

# A Positive Regulatory Role for the mSin3A-HDAC Complex in Pluripotency through Nanog and Sox2\*

Received for publication, October 3, 2008, and in revised form, December 19, 2008 Published, JBC Papers in Press, January 12, 2009, DOI 10.1074/jbc.M807670200

Gretchen A. Baltus, Michael P. Kowalski, Antonin V. Tutter, and Shilpa Kadam<sup>1</sup>

From the Department of Developmental and Molecular Pathways, Novartis Institute for Biomedical Research, Cambridge, Massachusetts 02139

Large networks of proteins govern embryonic stem (ES) cell pluripotency. Recent analysis of the critical pluripotency factors Oct4 and Nanog has identified their interaction with multiple transcriptional repression complexes, including members of the mSin3A-HDAC complex, suggesting that these factors could be involved in the regulation of Oct4/Nanog function. *mSin3A* is critical for embryonic development, but the mechanism by which the mSin3A-HDAC complex is able to regulate ES cell pluripotency is undefined. Herein we show that the mSin3A-HDAC complex positively regulates *Nanog* expression in ES cells through Sox2, a critical ES cell transcription factor and regulator of *Nanog*. We have identified the mSin3A-HDAC complex to be present at the *Nanog* promoter only under proliferating conditions concurrent with histone acetylation. We find that Sox2 associates with mSin3A-HDAC complex members both *in vitro* and *in vivo*, similar to the interactions found between Oct4/Nanog and the mSin3A-HDAC complex. Knockdown of mSin3A-HDAC complex members or HDAC inhibitor treatment reduces *Nanog* expression, and overexpression of mSin3A-HDAC complex subunits stimulates *Nanog* expression. Our data demonstrate that the mSin3A-HDAC complex can positively regulate *Nanog* expression under proliferating conditions and that this activity is complementary to mSin3A-mediated p53-dependent silencing of *Nanog* during differentiation.

Precise modulation of transcriptional activation and repression in ES<sup>2</sup> cells is crucial for proper development, lineage differentiation, and genomic stability. At the core of ES cell self-renewal are the transcription factors Oct4, Sox2, and Nanog. These factors are central to maintaining pluripotency, as well as directing appropriate lineage commitment (1, 2). In addition to regulating the transcription of other genes, Oct4, Nanog, and Sox2 also activate one another's transcription. Oct4 and Sox2 together recognize and bind to a highly conserved consensus sequence, which is essential for *Nanog* expression in both mouse and human ES cells (3, 4). In addition to Oct4 and Sox2, many other transcription factors have been linked to regulation

of *Nanog* expression and ES cell pluripotency. For example, Sall4 (5), FoxD3 (6), and STAT3 and T (7) transcription factors positively regulate *Nanog* expression, whereas p53 (8) and GCNF (9) suppress *Nanog* expression.

In addition to specific transcription factors, the active or inactive transcriptional state of genes is established through highly regulated modulation of underlying chromatin structure (10). This is achieved by a large family of histone-modifying and chromatin-remodeling enzymes (11). Nanog is known to interact with a variety of chromatin-modifying complexes. Analysis of the Nanog interaction network has shown that it is linked to repressor proteins (12). A recent protein interaction study identified the interaction of Oct4 and Nanog with a novel repressor complex, NODE, which contains several chromatin-associated proteins, including mSin3A, HDAC1, and HDAC2 (13). ES cells that have lost *Mbd3*, a component of the nucleosome-remodeling complex NuRD, show LIF-independent growth with no effect on *Nanog* expression or in the expression of lineage-specific genes (14), illustrating how chromatin-modifying complexes can regulate *Nanog* expression. In contrast, knockdown of Mta1/2-containing repression complexes led to ES cell differentiation by specifically up-regulating endoderm lineage markers (13). Collectively, these studies show that Nanog and Oct4 interact with multiple repression complexes to regulate their target genes and hence to control the fate of ES cells.

HDAC1 and HDAC2 are members of a number of deacetylase complexes, including the mSin3A-HDAC complex, the NuRD complex, the BCH10-containing complex, and the CoREST complex (15). Although they have broadly similar functions in regulating transcription, the mSin3A-HDAC complex can be distinguished from these complexes by the presence of the mSin3A protein. mSin3A has been well described as a core component of a multiprotein co-repressor complex known to silence gene expression by deacetylating histones (for review, see Ref. 16) and has been shown to play an essential role in early embryonic development (17). ES cells derived from *mSin3A*<sup>-/-</sup> blastocysts form significantly smaller colonies and eventually die in culture compared with wild-type or *mSin3A*<sup>+/-</sup> ES cells (18). Likewise, *HDAC1* null ES cells also show growth defects compared with wild-type ES cells, and loss of *HDAC1* contributes to embryonic lethality prior to E10.5 (19). *HDAC2*<sup>-/-</sup> mice display difficulty progressing through gestation (20). These observations allude to a function of the mSin3A-HDAC complex in ES cell survival and proliferation. However the mechanism by which mSin3A-HDAC complex regulates ES cells still remains unclear.

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<sup>1</sup> To whom correspondence should be addressed: Novartis Institutes for Biomedical Research, 250 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-871-7496; Fax: 617-871-4072; E-mail: shilpa.kadam@novartis.com.

<sup>2</sup> The abbreviations used are: ES, embryonic stem; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; siRNA, small interference RNA; qPCR, quantitative PCR; RA, retinoic acid; LIF, leukemia inhibitory factor; TSA, trichostatin A.

Our goal was to investigate the mechanisms underlying *Nanog* transcription. We sought out co-activator or co-repressor complexes (21) that could regulate *Oct4* or *Sox2*, and consequently *Nanog* itself. Using chromatin immunoprecipitation (ChIP) we have identified components of the mSin3A-HDAC complex present at the *Nanog* promoter under proliferating conditions, despite also finding acetylated histones at this loci. This binding is lost upon differentiation, concurrent with histone deacetylation. Depletion of mSin3A-HDAC activity by siRNA or treatment with HDAC inhibitors negatively affects *Nanog* transcription but does not similarly affect *Oct4*. Overexpression of mSin3A-HDAC complex members stimulates expression from the *Nanog* promoter in a reporter system. In addition, mSin3A-HDAC complex members interact with Sox2 *in vitro* and *in vivo*, providing a link between a factor known to positively regulate *Nanog* expression and the mSin3A-HDAC complex. Our studies demonstrate that the mSin3A-HDAC complex is required for ES cell proliferation due to its role in positively regulating the expression of *Nanog*.

## EXPERIMENTAL PROCEDURES

**ES Cell Culture**—Murine J1 ES cells were grown under typical ES cell conditions on gelatinized tissue culture plates in feeder-free conditions. For differentiation, ES cells were plated at  $1.5 \times 10^5$  cells/ml in differentiation media (Dulbecco's modified Eagle's medium (Invitrogen, 11965-092), 10% fetal bovine serum (HyClone, SH30070), penicillin/streptomycin (Invitrogen, 15140-122), and 5  $\mu$ M retinoic acid (Sigma, R2625)) for 5 days with a change in medium every other day. For drug treatments, adherent ES cells at 75% confluence were washed in phosphate-buffered saline, then incubated with normal ES cell media plus 5  $\mu$ M retinoic acid, 100 ng/ml trichostatin A (Millipore, 19-138), 5 mM sodium butyrate (Millipore, 19-137), or 3 mM valproic acid (Sigma, P4543) for 3 or 6 h as noted.

**Plasmid Construction**—To generate a pNusa-Sox2 bacterial expression vector, a Gateway Conversion Cassette, RFA isoform (Invitrogen) was inserted into pET43.1a(+) (Novagen) downstream of the NuaA coding sequence. Next, a C-terminal STREP-II purification tag sequence (IBA, Germany) was introduced downstream of the Gateway attR2 site, in-frame with cDNA sequences introduced by Gateway LR recombination (Invitrogen). Lastly, murine Sox2 cDNA was introduced into this plasmid by Gateway LR recombination with a Sox2 Gateway donor vector. To generate luciferase expression plasmids pGL3-TK-Luc and pGL3-O/S-TK-Luc, plasmids pGL3-Basic (Promega, E1751, GenBank<sup>TM</sup>/EMBL accession number U47295) and pRL-TK (Promega, E2241, GenBank<sup>TM</sup>/EMBL accession number AF025846) were digested with BglII/HindIII. The TK promoter from pRL-TK was subcloned into pGL3-Basic to generate pGL3-TK-Luc. pGL3-TK-Luc was digested with KpnI/BglII. Oligonucleotides containing tandem Oct4/Sox2 binding sites from the *Nanog* promoter were annealed together and ligated into the digested pGL3-TK to generate pGL3-O/S-TK-Luc. Forward primer: 5'-cttcttttgcattacaatgtccattctttgattacaatgtccattctttgattacaatgtcca-3'. Reverse primer: 5'-gattcttgacattgtaatgcaaaagaatggacattgtaatgcaaaagaatggacattgtaatgcaaaagaaggtaac-3'. To generate expression plasmids for Oct4, Sox2, mSin3A, and HDAC2, total mouse ES cell cDNA was

amplified using reverse-transcription (Bio-Rad, 170-8891) from total RNA. Gene-specific primers for *Oct4*, *Sox2*, *mSin3A*, and *HDAC2* were used to amplify the cDNA then recombined into a Gateway donor vector (Invitrogen), which was then sequence-verified. The cDNAs were introduced into pDEST26 (Invitrogen, 11809-019) by Gateway LR recombination.

**Chromatin Immunoprecipitation**—ChIP was performed using  $2.0 \times 10^7$  ES cells as described in the online protocol provided by Upstate (Millipore). Purified DNA was amplified with primers spanning the Oct4-Sox2 binding site (forward primer: 5'-ggatgtctttagatcagaggatgcc-3'; reverse primer: 5'-ccacagaagagcaagacaccaacc-3') and p53 binding site (forward primer: 5'-tcagacttgcgttaaaagccgcac-3'; reverse primer: 5'-gcttagggggcatcctctgatct-3') on the *Nanog* promoter and Oct4-Sox2 binding site (forward primer: 5'-ggcagccttaggctaacctg-3'; reverse primer: 5'-ctccactctgtcatgctcacctcc-3') on the *Oct4* promoter using Applied Biosystems SYBR PCR mastermix (Applied Biosystems, 4309155). Relative enrichment was calculated as  $2^{\Delta(\Delta C_T(\text{control CHIP}) - \Delta C_T(\text{experimental CHIP}))}$ , where  $\Delta C_T$  is equal to the  $C_T(\text{immunoprecipitated sample}) - C_T(\text{input})$ . Antibody sources were: Histone H3 Ac (Millipore, 06-942), H3 K4 2Me (Millipore, 07-030), H3 K4 3Me (Millipore, 07-473), H3 K9 1Me (Millipore, 07-450), H3 K9 2Me (Millipore, 07-441), H3 K27 3Me (Millipore, 05-851), H3 K79 2Me (Millipore, 05-835), Sox2 (Abcam, ab15830), RNAPII (Abcam, ab817), HDAC1 (Abcam, ab7028), HDAC2 (Abcam, ab7029), Oct4 (R&D Biosystems, AF1759), Nanog (Cosmo Bio Co., REC-RCAB0002P-F), and mSin3A (Santa Cruz Biotechnology, sc994).

**RNA Isolation, qPCR, and Analysis of Transcript Levels**—Total RNA was purified with an RNeasy Miniprep kit (Qiagen, 74106), DNase-treated (Promega, M6101), and reverse-transcribed (Bio-Rad, 170-8891). Quantitative PCR (qPCR) reactions were performed using 10 ng of cDNA in an ABI7500 Fast Real-Time PCR System as per the manufacturer's instructions (Applied Biosystems). TaqMan gene expression assays (all from Applied Biosystems) were as follows: *Oct4* (Mm00658129\_gH), *Sox2* (Mm00488369\_s1), *Hand1* (Mm00433931\_m1), *HoxA5* (Mm00439362\_m1), *Nestin* (Mm00450205\_m1), *mSin3A* (Mm00488255\_m1), *Rex1* (Mm01194090\_g1), *Bmp4* (Mm00432087\_m1), *Gata4* (Mm00484689\_m1), *Sox17* (Mm00488363\_m1), *Cdx2* (Mm00432449\_m1), *Eomes* (Mm01351985\_m1), *Fgf5* (Mm00438919\_m1), *p21* (Mm00432448\_m1), and *Mdm2* (Mm01233136\_m1). A custom TaqMan assay was designed for *Nanog* gene expression: forward primer, 5'-TCCTCGCCCTT-CCTCTGAA-3'; reverse primer, 5'-CAGGACTTGAGAGCT-TTTGTTTGG-3'; and, reporter sequence, 5'-CAGCCCTGATTCTTCT-3'. To calculate changes in gene expression, average threshold values ( $C_T$ ) were determined for three PCR reactions and calculated as -fold change compared with control samples using the comparative  $\Delta\Delta C_T$  method.

**RNA Interference**—SMARTpool siRNA targeting *HDAC1* (Dharmacon, M-040287-01), *HDAC2* (Dharmacon, M-046158-00), *mSin3A* (Dharmacon, M-044653-00), or control siRNA targeting Lamin (Dharmacon, D-001050-01) were incubated with DharmaFECT4 (Dharmacon, T-2004) in Opti-MEM (Invitrogen, 319850-62) for 30 min at room temperature to allow siRNA-lipid complexes to form.  $3.0 \times 10^5$  ES cells were

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added to the transfection reagents for final concentrations of 84 nM siRNA and 1.17  $\mu$ l/ml DharmaFECT4. For siRNA combinations (targeting 2–3 genes), the total siRNA concentration remained constant (42 nM of each of two or 28 nM of each of 3 targets). Total RNA and protein were harvested from cells after incubation with siRNA for 96 h.

**Protein Purification and Protein-Protein Interactions**—NusA-tagged Sox2 was expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen, C6000-03) and purified under native conditions using Streptactin resin (IBA, 2-1232-005). NusA pull-down assays were carried out using 15  $\mu$ g of NusA-Sox2 and 0.5 mg of ES cell nuclear extract. Endogenous Sox2 and interacting proteins were identified using 300  $\mu$ g of nuclear and cytoplasmic fractions (NE-PER, Pierce 78833) from drug-treated or untreated ES cells with 3  $\mu$ g of Sox2 antibody (Abcam, ab15830). Bound complexes were washed in Nonidet P-40 buffer, resuspended in SDS sample buffer, and electrophoresed on an SDS-PAGE gel and blotted onto a polyvinylidene difluoride membrane according to standard procedures. Blots were probed with primary antibodies: HDAC1 (Santa Cruz Biotechnology, sc7872), HDAC2 (Santa Cruz Biotechnology, sc7899), mSin3A (Santa Cruz Biotechnology, sc994), Nanog (Millipore, AB5731), and Sox2 (Millipore, AB5603).

**Size-exclusion Chromatography**—ES cell nuclear extract was prepared with Pierce NE-PER reagents. 11 mg of extract was dialyzed against 1 liter of Nonidet P-40 buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA), then concentrated with Amicon Ultra-15 concentrator columns (Millipore, UFC901096). 1 ml (~10 mg) of dialyzed extract was loaded onto two tandem Superose 6 10/300 GL columns (GE Life Sciences, 17-5172-01) under isotonic conditions with a flow rate of 0.2 ml/min using a Bio-Rad Duoflow FPLC. 150 0.4-ml fractions were collected, and 30  $\mu$ l from every fifth fraction was run on an SDS for Western blotting for mSin3A and Sox2.

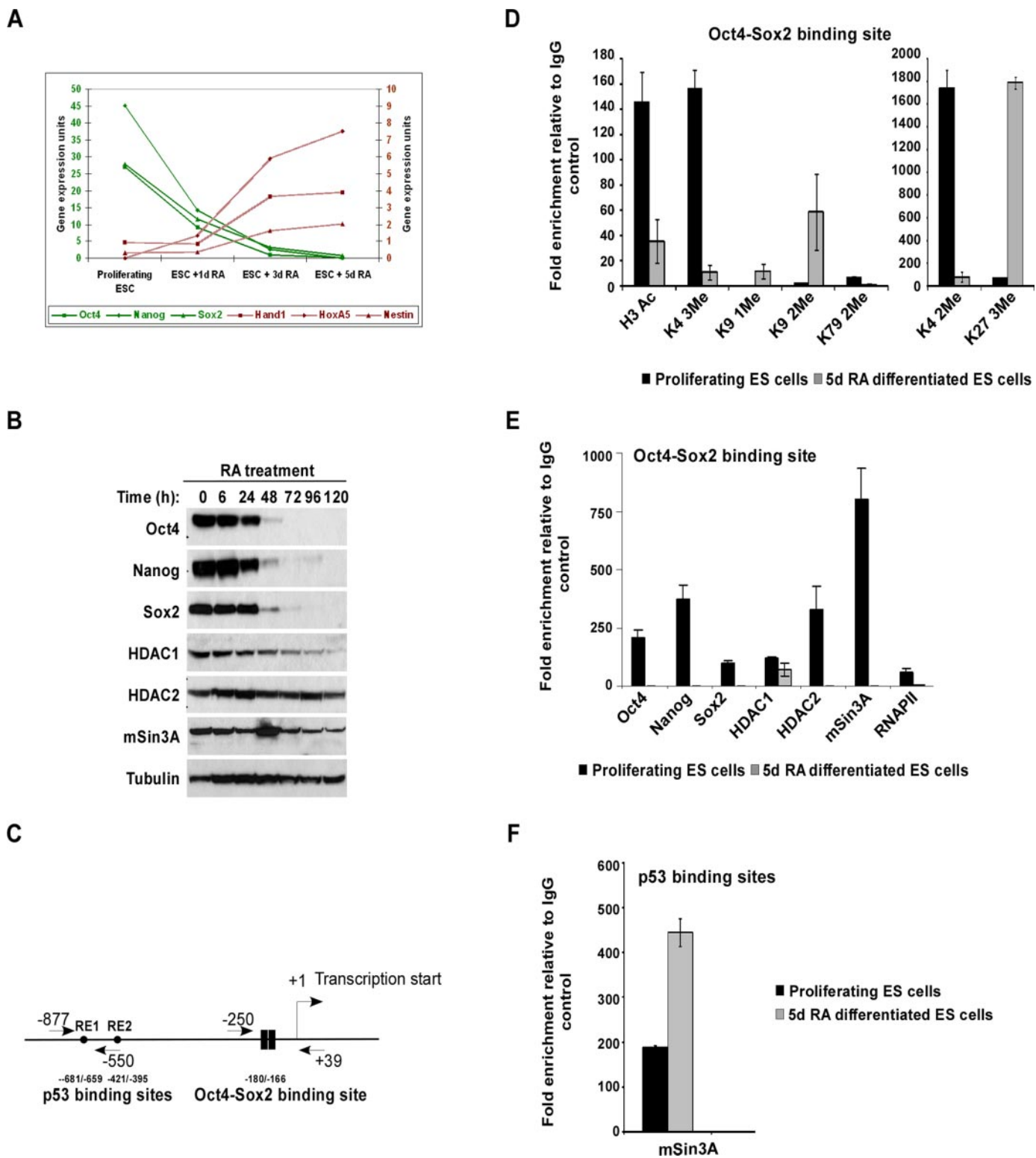
**Luciferase-reporter Assays**—NIH3T3 cells were seeded at a density of  $2.5 \times 10^5$  cells per well of a 6-well dish and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and  $1 \times$  penicillin/streptomycin and maintained at 37 °C and 5% CO<sub>2</sub>. DNA transfections were carried out using Lipofectamine 2000 (Invitrogen, 11668-019) as per the manufacturer's instructions. Cells were harvested after 48 h, and the luciferase activity of the lysate was measured with the Dual-Luciferase reporter assay system (Promega, E1960) using the Envision luminometer (PerkinElmer Life Sciences).

## RESULTS

**mSin3A-HDAC Complex Binds to the Nanog Promoter during ES Cell Proliferation**—To gain insight into *Nanog* transcriptional regulation in mouse ES cells, we identified factors that occupy the *Nanog* promoter during ES cell proliferation and differentiation by ChIP. To accomplish this, we established 5-day retinoic acid (RA) treatment minus LIF as a benchmark for differentiated ES cells. This treatment completely differentiates the cells as marked by loss of *Oct4*, *Sox2*, and *Nanog* expression (Fig. 1, A and B), and increased expression of lineage markers *Hand1*, *HoxA5*, and *Nestin* (Fig. 1A). We chose this method of differentiation over LIF withdrawal, because RA uni-

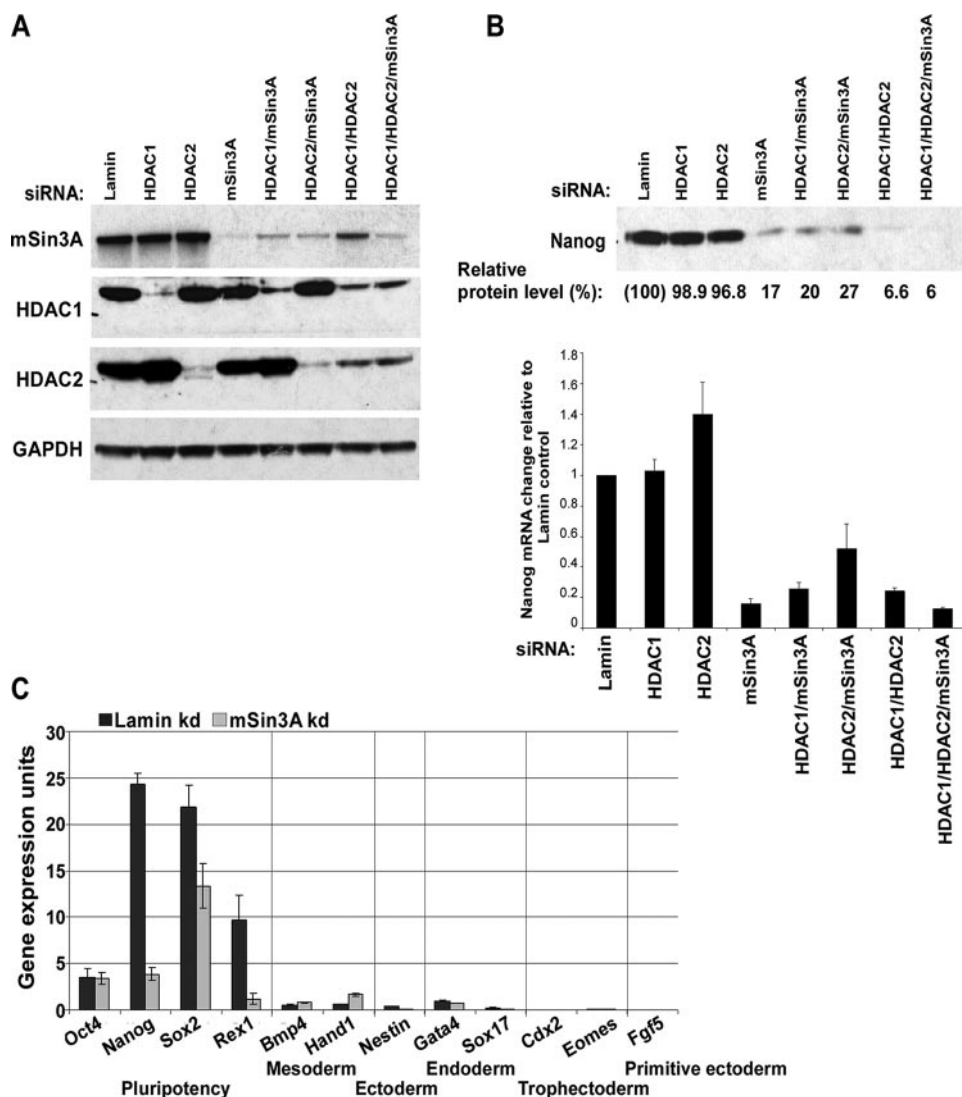
formly differentiates ES cells in to neural lineage (22), whereas LIF withdrawal results in heterogeneous expression of pluripotency and lineage markers. We compared histone modifications and binding of a panel of factors at the *Nanog* promoter between 5-day RA differentiated and proliferating ES cells, using qPCR primers that span the Oct4-Sox2 binding site (Fig. 1C). As expected, we found active histone modifications (H3Ac, H3K4 di- and trimethyl, and H3K79 dimethyl) (Fig. 1D), transcription factors (Oct4, Nanog, and Sox2), and RNA polymerase II (Fig. 1E) present at the *Nanog* in the promoter only under proliferating conditions, and not after differentiation. Additionally, repressive histone modifications (H3K9 mono- and dimethyl and H3K27 trimethyl) (Fig. 1D) were present only after differentiation. Surprisingly, we observed HDAC1, HDAC2, and mSin3A, members of the mSin3A-HDAC complex, at the Oct4-Sox2 binding site on the *Nanog* promoter under proliferating, but not differentiated conditions (Fig. 1E). Because the mSin3A-HDAC complex has been well characterized as a transcriptional repressor, we did not expect to find it at the promoter of an actively transcribed gene. We assayed the expression of mSin3A, HDAC1, and HDAC2 over a 5-day RA treatment of ES cells, and detected all components throughout the time course (Fig. 1B) indicating that the loss of the complex from the *Nanog* promoter is not due to a global loss of these proteins. As previously reported (8), we also observed increased mSin3A occupancy at the p53 binding sites (diagram of *Nanog* promoter in Fig. 1C) on the *Nanog* promoter following RA-induced differentiation (Fig. 1F). These data clearly demonstrate that occupancy of mSin3A at the Oct4-Sox2 binding site is distinct from that of the p53 binding site, and it is possible that mSin3A may have varying functions under different cellular conditions.

**Knockdown of mSin3A Diminishes Nanog Expression**—Because the mSin3A-HDAC complex plays a central role in transcriptional repression (15), it was intriguing to find it bound to the *Nanog* promoter during proliferation, especially because the histones at this site are heavily acetylated. To assay the possible role for this complex in *Nanog* regulation, we used siRNAs to inhibit the expression of mSin3A-HDAC complex subunits. If the mSin3A-HDAC complex is essential for positively regulating *Nanog*, loss of mSin3A-HDAC complex members would be expected to diminish *Nanog* expression. We knocked down *HDAC1*, *HDAC2*, and *mSin3A* (individually or in combination) with siRNAs for 96 h, and then analyzed *Nanog* transcript and protein levels. We saw efficient knockdown of target proteins (Fig. 2A) and found that levels of Nanog protein and mRNA dropped by ~80% in the presence of *mSin3A* siRNA (alone or in combination with *HDAC1/2*) with no significant change in *Nanog* levels by *HDAC1* or *HDAC2* siRNA alone (Fig. 2B). This suggests that mSin3A is the key member of the complex regulating *Nanog* expression. We did see a reduction in the level of *Nanog* by knocking down both HDAC1 and HDAC2 together, but this knockdown also resulted in roughly 60% reduction of mSin3A protein levels (Fig. 2A), further suggesting a key role for mSin3A in *Nanog* regulation. We also examined earlier time points of *mSin3A* siRNA knockdown and observed that mSin3A levels were reduced by ~80% within 24 h of siRNA treatment, and *Nanog* levels dropped by ~50% (data not



**FIGURE 1. Recruitment of the mSin3A-HDAC complex to the *Nanog* promoter during ES cell proliferation.** *A*, qPCR analysis of ES cells in the presence of 5  $\mu$ M retinoic acid (RA) showing a decrease in pluripotency markers *Oct4*, *Nanog*, and *Sox2* and an increase in lineage markers *Hand1*, *HoxA5*, and *Nestin*. *B*, Western blot showing levels of Oct4, Nanog, Sox2, HDAC1, HDAC2, and mSin3A upon RA treatment (5  $\mu$ M). Tubulin was used as a loading control. *C*, schematic of the proximal *Nanog* promoter showing positions of Oct4-Sox2 and p53 binding sites relative to the transcription start site. Arrows denote location of primers used for chromatin immunoprecipitation (ChIP) relative to the transcription start site. *D* and *E*, ChIP assays were performed for histone modifications, transcription factors, and deacetylase complex members as indicated using protein extracts of proliferating and 5-day RA-treated ES cells, with primers amplifying the Oct4-Sox2 binding site of the *Nanog* promoter. Values are expressed as -fold enrichment relative to IgG control ChIP. Results shown are the average of three independent PCR reactions. *F*, ChIP from proliferating and 5-day RA-treated ES cells using antibodies to mSin3A with primers amplifying the p53 binding site of the *Nanog* promoter.

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**FIGURE 2. RNA interference knockdown of mSin3A-HDAC complex members affects *Nanog* expression without inducing differentiation.** *A*, Western blot showing levels of mSin3A, HDAC1, and HDAC2 proteins in siRNA-treated ES cells. Lamin siRNA was used as a negative control. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. *B*, levels of Nanog protein (top) and mRNA (bottom) in response to knockdown of mSin3A and associated HDAC members; 96 h after indicated siRNA treatment. Relative protein levels were quantified by image densitometry. Values for Nanog mRNA and protein were normalized to Lamin siRNA. *C*, mRNA expression levels of pluripotency-related genes (*Oct4*, *Nanog*, *Sox2*, and *Rex1*), mesodermal lineage markers (*Bmp4* and *Hand1*), ectodermal lineage marker (*Nestin*), endodermal lineage markers (*Gata4* and *Sox17*), trophoctodermal lineage markers (*Cdx2* and *Eomes*), and primitive ectoderm lineage (*Fgf5*) after siRNA treatment for Lamin (control) or mSin3A.

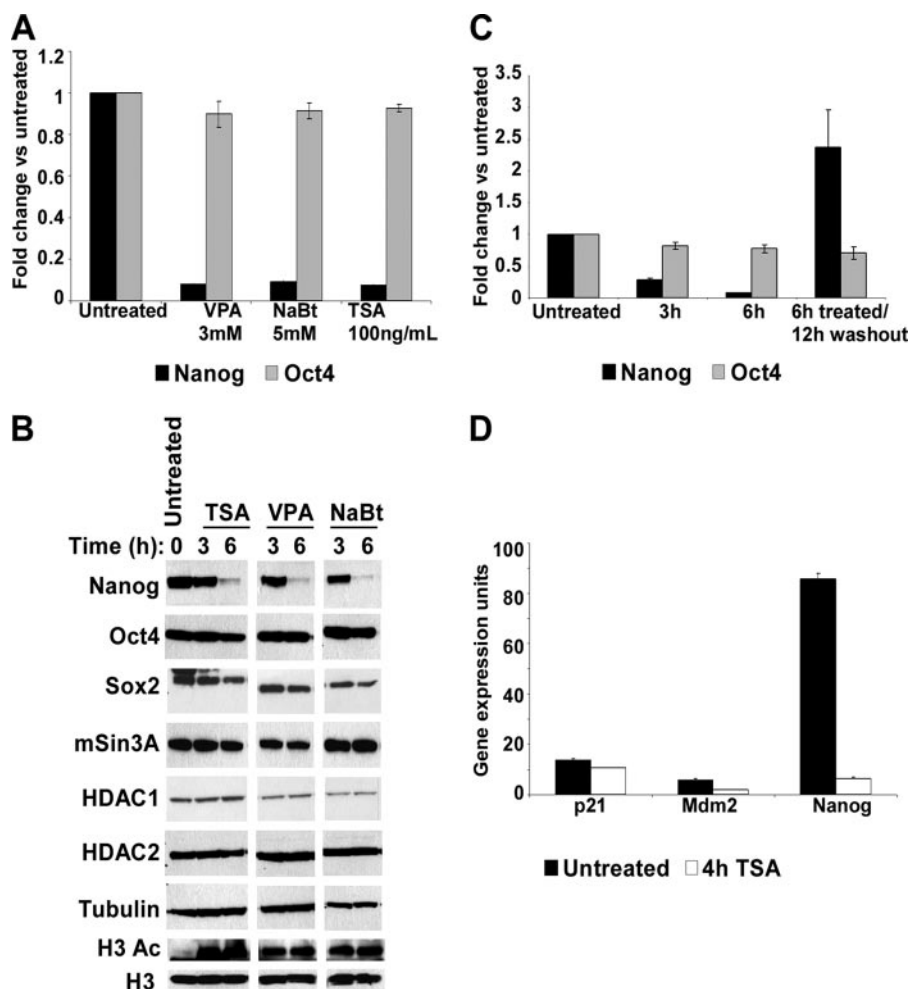
shown). To rule out the possible involvement of p53 in suppressing *Nanog* expression, we assayed expression of direct p53 targets *p21* and *Mdm2*. Activated p53 increases expression of both *p21* and *Mdm2*, but under these conditions we saw very little change in their expression (data not shown), indicating that p53 has not been activated. This suggests that mSin3A siRNA-mediated down-regulation of *Nanog* is independent of the p53 pathway. We next wanted to examine the expression of differentiation markers in the mSin3A knockdown ES cells, to eliminate the possibility that changes in *Nanog* levels are being caused by differentiation due to the loss of mSin3A. As seen previously (Fig. 2*B*), mSin3A knockdown resulted in decreased expression of not only *Nanog* but also in *Sox2* and *Rex1* levels (Fig. 2*C*). However, mSin3A knockdown had no effect on

expression of *Oct4* or a variety of lineage markers (*Bmp4*, *Hand1*, *Gata4*, *Sox17*, *Cdx2*, *Eomes*, and *Fgf5*) (Fig. 2*C*). Taken together, these results suggest that reduction in mSin3A levels reduces *Nanog* expression, and this effect is not due to an induction of differentiation either through p53 activation or mSin3A itself.

**Enzymatic Activity of the mSin3A-HDAC Complex Is Vital for *Nanog* Expression**—Because knockdown of the mSin3A-HDAC complex suggests a positive role for this co-repressor complex in *Nanog* regulation, we questioned if the enzymatic activity of the complex is important for regulating *Nanog*. To test this, we blocked deacetylase activity in ES cells by treating them with the HDAC inhibitors valproic acid, sodium butyrate, or trichostatin A (TSA). We found that, within 6 h, ES cells treated with any of these inhibitors showed significantly lower *Nanog* transcript (Fig. 3*A*) and protein levels (Fig. 3*B*) as measured by qPCR and Western blot analysis, respectively. Global histone acetylation was robustly increased (Fig. 3*B*), demonstrating the potent activity of the deacetylase inhibitors. The effect of these inhibitors did not permanently impact pluripotency, because *Nanog* expression could be rescued by drug withdrawal (Fig. 3*C*), and *Oct4* levels were largely unaffected (Fig. 3, *A–C*). In addition, alkaline phosphatase staining and morphology analysis were similar to untreated ES cells (data not shown). To further validate that the reduction in *Nanog*

is p53-independent, we assayed the expression of p53 target genes *p21* and *Mdm2*. Neither of these two targets showed any change in their expression in the TSA-treated ES cells (Fig. 3*D*), highlighting that prevention of deacetylase activity directly leads to the reduction of *Nanog*, independent of p53 activity.

We next examined histone modifications at the *Nanog* and *Oct4* promoters after treatment with deacetylase inhibitors. The *Oct4* promoter still displayed active histone modifications in the presence of deacetylase inhibitors (Fig. 4*A*), consistent with a transcriptionally active locus. Following inhibitor treatment, the *Nanog* promoter showed loss of active histone modifications (Fig. 4*B*), an increase in repressive chromatin modifications (Fig. 4*C*), and complete loss of



**FIGURE 3. HDAC inhibitors reduce *Nanog* gene expression.** *A*, qPCR analysis of *Nanog* and *Oct4* gene expression in the presence of valproic acid (VPA), sodium butyrate (NaBt), and trichostatin A (TSA) for 6 h expressed as -fold change over those untreated. *B*, Western blot showing levels of Nanog, Oct4, Sox2, mSin3A, HDAC1, and HDAC2 protein levels in the presence of TSA, valproic acid, and sodium butyrate for the times indicated. Total acetylated histone H3 levels were analyzed using H3Ac-specific antibodies as a positive control for HDAC inhibitor treatment. Tubulin and total histone H3 were used as loading controls. *C*, qPCR analysis of *Nanog* and *Oct4* mRNA in the presence of 100 ng/ml TSA expressed as -fold change over those untreated. Duration of treatment and washout is indicated at the bottom. *D*, qPCR analysis of *Nanog*, *p21*, and *Mdm2* mRNA in the presence of 100 ng/ml TSA for 4 h expressed as gene expression units relative to glyceraldehyde-3-phosphate dehydrogenase.

Oct4, Nanog, Sox2, mSin3A-HDAC complex, and RNA polymerase II (Fig. 4D). These results indicate that there is an HDAC-sensitive step in the regulation of *Nanog* and that the enzymatic activity of the mSin3A-HDAC complex is critical to *Nanog* expression.

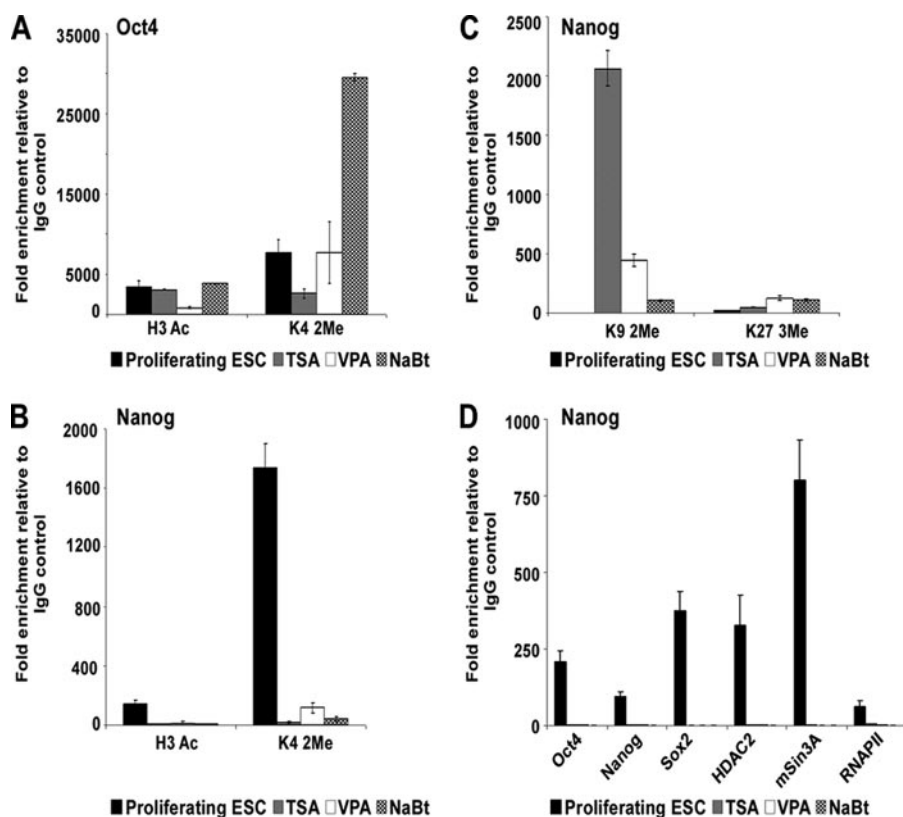
**mSin3A-HDAC Complex Interacts with Sox2 to Positively Regulate Nanog Expression**—Given that deacetylase activity is important for *Nanog* expression, we wanted to determine the target of the deacetylase activity of mSin3A-HDAC complex. We reasoned that it might be a non-histone factor present at the *Nanog* promoter, because the histones at the *Nanog* promoter remain acetylated in the presence of the mSin3A-HDAC complex under proliferating conditions. We focused our analysis on Sox2, because previous studies have shown that activity of SRY, a prototypical member of the *Sox* family, is regulated by HDAC3 during mammalian sex determination (23). A recent study by Liang and colleagues (13) has shown that both Nanog and Oct4 associate with multiple

deacetylase-containing complexes, including NuRD, mSin3A, and Pml, but did not demonstrate the mechanism by which these complexes might regulate Nanog or Oct4 activity. Pull-down experiments using NusA-tagged Sox2 and ES cell nuclear extract showed that Sox2 interacts with the mSin3A-HDAC complex (Fig. 5A). To confirm these observations, we co-immunoprecipitated endogenous Sox2 from nuclear and cytoplasmic fractions of ES cells treated for 6 h with either RA or vehicle control. We confirmed the purity of the nuclear and cytoplasmic fractions using Western blots for histone H3 and  $\alpha$ -tubulin. Sox2 interacted strongly with mSin3A, HDAC1, and HDAC2 in the nuclear fraction of vehicle-treated ES cells (Fig. 5B), and to a lesser extent with mSin3A and HDAC2 in the cytoplasm of vehicle-treated ES cells. Following RA treatment, there was a marked reduction in the robustness of this interaction. These results demonstrate that Sox2 interacts with the mSin3A-HDAC complex *in vitro* and *in vivo*, and this interaction is more prominent under proliferating conditions.

To further confirm the interaction of Sox2 with mSin3A, we performed size-exclusion chromatography on ES cell nuclear extract. We fractionated the nuclear extract into 150 fractions, and probed every 5th fraction for mSin3A and Sox2. Most of the fractions that contained Sox2 also contained mSin3A, confirming that they are part of the large complex (Fig. 5C). We do find some fractions with only mSin3A, or only Sox2, indicating that these two proteins likely have biological functions independent of one another as well.

Because we find both the mSin3A-HDAC complex and Sox2 at the *Nanog* promoter during active transcription of *Nanog*, we wanted to determine if the mSin3A-HDAC complex cooperates with Sox2 to promote *Nanog* expression. To address this, we took advantage of a luciferase reporter system in NIH3T3 cells. These cells do not normally express Oct4, Nanog, or Sox2, which allows us to more finely control for which factors are affecting *Nanog* expression. We engineered three tandem repeats of the Oct4/Sox2 binding sites into the pGL3-TK-Luc reporter vector (Fig. 5D), where the thymidine kinase promoter drives a low level of luciferase expression. This vector, pGL3-O/S-TK-Luc, or its parental

## mSin3A-HDAC Complex Regulates Nanog



**FIGURE 4. Analysis of *Nanog* and *Oct4* promoters in the presence of HDAC inhibitors.** A and B, ChIP analysis for active histone modifications (*H3 Ac* and *H3 K4 2Me*) at the *Oct4* (A) and *Nanog* (B) promoters in the presence of HDAC inhibitors. C, ChIP analysis for repressive histone modifications (*H3 K9 2Me* and *H3 K27 3Me*) at the *Nanog* promoter in the presence of HDAC inhibitors. D, ChIP analysis for transcription and chromatin modifying factors at the *Nanog* promoter in the presence of HDAC inhibitors.

vector was transfected into NIH3T3 cells, along with expression vectors for Oct4 and Sox2. Transfection of pGL3-O/S-TK-Luc together with Oct4/Sox2 expression vectors more than doubled the luciferase activity over the parental vector with Oct4/Sox2 (Fig. 5E), verifying that addition of the binding sites increased Oct4/Sox2-mediated transcription. We next transfected the mSin3A-HDAC complex members into the assay system either with or without Oct4/Sox2. Although the absolute increase in luciferase varied between HDAC1, HDAC2, or mSin3A, the addition of any of the complex members increased luciferase activity ~3-fold over the complex member without Oct4/Sox2. The increased luciferase activity with HDAC1 or HDAC2 compared with mSin3A may be due to interaction of these factors with endogenous mSin3A protein in 3T3 cells. We next added all three mSin3A-HDAC complex members together ( $\pm$ Oct4/Sox2), however we did not see any further increase in luciferase activity compared with that seen with each factor alone. Taken together, these results suggest that the mSin3A-HDAC complex positively regulates *Nanog* expression, and this is enhanced by the presence of Oct4 and Sox2.

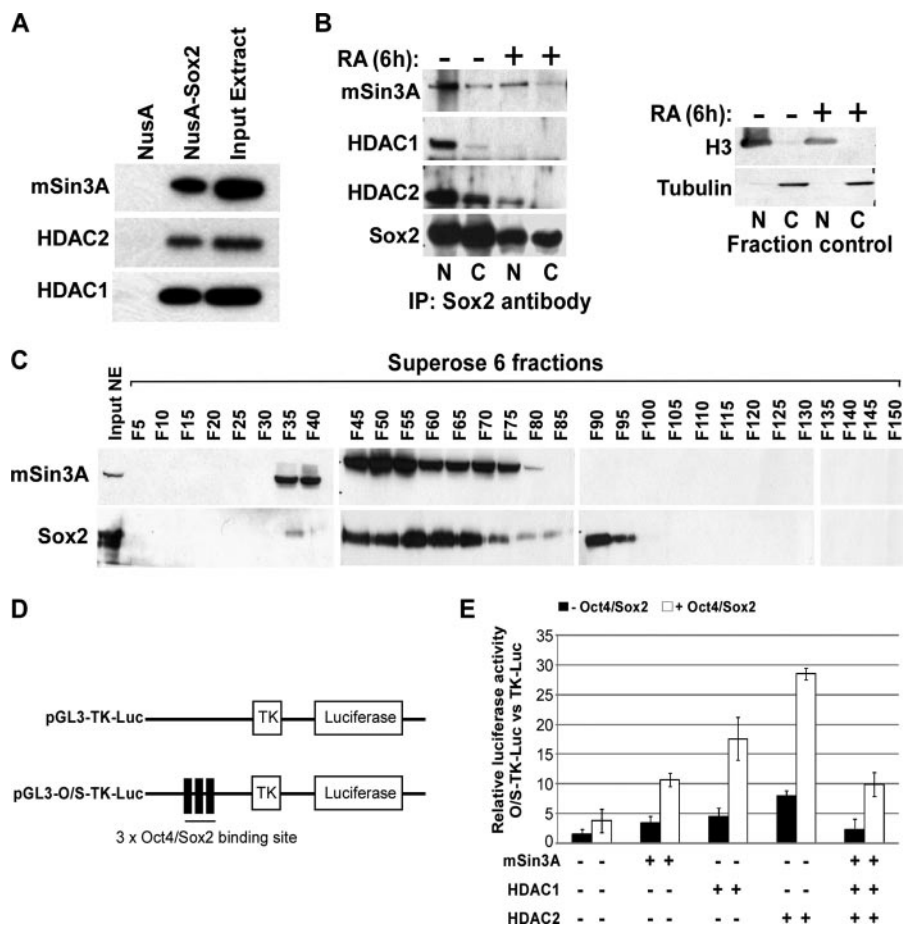
## DISCUSSION

Broad cohorts of proteins are involved in the regulation of *Nanog* (10). We reveal a novel role for the mSin3A-HDAC complex in *Nanog* transcriptional regulation, whereby the mSin3A-

HDAC complex binds to the *Nanog* promoter at the Oct4/Sox2 binding sites, and its occupancy at the promoter correlates with active *Nanog* transcription. Luciferase assays confirm that the mSin3A-HDAC complex increases the transcriptional activity from these binding sites upon co-expression with Oct4/Sox2. This transcriptional activator role of mSin3A is different from its p53-dependent role in *Nanog* silencing (8). When we examined the region of p53 binding site on the *Nanog* promoter, we also observed increased mSin3A occupancy following RA-induced differentiation (Fig. 1F). It is not unexpected that the mSin3A-HDAC complex could operate in different capacities at these distinct sites to positively or negatively regulate *Nanog* expression, perhaps through interactions with specific transcription factors. However, the reduction in *Nanog* expression we see with mSin3A siRNA is independent of the p53 pathway.

Although core histones are the primary substrate of the mSin3A-HDAC complex, increasing evidence points to the importance of regulation of non-histone proteins by this complex (for review, see Ref. 24). Interaction of the mSin3A-HDAC complex with various factors impairs their ability to activate transcription of target genes (25–28). Recent protein interaction studies identified *Nanog* and *Oct4* as novel interactors of transcriptional repressor complexes, including mSin3A-HDAC (12, 13). Wang and colleagues describe both *Nanog* and *Oct4* as interactors of HDAC2 in proliferating ES cells (12), and Liang and colleagues demonstrate that *Nanog* and *Oct4* associate with additional repressor proteins, including mSin3A-containing complexes (13). Our studies focused primarily on the mSin3A-HDAC complex and found that it interacts with Sox2. We show that this interaction can be largely disrupted by inducing differentiation in ES cells. The presence of the mSin3A-HDAC complex at the *Nanog* promoter can also be disrupted by inducing differentiation or by deacetylase inhibitor treatment, suggesting that acetylation may be important in the regulation of these pluripotency factors.

The activities of both histone deacetylases and acetyltransferases have been shown to play a role in ES cell differentiation. Blocking deacetylation of histones using small molecules such as TSA delays the formation of embryoid bodies, indicating that histone deacetylation is necessary for full progression through differentiation (29). However, analysis by McCool and colleagues (30) indicated that there is a



**FIGURE 5. The mSin3A-HDAC complex interacts with Sox2 to cooperatively stimulate *Nanog* transcription.** *A*, *in vitro* NusA-Sox2 pull-down from ES cell nuclear extract. mSin3A-HDAC complex subunits were detected by Western blot. *B*, immunoprecipitation of endogenous Sox2 from nuclear and cytoplasmic fractions of ES cells treated for 6 h with 5  $\mu$ M RA or vehicle. mSin3A-HDAC complex subunits were detected by Western blot using the indicated antibodies. Western blots for H3 and tubulin indicate the purity of the fractions. *C*, fractionation of ES cell nuclear extract. Western blots of every fifth fraction indicate that mSin3A and Sox2 are present in majority of the same fractions. *D*, schematic diagram of the pGL3-TK-Luc and pGL3-O/S-TK-Luc constructs. pGL3-TK-Luc has luciferase driven by the TK promoter. pGL3-O/S-TK-Luc contains three tandem copies of the Oct4-Sox2 binding site from the *Nanog* promoter inserted upstream of the TK promoter. *E*, increase in luciferase activity by the addition of Oct4/Sox2, and mSin3A-HDAC complex members. The graph represents the -fold increase in luciferase activity between pGL3-O/S-TK-Luc and pGL3-TK-Luc after adding the indicated factors.

global increase in acetylation over the course of differentiation of ES cells and that changes in histone acetylation during differentiation vary across different promoters. Additionally, the authors demonstrate changes in gene expression can be seen within 2 h of TSA treatment. In our study, we examine early stages of deacetylase inhibition by assaying levels of pluripotency factors after 3 or 6 h of TSA treatment. We find that one of the earliest events during deacetylase inhibition (3 h) is the down-regulation of *Nanog* expression without a concurrent change in *Oct4* levels, and we believe that this change is tied to the activity of the deacetylase enzymes. We have also found that long term TSA treatment differentiates ES cells (data not shown). There are clearly diverse roles for deacetylases in ES cells, and it will be interesting to see how different genes are regulated by deacetylases during development.

Histone deacetylase enzymes are most commonly associated with the suppression of eukaryotic gene transcription

(for review, see Ref. 31). However, there are a few reports documenting a role for deacetylases in gene activation. For example, Sin3p, the yeast homologue of mSin3A, has been shown to regulate transcriptional activation of *Hog1* target genes by deacetylating target promoters and conferring resistance to osmotic stress (32). YY1 is actively acetylated and deacetylated, and acetylation of its zinc finger domains decreases its ability to bind DNA (33). We observed that the mSin3A-HDAC complex stimulates *Nanog* expression, and we surmise that this effect is mediated by interaction with Sox2. It will be interesting to determine if the mSin3A-HDAC complex is involved in regulating the activity of Sox2. In addition to repressor complexes, recent reports using Chip-seq technology demonstrate that the co-activator CBP/p300 is recruited to genomic sequences bound by clusters of transcription factors that include *Nanog*, *Oct4*, and *Sox2* (34). Depletion of *Nanog*, *Oct4*, and *Sox2* by RNA interference reduced binding of CBP/p300 to these genomic clusters in ES cells. This finding suggests that there is an active balance of HAT and HDAC activity regulating *Nanog* and its targets. Further analysis will be needed to determine how different families of co-activator and co-repressor complexes regulate the pluripotency transcription factor network in response to the cellular environment.

*Acknowledgments*—We thank B. Emerson at the Salk Institute for Biological Studies; A. Baltus, J. Doench, and H. Arthanari at Harvard University for helpful comments; and colleagues at Novartis Institute for Biomedical Research, particularly E. Li, T. Chen, F. Gaudet, and T. Gebuhr, for insightful discussions. The pClG-HDAC1 cDNA was a kind gift from Dr. Ramesh Shivdasani (Dana Farber Cancer Institute).

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