The Oncoprotein SS18-SSX1 Promotes p53 Ubiquitination and Degradation by Enhancing HDM2 Stability

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Abstract

Mutations of the *p53* gene are uncommon in synovial sarcoma, a high-grade tumor genetically characterized by the chromosomal translocation t:(X;18), which results in the fusion of *SS18* with members of *SSX* gene family. Although implicated in tumorigenesis, the mechanisms by which SS18-SSX promotes tumor growth and cell survival are poorly defined. Here, we show that SS18-SSX1 negatively regulates the stability of the tumor suppressor p53 under basal conditions. Overexpression of SS18-SSX1 enhanced p53 ubiquitination and degradation in a manner dependent on the ubiquitin ligase activity of HDM2. The negative effect of SS18-SSX1 expression on p53 was mediated by its ability to promote HDM2 stabilization through inhibition of HDM2 autoubiquitination. Furthermore, SS18-SSX1 expression altered the induction of p53-regulated genes in response to cellular stress by abrogating the transactivation of HDM2, PUMA, and NOXA but not p21. Our data uncover a novel mechanism whereby SS18-SSX1 can negatively regulate p53 tumor-suppressive function by increasing the stability of its negative regulator HDM2 and suggest that chemical compounds that target the p53-HDM2 regulatory axis may be of therapeutic benefit for the treatment of synovial sarcoma. (Mol Cancer Res 2008;6(1):127–38)

Introduction

Disruption of p53-mediated tumor surveillance is a common motif in the development of cancer. In response to a variety of cellular stresses, p53 acts as an intracellular mediator of cell fate by inducing cell cycle arrest, DNA repair, or apoptosis, thus allowing cells to cope with situations that could otherwise lead to enhanced tumorigenesis (1). The potent tumor-suppressive function of p53 is kept in check by the action of its negative regulator HDM2, which catalyzes the ubiquitination of conserved lysine residues in the COOH-terminal domain of p53, promoting nuclear export, inhibiting p53 transcriptional activity, and promoting proteasome-mediated degradation (2-4). Intracellular p53 levels are regulated by an elegant feedback loop whereby increased levels of p53 result in enhanced transcription of HDM2, leading to the subsequent down-regulation of p53 protein levels by proteasomal-mediated degradation (5). The discovery that HDM2 was commonly overexpressed in a variety of tumors, particularly sarcomas, further provided a mechanism of deactivating p53 in the absence of discernible p53 mutations (6). Consequently, disruption of the HDM2-p53 feedback loop has been suggested as a novel target for cancer therapy particularly in tumors where the tumor-suppressive function of p53 is dampened by enhanced HDM2 expression.

Synovial sarcoma, a high-grade soft tissue tumor, displays a reciprocal chromosomal translocation t(X;18), which results in the fusion of two disparate genes: *SS18* from chromosome 18 and *SSX* from the X chromosome. This event results in the expression of a putative fusion protein, which contains all but the last eight amino acids of SS18 and the COOH terminus of SSX (7-10). SS18 is a transcriptional coactivator, whereas SSX has been implicated in transrepression (11-13). In its role as a transcriptional activator, SS18 interacts with an array of transcription factors implicated in gene regulation, such as the histone acetyl transferase p300, the BRM and BRG1 components of the SWI/SNF complex, the leucine zipper transcription factor AF10, and the histone deacetylase Sin3a (13-17). Conversely, members of the SSX gene family are potent corepressors and have been shown to interact with polycomb group proteins, core histones, and matrix metalloproteinase-2 (11, 13, 18-20). In synovial sarcoma, the chromosomal translocation results in the production of a proto-oncogene with dual features of a transactivator and repressor. The resultant fusion protein is presumed to function as an aberrant transcription factor through the misdirected targeting of interacting partners and alteration of epigenetic control (21, 22). Interestingly, in comparison with other tumor types, synovial sarcomas display a remarkably low number of mutations in the *p53* gene, implying that defects in upstream pathways may be responsible for loss of p53-mediated tumor suppression (23, 24).

In this study, we show that SS18-SSX expression promotes the ubiquitination and degradation of p53 in an HDM2-dependent manner. Expression of SS18-SSX1 reduced p53 half-life, promoted cell survival, enhanced colony formation ability, and abrogated the transactivation of p53 target genes in response to cellular stress. The negative effects of SS18-SSX1 on p53 stability were due to enhanced ubiquitination of p53 in a

manner dependent on the activity of HDM2. Our results highlight an important oncogenic function of SS18-SSX1 as a positive regulator of HDM2 stability.

Results

SS18-SSX Expression Negatively Attenuates p53 Stability

Previous reports have shown that synovial sarcomas harbor a remarkably low number of mutations in the *p53* gene in comparison with other tumor types (23, 24). Consistent with this, we analyzed the p53 status in a set of 20 primary synovial sarcoma tumors and found no discernible mutations of the *p53* gene (results not shown). In light of this, we investigated whether a potential oncogenic mechanism of SS18-SSX expression is the abrogation of p53 function. Transfection of increasing amounts of SS18-SSX1 into U2OS or cotransfection with p53 in p53-null Saos-2 cells resulted in a concomitant decrease in the levels of p53 (Fig. 1A and B). Because no reliable antibodies for the detection of SS18-SSX expression are available, SS18-SSX expression was determined using real-time PCR. Cotransfection with a green fluorescent protein (GFP) vector was used as a determinant of transfection efficiency. Transfection with SS18-SSX1 resulted in increased levels of SS18-SSX1 mRNA with minimal changes in the levels of *p53* transcripts, indicating that the negative effect of SS18-SSX is a posttranscriptional mechanism (Fig. 1A and B, *bottom*). The relationship between the expression of SS18-SSX and p53 was further investigated following transfection of a synovial sarcoma cell line CME1 with doxycycline-inducible small interfering RNA (siRNA) vector containing a short hairpin RNA cassette targeting endogenous SS18-SSX. Knockdown of SS18-SSX was confirmed by real-time PCR (Fig. 1C). siRNA targeting of endogenously expressed SS18-SSX in a synovial sarcoma cell line increased p53 levels following induction of siRNA expression with doxycycline (Fig. 1D). To determine if SS18-SSX expression could affect p53 stability, the half-life of p53 was determined by a pulse-chase analysis by immunoprecipitating p53 from [^{35}S]methionine-labeled U2OS cells expressing SS18-SSX1 or a control vector. As seen in Fig. 1E, the half-life of p53 in SS18-SSX1–expressing cells was significantly reduced when compared with control U2OS cells.

SS18-SSX Expression Enhances Cell Survival

To further investigate if the expression of SS18-SSX confers cell survival, we did clonogenic survival assays on a variety of cell lines. U2OS (p53$^+$) cells were transfected with an SS18-SSX1 expression vector or a control plasmid and selected in the presence of G418. Following selection, SS18-SSX–expressing clones appeared more numerous and formed larger foci when compared with cells transfected with empty vector (Fig. 2A). To confirm our results, Saos-2 cells (p53 null) were transfected with expression vectors for p53 or SS18-SSX1 alone or cotransfected with p53 and SS18-SSX1 and grown in G418 selection for 2 weeks. Following selection, p53-transfected Saos-2 cells produced low numbers of colonies, presumably due to the induction of p53-dependent cell cycle arrest or apoptosis. However, coexpression of SS18-SSX1 with p53 could effectively counteract the antisurvival effect of p53,

resulting in the appearance of numerous foci comparable with those produced by vector-only controls (Fig. 2B). The functional consequence of SS18-SSX expression was further analyzed by RNA interference against SS18-SSX in the synovial sarcoma cell line CME1, which endogenously expresses SS18-SSX2. CME1 cells, in which SS18-SSX was depleted, had a decreased survival advantage when compared with the parental cells (Fig. 2C). *In vitro* growth curves, determined by counting the number of viable cells, showed that SS18-SSX knockdown displayed significantly slower growth times (Fig. 2D) and become arrested in G$_1$ (Fig. 2E). Taken into consideration, these results show that SS18-SSX has pro-survival activity and growth-stimulating properties.

SS18-SSX Attenuates the p53 Response

To evaluate the effect of cell stress on p53 stability and transactivation function, p53-proficient U2OS cells or U2OS cells expressing SS18-SSX1 were exposed to doxorubicin or actinomycin D. Actinomycin D induces p53 stability by activating a ribosomal stress response, which activates p53 function without triggering p53 NH$_2$-terminal phosphorylation, whereas doxorubicin activates p53 function via the induction of DNA strand breaks, which promotes p53 NH$_2$-terminal phosphorylation and stabilization (25, 26). Treatment of control U2OS cells with low-dose actinomycin D resulted in the rapid time-dependent induction of p53, with p53 levels peaking after 6 h of actinomycin D treatment. In contrast, actinomycin D treatment induced a significantly slower kinetic of p53 stabilization in U2OS cells expressing SS18-SSX1, with p53 levels reaching a peak at 12 h (Fig. 3A). We also analyzed the levels of protein induction of p53's negative regulator and target HDM2. Analysis of HDM2 expression revealed a time-dependent induction in control U2OS cells, which corresponded with the increase in p53 levels. Interestingly, SS18-SSX1–expressing cells had a constitutively higher level of HDM2 expression when compared with control cells under basal conditions (Fig. 3A). Because p53 can be differentially stabilized by different stresses, we analyzed the induction of p53 in response to DNA damage induced by doxorubicin. Addition of doxorubicin induced a rapid time-dependent stabilization of p53 in both control and SS18-SSX1–expressing U2OS cells (Fig. 3B). In contrast, the negative effect of SS18-SSX1 on p53 induction in response to doxorubicin treatment was relatively weak when compared with that of actinomycin D, with p53 levels peaking at 12 h in both control and SS18-SSX1–expressing cells. Taken into consideration, these results suggest that SS18-SSX expression can hinder the induction of p53 stability in response to ribosomal stress induced by actinomycin D but not in response to genotoxic stress induced by doxorubicin. Similarly to previous results, HDM2 levels were constitutively elevated in SS18-SSX1–expressing cells. To investigate the effect of SS18-SSX1 on p53 stability after the induction of a ribosomal stress response, a pulse-chase analysis of p53 half-life was done on U2OS cells expressing SS18-SSX1 or a control vector. Cells were pretreated with actinomycin D for 24 h before pulse chase with [^{35}S]methio-ionine. Control U2OS cells displayed steady levels of p53 during the time course (90 min). In contrast, p53 levels were significantly lower in SS18-SSX1–expressing cells. These data
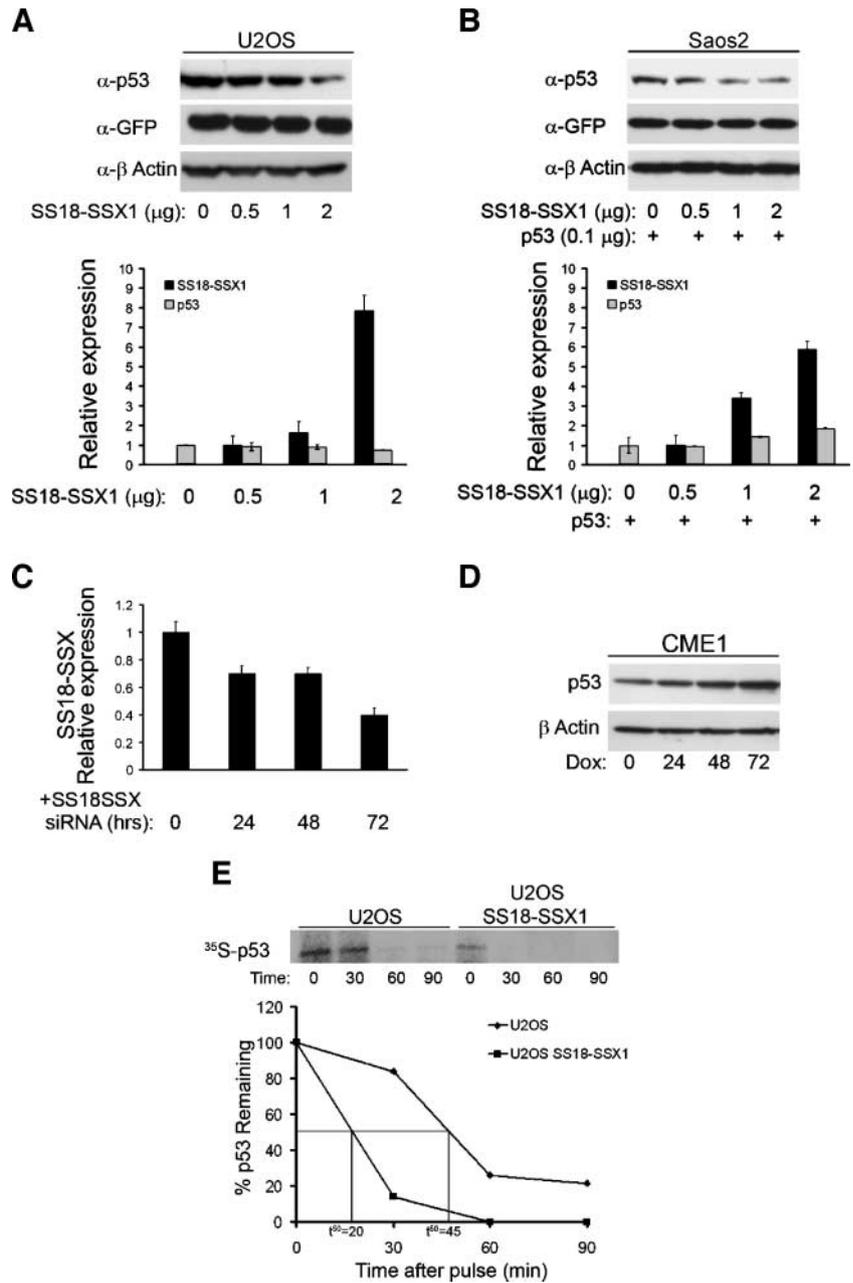
FIGURE 1. SS18-SSX negatively regulates p53 stability. **A.** U2OS cells were transfected with the indicated amounts of SS18-SSX1 and GFP as indicated. Cells were collected and harvested for mRNA or protein expression. Levels of relative expression of SS18-SSX1 or p53 were determined using real-time PCR. **B.** Saos-2 cells were cotransfected with p53 and increasing amounts of SS18-SSX1 as in **A**. **C.** siRNA targeting of SS18-SSX in a doxycycline-inducible synovial sarcoma cell reduces SS18-SSX expression. Cells were treated with doxycycline (2 μg/mL) for indicated time points and prepared for real-time PCR analysis. **D.** siRNA targeting of SS18-SSX in a doxycycline (*Dox*)-inducible synovial sarcoma cell induces increases in p53 levels. Cells were treated with doxycycline (2 μg/mL) for indicated time points and prepared for Western blot analysis. **E.** Stable clones of U2OS expressing SS18-SSX or empty vector were treated with cycloheximide and harvested at indicated time points. Top, cells were lysed at indicated time points and the level of p53 expression was determined by Western blotting; bottom, the levels of p53 were quantified by densitometry and the levels of p53 remaining were plotted.

indicate the SS18-SSX1 can interfere with the ability of cells to maintain high p53 levels following a ribosomal stress response induced by low-dose actinomycin D treatment (Fig. 3C). To evaluate the effect of cell stress on p53 transactivation function, control or SS18-SSX1–expressing U2OS cells were exposed to doxorubicin or actinomycin D for the indicated time points and real-time PCR analysis of the p53-induced genes *p21*, *HDM2*, *NOXA*, and *PUMA* was done. The expression of the cell cycle inhibitor p21 was not significantly altered in either U2OS or U2OS SS18-SSX1–expressing cells in response to either doxorubicin or actinomycin D treatment (Fig. 3D, *top left*). SS18-SSX1–expressing cells did, however, display a slight reduction in the fold activation of p21 mRNA over the duration of the experiment in response to both treatments. In

contrast to *p21*, the expression of *HDM2, NOXA*, and *PUMA* was significantly abrogated in the presence of SS18-SSX1. U2OS cells displayed a strong fold induction of *HDM2, PUMA*, and *NOXA* in response to treatment with either doxorubicin or actinomycin D; however, the ability of SS18-SSX1–expressing cells to activate transcription of these genes was severely reduced. The induction of *p21* in response to stress in SS18-SSX1–expressing cells suggests that p53 is transcriptionally active with respect to *p21* but that the induction of other p53 target genes is significantly altered. Interestingly, the negative effect of SS18-SSX1 on the p53-mediated transactivation of *HDM2, NOXA*, and *PUMA* seems to be independent of p53 stability because p53 is efficiently stabilized by doxorubicin in SS18-SSX1–expressing cells

(Fig. 3B). Taken into consideration, these results show that SS18-SSX can inhibit p53 stabilization in response to ribosomal stress and alter the spectrum of genes activated by p53 independent of p53 stability.

SS18-SSX1 Promotes Ubiquitination and Nuclear Export of p53

In light of our observation that SS18-SSX can negatively modulate the levels of p53, we sought to determine if this effect was mediated via the ubiquitin-proteasome system. Consistent with this hypothesis, transient transfection of U2OS cells with SS18-SSX1 resulted in enhanced levels of p53 ubiquitination as

visualized by the appearance of a high molecular weight smear of p53-ubiquitin conjugates (Fig. 4A). This result was also repeated using exogenously expressed p53 and SS18-SSX1 in p53-null Saos-2 cells (Fig. 4B). Conversely, siRNA-mediated down-regulation of endogenous SS18-SSX resulted in decreased levels of p53 ubiquitination (Fig. 4C). To confirm these observations, we did an *in vivo* ubiquitination assay using $6\times$ His-tagged ubiquitin and Ni^{2+} affinity chromatography to purify ubiquitinated proteins according to a previous report (27). p53-null H1299 cells were cotransfected with plasmids encoding wild-type p53, GFP, and $6\times$ His-ubiquitin in the presence or absence of increasing amounts of SS18-SSX1 expression vector. Cells were collected 48 h after transfection
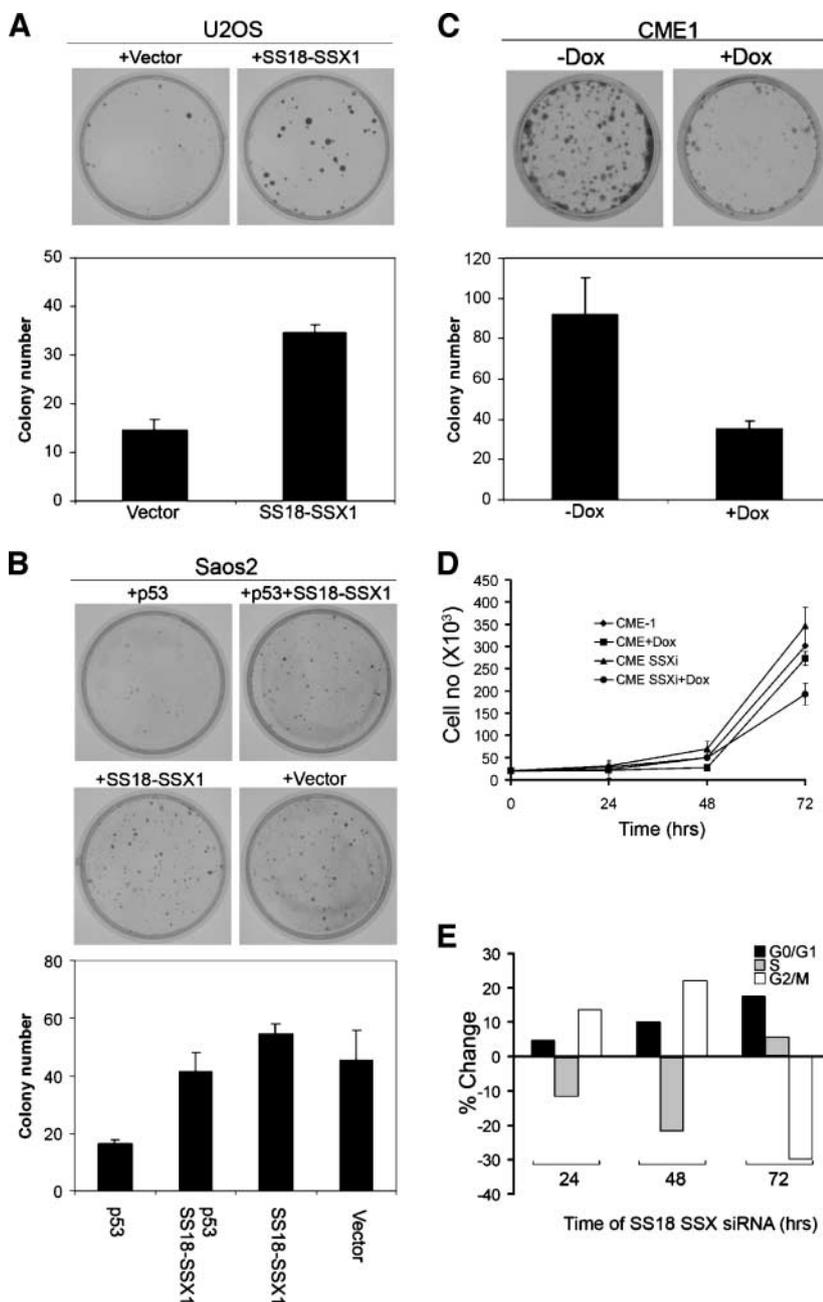

FIGURE 2. SS18-SSX expression increases clonogenic survival. **A.** U2OS cells were transfected with SS18-SSX or empty vector alone and grown in G418 selection for 2 wk. **B.** Saos-2 cells were transfected with p53 alone, SS18-SSX, and p53 or empty vector. **C.** For siRNA knockdown of endogenous SS18-SSX2, CME1 cells were plated at low density and grown in the presence of doxycycline (2 μg/mL) for 2 wk. Colonies were visualized by Giemsa staining. Columns, mean of triplicate plates; bars, SD. **D.** CME1 cells were grown in the presence of doxycycline (2 μg/mL), harvested, and counted or collected for fluorescence-activated cell sorting analysis at the indicated time points (**E**).
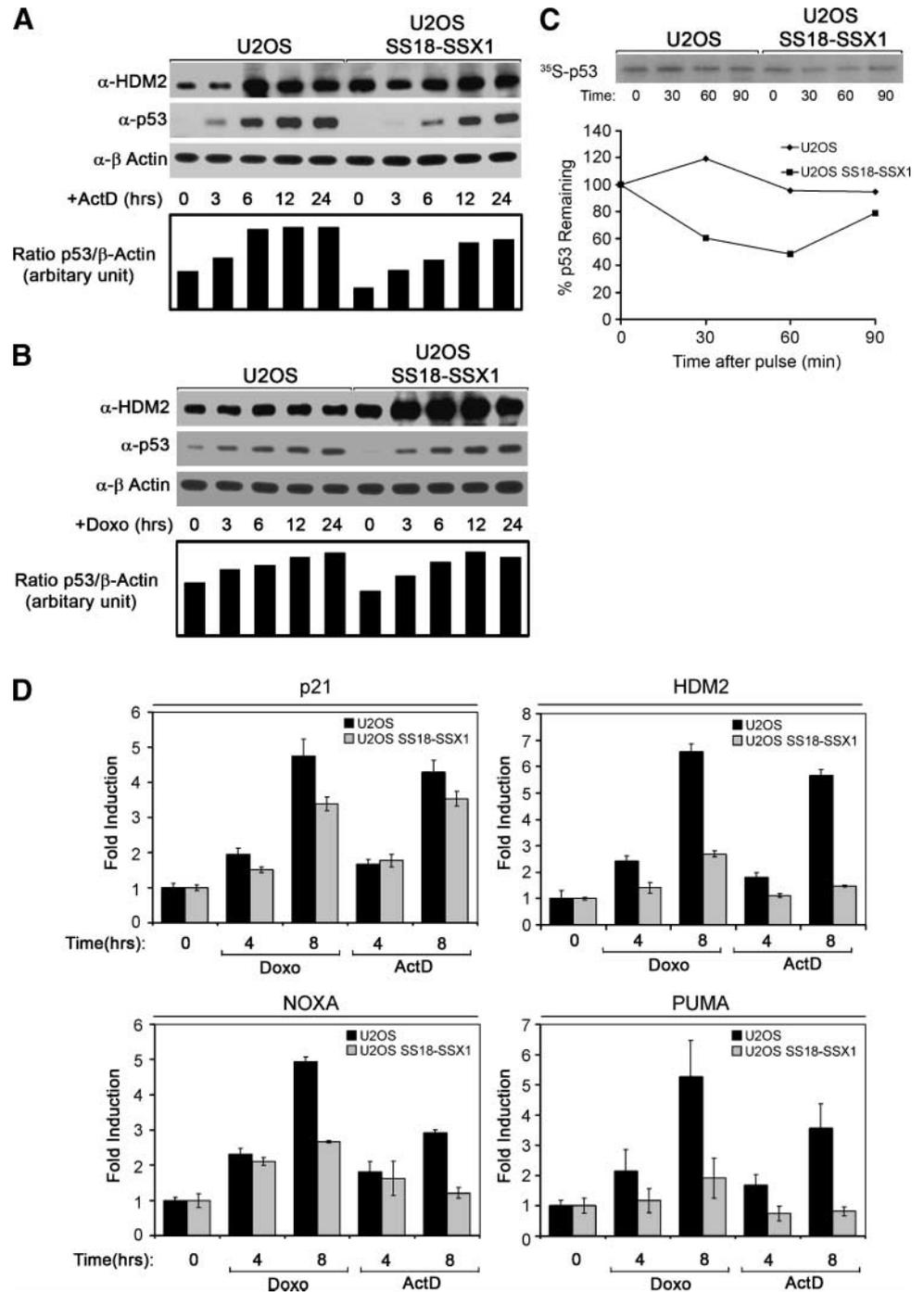
FIGURE 3. SS18-SSX attenuates the p53 response. Effect of SS18-SSX on p53 levels following actinomycin D (**A**) or doxorubicin (**B**) treatment. U2OS cells expressing SS18-SSX1 or control vector were treated with actinomycin D for indicated time points. Cell lysates were analyzed by Western blotting using antibodies as indicated. Quantification of p53 level was based on the ratio of p53 to β-actin. **C.** Cells were treated with 5 nmol/L actinomycin D for 24 h, pulse chased with [^{35}S] methionine, and harvested at indicated time points. **D.** Transcriptional regulation by p53. mRNA expression levels of p53 target genes *p21, HDM2, NOXA*, and *PUMA* in response to treatment with doxorubicin (0.1 μg/mL) or actinomycin D (5 nmol/L).

and analyzed for His-ubiquitin–conjugated p53 and *SS18-SSX* expression as described in Materials and Methods. As shown in Fig. 4D, transfection with increasing amounts of SS18-SSX resulted in a subsequent increase in the levels of p53 ubiquitination as visualized by the appearance of a high molecular weight ladder of p53-ubiquitin conjugates. The enhancement in the levels of p53 ubiquitination in the presence of SS18-SSX1 was not due to differences in transfection efficiency because the levels of GFP protein expression were equivalent in all transfections. The increased levels of p53

ubiquitination observed were concomitant with an overall decrease in total p53 levels and increased *SS18-SSX* expression (Fig. 4D, *bottom*). Because p53 ubiquitination has been implicated in the shuttling of p53 from the nucleus to the cytoplasm, we looked at the subcellular localization of p53. As shown in Fig. 4E, Saos-2 cells expressing p53 alone displayed predominantly nuclear p53 staining, whereas cells coexpressing p53 and SS18-SSX1 displayed a higher proportion of cytoplasmic sequestered p53, with ∼40% of cells displaying high levels of extranuclear staining. Collectively, these results
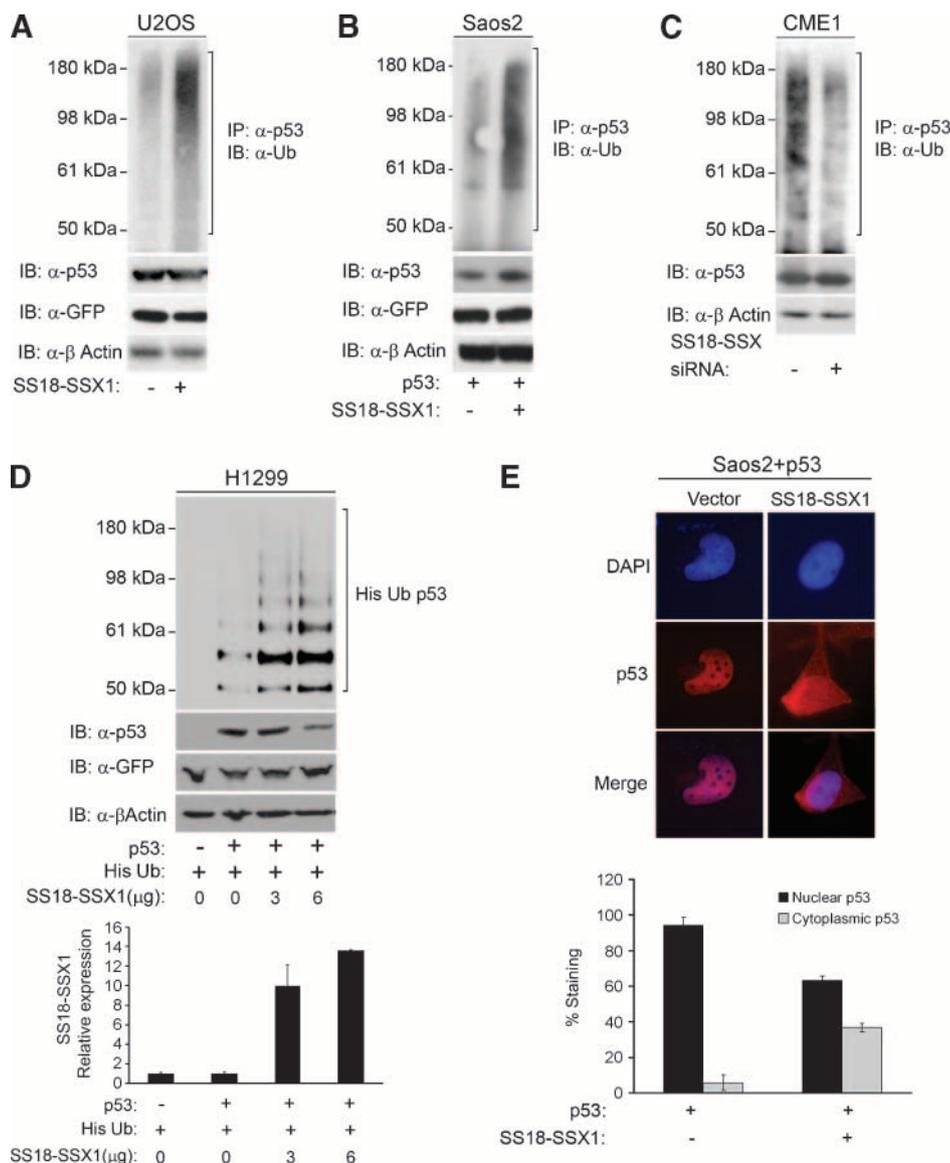
FIGURE 4. SS18-SSX promotes p53 ubiquitination. U2OS (**A**) or Saos-2 (**B**) cells were transfected with indicated plasmids. Thirty-six hours after transfection, cells were treated with epoxomycin (500 nmol/L) for 4 h before harvesting to allow accumulation of ubiquitin conjugates. Endogenous p53 was immunoprecipitated and levels of ubiquitination were visualized using α-ubiquitin (α-*Ub*) antibody. Crude lysates were used as loading controls with the indicated antibodies. **C.** siRNA knockdown of endogenous SS18-SSX was initiated by addition of doxycycline to culture medium. The level of p53 ubiquitination was determined as above. **D.** H1299 cells were cotransfected with 1 μg p53, 1 μg GFP, 6 μg His-ubiquitin, and the indicated amount of SS18-SSX1 expression vectors. Ubiquitinated proteins were purified with Ni^{2+} agarose beads and analyzed by Western blotting using α-p53 antibody. The relative expression of SS18-SSX1 from H1299 cells is shown below. **E.** Saos-2 cells were transfected with p53 and SS18-SSX or p53 and empty vector. Thirty-six hours after transfection, cells were fixed and analyzed for p53 immunofluorescence. The level of cytoplasmic p53 staining was determined visually. DAPI, 4′,6-diamidino-2-phenylindole. Columns, mean of triplicate slides; bars, SD.

show that SS18-SSX1 can enhance the ubiquitination and nuclear export of p53.

SS18-SSX–Mediated p53 Ubiquitination Is HDM2 Dependent

Because HDM2 is a key mediator of p53 ubiquitination, we investigated if the increased p53 ubiquitination was dependent on the ubiquitin ligase activity of HDM2. Control or SS18-SSX1–expressing U2OS cells were transfected with siRNA molecules targeting endogenous HDM2. Before harvesting, cells were treated with the proteasomal inhibitor epoxomycin to inhibit degradation of p53 and allow direct comparison of protein levels. As expected, knockdown of endogenous HDM2 levels decreased the levels of ubiquitinated p53 (Fig. 5A, *lanes 1* and *2*). Expression of SS18-SSX1 increased p53 ubiquitination (Fig. 5A, *lane 3*), which could be decreased by siRNA knockdown of endogenous HDM2 (Fig. 5A, *lane 4*). The efficiency of siRNA against HDM2 was confirmed by Western blot (Fig. 5B).

SS18-SSX1 Increases HDM2 Half-life and Inhibits HDM2 Autoubiquitination

We questioned whether the HDM2-mediated degradation of p53 in SS18-SSX–expressing cells was associated with an increased half-life of HDM2. We quantified the levels of HDM2 protein in the presence or absence of SS18-SSX1 by methionine pulse chase. As shown in Fig. 6A, the half-life of HDM2 in control U2OS was estimated at ∼ 20 min; however, this was increased to ∼ 50 min in U2OS cells expressing SS18-SSX1. Because HDM2 stability is primarily determined by its own intrinsic autoubiquitination activity, we postulated that SS18-SSX1 may increase the stability of HDM2 by inhibiting autoubiquitination. To determine this, U2OS cells were transfected with expression vectors for SS18-SSX1, HDM2, or both. Thirty-six hours after transfection, cells were treated with epoxomycin to block proteasome-dependent degradation of HDM2. Expression of SS18-SSX1 remarkably reduced the endogenous levels of HDM2 ubiquitination (Fig. 6B, *lanes 1*

and *2*). Consistent with its autoubiquitination activity, over-expression of HDM2 resulted in significantly increased HDM2 ubiquitination (Fig. 6B, *lanes 3* and *4*). These findings were confirmed using the His-ubiquitin system described in Fig. 4D. H1299 cells were cotransfected with plasmids encoding GFP and $6\times$ His-ubiquitin together with the indicated amounts of SS18-SSX1 expression vector, and the levels of His-ubiquitin-HDM2 conjugates were analyzed following inhibition of the proteasome activity with MG132. As can be seen in Fig. 6C, expression of SS18-SSX1 remarkably reduced the levels of HDM2 ubiquitination in a dose-dependent manner. Increasing levels of SS18-SSX1 expression (Fig. 6C, *bottom*) coincided with decreased HDM2 ubiquitination. Because activation (by phosphorylation) of the serine-threonine kinase phosphatidylinositol 3-kinase/Akt is important for HDM2 stability, we blocked Akt phosphorylation with the inhibitor LY294002 in U2OS and U2OS SS18-SSX and analyzed the levels of HDM2. We observed that the levels of phosphorylated Akt were efficiently reduced by exposure to the LY294002 inhibitor (Fig. 6D). Slightly higher levels of phosphorylated Akt were, however, observed in the presence of SS18-SSX at this time point of the experiment (6 h).

## SS18-SSX1 Inhibits Apoptosis in Response to Genotoxic Stress

To investigate whether the prosurvival function of SS18-SSX1 was linked to apoptosis evasion, we exposed control or SS18-SSX1-U2OS cells to high concentrations of actinomycin D. Addition of actinomycin D resulted in a dramatic increase in the sub-G_1 population of control U2OS cells, with $\sim 39\%$ of cells displaying apoptosis induction (Fig. 7A). In contrast, SS18-SSX1–expressing cells were relatively resistant to the onset of DNA fragmentation, with only $\sim 9\%$ of the cells displaying a sub-G_1 population. In addition, SS18-SSX1–expressing cells tended to accumulate in the G_2 phase of the cell cycle. Similar results were obtained when p53-positive HT1080 or SS18-SSX1-HT1080 cells were treated (results not shown). Consistent with induction of apoptosis, the high levels of DNA fragmentation were concomitant with an increase in poly-caspase activity in control U2OS cells, which was inhibited in cells expressing SS18-SSX1 (Fig. 7B). To investigate whether SS18-SSX1 expression can inhibit apoptosis induced by other genotoxic agents, control and SS18-SSX1–expressing U2OS cells were exposed to doxorubicin, mitomycin C (MMC), etoposide, and UV. As can be seen from Fig. 7C, SS18-SSX expression reduced the onset of apoptosis in response to MMC, doxorubicin, and etoposide but not to UV irradiation. Analysis of sub-G_1 levels showed that MMC and actinomycin D were potent inducers of apoptosis in control U2OS cells, with the number of sub-G_1 cells in response to MMC and actinomycin D approaching 40%. Expression of SS18-SSX resulted in a statistically significant reduction in apoptosis, with the number of cells displaying a sub-G_1 population remaining below 10%.

In light of these data, we postulated that inhibiting HDM2 could counteract the protective effect of SS18-SSX1 expression on apoptosis induction. HDM2 expression was depleted using RNA interference molecules in both wild-type and SS18-SSX1–expressing cells. Seventy-two hours after RNA interference transfection, cells were treated with actinomycin D or MMC and the levels of apoptosis were determined as previously described. Knockdown of HDM2 levels produced minimal changes in apoptosis and cell cycle profile in both control and SS18-SSX1–expressing cells. However, siRNA knockdown of HDM2 in SS18-SSX1–expressing cells could effectively enhance actinomycin D–induced or MMC-induced apoptosis; although the levels of apoptosis failed to reach that observed in control cells, this may be due to the efficiency of the siRNA protocol (Fig. 7D). In light of our observation that Akt phosphorylation was higher in SS18-SSX–expressing cells and that inhibition of Akt could decrease HDM2 levels in both control and SS18-SSX–expressing cells (Fig. 6D), we postulated that inhibiting the Akt pathway could counteract the protective effect of SS18-SSX1 expression on apoptosis induction. Control cells or cells expressing SS18-SSX1 were treated with the Akt inhibitor LY294002 for 6 h before the addition of actinomycin D or MMC. This treatment produced minimal changes in the sub-G_1 content of these cells; however, it did induce a potent G_1 arrest in both cell lines (results not shown). Interestingly SS18-SSX1–expressing cells, which were relatively resistant to actinomycin D–induced or MMC-induced apoptosis, were sensitized by prior treatment with LY294002 (Fig. 7E). Collectively, these results show the involvement of SS18-SSX in promoting resistance to apoptosis.
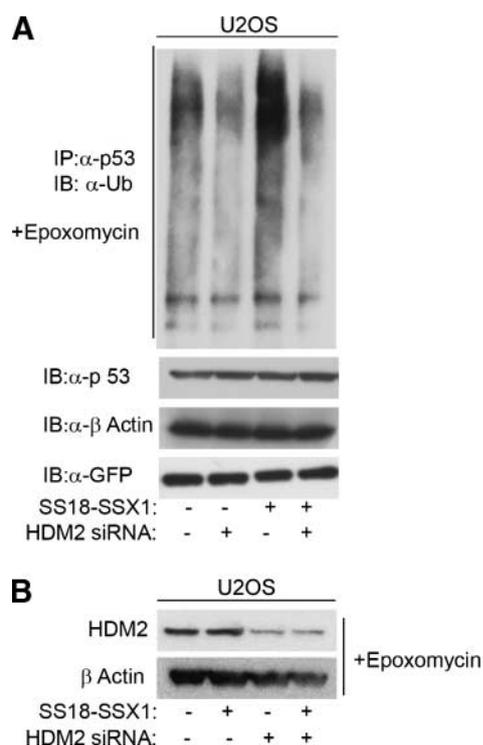
FIGURE 5. SS18-SSX1–induced p53 ubiquitination is dependent on HDM2. **A.** U2OS cells expressing SS18-SSX or empty vector alone were transfected with siRNA molecules targeting HDM2. Seventy-two hours after transfection, cells were treated with epoxomycin to block proteasomal degradation and analyzed for p53 ubiquitination and HDM2 expression (**B**).
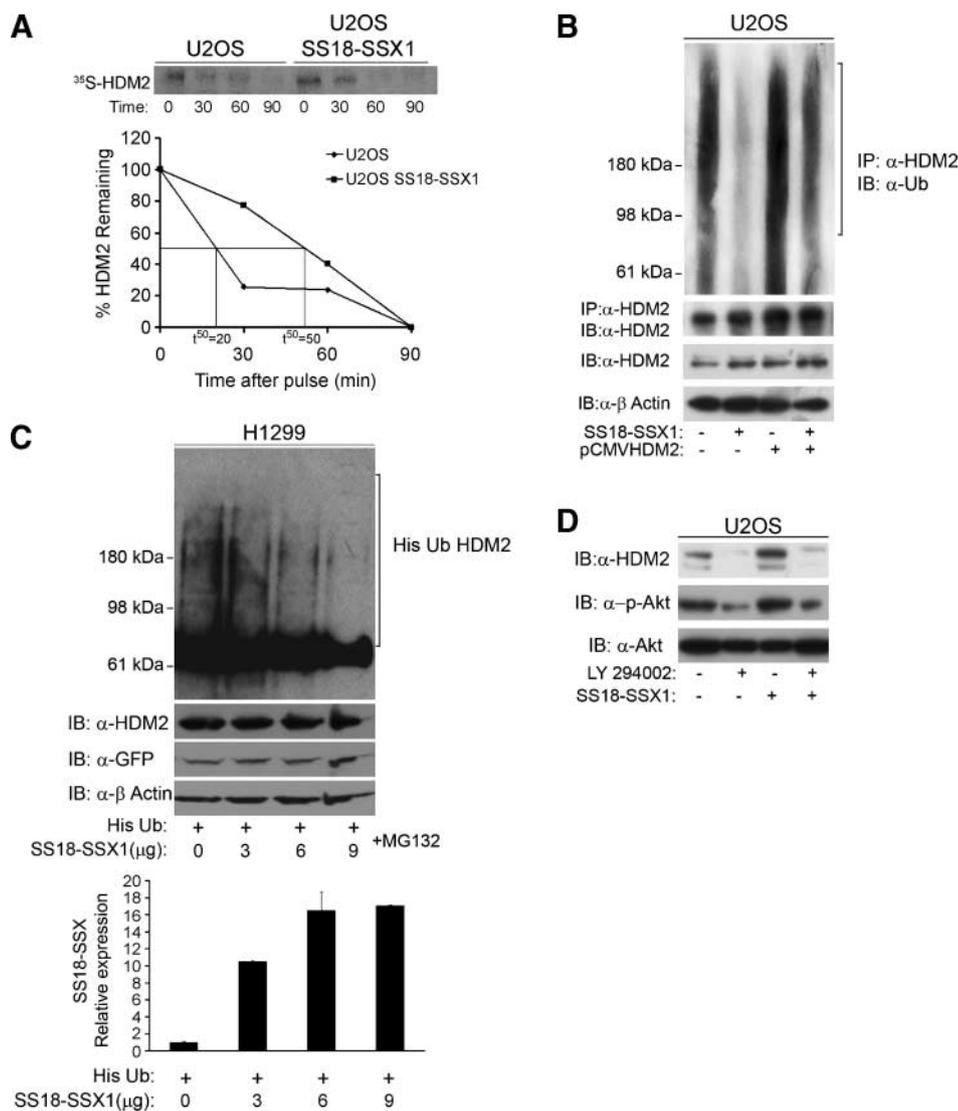
FIGURE 6. SS18-SSX1 increases HDM2 stability by abrogating HDM2 autoubiquitination. **A.** U2OS or SS18-SSX1–expressing U2OS cells were pulse chased with [^{35}S]methioionine and endogenous HDM2 was immunoprecipitated and half-life was determined. **B.** U2OS cells expressing SS18-SSX or control vector were transfected with HDM2 as indicated. Cells were treated with epoxomycin for 4 h before lysis. HDM2 was immunoprecipitated and levels of HDM2 ubiquitination were determined. **C.** H1299 cells were transfected with His-ubiquitin expression vector and the indicated amount SS18-SSX1 expression plasmid. Thirty-six hours after transfection, cells were treated with MG132 for 4 h before harvesting. **D.** Stable clones of U2OS expressing SS18-SSX or empty vector were treated with LY294002 or DMSO for 6 h and analyzed by immunoblotting with indicated antibodies.

Discussion

The fusion of *SS18* on chromosome 18 with one of the *SSX* genes, either *SSX1, SSX2*, or *SSX4*, on the X chromosome is the main genetic event in synovial sarcoma (7, 8, 10, 28) and probably an early event in the tumorigenesis process. Although some molecular mechanisms on the SS18-SSX–induced transformation process have been elucidated, a role of this fusion gene in tumor initiation has not been proposed (29).

The role of p53 in synovial sarcoma development has been previously studied by mutation analysis and expression levels of p53, leading to the conclusion that p53 mutations are rare events (~ 10%) in synovial sarcomas (22, 23). In this study, we propose a novel mechanism through which SS18-SSX blocks the tumor-suppressive function of p53 in the absence of inactivating p53 mutations. Our data have established a role for SS18-SSX1 in the negative regulation of p53 by promoting its degradation (Fig. 1). We show that SS18-SSX1 could effectively reduce p53 half-life by enhancing p53 ubiquitination and nuclear to cytoplasmic relocalization (Fig. 4). Coexpression

of SS18-SSX1 with p53 in a clonogenic survival assay could effectively overwrite the apoptotic activity of p53, highlighting a pro-oncogenic function of SS18-SSX in disabling p53 tumor suppression (Fig. 2). Cellular levels of p53 are primarily regulated by the ubiquitin-proteasome system whereby p53 is tagged for degradation by the HDM2 catalyzed covalent attachment of ubiquitin moieties to lysine residues in the COOH terminus of p53, which target it to the 26S proteasome (2, 3). The *MDM2* gene (*HDM2* in humans) was initially identified from chromosomal fragments derived from spontaneously transformed murine fibroblasts, where its transforming function was subsequently found to be the ability of MDM2 to bind to and inhibit the function of p53 (4, 30, 31). Overexpression of HDM2 is common in numerous sarcomas, particularly in tumors that retain wild-type p53, where the overexpression of HDM2 removes the requirement for acquired p53 mutations in tumor progression (6, 32). Our results implicate increased HDM2 stability induced by SS18-SSX1 as a molecular mechanism by which SS18-SSX1 can decrease

p53 levels in synovial sarcoma. Indeed, such a mechanism would remove the selective pressure for acquiring p53 mutations and may explain the relatively low frequency of p53 mutations in these tumors. In addition to its role of a negative regulator of p53, HDM2 also has numerous additional pro-oncogenic functions involved in cell cycle regulation, differentiation, DNA repair, and transcriptional regulation (33). Thus, SS18-SSX–induced HDM2 expression would have far-reaching consequences in relation to cell growth and survival functions, which would be independent of the classic p53-HDM2 pathway. In addition, we also show that SS18-SSX1 expression can compromise the ability of p53 to be stabilized in response to ribosomal stress induced by low doses of actinomycin D, which specifically inhibits RNA polymerase I activity by perturbation of ribosomal biogenesis through abrogation of rRNA processing (34, 35). Although the mechanism of p53 stabilization in response to ribosomal stress in not fully understood, several classes of ribosomal proteins have been shown to stabilize p53 by binding with HDM2 and inhibiting its ubiquitin ligase activity (36-40). In the case of

ribosomal stress, p53 is stabilized via a mechanism independent of the canonical NH_2-terminal phosphorylation cascade; thus, HDM2 can still bind p53. However, its ability to ubiquitinate p53 is severely restricted (35). In light of such, it is tempting to speculate that SS18-SSX can interfere with the maintenance of high p53 levels in response to cellular stresses that do not induce p53 NH_2-terminal phosphorylation and inhibit HDM2 binding. Remarkably, the presence of SS18-SSX abrogated the transcription of *HDM2, PUMA*, and *NOXA*, but not *p21*, when cells were treated with either doxorubicin or actinomycin D. We find that this result is highly significant for several reasons. First, it implies that stabilization of p53 is insufficient to activate p53-responsive genes in the presence of SS18-SSX. For example, doxorubicin induced p53 stability to similar levels in both control and SS18-SSX1–expressing cell lines, yet the induction of HDM2, PUMA, and NOXA mRNA was significantly reduced in SS18-SSX1–expressing cells (compare p53 levels in Fig. 3A and B with real-time PCR results in Fig. 3D). An intriguing observation was the fact that the induction of p21 transcription was not radically altered in

FIGURE 7. SS18-SSX1–promoted cell survival depends on HDM2. **A.** Decreased sub-DNA content of U2OS cells expressing SS18-SSX1 following exposure to actinomycin D. Clones of U2OS expressing SS18-SSX or vector alone were treated as indicated and harvested for propidium iodide staining 24 h later. **B.** Reduced poly-caspase activity in SS18-SSX–expressing cells. Caspase activity was determined 4 h after treatment with actinomycin D. **C.** Expression of SS18-SSX attenuates apoptosis. U2OS control or SS18-SSX1–expressing cells were treated with doxorubicin (2 μmol/L), MMC (10 μg/mL), actinomycin D (500 ng/mL), etoposide (10 μmol/L), or UV (50 J/M^2) for 36 h and harvested for PI staining and FACS analysis. **D.** U2OS expressing SS18-SSX or vector alone were transfected with siRNA molecules targeting HDM2. 72 h post transfection cells were treated with 500 ng/mL actinomycin D for 24 h and analysed as above. **E.** U2OS control or SS18-SSX1–expressing cells were treated with LY294002 for 4 h followed by treatment with MMC (10 μg/mL) or actinomycin D (500 ng/mL) for 24 h and analyzed by fluorescence-activated cell sorting and propidium iodide as above. Columns, mean of triplicates; bars, SD.
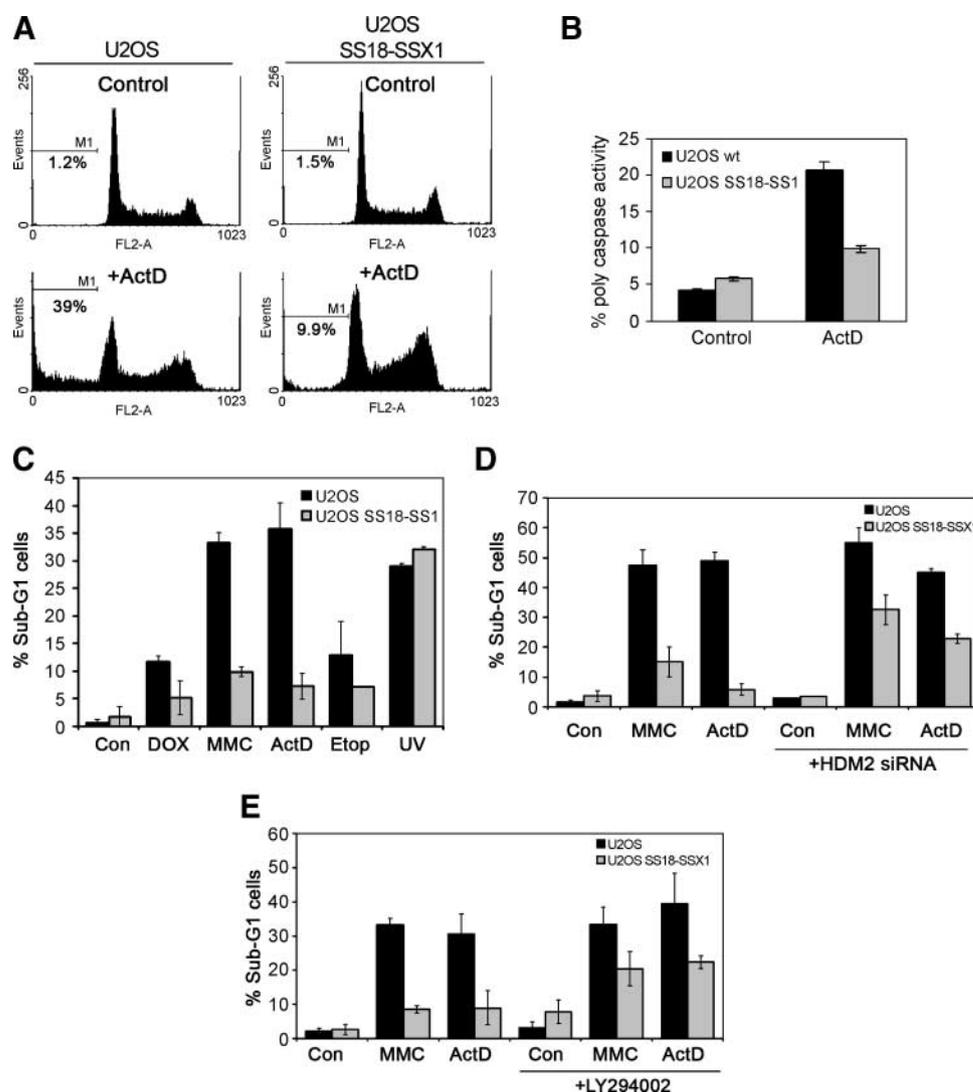
comparison with the other p53-responsive genes in the presence of the SS18-SSX1 (compare the induction of p21 with that of HDM2 in Fig. 3D). Taken into consideration, our results suggest that SS18-SSX1 expression can affect p53 stability under basal conditions by promoting HDM2-mediated ubiquitination and proteasomal-mediated degradation but can also modulate the spectrum of genes induced by p53 in response to cellular stress favoring those that promote growth arrest over those that promote apoptosis. For example, several independent groups have reported that ubiquitination of p53 during a stress response can have functions independent of the canonical proteasomal-mediated degradation pathway. Several studies have shown that ubiquitinated species of p53 are still proficient for transactivation and are found at the promoter of genes involved in growth arrest and DNA repair (*Gadd45* and *p21*) but not those involved in apoptosis (41-43). In light of our results showing that SS18-SSX1 can promote the ubiquitination of p53, it would be tempting to speculate that this ubiquitination may also alter the p53 response promoting the activation of a subset of p53 target genes. Additionally, Tsuda et al. (44) showed that SS18-SSX1 could activate the p21 promoter under basal conditions and in a p53-independent manner. Interestingly, we have previously shown that SS18-SSX1 can induce the transcription of the DNA repair protein XRCC4 (45). One could envision that SS18-SSX–mediated activation of XRCC4 expression accompanied with p21 expression may allow synovial sarcoma cells to survive gross genotoxic insults and evade apoptotic surveillance.

So what are the mechanisms in which SS18-SSX promotes stability of HDM2? We cannot rule out direct interactions of SS18-SSX with either p53 or HDM2. However, attempts to detect SS18-SSX-HDM2 interactions via coimmunoprecipitation or to produce recombinant protein have been unsuccessful in our hands, which may be due to the unstable nature of the SS18-SSX protein. In the absence of direct interactions, SS18-SSX may induce posttranslational modifications of HDM2, which promote its stability or deflect its autoubiquitination activity from itself to other proteins. Phosphorylation of serine residues in the central domain of HDM2 serves to promote HDM2 nuclear localization and increase HDM2 stabilization. For example, the growth factor–activated Akt kinase is a key modulator of HDM2 function (46). Phosphorylation of HDM2 by Akt promotes its nuclear localization, resulting in enhanced stability and increased p53 ubiquitination and degradation (46, 47). Indeed, in light of our results, the stabilizing effect of SS18-SSX1 on HDM2 could be counteracted by treatment with inhibitors of Akt activity, suggesting that the Akt-HDM2 pathway may be a node disrupted by SS18-SSX expression; however, a deeper analysis of this phosphorylation pathway is needed to confirm this hypothesis. Interestingly, recent studies by several groups have shown that the expression of SS18-SSX1 can induce the expression of the growth factor ligand insulin-like growth factor-II in a mechanism involving epigenetic deregulation (22, 48, 49). Because insulin-like growth factor-II can activate the insulin-like growth factor-I receptor, which is a known activator of the Akt and extracellular signal-regulated kinase pathways, deregulated insulin-like growth factor-II expression induced by SS18-SSX may provide an autocrine mechanism of promoting HDM2 stability and enhanced p53 ubiquitination and cell survival in addition to the extracellular signal-regulated kinase–mediated activation of cell cycle regulators.

In light of the role of SS18-SSX in promoting HDM2 stability, it raises the possibility of using small molecular weight compounds that target HDM2 as potential therapeutic treatments for synovial sarcoma. Several classes of HDM2 inhibitors have been developed, which abrogate the ubiquitin ligase activity of HDM2 or inhibit HDM2-p53 interactions (50-52). One could envision that such compounds would drastically inhibit cell growth and survival by promoting p53-induced growth arrest and apoptosis in synovial sarcoma cells, which retain wild-type p53, and as such may provide a therapeutic option for the treatment of synovial sarcoma.

Materials and Methods

Vector Constructs and siRNA

All plasmids were generated by standard cloning procedures and verified by DNA sequencing. For mammalian expression studies, inserts encoding SS18-SSX1 were PCR amplified from tumor cDNA and cloned into pTarget (Promega) as previously described (53). Vectors encoding wild-type p53 and HDM2 were generated as described (54, 55). For siRNA knockdown of endogenous HDM2, siRNA molecules were generated using Silencer siRNA construction kit (Ambion) and oligonucleotides (sense, 5′-AATGATCTTCTAGGAGATTTGCCTGTCTC-3′ and antisense, 5′-AACAAATCTCCTAGAAGATCACCTGT-CTC-3′). For siRNA expression vectors, suitable target oligonucleotides specific for the 3′ untranslated region of SS18-SSX were selected using established criteria for siRNA design. The SS18-SSX targeting sequence was designed to contain a complementary and reverse compliment 19-nucleotide sequence separated by a 9-nucleotide noncomplementary spacer.

Sense and antisense oligo inserts (5′-GGCTACGA-TAGGCCTTATGttcaagagaCATAAGGCCTATCGTAGCC-3′) were annealed and subsequently cloned into the *Bgl*II and *Hin*dIII sites of the pSuperior. A control vector containing a scrambled sequence was generated as a control. The 6× His-ubiquitin constructs were a kind gift from Dr. Sonia Lain (University of Dundee, Dundee, Scotland).

Cell Culture and Reagents

Osteosarcoma cells U2OS (p53$^+$) and Saos-2 (p53$^-$) and the fibrosarcoma cell line HT1080 (p53$^+$) were maintained in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin (Sigma-Aldrich). The synovial sarcoma cell line CME1 expressing the SS18-SSX2 fusion gene was cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% reduced Tet fetal bovine serum (Clontech), glutamine, penicillin, and streptomycin. Cell transfection was done using GeneJuice (Novagen) or calcium precipitation using standard protocols. Stable clones of U2OS and HT1080 were selected based on the expression levels of SS18-SSX in comparison with synovial sarcoma cell lines. For the generation of siRNA Tet-On lines, cells were transfected with pcDNA6 TR and selected with 8 μg/mL blasticidin (Sigma-Aldrich). Following selection of stable clones, cells were subsequently transfected with pSuperior RNA interference vector and selected with 1 μg/mL puromycin. Doxorubicin, actinomycin D, MMC, and etoposide were purchased from Sigma-Aldrich.

Real-time PCR for Detection of SS18-SSX

Total RNA was isolated from adherent cells using RNeasy kit (Qiagen). Total RNA (200 ng) was reverse transcribed to cDNA using random primers (Promega) in a 20 μL reaction containing 500 μmol/L of each deoxynucleotide triphosphate (Invitrogen) and using SuperScript II reverse transcriptase (Invitrogen). For amplification, the following primers were used: SS18, 5′-CCTCCAGAAGGCATGAACC-3′ (forward); SSX, 5′-CTCGTCATCTTCCTCAGGGTC-3′ (reverse); and p53, 5′-GCTTTCCACGACGACGGTGAC-3′ (forward) and 5′-GCTCGACGCTAGGATCTGAC-3′ (reverse). Real-time PCR was done using SYBR Green and ABI 7500 Light Cycler (Applied Biosystems) according to the manufacturer's guidelines. For Taqman gene expression analysis, 1 μL of the appropriate probe and 10 μL of 2× Taqman Universal PCR master mix were used in a total volume of 20 μL.

Western Blotting and Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Roche) and 20 mmol/L NEM (Calbiochem) followed by centrifugation at 12,000 rpm for 10 min. Protein concentration was determined by Bio-Rad protein assay. For immunoprecipitation reactions, samples were incubated with 1 μg primary antibody for 4 h at 4°C with Dynabead-conjugated protein G (Dynal). Samples were fractionated on 4% to 12% SDS-polyacrylamide gels (Novex) and transferred to polyvinylidene difluoride membranes (Amersham). The following antibodies were used: α-β-actin (AC-15; Sigma-Aldrich), α-GFP (Clontech); α-p53 (DO1; Santa Cruz Biotechnology), α-p53 (E19; Santa Cruz Biotechnology), α-MDM2 (N20; Santa Cruz Biotechnology), and α-MDM2 (D12; Santa Cruz Biotechnology). Detection was done with enhanced chemiluminescence (Amersham) or West Femto maximum sensitivity substrate (Pierce).

Colony Formation Assays

U2OS cells were transfected with SS18-SSX or empty vector as described. Twenty-four hours after transfection, cells were split and 10,000 cells were seeded in a 60-mm dish and selected in medium containing 500 μg/mL G418. CME1 Tet-On siRNA cells were plated at a density of 5,000 in a 60-mm dish and cultured in the presence or absence of doxycycline (1 μg/mL) for 2 weeks. Colonies were stained with Giemsa and scored for colony formation.

Ubiquitination Assays

For *in vivo* ubiquitination assays, cells were cotransfected with plasmids expressing His-tagged ubiquitin, SS18-SSX1, p53, and GFP using the calcium precipitation method. His-tagged ubiquitin-protein conjugates were purified using Ni^{2+} NTA agarose using the previously described protocol by Xirodimas et al. (27). In brief, cells were collected 48 h after transfection and 20% of the total suspension was retained for RNA extraction and total protein analysis. The remaining cell fraction was lysed under denaturing conditions to inhibit deubiquitination and prevent the formation of nonspecific protein-protein aggregates. Total His-ubiquitin protein conjugates were purified by affinity chromatography using Ni^{2+} NTA agarose beads and the levels of ubiquitination were determined using anti-p53 antibody (DO1) or anti-HDM2 antibody (N20).

Fluorescence-Activated Cell Sorting Analysis

For analysis of sub-G_1 content, cells were collected by centrifugation and fixed in 70% ethanol for 24 h. DNA was stained with propidium iodide solution (50 μg/mL) containing 100 μg/mL RNase A. Samples were analyzed by fluorescence-activated cell sorting scan (FACSCalibur, Becton Dickinson). For caspase activation, cells were treated with actinomycin D for 4 h, harvested, and labeled with CaspaTag Pan Fluorescein Caspase (InterGen).

Kinetics of p53 and HDM2 Stability

[^{35}S]methionine labeling was carried out by culturing the cells in methionine-free medium containing 10% dialyzed FCS for 30 min. Cells were pulsed with 0.1 mCi of [^{35}S]Promix (Amersham) for 40 min followed by incubation in fresh medium containing 10% FCS and 10 mmol/L nonlabeled methionine for the indicated time points. p53 and HDM2 proteins were immunoprecipitated and separated by SDS-PAGE. The resulting signals were quantified using Fluor-S multi-imager (Bio-Rad) and represented as % change over the amount of protein at time zero.

Immunofluorescence

Cells were grown to 50% confluence and transfected as described. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.4% Triton X-100 for 20 min, and blocked in 5% bovine serum albumin. Cells were incubated with primary antibody overnight and counterstained with Texas read–conjugated anti-mouse (Jackson).

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The Oncoprotein SS18-SSX1 Promotes p53 Ubiquitination and Degradation by Enhancing HDM2 Stability

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