HDAC2 Regulates Site-Specific Acetylation of MDM2 and Its Ubiquitination Signaling in Tumor Suppression

MULE undergoes MDM2/HDAC2-mediated degradation in synovial sarcoma
HDAC inhibition promotes MULE stabilization by acetylation and dissociation of MDM2
SS18-SSX fusion oncoprotein is a target of MULE E3 ligase
HDAC inhibitors downregulate SS18-SSX stability through the ubiquitin system
HDAC2 Regulates Site-Specific Acetylation of MDM2 and Its Ubiquitination Signaling in Tumor Suppression

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SUMMARY
Histone deacetylases (HDACs) are promising targets for cancer therapy, although their individual actions remain incompletely understood. Here, we identify a role for HDAC2 in the regulation of MDM2 acetylation at previously uncharacterized lysines. Upon inactivation of HDAC2, this acetylation creates a structural signal in the lysine-rich domain of MDM2 to prevent the recognition and degradation of its downstream substrate, MCL-1 ubiquitin ligase E3 (MULE). This mechanism further reveals a therapeutic connection between the MULE ubiquitin ligase function and tumor suppression. Specifically, we show that HDAC inhibitor treatment promotes the accumulation of MULE, which diminishes the t(X;18) translocation-associated synovial sarcomagenesis by directly targeting the fusion product SS18-SSX for degradation. These results uncover a new HDAC2-dependent pathway that integrates reversible acetylation signaling to the anticancer ubiquitin response.

INTRODUCTION
Synovial sarcoma is an incurable malignant soft tissue tumor, which primarily affects children and young adults. Like many other sarcomas, this disease is histologically composed of mesenchymal cells. However, synovial sarcoma displays variable degrees of epithelial differentiation and contains a unique chromosomal translocation t(X;18), which most commonly fuses the SS18 gene with SSX1 or SSX2 (Nielsen et al., 2015). Depletion of SS18-SSX by small interfering RNAs (siRNAs) causes apoptotic cell death of human synovial sarcoma cells (Peng et al., 2008; Cai et al., 2011; Carmody Soni et al., 2014). Conversely, overexpression of SS18-SSX in noncancerous rat fibroblast cells shows transforming activity in a xenograft model (Nagai et al., 2001). Notably, mice conditionally expressing the SS18-SSX fusion gene in certain cell lineages develop tumors that are pathologically indistinguishable from and molecularly consistent with synovial sarcoma in humans (Haldar et al., 2007), thus confirming the critical role for SS18-SSX in the pathogenesis of synovial sarcoma.

Fundamental progress has been made in understanding how the SS18-SSX fusion protein promotes tumorigenesis, which indeed involves multiple parallel mechanisms, such as epigenetic remodeling (Su et al., 2012; Kadoch and Crabtree, 2013; Banito et al., 2018; McBride et al., 2018), cellular adhesion (Eid et al., 2000), mesenchymal-to-epithelial transition (Saito et al., 2006; Barrott et al., 2015), protein translocation (Pretto et al., 2006), and microRNA regulation (Hisaoa et al., 2011; Minami et al., 2014). Such complexities in SS18-SSX action make the development of targeted therapies for synovial sarcoma extremely challenging. Despite the lack of effective treatment options, several lines of evidence have shown that human synovial sarcoma cells are highly sensitive to histone deacetylase (HDAC) inhibitors in cell cultures and in a cell-line-based xenograft model (Ito et al., 2005; Laporte et al., 2017a). One well-supported explanation for the action of HDAC inhibitors is histone acetylation through which key tumor suppressor genes become epigenetically reactivated (Lubieniecka et al., 2008; Su et al., 2010, 2012; Laporte et al., 2017b). In the present study, we propose an additional, transcription-independent mechanism whereby HDAC inhibition facilitates proteasomal degradation of the SS18-SSX fusion protein. This action relies mostly on a novel combination of HDAC2 and MDM2 activities in concert with the MULE E3 ligase function. Our findings connect HDAC2 activity to oncogenic protein stabilization via a series of post-translational events, which constitute an acetylation-dependent ubiquitin pathway that may serve as a common therapeutic target in human cancers.
RESULTS

HDAC Inhibitor Treatment Reduces SS18-SSX Levels through the Ubiquitin System

To assess the in vivo efficacy of HDAC inhibition in synovial sarcoma, we generated transgenic mice expressing human SS18-SSX2 fusion oncogene within the myogenic factor 5 (Myf5) lineage (Haldar et al., 2007). Treatment with the HDAC inhibitor FK228 on a weekly basis significantly reduced growth of mouse synovial sarcomas (Figures S1A and S1B), associated with remarkable cytoreductive activity (Figures S1C–S1H). In addition to the histological observations, we noticed that SS18-SSX2 protein abundance was substantially decreased in FK228-treated tumors (Figures 1A and 1B). Considering the fusion oncogene dependency in synovial sarcoma, we decided to examine the molecular mechanism of SS18-SSX downregulation upon HDAC inhibition. To this end, we first developed a CRISPR/Cas9-based genome editing approach for FLAG epitope tagging of endogenous SS18-SSX2 fusion oncprotein in patient-derived SYO-1 cells (Figures S1I–S1K). Anti-FLAG western blots revealed that SS18-SSX levels remained constant through the early time points of FK228 treatment, but fell drastically after overnight stimulation (Figure S1L). Similar results were obtained in cells treated with other structurally different HDAC inhibitors, such as SB939 and PCI-24781 (Figure 1C). We also tested this in SS18-SSX1-associated synovial sarcoma cells (Yamato-SS) and found that treatment with the HDAC inhibitors FK228 and SB939 led to a marked reduction of SS18-SSX protein levels, coupled with impaired tumor cell growth (Figures 1D and 1E). Importantly, the mRNA levels of SS18-SSX remained unchanged (Figures 1F and 1G). This effect was efficiently blocked by the proteasome inhibitor MG-132 (Figure 1C), and restoration of SS18-SSX levels correlated positively with increased conjugation of poly-ubiquitin chains (Figure 1H). Our findings indicate the existence of as-yet-undefined ubiquitin ligases that target SS18-SSX for proteasomal degradation.

MULE Ubiquitin Ligase Binds to SS18-SSX and Promotes Its Degradation

Next, we performed anti-FLAG immunoprecipitation to purify SS18-SSX-interacting proteins. Mass spectrometric analysis of the peptides uniquely enriched after HDAC inhibitor stimulation led to identification of MCL-1 ubiquitin ligase E3 (MULE), a HECT-type enzyme that plays a central role in the regulation of cell proliferation and tumorigenesis (Shmueli and Oren, 2005; Kao et al., 2018) (Figures 2A and 2B). Its interaction with the fusion oncprotein was readily detected in both proximity ligation and immunoprecipitation assays (Figures 2C–2E). This binding event seemed to involve two fundamental domains in MULE, WWE and UBM, which co-occupied the repression domain (SSXRD) at the C-terminal end of SS18-SSX (Figures 3A–3D). Interestingly, there exists an alternatively spliced product of the SSX2 gene (dos Santos et al., 2000), containing a different C-terminal region (Figure S2A). We found that this variant failed to interact with MULE in SYO-1 cells, regardless of HDAC inhibitor stimulation (Figures S2B–S2D). Given the fact that MULE neither binds wild-type SS18 nor does its deletion affect SS18 protein expression (Figures S2E and S2F), we reasoned that the C-terminal SSXRD may play an indispensable role for SS18-SSX recognition by MULE E3 ligase.

We continued to assess whether SS18-SSX is a substrate of MULE, and found that in vitro synthesized fusion oncprotein could be poly-ubiquitinated by cell extracts prepared from wild-type but not Mule-knockout mouse embryonic fibroblasts (Figures 4A–4C). Consistently, depletion of MULE by short hairpin RNA in SYO-1 cells prevented endogenous SS18-SSX ubiquitination upon HDAC inhibitor stimulation (Figure 4D). Removal of MULE-binding SSXRD domain strikingly reduced SS18-SSX ubiquitination levels (Figure 3E), suggesting that MULE ubiquititates the fusion oncprotein largely through their physical interaction. It should be noted that the SYT N-terminal Homolog (SNH) motif of SS18-SSX also contributed to MULE-mediated ubiquitination (Figure 3E). Given that the SNH-deleted (ΔSNH) mutant retained binding to MULE (Figure 3D), we reasoned that the critical SS18-SSX ubiquitination sites may be located within the SNH region. Indeed, previous studies have identified a lysine residue (K13) within wild-type SS18 protein that is ubiquitinated in human cell lines (Mertins et al., 2013; Udeshi et al., 2013). However, among four lysines, only mutating K23 to arginine (K23R) effectively blocked SS18-SSX ubiquitination (Figure 3E). Thus the mechanism underlying SS18-SSX ubiquitination likely differs from that for wild-type SS18. Together, these results provide molecular insights into MULE E3 ligase activity toward the fusion oncprotein (Figure 3F).

To elucidate the biological significance of these findings, we examined the effect of the presence or absence of MULE on SS18-SSX protein expression upon HDAC inhibitor treatment. In SYO-1 cells,
SS18-SSX levels fell after addition of FK228 and SB939. However, under the same conditions, the fusion oncoprotein avoided downregulation by MULE depletion (Figure 4E). More importantly, removal of MULE reduced the sensitivity of SYO-1 cells to HDAC inhibitors (Figures 4F and 4G). This resistance was neither due to the reported role of MULE in degrading histones (Liu et al., 2005) nor due to epigenetic changes in global histone acetylation upon HDAC inhibitor treatment (Figures S3A–S3C). This might result at least partly from impaired turnover of SS18-SSX, which retained the ability to promote cell proliferation and transformation via HDAC-independent mechanisms. In addition, among several known substrates of...
MULE, we found that HDAC inhibitor treatment downregulated CTCF and β-catenin protein expression, and this reduction was closely dependent on MULE (Figures S3D and S3E). CTCF is a multifunctional transcription factor that organizes chromatin architecture and controls genomic stability in various biological processes involved in tumorigenesis (Qi et al., 2012; Ghirlando and Felsenfeld, 2016; Song and Kim, 2017).

β-Catenin acts as a central regulator of the WNT signaling pathway, which has been extensively studied in many human cancer types and directly linked to the progression of synovial sarcoma (Barham et al., 2013; Trautmann et al., 2014; Barrott et al., 2015, 2018; Cironi et al., 2016; Sanchez-Vega et al., 2018). Collectively, our findings point to MULE-mediated ubiquitination signaling as a potential fundamental target for HDAC inhibitors in tumor suppression.

HDAC Inhibition Stabilizes MULE by Acetylation and Dissociation of MDM2

We noted that MULE protein levels rose upon HDAC inhibitor treatment, without mRNA level changes (Figures S4A and S4B). Similar effects were also observed after proteasome inhibition (Figures 5A and S4C), indicating the unstable status of MULE protein in synovial sarcoma cells. Consistent with this notion, recent studies have reported MULE degradation by the oncogenic E3 ligase MDM2 in human cancers (Kurokawa et al., 2013; Canfield et al., 2016). We confirmed the endogenous interaction between MDM2 and MULE in both SYO-1 and Yamato-SS cells (Figures 5B and S4D). Knockdown of MDM2 by siRNAs led to increased levels of MULE, which inversely correlated with SS18-SSX downregulation and impaired cell viability (Figures 5C, 5D, S4E, and S4F). This effect seems independent of the p53 tumor suppressor, a key target of MDM2, because SYO-1 cells express wild-type p53, but Yamato-SS cells harbor a mutant p53 (R273C) (Vlenterie et al., 2016). Consistent with this view, depletion of endogenous p53 failed to rescue SYO-1 cells.
from MDM2 knockdown (Figures S4G–S4I). These results indicate a predominant role for MDM2 control of MULE (but not p53) signaling in synovial sarcoma cells. Notably, this action could be reversed by the addition of FK228 and SB939, which suppressed MDM2 binding to MULE (Figure 5E). As a result, HDAC inhibition led to a decrease in MULE ubiquitination and an increase in its protein stability (Figures 5F, 5G, S4J, and S4K). In addition, we found that the response of SYO-1 cells to FK228 and SB939 treatment, similar to MDM2 knockdown, remained unaffected after p53 deletion (Figures S4L–S4N), further supporting the notion that HDAC-inhibitor-induced anticancer action relies largely on MDM2 regulation of MULE, rather than p53, in synovial sarcoma.

Given that HDAC inhibition affects biological functions mostly through protein acetylation (Verdin and Ott, 2015), we investigated whether the same mechanism controls the MDM2-MULE interaction. Recently, it has been discovered that acetylation of the lysine-rich domain (KRD) can remove its positive charge and interrupt its association with the negatively charged acidic domain (Wang et al., 2016, 2017). Indeed, there does exist a lysine-rich stretch (amino acids 460–476) in the MDM2 protein, whereas MULE has an acidic domain between the residues 2425 and 2469. In support of these clues, we found structural evidence of MULE's acidic domain bound to the MDM2 KRD (Figures 6A, S5A, and S5B). This interaction likely involved five evolutionarily conserved lysine residues within KRD (Figures S5C–S5F), which were acetylated in endogenous and ectopically expressed MDM2 upon HDAC inhibition.
inhibitor treatment (Figures 6B, 6C, S5G, and S5H). According to these properties, we generated a mutant MDM2 (5KR) in which all KRD lysine sites were replaced by arginine (Figure 6D). This acetylation-deficient mutation retained the ability of MDM2 binding to MULE, but exhibited resistance to HDAC-inhibitor-stimulated dissociation (Figure 6E). Point mutation analysis identified two acetylated...
lysines, K469 and K470, responsible for the release of MDM2 from MULE (Figures 6F, 6G, S5I, and S5J).

In line with this observation, K469 and K470 appeared to be the most effective sites of MDM2 acetylation after FK228 addition (Figure S5H). To further test this idea, we generated another MDM2 mutation in which the K469 and K470 residues were substituted to glutamine. This acetylation-mimicked mutation resulted in a dramatic loss of the MDM2-MULE interaction even without HDAC inhibitor treatment (Figures 6H and 6I). Intriguingly, Moshe Oren and colleagues reported that acetylation of the neighboring lysines K466 and K467 impairs MDM2’s E3 ligase activity (Wang et al., 2004).

However, a similar defect was not found when mimicking the acetylation of K469 and K470 (Figure S5K). Therefore different acetylation modifications in the KRD region might have distinct and cooperative roles in limiting MDM2 function.

**HDAC2 Regulates the MDM2-MULE Interaction and Maintains SS18-SSX Protein Stability**

Finally, it is important to determine which of the 11 HDAC family members act upstream of MDM2 (Figure S6A). We focused on two highly homologous members, HDAC1 and HDAC2, because they are...
Figure 6. MDM2 Acetylation at Specific Lysine Sites Negatively Regulates its Interaction with MULE

(A) Structural interface showing the interaction between MDM2 (lysine-rich domain; KRD) and MULE (acidic domain).

(B) Acetyl-lysine western blots showing increased signals in endogenous MDM2 immunoprecipitates prepared from HDAC inhibitor (FK228/SB939)-treated SYO-1 and Yamato-SS cells, compared to DMSO (–). Rabbit IgG was used as a negative control.

(C) MS/MS spectra of the MDM2 lysine-rich domain (KRD) peptides identified in anti-MDM2 immunoprecipitates prepared from SYO-1 cells under the treatment of FK228, but not DMSO.

(D) Schematic of HA-tagged MDM2 (WT), acetylation-deficient (KR) and -mimicked (KQ) mutations.

(E) Immunoprecipitation analysis of MULE association with ectopically expressed MDM2 (WT) and its 5KR mutant in HEK293 cells treated with DMSO (–) or FK228, in the presence of MG-132. Input serves as a loading control.
most abundantly expressed in synovial sarcoma cells (Figure 7A) (Pacheco and Nielsen, 2012), and because the compound FK228 mainly inhibits HDAC1/2 activity. Interestingly, MDM2 associated with both HDAC1 and HDAC2 (Figure S6B), and depletion of neither HDAC1 nor HDAC2, affected MDM2 protein abundance (Figures S6C and S6D). However, MDM2-MULE interaction was diminished in HDAC2-deficient cells (Figures 7B and S6E). Moreover, the amount of SS18-SSX protein (but not mRNA) was drastically reduced upon HDAC2 knockdown (Figures 7C, S6F, and S6H), similar to the results obtained in MDM2-knockdown cells. We did not observe any significant effects after depletion of either HDAC1 or its Class I homolog HDAC3 (Figures S6G–S6I). Therefore unlike HDAC1, which contributes to SS18-SSX-mediated gene regulation (Su et al., 2012; Cironi et al., 2016), HDAC2 performs a nonredundant role in safeguarding the fusion oncoprotein from ubiquitin-mediated degradation.

DISCUSSION
The experiments presented here demonstrate a new mechanism for MDM2 inactivation, which is achieved through site-specific acetylation (Figure 7D). Repression of this acetylation is crucial to MDM2 interaction with its substrate MULE. In synovial sarcoma, the HDAC2 enzyme governs MDM2 substrate-binding activity; suppression of HDAC2 by RNA interference or small-molecule inhibitors allows dissociation and accumulation of the ubiquitin ligase MULE, leading to subsequent degradation of the SS18-SSX fusion oncoprotein, β-catenin, and CTCF. We provide mechanistic and functional evidence pointing toward the therapeutic implication for using HDAC inhibitors in synovial sarcoma treatment. Notably, this may not be limited to synovial sarcoma. For example, MDM2-mediated MULE downregulation has been reported to confer breast cancer resistance to the human epidermal growth factor receptor 2 inhibitor lapatinib (Kurokawa et al., 2013). It is tempting to speculate that use of HDAC inhibitors in combination therapy may resensitize some currently incurable cancers to conventional treatment.

Parallel with our current work, we recognize that MDM2 can also interact with MULE in an HDAC2-independent manner. This binding event requires the hydrophobic p53-binding pocket of MDM2, which in turn provides a targeting site for the small-molecule antagonist Nutlin-3a to prevent MULE accommodation and degradation (Kurokawa et al., 2013). In synovial sarcoma cells, however, Nutlin-3a treatment does not disrupt the MDM2-MULE complex, nor does it influence MULE protein expression (Figures S6J and S6K). These different results indicate that instead of a two-site binding model, there exist at least two levels of MDM2 control of MULE—one acting in the forward direction involving the intermolecular contact, whereas the second acting in the reverse direction involving lysine acetylation to unlock the binding interface. It will be important to further examine if any of the other post-translational modifications have a similar role as MDM2 in the regulation of MULE stability and activity. These efforts may also lead the way to a better understanding of how MDM2 integrates diverse input signals to execute its ubiquitination function with a high degree of specificity.

Limitations of the Study
We do not know if HDAC2 regulates MDM2 acetylation in any cancer forms other than synovial sarcoma. With our experiments, it is also not known if HDAC2 has a similar role in noncancerous context. Under different physiological conditions, HDAC2 and other related HDAC members may distinctly target the same lysine residues for control of MDM2 acetylation and function. Another limitation of our current study is the inability to answer whether HDAC inhibitors also control the substrate targeting of MULE E3 ligase through lysine acetylation. At this moment, the importance of post-translational modifications in the differential regulation of MULE downstream targets remains an open issue for further investigation.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and six figures and can be found with this article online at https://doi.org/10.1016/j.isci.2019.02.008.

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AUTHOR CONTRIBUTIONS

N.P., J.W., Y.Z., and G.G.D. designed and performed the experiments. K.S. and T.K. conducted mass spectrometry. K.B.J., Z.H., M.R.C., and T.W.M. carried out the animal studies. J.W.P. performed the structural analysis. N.T.N. assisted in characterizing MDM2-MULE interaction. D.W., A.V.S., R.V.W., M.E., K.M.M., T.O.N., and T.M.U. performed the experiments in the initial stage of this study. L.B., S.K.M., and R.M.M. guided CRISPR/Cas9 genome editing. L.S. supervised the study and wrote the manuscript with input from the other authors.

Figure 7. HDAC2 Contributes to MDM2-MULE Interaction and Governs SS18-SSX Protein Stability

(A) RNA sequencing analysis for the expression of HDAC family members in human synovial sarcoma cell lines, SYO-1 and Yamato-SS. FPKM, fragments per kilobase million.
(B) Immunoprecipitation analysis of the MDM2-MULE interaction in MG-132-treated SYO-1 cells depleted of HDAC1 or HDAC2. Cell lysate (input) and rabbit IgG serve as positive and negative controls, respectively.
(C) Western blot analysis of the lysate from nonspecific and HDAC2 knockdown CRISPR/Cas9-modified SYO-1 cells. Actin serves as a loading control.
(D) Proposed model for MDM2 control of MULE stability and activity through HDAC-inhibitor-induced lysine acetylation.
The authors declare no competing interests.

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Supplemental Information

HDAC2 Regulates Site-Specific Acetylation of MDM2 and Its Ubiquitination Signaling in Tumor Suppression

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**Transparent Methods**

*Cell culture and chemicals*

Human synovial sarcoma cell lines SYO-1 (Akira Kawai, National Cancer Center, Tokyo, Japan) and Yamato-SS (Kazuyuki Itoh, Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan) were cultured in RPMI-1640 and DMEM (Invitrogen) respectively, supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Human embryonic kidney 293 (HEK293) cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM with 10% FBS. All cell lines were grown at 37°C, 95% humidity and 5% CO2.

All chemicals used in this study are commercially available: FK228 (Selleck Chemicals), SB939 (Abcam), PCI-24781 (Santa Cruz Biotechnology), Geneticin/G418 (Invitrogen), MG-132 (Santa Cruz Biotechnology), Cycloheximide (BioVision) and DMSO (Sigma-Aldrich).

To achieve stable knockdown of human MULE and p53 genes, SYO-1 cells were infected with control or MULE/p53-specific shRNA lentiviruses in regular growth medium, supplemented with 5 µg/ml Polybrene (Santa Cruz Biotechnology). Selection was carried out using Puromycin (Santa Cruz Biotechnology) (at the final concentration of 10µg/ml) to remove non-transduced cells. Resistant cells were then expanded and maintained in Puromycin-containing growth medium. The efficiency of knockdown was confirmed at the mRNA or protein levels. Control, MULE and p53 shRNA lentiviral particles were purchased from Santa Cruz Biotechnology.

To construct the 3×Flag tagging donor plasmid, we designed the dsDNA genomic blocks (IDT) corresponding to homolog arms of individual SSX2 isoform and sub-cloned them into the pFETCh plasmid. Cas9/gRNA oligos (IDT) were designed nearby the 3’ stop codons of SSX2 and integrated into the PX330 plasmid. 2×10^6 cells were transfected with the two constructs (5µg each), using the Nucleofector Kit V (Lonza). After overnight incubation in regular medium, cells were
subjected to G418 selection at the concentration of 400 µg/ml for 7-10 days. Resistant cells were expanded in regular medium for downstream applications. The sequences of gRNAs and genomic blocks are listed below:

gRNAs and genomic blocks for SSX2A

| gRNAs      | Genomic blocks              |
|------------|----------------------------|
| **forward**: caccGACTGCGTGAGAAAGACGC | **HOM1**: tccccgacgctagcccagctAAGTGTTAAATGGATTGTTGTTGTAACACAAGGATAAATGCTTGAGGATGATCTTCTATATTTCATTGATGATTATCCCATGCTATGCTGTATATTACTGTATCTACGCCATCCCTATTAAAAACTTAAAGAAATGCTGAGCTCTGACCAAGGAGGCTAAAACTTTAATTGGCATTTGGCCTATGTTGGTGTGGATCTAAGGTCTCAGCCTCTCTAAAGAGAATGTGAAAAACTGGATGAAGAAGGCCCATGGGCACTTGAGGGAAGCAGGCATCTCCTTTTTTTGAGTAAACAGAGCCTAACACTCTCCAACCTACCCCAACCTCACCTTTCCACTTACCTTACAGGCACTGTGGCATGAACAGATGGTTCCCTGAGGAACCGTGGAAGGCCTAGGTCCTGAGATAGGGCAGTTGCCACGTGGTGGGAACTCACTGCCACTTATTTCCTTCTCTCTTCCTGCCTCAGCCTCGGGATACGACACCATGCATGATGAGAAGCAGAACGTGGTGACCTTTCACGAACATGGGCATGGCTGCGGACCCCTCGTCATCAGGTGCATAGCAAGTGAAAGCAAGTGTTCACAACAGTGAAAAGTTGAGCGTCATTTTTCTTAGTGTGCCAAGAGTTCGATGTTAGCGTTTAGCTGTTGTATTTTCTTACACTcttggaaagtcctctccactg |
| **reverse**: aaacGCTGTTTCTCCTCAGCGACGTC |

| Genomic blocks |
|----------------|
| **HOM2**: agtctcttgattcaactcTTATGAGAGATCAGCGACCTCTGAGGAAGATGACGAGTAACCTCCGTAAAGAGAAGGCTTTTTCAACTTCCACATCCCATCCCACATCCCTGCAGACGTGCTATTCTGTTATGATACTGTATCTTCTACCTGGTCCATATCAAAACATTTCTTTCTACTCTGACATGAGGGAAGTCACTGTGGCATGAACAGATGGTTCCCTGAGGAACCGTGGAAGGCGTGTGCAGGTCCTGAGATAGGGCAGTTGCCACGTGGTGGGAACTCACTGCCACTTATTTCCTTCTCTCTTCCTGCCTCAGCCTCGGGATACGACACCATGCATGATGAGAAGCAGAACGTGGTGACCTTTCACGAACATGGGCATGGCTGCGGACCCCTCGTCATCAGGTGCATAGCAAGTGAAAGCAAGTGTTCACAACAGTGAAAAGTTGAGCGTCATTTTTCTTAGTGTGCCAAGAGTTCGATGTTAGCGTTTAGCTGTTGTATTTTCTTACACTcttggaaagtcctctccactg |
### gRNAs and genomic blocks for SSX2B

| gRNAs                          |
|--------------------------------|
| forward: caccGGATGTGAGGATACCT   |
| reverse: aacaTCACCATCCACCATCACA|

| Genomic blocks                  |
|--------------------------------|
| HOM1                           |
| tcccccagctgcaagccctgcatGGAGGATG|
| TATACACCAGCTGGCTACCCGCTAAGAAC    |
| AACTAATGAAAAATAAAATTACATTGCT    |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
| GGCAGTTGCTGGGCACAGCTTCTCTCTCT  |
| CAGTGAGGTGCGTGTGCGGTTGAGGATTT   |
| TGTGGATGCCACAGCTGATGAGAAGTGGAT  |
| TCAGAGGCTGCGTGGTGTGCTTCTCTCTG  |
| TACGACAGATCCAGATACACAGCTAGCT    |
| TATATGCTGACGATACGAGAACGTGGTGAG  |
| TTTCCCATGCTCTTTCTACTCTCTGCGTCT  |
| GCCCTGATGTAAGGAGACTCTCTTGATGTT  |
| AGGGCTGCTGGTGTGTGTTGGTGTGGATT   |
| GAGGAAGTCACTGTGGCATGAACAGATGG   |
| TCACTGCACTGCAGATACCCCTGCTGATG  |
| GCGATGGGCGAATCCGCGAGGCTGCTGGA  |
were bred to Myf5-Cre mice, so as to express SS18-SSX2 in myoblasts and mesenchymal precursor cells (Haldar et al. 2007). The resulting Myf5-Cre/SSM2 progenies at age 14 weeks were subjected to weekly administration of FK228 or vehicle, and sacrificed at age 17 weeks for tumor collection. Tumor tissues were fixed in formalin and paraffin-embedded as standard pathology blocks. Blocks were serially sectioned at 6-µm thickness onto Fisher Plus microscope slides, prior to Hematoxylin and Eosin (H&E) staining. For immunofluorescence assay, slides were deparaffinized and rehydrated, prior to heat-induced antigen retrieval in Tris-EDTA buffer (pH=9). Non-specific staining was blocked by 10% goat serum in PBS for 60 min at room temperature, followed by incubation with human SSX2 antibody (BioSS Antibodies; 1:100 dilution in PBS with 10% goat serum) overnight at 4°C. After three PBS wash steps of 10 min, slides were incubated with the secondary goat anti-rabbit Alexa 594 antibody (Invitrogen) at 1:500 dilution for 30 min. After three PBS wash, slides were counterstained with DAPI (1:500 dilution) for 5 min, and mounted with Cytoseal-60 (Thermo Scientific). Microscopy analysis was taken with an Olympus BX-63 microscope and an Olympus XM-10 camera.

Mule-knockout mice, Mule^{fl/+} (female) and Mule^{fl/y} (male), were crossed to generate the E14.5 embryos for mouse embryonic fibroblast (MEF) isolation (Hao et al. 2012). Mule^{fl/y} MEFs were transformed with pLPC-E1A-IRES-RasV12, before transduction with His-TAT-NLS-Cre, to induce Cre-based deletion of the floxed Mule allele. Loss of Mule expression was confirmed at the protein level by western blotting analysis.

**Proximity ligation assay (PLA)**

Cells were incubated on 8-well chamber slides (Sigma-Aldrich) overnight to 80% confluent. After 16-hr treatment with HDAC inhibitors, in the presence of MG-132 (1µM), cells were washed with
PBS, and fixed in 4% paraformaldehyde (PFA) (Santa Cruz Biotechnology) for 10 min at room temperature. Permeabilization was conducted with 0.5% Triton X-100 (in PBS) for 10 mins at room temperature. We used the Duolink kit (Sigma-Aldrich) for PLA experiments. Briefly, cells were blocked in Duolink Blocking Solution for 30 min at 37 °C, and incubated with primary antibodies (mouse anti-Flag, 0.5 µg/ml; rabbit anti-MULE, 1.0 µg/ml) for 90 min at room temperature, followed by 60-min incubation of the PLUS and MINUS probes at 37 °C. The probes were hybridized in the Duolink Ligation buffer for 30 min at 37°C, and PLA signals were strengthened by the amplification reaction (100 min at 37°C). Negative controls were set up with no primary antibodies. Slides were mounted using Duolink in situ mounting medium with DAPI and subjected to microscopy analysis.

**Immunoprecipitation and western blots**

Cell extracts were prepared using the RIPA Lysis Buffer system (Santa Cruz Biotechnology), and protein concentrations were measured by the Bradford protein assay (Bio-Rad). For immunoprecipitation, cell lysates were prepared using the Pierce IP Lysis Buffer (Thermo Scientific) with protease inhibitor cocktails (Roche). Equal amounts of cell lysates were incubated with Bio-Rad Protein-A/G magnetic beads (75 µl) coupled with primary antibodies (3-4 µg). After overnight rotation at 4°C, the beads were subjected to two washes with the IP buffer and two washes with PBS/T (0.1% Tween-20). Immunoprecipitates were eluted in 2× Laemmli buffer (10 min at 70°C). For western blot analysis, protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to 0.45 µm (or 0.2 µm) nitrocellulose membranes (Bio-Rad). Blots were incubated with the indicated primary antibodies (see below) and visualized using LI-COR Odyssey Infrared System.
List of primary antibodies used in this study

| Antibody Type                  | Vendor               | Antibody Type                  | Vendor               |
|-------------------------------|----------------------|-------------------------------|----------------------|
| Histone H3                   | Abcam                | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# ab10799)                  |                      |
| MDM2 [SMP 14]                | Abcam                | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# ab3110)                   |                      |
| Myc-tag [9E10]               | Abcam                | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# ab32)                     |                      |
| Acetylated-Lysine            | Abcam                | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# 61637)                    |                      |
| Lasu1/Ureb1/MULE             | Bethyl Laboratories  | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# A300-486A)                |                      |
| human SSX2/CT5.2             | Bioss                | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# 11312R)                   |                      |
| Acetylated-Lysine            | GeneTex              | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# 9814)                     |                      |
| GFP-tag                      | GeneTex              | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# GTX113617)                |                      |
| MDM2                         | Genetex              | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# GTX100531)                |                      |
| SS18                         | Genetex              | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# GTX129428)                |                      |
| CTCF                         | Millipore/Sigma      | Rabbit polyclonal antibody    | Active Motif         |
|                               |                      | (# 07-729)                   |                      |
| FLAG-tag (M2)                | Millipore/Sigma      | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# F1804)                    |                      |
| α-Tubulin (DM1A)             | Millipore/Sigma      | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# T9026)                    |                      |
| β-Actin (C4)                 | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-47778)                 |                      |
| β-catenin (H-1)              | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-133240)                |                      |
| HDAC2 (3F3)                  | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-81599)                 |                      |
| HDAC3 (A-3)                  | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-376957)                |                      |
| Mcl-1 (22)                   | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-12756)                 |                      |
| Ubiquitin (P4D1)             | Santa Cruz Biotechnology | Mouse monoclonal antibody | Active Motif         |
|                               |                      | (# sc-8017)                  |                      |
| HA-tag (2-2.2.14)            | Thermo Scientific    | Mouse monoclonal antibody    | Active Motif         |
|                               |                      | (# 26183)                    |                      |
| HDAC1                        | Thermo Scientific    | Rabbit polyclonal antibody   | Active Motif         |
|                               |                      | (# PA1-860)                  |                      |
| DYKDDDDK/FLAG-tag            | Thermo Scientific    | Rabbit polyclonal antibody   | Active Motif         |
|                               |                      | (# 740001)                   |                      |
Protein structure modeling

Models for MULE (amino acids 2261-2970) were generated using I-TASSER hybrid modeler, and for MDM2 (amino acids 418-491) using YASARA homology modeling. Docking analysis was performed using the HADDOCK server, followed by energy minimization of each predicted interaction using both the AMBER03 and YASARA2 force fields. Evolutionary analysis and codon selection work was done using our published Sequence-to-Structure-to-Function methods (Prokop et al. 2017).

Ubiquitination analysis

For in vitro ubiquitination analysis, Myc-tagged SS18-SSX2 fusion protein was made by the TnT Quick Coupled Transcription/Translation system (Promega) and mixed with 35µg of either wild-type (WT) or Mule-null MEF cell extracts in the reaction buffer (50mM Tris-HCl, 10mM NaCl, 5mM MgCl$_2$, 1mM DTT, 20µM MG-132, 5mM ATP). After 60-min incubation at 30°C, samples were subjected to anti-Myc immunoprecipitation using antibody-coated magnetic beads (crosslinking with 20mM dimethyl pimelimidate/DMP, Sigma-Aldrich). Proteins in the eluent were separated on 8% SDS-PAGE, followed by western blotting against Myc and ubiquitin. For in vivo ubiquitination study, cells were treated with HDAC inhibitors for 12 hr, followed by adding 20µM MG-132 for 6 hr. The lysate was prepared using RIPA Lysis Buffer (Santa Cruz Biotechnology), sonicated by the Bioruptor (10 sec × 3 cycles, low power) and subjected to immunoprecipitation (primary antibody: 4µg/assay). Beads were washed twice with the RIPA buffer, followed by three PBS/T washes, and boiled in Bio-Rad sample buffer for SDS-PAGE and western blot analysis.
Mass spectrometry

The protein samples were separated by 12.5% SDS-PAGE and subjected to in-gel digestion using trypsin (Kondo and Hirohashi 2006). The tryptic digests were then subjected to liquid chromatography coupled with nanoelectrospray tandem mass spectrometry (Finnigan LTQ Orbitrap XL mass spectrometer; Thermo Fisher). The Mascot software package (version 2.5.1; Matrix Science) was used to search for the mass of each peptide ion peak against the SWISS-PROT database (Homo sapiens, 20205 sequences in the Swiss prot_2015_09.fasta file) using the following parameters – 3 or 4 missed cleavage; variable modifications: oxidation (Met) and acetylation (Lys); peptide tolerance: 10 p.p.m.; MS/MS tolerance: 0.8 Da; peptide charge: 2⁺, 3⁺ and 4⁺.

Cell viability and colony formation assays

Human synovial sarcoma cells were cultured at 60% confluence, and transfected with indicated siRNAs using Lipofectamine RNAiMAX (Invitrogen). After 72 hours, cell cultures were incubated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich), and MTT formazan was solubilized in acidic isopropanol with 0.04N HCl. Absorbance was read at 570 and 630 nm with a colorimeter. Cell viability was calculated as the relative absorbance (570/630 nm), and normalized to control cells. To study drug effects, cell viability was examined at the 48-hr time point after treatment with HDAC inhibitors. For colony formation assay, control and MULE-deficient SYO-1 cells were grown to 80% confluence, and treated with HDAC inhibitors for 24 hours. Cells were seeded into 6-well plates at the density of 600 cells/well in regular growth medium, and cultured for 10 days with medium change every 2-3 days. Finally, cells were fixed with 10% formaldehyde for 30 min at room temperature and
stained with 0.5% crystal violet solution (20% methanol) for 10 min at room temperature. Plates were washed with water and air-dried overnight, prior to scanning.

**RNA interference (RNAi)**

Two individual small interfering RNAs (siRNAs) were purchased from Thermo Stealth RNAi collection, for MDM2 (HSS142909, HSS142911), HDAC1 (HSS104725, HSS104726), HDAC2 (HSS104728, HSS104729) and HDAC3 (HSS113050, HSS113051). At 60% confluence, cells were transfected with indicated siRNAs at the final concentration of 20 nM, using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. For human SS18 (Santa Cruz Biotechnology) and SS18-SSX2 (sense: CAAGAAGCCAGCAGAGGAATT, antisense: UUCCUCUGCUUGCUUCUUGTT), RNAi reactions were carried out with 40-80 nM siRNAs. Knockdown efficiency was confirmed by western blots.

**Plasmid DNA construction and transfection**

Flag- and HA-tagged human MULE constructs have been used in previous studies (Zhang et al. 2011; Yi et al. 2015). SS18-SSX2 cDNA and deletion mutants (ΔSNH, ΔQPGY and ΔRD) were synthesized via Integrated DNA Technologies (IDT) and inserted into a Gateway pENTR1A vector. To make Myc-tagged SS18-SSX2, EcoR1-Not1 fragment was sub-cloned into the pcDNA4 mammalian expression vector (Invitrogen). To generate GFP-tagged fusions, pENTR1A constructs were subjected to Gateway recombination reactions, creating pcDNA-DEST47 (Invitrogen) expression clones. For lysine-to-arginine (KR) and -glutamine (KQ) substitution experiments, we used the Q5 Site-Directed Mutagenesis kit (New England Biolabs) with the PCR primers listed below. For overexpression, cells were transiently transfected with indicated plasmid
DNA using Lipofectamine 3000 reagent (Invitrogen). After 48 hours post transfection, cells were harvested for downstream applications.

The sequences of PCR primers used for site-directed mutagenesis

| SS18-SSX2 constructs |  |
|---------------------|--|
| K13 - forward:      | GAGGCAGCGAGGCAGGGGGAGATCA |
| K13 - reverse:      | GGGGCCGCGAAGCCACAG        |
| K23 - forward:      | CTGCAGATTCAGAGGATTTGGATGAC |
| K23 - reverse:      | CGGGAGTGATCTCCCCCT        |
| K41/43 - forward:   | AAGGACCTCAGAGTGTTCAG      |
| K41/43 - reverse:   | CCTCTATTCTGAGAGTCATTATAC  |

| MDM2 constructs    |  |
|--------------------|---|
| 5KR - forward:     | agaaggaataggCCCTGCCAGATATGTAAG |
| 5KR - reverse:     | ccttagctctTGACATGTAAAGCAGGC   |
| KR1 - forward:     | aaaaaggaataagCCCTGCCAGATATGTAAG |
| KR1 - reverse:     | ctttagctctTGACATGTAAAGCAGGC   |
| KR2 - forward:     | aaaaaggaataagCCCTGCCAGATATGTAAG |
| KR2 - reverse:     | ctttagctctTGACATGTAAAGCAGGC   |
| KR3 - forward:     | aaaaaggaataagCCCTGCCAGATATGTAAG |
| KR3 - reverse:     | ctttagctctTGACATGTAAAGCAGGC   |
| KQ1 - forward:     | aaaaaggaataagCCCTGCCAGATATGTAAG |
| KQ1 - reverse:     | ctttagctctTGACATGTAAAGCAGGC   |
| KQ2 - forward:     | ctttagctctTGACATGTAAAGCAGGC   |
| KQ2 - reverse:     | ctttagctctTGACATGTAAAGCAGGC   |

**Real-time qPCR and RNA-sequencing**

Total RNA was isolated and then transcribed to cDNA using the Qiagen RNeasy kit and the high-capacity cDNA reverse transcription kit (Applied Biosystems), respectively. Taqman gene expression assays were performed on an ABI-7500 Fast Real-Time PCR System with human
MULE-specific primer/probe sets (Applied Biosystems). SS18-SSX2 fusion oncogene expression (5’-TGACCAGATCATGCCAAG-3’; 5’-GGGTCCAGATCTCTCGTGAA-3’) was measured by SYBR Green qPCR assay (Applied Biosystems). All transcript levels were normalized to expression of 18S ribosomal RNA (rRNA). For sequencing analysis, RNA isolated with RNAzol (Sigma-Aldrich) was quality-checked for the integrity scores greater than 8 on the Agilent Bioanalyzer, and then prepared and indexed with Truseq Stranded mRNA kit (Illumina). Sequencing was done on an Illumina HiSeq 2500 50 Paired End run. All data were aligned to hg19 using TopHat2, and differential expression was demonstrated through Cufflinks and normalized FPKM numbers.

**Histone extraction**

Cells were suspended at the density of $4 \times 10^7$ cells per ml in the solution (10mM HEPES, 10mM KCl, 1.5mM MgCl$_2$, 0.34M sucrose, 10% glycerol, 1mM DTT, 0.2% Triton X-100) and incubated for 5 min on ice. Nuclei were collected in pellet by low-speed centrifugation (4 min, 1,300×g, 4°C), washed once with PBS/T (0.5% Triton X-100) and resuspended in 0.2N HCl. After overnight incubation at 4°C, the supernatant was collected by high-speed centrifugation (10 min, 6,500×g, 4°C) and neutralized with 1M Tris-HCl (pH=8). Histone samples were separated on 15% SDS-PAGE and analyzed by coomassie blue staining (Protea SuperBlue) and western blotting.

**Data and Software Availability**

Original mass spectrometry files are deposited in the ProteomeXchange Consortium (the accession #PXD012540) and Japan Proteome Standard Repository/Database (#JPST000550). Raw data from RNA-sequencing are deposited in Gene Expression Omnibus (GEO) under #GSE126152.
Supplemental Figure Titles and Legends

**Figure S1.** HDAC inhibition reduces synovial sarcoma growth in a genetic mouse model of and downregulates SS18-SSX fusion oncoprotein in human synovial sarcoma cells, Related to Figure 1. (A) Outline of drug administration. (B) Total mass of tumors present at age 17 weeks in the thoracic cage of mice bearing the human SS18-SSX2 fusion oncogene induced by Myf5-Cre, after treatment with 3 weekly doses of FK228 or vehicle, beginning at age 14 weeks (mean of 5-8 samples per group ± SD). *, $P < 0.05$ by two-tailed Student’s t-test. (C-H) Hematoxylin and eosin photomicrographs of tumors obtained from vehicle (C-E) or FK228 (F-H) treated mice demonstrating the normal synovial sarcoma morphology of control tumors, including monophasic (C) and biphasic (D) patterning, including frank gland formation (solid black arrows) that was not identified in any treated tumors, as well as infiltrative growth between muscle fibers (E). FK228-treated tumors had no glands remaining, and showed areas with inflammatory cells and hemosiderin laden macrophages (white arrows in F), areas where tumor cells were scant and collagen matrix was plentiful (G), and regions where apparent prior intramuscular infiltration was replaced with paucicellular fibrosis (open black arrows in H). All magnification bars are 50µm in length. (I) Outline of the CRISPR/Cas9 approach targeting the SSX2B gene. (J) Anti-Flag western blot analysis of fusion oncoprotein levels in parental and CRISPR cells expressing control or SS18-SSX2-specific siRNA. Actin serves as a loading control. (K) Immunoprecipitation of Flag-SS18-SSX2 from parental and CRISPR-modified SYO-1 cells. The lysates (Input) and mouse IgG serve as positive and negative controls, respectively. (L) Anti-Flag western blot analysis of SS18-SSX2 protein abundance in FK228-treated CRISPR cells at different time points. Tubulin was used as a loading control; parental cell lysate serves as a negative control. (M) RT-qPCR analysis of SS18-
SSX2 transcript levels in SYO-1 cells treated with HDAC inhibitors (FK228, SB939 and PCI-24781) at the 8- and 16-hour time points.

**Figure S2. MULE does not interact with the SSX2A isoform in human synovial sarcoma cells, Related to Figure 3.** (A) Amino-acid sequence comparison of the SSX2-A and -B isoforms. (B) Outline of the CRISPR/Cas9 approach targeting the SSX2A gene. (C) Western blot assay showing SSX2A protein (anti-Flag) immunoprecipitated from CRISPR-edited SYO-1 cells. Parental cells serve as a negative control. (D) Western blotting analysis of anti-Flag/-MULE immunoprecipitates from CRISPR cells treated with DMSO (−) or FK228 (30nM), in the presence of MG-132. Mouse IgG was used as a negative control. (E) Reciprocal immunoprecipitation showing no interaction between MULE and wild-type SS18 in HEK293 cells transfected with the plasmid encoding full-length MULE. Rabbit IgG serves as a negative control. (F) Western blot analysis of SS18 protein expression in wild-type and Mule-knockout MEF cells. Actin serves as a loading control.

**Figure S3. Effect of MULE knockdown on histone acetylation and its known substrates upon HDAC inhibition, Related to Figure 4.** (A) Schematic of a biochemical approach to isolate core histones. (B) Coomassie blue staining of total histone proteins isolated from indicated SYO-1 cell lines upon treatment of DMSO (−) or HDAC inhibitors (FK228 and SB939). (C) Under the same conditions, western blot analysis was performed to show the levels of total histone H3 and global histone H3 acetylation. (D) Western blot analysis of several known MULE substrates in response to HDAC inhibition (FK228 and SB939). Tubulin serves as a loading control. (E) Western blotting analysis showing the levels of CTCF, β-catenin and HDAC2 in control and MULE-knockdown SYO-1 cells treated with DMSO (−) or HDAC inhibitors. HDAC2 was used as a negative control.
which stays constant upon HDAC inhibitor treatment, regardless of the MULE status. Tubulin and Actin serve as loading controls.

Figure S4. HDAC inhibition induces MULE upregulation through MDM2, Related to Figure 5. (A and B) Analysis of MULE mRNA (A) and protein (B) levels in SYO-1 and Yamato-SS cells treated with DMSO, FK228 or SB939. RT-qPCR data represent mean ± SD of three independent experiments; Tubulin was used as a loading control. (C) Anti-MULE western blot analysis of the lysate prepared from Yamato-SS cells treated with or without MG-132. Tubulin serves as a loading control. (D) Western blot analysis of anti-MULE immunoprecipitates from Yamato-SS cells in the presence of MG-132. Cell lysate (Input) and rabbit IgG serve as positive and negative controls, respectively. (E) Western blot analysis of the lysate from Yamato-SS cells transfected with control (−) or MDM2 siRNAs. Tubulin was used as a loading control. (F) Viability of Yamato-SS cells in response to MDM2 knockdown by two individual siRNAs. Values were normalized to control (−) knockdown; results represent mean ± SD of three independent experiments. (G) Western blotting of MDM2-knockdown SYO-1 cells stably expressing control or p53 shRNAs. Actin was used as a loading control. (H and I) Viability (H) and colony formation (I) assay of stable cell lines (as in G) in response to control or MDM2-specific siRNA transfection. Results represent mean ± SD of three independent experiments; representative image of colony formation assay shown in the right panel. (J) Ubiquitination analysis of endogenous MULE protein in vehicle (−), FK228- and SB939-treated Yamato-SS cells. Cell lysate (Input) was applied to show equal amounts of ubiquitin under all conditions. (K) Western blotting analysis of MULE protein abundance in DMSO- and FK228-treated Yamato-SS cells, upon exposure to cycloheximide (CHX) for the indicated time. Tubulin was used as a loading control. (L and M) Viability analysis of SYO-1 stable cell lines in response
to FK228 (L) and SB939 (M) at different concentrations. All results represent mean ± SD of three independent experiments. (N) Colony formation analysis of SYO-1 stable cell lines in response to FK228 and SB939 treatment. The image represents three independent experiments.

**Figure S5. Effect of site-specific lysine acetylation on MDM2-MULE interaction in response to HDAC inhibition, Related to Figure 6.** (A-E) Evolutionary and interaction data for MDM2 and MULE. (A) Predictions of 100 global HADDOCK docking for models of MDM2 (red) with MULE (gray). (B) Following one round of energy minimizations the using multiple force fields the top binding energy conformation was selected. Negatively charged amino acids are shown in red for MULE and positive charged residues in blue for MDM2. (C) Deep evolutionary analysis of MDM2 using open reading frame sequences of 180 species for codon alignment. Four species (mouse, human, zebrafish, and chicken) are highlighted to show the depth of vertebrate species used for the alignment. (D) Codon selection scores put on a 21-codon sliding window mapping functional domains and motifs of the MDM2 protein. (E) Zoomed view of MULE-interacting amino acids in the RING domain of MDM2, with scores for codon selection (dN-dS, rate of nonsynonymous variants minus rate of synonymous variants). (F) Alignment of the MDM2 KRD, showing highly conserved lysine residues across different species. (G) Schematic of wild-type and mutant MDM2 constructs with lysine-to-arginine substitution highlighted in red. (H) Western blot analysis of lysine acetylation in anti-HA immunoprecipitates from HEK293 cells expressing HA-MDM2 constructs (as in G) under the treatment of FK228. To calculate MDM2 acetylation levels, acetyl-lysine/total ratios of FK228-treated samples were first normalized to individual DMSO (−) controls, and then normalized to the wild-type (WT) level. All results represent the average of two independent experiments. (I) Western blot analysis of anti-HA immunoprecipitates from DMSO-
and FK228-treated HEK293 cells expressing HA-MDM2 (WT) or KR3, in the presence of MG-132. Input serves as loading control. (J) Band intensities of MULE-bound MDM2 were quantified by ImageJ and normalized to Input. Values of FK228-treated samples were further normalized to DMSO (−). Data represent mean ± SD of three independent experiments. (K) Western blot analysis of HEK293 cells expressing empty vector (−) or Flag-p53, along with the HA-MDM2 constructs. Actin was used as a loading control.

**Figure S6. HDAC2, rather than HDAC1 and HDAC3, regulates MDM2-MULE association and SS18-SSX protein expression, Related to Figure 7.** (A) Model proposing a role for HDAC enzyme(s) in stabilizing the MDM2-MULE complex through removal of acetylation signals. (B) Western blot analysis of anti-MDM2 immunoprecipititates from SYO-1 and Yamato-SS cells. The lysate (Input) and rabbit IgG serve as positive and negative controls, respectively. (C and D) Western blotting analysis of SYO-1 (C) and Yamato-SS (D) cell extracts showing the knockdown efficiency of HDAC1 and HDAC2 siRNAs. MDM2 protein levels remain constant in control and knockdown cells. Actin and Tubulin serve as loading controls. (E) Immunoprecipitation analysis of MDM2-MULE interaction in MG-132-treated Yamato-SS cells depleted of HDAC1 or HDAC2. Input and rabbit IgG serve as positive and negative controls, respectively. (F) RT-qPCR analysis of SS18-SSX2 transcript levels in SYO-1 cells expressing control (−) or HDAC2 siRNAs. Results represent mean ± SD of three independent experiments. (G) Western blot analysis of the lysate from control (−) and HDAC1-knockdown SYO-1 cells. Actin was used as a loading control. (H) Western blot assay showing the levels of SS18-SSX1 protein in Yamato-SS cells transfected with control (−), HDAC1 or HDAC2 siRNAs. Tubulin serves as a loading control. (I) Western blotting analysis of the lysate from SYO-1 (left) and Yamato-SS (right) cells depleted of HDAC3. Tubulin
serves as a loading control. (J and K) Nutlin-3a does not interfere with MDM2 association and
downregulation of MULE in human synovial sarcoma cells. (J) Immunoprecipitation analysis of
MDM2 binding to MULE in Nutlin-3a treated SYO-1 cells at the indicated concentrations, in the
presence of MG-132. Rabbit IgG serves as a negative control. (K) Western blotting analysis of the
lysate of SYO-1 cells treated with DMSO, Nutlin-3a (10µM) or FK228 (30nM). Tubulin was used
as a loading control.
**A** Myf5-Cre; SSM2 mice

Weekly treatment

#1 #2 #3 Analysis

Age (weeks)

13 14 15 16 17

**B**

Tumor mass (mg)

| FK228 (mg/kg) | 0.3 | 1.0 | 3.0 |
|---------------|-----|-----|-----|
| *             |     |     |     |
| *             |     |     |     |

**C**

**D**

**E**

**F**

**G**

**H**

**I**

Scale: chrX: 5 kb hg19

52,781,000 52,782,000 52,783,000 52,784,000 52,785,000 52,786,000 52,787,000 52,788,000 52,789,000 52,790,000

**J**

SYO-1: CRISPR Parental

si-SS18-SSX2

SS18-SSX2 (anti-Flag)

Actin

**K**

IP:

Flag IgG Input

CRISPR:

+ - + -

SS18-SSX2 (anti-Flag)

**L**

SYO-1 cells

| CRISPR | Parental |
|--------|----------|
| FK228: | 0 4 8 16 | 0 4 8 16 |
| SS18-SSX2 (anti-Flag) | | |

**M**

SS18-SSX2 mRNA levels

| Percent of vehicle | FK228 | SB939 | PCI-24781 |
|--------------------|-------|-------|-----------|
| 8 Hr               | -     | -     | -         |
| 16 Hr              | -     | -     | +         |
A

**Overlap (1-156 aa)**

**No significant similarity**

SSX2 isoform A: 1

KRAB [223]

SSX2 isoform B: 1

KRAB [223] RD [188]

111 (breakpoint)

B

![Genomic sequence diagram](image)

**gRNA cuts genomic backbone near stop codon**

Intron of genomic backbone

**Homology arm 1**

**Homology arm 2**

Insert Flag tag using a donor plasmid for Homology directed repair

C

SYO-1 cells:

CRISPR

X

Parental

SSX2A (anti-Flag)

IgG

IP: Flag

**D**

IP: IgG  IP: Flag  IP: MULE

FK228  MG-132

-  +  -  +  -  +

MULE

SSX2A (anti-Flag)

250

37

E

MULE

SS18WT

**F**

MEF:

Mule

WT

50

37

SS18WT

Actin
**A** MDM2 Conservation (21 codon window)

**B** MDM2-MULE interaction (Percent of vehicle)

**C** MDM2 (a.a. 460 – 476)

**D** MDM2 Conservation (21 codon window)

**E** Normalized dN-dS

**F** MULE interacting amino acids

**G** MDM2 (acetyl-Lysine) vs MDM2 (Total)

**H** FK228-induced MDM2 acetylation

**I** HA-MDM2 constructs

**J** MDM2-MULE interaction (Percent of vehicle)

**K** MDM2 (anti-HA) vs p53 (anti-Flag)
