX, another critical component of the DNA damage response, thought to be rapidly phosphorylated at Ser139 located on its COOH-terminal SQ motif in chromatin regions surrounding a double-stranded break by any number of phosphoinositide-3-kinase-related kinases, showed increased phosphorylation as well.

The mechanism of increased p53 mRNA and total p53 protein likely could be due to either a direct or indirect effect of...
SNF2L inhibition on p53 transcription. Because NURF has been shown to modulate Wnt signaling, because there is a known cross-talk between the Wnt pathway and p53 (44), because the lack of p53 cooperates with activation of the Wnt pathway (44), and because overexpression of β-catenin can increase the transcriptional activity of p53 (45), SNF2L or its complexes may regulate the expression of p53 gene through modulating the Wnt pathway. Another possible mechanism is that SNF2L or its complexes may directly associate with the regulatory regions (promoter) of the p53 gene to regulate its transcription. SNF2L may therefore act upstream of both ATR as well as p53.

Our studies indicated that SNF2L knockdown leads to DNA damage and a DNA damage response, which results in the activation of key checkpoints that lead, in turn, to a G2-M cell cycle arrest and apoptosis, although no sub-G1 cell population was evident. The explanation for this was as follows: the cell cycle experiments were done 12 to 24 hours after transfection with SNF2L siRNA, whereas the apoptosis experiments were done 48 to 72 hours after transfection with SNF2L siRNA. This is probably why a sub-G1 population is not present in the cell cycle histograms.

The apoptosis is not the cause of the DNA damage nor the DNA damage response but clearly the result of it. PARP, a 116-kDa nuclear PARP, appears to be involved in DNA repair in response to environmental stress. This protein can be cleaved by many caspases in vitro and is one of the main cleavage targets of caspase-3 in vivo. Usually, caspase-3 is considered the main enzyme that cuts PARP. Compared with the negative control, caspase-3, 8, and 9 inhibitors had only a small inhibitory effect on the cleavage of PARP (Fig. 7C). The general caspase inhibitor I, on the other hand, showed significant inhibition of PARP cleavage. This was not surprising because the general caspase inhibitor has a wide spectrum of inhibition of many caspases. And PARP can be cleaved by many caspases. So the general caspase inhibitor proved to be a good tool for investigating the “chicken versus egg” question of whether apoptosis caused the DNA damage and the DNA damage response or was the consequence of this.

Based on the observation that SNF2L knockdown in cancer cells caused increased levels of expression of Apaf-1 and caspase 9, we postulate that SNF2L knockdown–induced apoptosis may, in part, be mediated by this pathway. However, inhibition of caspase 9 using an inhibitor only slightly inhibited a surrogate marker of apoptosis, cleaved PARP, whereas the use of a general caspase inhibitor had a much greater effect in inhibiting cleaved PARP. These apparently contradictory results can be reconciled by postulating that caspase 9 still could be the major caspase involved, but its elevated levels are more difficult to inhibit with a caspase 9 inhibitor or that other caspases are also involved. Because we did not directly examine the role of Apaf-1/caspase 9 in the death of the SNF2L-depleted cells, other caspases may be involved in mediating apoptosis.

The most important question of all is why are the HM lines so dependent on the SNF2L ATPase and so sensitive to its knockdown. One possibility to explain the dependency of HM lines on this protein is that HM lines have a higher endogenous rate of DNA damage at baseline due to whatever other pathways are disrupted in them. The differential effects noted were certainly not related to growth rate per se because many of the LG and NU lines grew as rapidly as the HM lines.

All known ATP-dependent chromatin-remodeling enzymes belong to SFN2 (1). Based on distinct domain structures, there are four well-characterized families of mammalian chromatin remodeling ATPases: the SWI/SNF family, the ISWI family, the nucleosome remodeling and deacetylation/chromodomains, helicase, DNA binding family, and the INO80 family. Both members of the SWI/SNF family of ATPases, BRM (homologue of Drosophila protein “brahma”) and BRG1 (BRM/SWI2-related gene 1), contain a COOH-terminal bromodomain that binds to acetylated histone tails. ISWI family members, SNF2H and SNF2L, have a SANT (“SW13, ADA2, NCOR, and TFIIB” DNA-binding domains) and a SLIDE (SANT-like ISWI) domain that mediate interaction with unmodified histone tails and linker DNA. Nucleosome remodeling and deacetylation/chromodomains, helicase, DNA binding family members, CHDs 1 to 5, have unique tandem chromodomains that specifically recognize methylated histone tails. Inositol requiring 80 family members, INO80, SNF2-related cAMP-responsive element binding protein activator protein, and p400, are characterized by split ATPase domains (46).

Another possibility then which was suggested by our microarray data were that HM lines and cases of human cancer uniformly exhibit globally low or undetectable levels of chromatin remodeling and SWI/SNF family gene expression and, therefore, are more dependent on SNF2L. NU and LG lines and normal human tissues then have sufficient compensatory levels of expression of other chromatin remodeling or SWI/SNF genes not to be dependent on SNF2L. But this is only a hypothesis. Clearly there could be possibly other more subtle differences between chromatin remodeling and SWI/SNF family complexes in HM lines versus LG and NU lines that could account for their differences in sensitivity to SNF2L knockdown in addition to mere expression levels.

Materials and Methods
Reagents and Antibodies

All antibodies used were rabbit polyclonal unless otherwise indicated and included an antibody to SNF2L (SMARCA1; ab37003; Abcam, Inc.). Antibodies used for detection of DNA damage were obtained from the DNA Damage Antibody Sampler (Cell Signaling Technology, Inc.), which included Phospho-ATR (Ser428), Phospho-ATM (Ser1981) mouse monoclonal antibody (mAb), Phospho-BRCA1 (Ser1524), Phospho-CHK1 (Ser296), Phospho-CHK2 (Thr68), Phospho-Histone H2AX (Ser139), and Phospho-p53 (Ser15; 16G8) mouse mAb. p53 (7F5) rabbit mAb and Histone H2AX (Ser139), and Phospho-p53 (Ser15; 16G8) mouse mAb, p53 (7F5) rabbit mAb and β-actin (13E5) rabbit mAb were also used (Cell Signaling Technology, Inc.). Antibodies for detection of cell cycle checkpoints used the Cell Cycle/Checkpoint Sampler kit (Cell Signaling Technology, Inc.), which included Phospho-cdc2 (Tyr15) and Phospho-Rb (Ser795). Additional antibodies that recognized total protein levels of the respective DNA damage proteins (CHK1, CHK2, BRCA1, ATR, and H2AX) and cell cycle checkpoint proteins (cdc2, Rb, mouse mAb) were also obtained (Cell Signaling Technology, Inc.). A mouse mAb (human specific;
Cell Signaling Technology, Inc.) directed against cleaved PARP (Asp214) was used for the detection of apoptosis. Secondary antibodies and Western blotting substrates were obtained (Pierce Biotechnology, Inc.). All primary and secondary antibodies were used as recommended by their manufacturers. Reagents used included Caspase Inhibitor Set IV (EMD Chemicals, Inc.), including caspase-3 inhibitor II, caspase-8 inhibitor II, caspase-9 inhibitor I, general caspase inhibitor, and the negative control. Reagents also included an Annexin-V-fluos staining kit (Roche). Human tumor total RNAs and the FirstChoice Human Total RNA Survey Panel were used (Ambion, Inc.) as a source of tumor and normal tissue RNA. All other reagents were obtained from vendors (Invitrogen Corporation, Inc., and Qiagen, Inc.).

Cell Lines, Murine, and Human Tissues
All the cell lines were grown under standard conditions in DMEM with 10% fetal bovine serum with the exception of the fibroblast lines, which were grown in MEM-α medium with 10% fetal bovine serum, and the myoepithelial lines, which were grown in KSFM with supplements (Life Technologies, Inc.). All lines, unless otherwise indicated, were obtained from a single source (American Type Culture Collection) and were human and consisted of the following: HM: estrogen receptor-negative breast cancer lines (MDA-MB-231, MDA-MB-468), Her-2/neu-amplified breast cancer lines (HCC202, HTB20; HTB27), an inflammatory breast cancer xenograft, MARY-X, established by us (42, 43), a cervical squamous cell carcinoma (HeLa), a rhabdomyosarcoma (RB), a leiomyosarcoma (SKLMS-1), and an osteosarcoma (U2-08) line; NU: three fibroblast lines including dermal HDF and pulmonary HLF; (gifts of Dr. Issekutz, Dalhousie University, Halifax, Canada) and skin OSU-2 (a gift of Altaf Wani, Ohio State University, Columbus, OH), a mammary epithelial line (HMEC; Clonetics), and the nontumorigenic estrogen receptor-negative MCF-10A line; and benign or LG lines and xenografts: HMS-X and HMS-1, derived by us from a benign human myoepithelial salivary gland tumor (47) and HMS-3X, HMS-4X, and HMS-6X, derived from other benign matrix-secreting human myoepithelial tumors from salivary gland, breast and pulmonary sources (48), and the estrogen receptor-positive breast carcinoma MCF-7. All the lines were grown under standard conditions. Cases of primary breast–infiltrating ductal carcinomas with normal tissue counterparts were obtained from the frozen tissue bank of the Human Tissue Network at the Ohio State University. Murine tissues were obtained from sacrificing athymic (nude) mice and harvesting their organs. Cell Signaling Technology, Inc.) as a source of tumor and normal tissue RNA.

cDNA Transfections
The cDNAs coding either SNF2L or SNF2H, synthesized and amplified by reverse transcription and PCR, were separately cloned into expression vector pcDNA6/Myc-His-A (Invitrogen Corporation, Inc.) to produce pcDNA6-SNF2L or pcDNA6-SNF2H, respectively. The inserts of both SNF2L and SNF2H were verified by sequencing. pcDNA6-SNF2L and pcDNA6-SNF2H were separately transfected following, concurrently, or preceding SNF2L knockdown. These transfection and cotransfection experiments used Lipofectamine RNAiMAX Transfection Reagent (Invitrogen Corporation, Inc.) according to the manufacturer’s instructions.

Cell Growth, Cell Cycle, and Apoptosis Experiments
Cells were transfected with siRNA, cDNA, or a combination of both and seeded in 24-well cell culture plates. The number of viable cells in each well was counted every 24 h for 3 d using trypan blue exclusion. The cell growth study was carried out in triplicate and repeated at least thrice. For cell cycle analysis, the cells were collected 12 to 24 h after transfection and fixed in 70% ethanol at −20°C, followed by washing once in PBS and staining in PI solution (69 nmol/L PI, 388 nmol/L sodium citrate, 100 μg/mL RNase A) for 15 min at room temperature. Ten thousand cells were analyzed on Coulter Epics XL flow cytometer (Beckman Coulter). For the apoptosis, assay cells were harvested at 48 to 72 h following transfection. The apoptosis assay used Annexin V-FITC and PI (kit PN IM2375; Beckman Coulter, Inc.) with flow cytometric analysis.

DNA Damage and DNA Damage Response with Apoptosis Inhibition
To determine the order of cellular events with SNF2L knockdown, selected cell lines, e.g., MDA-MB-468 cells, were seeded in six-well plates and incubated in 37°C overnight. Cells were treated first with caspase inhibitors for 45 min and then with SNF2L siRNA for 24 h. Treated cells were collected and divided into three aliquots. One aliquot was analyzed for apoptosis by dual staining with Annexin V and PI (Annexin-V-fluos staining kit; Roche), following the manufacturer’s protocol. Ten thousand cells were analyzed with apoptotic ratio using the Coulter Epics XL flow cytometer (Beckman Coulter). One aliquot was analyzed for DNA damage by using the CometAssay (single cell gel electrophoresis assay; Trevigen, Inc.). One aliquot was analyzed for protein levels of p-H2XA and cleaved PARP by Western Blot.
Alkaline Comet Assay

The CometAssay (single-cell gel electrophoresis assay; Trevigen, Inc.) was used to evaluate DNA damage according to the manufacturer’s instructions. The technique used electrophoresis of lysed cells embedded in an agarose gel and DNA fluorescence. Cells with damaged DNA exhibited migration of their DNA outside of the nucleus, producing a comet tail.

RNA Isolation and cDNA Synthesis

The total RNA was isolated from cultured cells or tissues using RNeasy Mini kit (Qiagen, Inc.) per manufacturer’s instructions. SuperScript III First-Strand Synthesis System (Invitrogen Corporation, Inc.) was used for the first strand cDNA synthesis. cDNA was combined using RNeasy Mini kit (Qiagen, Inc.) was used. Gene expression levels were calculated relative to the housekeeping gene β-actin by using 7500 Real-time PCR System (Applied Biosystems, Inc.). cDNA was combined with primer sets and Power SYBR Green PCR Master Mix PCR System (Applied Biosystems, Inc.). cDNA was combined with primer sets and Power SYBR Green PCR Master Mix (Applied Biosystems, Inc.) was used. Gene expression levels were calculated relative to the housekeeping gene β-actin by using 7500 System SDS software (Applied Biosystems, Inc.).

RT and Real-time PCR

An aliquot of 20 ng cDNA was used in each 25 μL PCR reaction, using Platinum Taq DNA Polymerase High fidelity (Invitrogen Corporation, Inc.). The following conditions were used as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 68°C for 1 min for a total of 25, 30, or 35 cycles. PCR products were analyzed by 2% agarose gel. Real-time PCR was done on a ABI 7500 Real-time PCR and Real-time PCR was done on a ABI 7500 Real-time PCR System (Applied Biosystems, Inc.). 25, 30, or 35 cycles. PCR products were analyzed by 2.0% agarose gel. Real-time PCR was done on a ABI 7500 Real-time PCR System (Applied Biosystems, Inc.).

Primer sets (forward and reverse) used for either RT-PCR or real-time PCR included the following forward (forward):

Human SNF2L: 5'-ACGGCCTCCCAAAACAGGCAAAATG-3', 5'-TGAGCCGAGCCTGGAATTGGGA-3',
Human SNF2H: 5'-AGAGCCGCGAGATGGAGGAATA-3', 5'-TCCCTACGGGTGTAATCGCCC-3',
Murine SNF2L: 5'-AGCTGTTGCGATCAGGTAGTAGG-3', 5'-GGAGTCTAAGAGGCGCTGG-3',
ATM: 5'-TGGATACGACCTTCTTGTTTGG-3', 5'-CCAAGTATGAACAAATAGAAGTAG-3',
ATR: 5'-TGGCTCTGATCTCCTCCAGCAGTGT-3', 5'-AGAGTCCCACATGCGCTGTT-3',
CHK1: 5'-GGTGAATATATGTCTGCTATGGAC-3', 5'-TTGGATATAAAGGGGAAGTGAC-3',
CHK2: 5'-AGTGAAGAGACTGGCCTGGAGTT-3', 5'-CCCAAGGGTCTCCTCCTACA-3',
TP53: 5'-TCAAACAGAGTATTTTGGGAACTG-3', 5'-ATGGTGCCTGTGCTCCTGAGT-3',
p21: 5'-CTCTACATTTTGTTGCTTTTTG-3', 5'-GTACACCAGCGGACACGAA-3',
14-3-3α: 5'-TGCTGCTCTGATCTGAATTTG-3', 5'-TTCCCTACATCTGGCTTGGC-3',
GADD45α: 5'-CAGCCGCACTCAGCTGTC-3', 5'-CCAGCAGCGACACAAAC-3',
APAF-1: 5'-GCATCACCCTTTTGATAAC-3', 5'-CCCAGCCTTTTGTGAT-3',
BAD: 5'-TTAACACTGCTGACGACTT-3', 5'-GCTGCTCCTGCCAGAGG-3',
BAX: 5'-CCTTTTCTACTTGTGGAAC-3', 5'-GAGGCCCTCCCAACCAC-3'.

Preparation of Protein Lysates and Western Blot Analysis

To prepare protein lysates from cell lines, cells were lysed using ice-cold radioimmunoprecipitation assay buffer (Pierce Biotechnology, Inc.). For Western blot analysis, boiiled protein was loaded onto a 4% to 12% precast gel gradient, transferred to nitrocellulose membranes (Invitrogen Corporation, Inc.) and incubated with antibodies. Western blots were initially probed with antibodies that recognized only the phosphoforms of the respective DNA damage and cell cycle checkpoint proteins. These blots were then stripped and re-probed with the antibodies that recognized total protein levels of the respective proteins. Bound antibodies were detected by a chemiluminescent detection system (West Femto; Pierce Biotechnology, Inc.).
Gene Profiling

Ten micrograms of total RNA extracted from each cell line were reverse transcribed and amplified by standard in vitro transcription methods and chromogen labeled. Hybridization was carried out according to Affymetrix protocols and chips were scanned and gene intensities were recorded. All data were log 2 transformed and then each array was normalized by using median values over the entire array. The global gene expression profiles (22,000 genes) were examined using Affymetrix Microarray Gene Chips (Affymetrix) and analyzed with BRB ArrayTools, an integrated package for the visualization and statistical analysis of DNA microarray gene expression data available on the WorldWide Web. We used dChip software to search for “Chromatin remodeling gene list” by annotation and “SWI/SNF family gene list” by keywords. Then hierarchical clustering was used to compare the gene expression of those two “gene list” groups among the samples examined. We examined representative HM, LG, and NU cell lines. We also examined primary human breast–infiltrating ductal carcinomas and their corresponding adjacent normal tissue counterparts. For representative genes from each group, levels of expression as determined by microarray chip were confirmed by real-time PCR studies.

Institutional Approvals and Human Tissues

Use of human tissues was approved by The Ohio State University Cancer Institutional Review Board under protocol 2006C0042. Specifically, cases of primary breast–infiltrating ductal carcinomas with normal tissue counterparts obtained from a frozen tissue bank and anonymized were used. All animal and in vitro studies were approved by The Ohio State University’s Animal Care and Use Committee (Institutional Animal Care and Use Committee), protocol 2007A0218, and by the Institutional Biosafety Committee, protocol 2007R0057.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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Inhibition of Expression of the Chromatin Remodeling Gene, SNF2L, Selectively Leads to DNA Damage, Growth Inhibition, and Cancer Cell Death

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