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Smarcc1/Baf155 Couples Self-Renewal Gene Repression with Changes in Chromatin Structure in Mouse Embryonic Stem Cells

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Abstract

Little is known about the molecular mechanism(s) governing differentiation decisions in embryonic stem cells (ESCs). To identify factors critical for ESC lineage formation, we carried out a functional genetic screen for factors affecting Nanog promoter activity during mESC differentiation. We report that members of the PBAF chromatin remodeling complex, including Smarca4/Brg1, Smarcb1/ Baf47, Smarcc1/Baf155, and Smarce1/Baf57, are required for the repression of Nanog and other self-renewal gene expression upon mouse ESC (mESC) differentiation. Knockdown of Smarcc1 or Smarce1 suppressed loss of Nanog expression in multiple forms of differentiation. This effect occurred in the absence of self-renewal factors normally required for Nanog expression (e.g., Oct4), possibly indicating that changes in chromatin structure, rather than loss of self-renewal gene transcription per se, trigger differentiation. Consistent with this notion, mechanistic studies demonstrated that expression of Smarcc1 is necessary for heterochromatin formation and chromatin compaction during differentiation. Collectively, our data reveal that Smarcc1 plays important roles in facilitating mESCs differentiation by coupling gene repression with global and local changes in chromatin structure.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Introduction

Embryonic stem cells (ESCs) are cell lines derived from the inner cell mass (ICM) of blastocyst stage mammalian embryos [1–3]. They can grow indefinitely in culture and give rise to cells of all three embryonic germ layers as well as germ cells [4]. For these reasons, ESCs hold great promise for regenerative medicine. Derivatives of ESCs could provide cells for transplantation therapies, avenues to develop improved diagnostic and pharmaceutical compounds, and model systems for understanding the etiologies of complex diseases. Whereas some molecular details of the ESC self-renewal network have emerged, more in-depth knowledge will be required to facilitate future ESC-based clinical applications.

ESC pluripotency and self-renewal are maintained by a network of transcription factors including Oct4, Sox2, Nanog, Esrrb, Tbx3, Tcl1, Nac1, Dax1, Zfp281, and Zic3, which participate in auto- and cross-regulatory interactions to increase their own expression and that of other self-renewal genes [5–11]. Removal of individual factors collapses the self-renewal network and triggers differentiation to single or mixed embryonic lineages [8–10, 12]. For example, loss of Oct4 expression strongly induces trophectoderm in both ICM and ESCs [13– 17], although loss of Nanog expression triggers the formation of primitive endoderm and mixed epiblast-derived lineages [18]. Thus, the interplay between these transcription factors, and likely others, is required for maintaining the developmental “potency” of ESCs and repressing specific embryonic lineages.

Environmental factors, such as serum, LIF, and BMP4, or bFGF (human ESCs), also play well defined, if poorly understood, roles in controlling ESC self-renewal and pluripotency. Despite considerable knowledge of the underlying molecular pathways, how external signals feed into the self-renewal circuit is largely a mystery. For example, in mouse ESCs (mESCs), the self-renewal transcription factor, Nanog, was, in part, discovered through a forward genetic screen by-passing LIF-Stat3 signaling [12]. But although Nanog overexpression was sufficient for self-renewal in the absence of LIF, Nanog itself is not a direct target of Stat3 [12]. Complicating matters further is that most of the signaling pathways involved in ESC self-renewal can also promote differentiation, such as, LIF-gp130-MAPK-ERK and BMP-TGFβ-Smad [5, 19]. Recent studies have shown that small-molecule inhibition of MAPK-ERK and GSK3 is sufficient for self-renewal and pluripotency in the absence of external signals [19]. Therefore, cooperation and antagonism between signaling pathways is likely a salient feature of self-renewal.

Even less is known about the molecular mechanism(s) regulating differentiation decisions and how the self-renewal circuit is dismantled during differentiation. It has been suggested that the same triad of Oct4, Sox2, and Nanog represses differentiation and lineage-specific gene expression in ESCs [9, 11, 20]. However, this thesis was largely based on transcription factor binding, rather than functional studies. An alternative hypothesis is that these factors contribute to pluripotency by “priming” transcription of lineage-specific genes, specifically

at chromatin regions harboring both repressive and activating histone marks—so-called bivalent regions [21, 22]. The only known physiological repressor of self-renewal gene expression, Tcf3, appears to act as a "dampener" rather than a differentiation- or lineage-specific inhibitor and does not appear to be regulated by differentiation signals [23–25].

To identify factors critical for ESC lineage formation, we carried out an RNAi screen in differentiating mESCs, specifically examining Nanog expression. We find that members of a SWI/SNF sub-complexes, PBAF, are required to repress Nanog expression during mESC differentiation. Mechanistic studies demonstrate that Smarcc1/Baf155 expression is necessary for heterochromatin formation and chromatin compaction during differentiation. These results along with others suggest that SWI/SNF complex members have dual roles in maintaining mESC pluripotency: on the one hand, in promoting self-renewal gene expression [26, 27] and, on the other, providing functions critical for lineage formation.

Materials and Methods

Generation of Nanog-GFP ESCs

To generate NG4 cells, we first "recombineered" a bacterial artificial chromosome (BAC) containing the mouse Nanog locus (RP23-180N22; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) so that the exon one of *Nanog* was replaced with a Hygro-enhanced green fluorescent protein (EGFP) fusion gene followed by a SV40 polyA sequence (Clontech, Palo Alto, CA, http://www.clontech.com). BAC modification was performed according to standard procedures [28]. High-resolution fingerprinting of the original or modified BAC revealed no unexpected deletions or recombinations. The modified BAC was digested with BsiWI and electroporated into wild-type CCE ESCs. Hygromycin resistant GFP+ ESC colonies were isolated and characterized further (see supplemental online Figs. S1 and S2).

ESC Growth and Embryoid Body Formation and Differentiation

ESCs were maintained on gelatin-coated tissue culture plates in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 15% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 1,000 U/ml leukemia inhibitory factor (Millipore, Billerica, MA, http://www.millipore.com). To induce non-retinoic acid (non-RA) mediated neuronal differentiation, cells were cultured in a 1:1 mixture of DMEM/F12 supplemented with N2 and Neurobasal medium supplemented with B27 (Invitrogen).

RNA shRNA and siRNA Transfection

RNA shRNAs were provided by Open Biosystems (Huntsville, AL, http://www.openbiosystems.com) and Operon (Huntsville, AL, http://www.operon.com). ShRNAs were designed using the RNAi oligo retriever (katahdin.cshl.org:9331/RNAi/html/ rnai.html) and were named (e.g., "Oct4_599") to denote target gene and the 5′ nucleotide of the target sequence relative to the transcriptional start site. For the primary screen and follow-up experiments, 20 pmoles of shRNA oligo were mixed with 1 μl Lipofectamine 2000

(Invitrogen) and incubated with a suspension of 5×10^4 mESCs, as described in [29]. After 24 hours, transfected cultures were treated with 2 μM RA for 48 hours and subjected to fluorescence-activated cell sorting (FACS) analysis or western blot analysis. SiRNAs were purchased from Qiagen (Hilden, Germany, http://www1.qiagen.com) and used at 60 pmoles of siRNA per 2 μl Lipofectamine per 5×10^4 cells. GFP expression was quantified using either a FACS sort or LSR II cell analyzer (Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com)

Western Blots

Western Blots were carried out using standard laboratory practices (www.cshprotocols.org), except that a modified RIPA buffer was used for protein extraction (150 mM NaCl, 50 mM Tris, 2 mM MgCl2, .1% SDS, .4% DOC, .4%Triton X-100, 2 mM DTT, and complete protease inhibitors (Roche, Basel, Switzerland, http://www.roche-applied-science.com)) followed by a 15 minutes digestion with 125 units of Benzonase (Merck, Whitehouse Station, NY, http://www.merck.com) at room temperature. The following antibodies were used for blotting at 1:1000 dilution, overnight at 4°C in TBST+3% dried milk: Nanog (Novus Littleton, CO, www.novusbio.com), Oct4 (Santa Cruz, Santa Cruz, CA, http://www.scbt.com), Sox2 (Abcam, Cambridge, U.K., www.abcam.com), Baf155/Smarcc1 (Santa Cruz), and Baf57/ Smarce1 (Santa Cruz).

Immunofluorescence

Each well of a 12-well culture plate was overlaid with glass coverslip and precoated with fibronectin overnight. shRNA oligos (20 pmol) were reverse transfected into 5×10^4 CCE cells as above and treated with 2 μM RA for 48 hours. RNAi treated cells were grown on glass slides and were harvested after 72 hours, fixed with 4% paraformaldehyde and immunostaining performed with antibodies against Oct4 (Santa Cruz sc9081; 1:500) and H3K9me3 (Millipore 07-442; 1:500), indirectly detected with a secondary Alexa-555 donkey anti-rabbit (Molecular Probes 1:1000) antibody. The coverslips were then mounted onto slides using Vectashield (with DAPI) and observed on a Nikon TE2000-U microscope using a Photometrics CoolSNAP charge-coupled device camera and MetaVue software. Images were analyzed with the ImageJ software (http://rsb.info.nih.gov/ij/) using the WCIP plug-in compilation (www.uhnresearch.ca/WCIF) as previously described [30]. Median filtering (two pixels radius) was used to reduce noise in the presented images. The foci of a nucleus were identified using the “Nucleus Counter” in the ImageJ plug-in, which was set to (1) exclude regions below a size of 20 pixels, (2) use a “maximum entropy threshold”, (3) use a “3 × 3 pixels median smoothing”, and (4) use a “watershed algorithm” that divides neighboring regions. The intensity and standard deviation of the signal of interest was measured inside each foci using the “multi measure” ImageJ plug-in. Mann-Whitney U statistical tests were performed at http://elegans.swmed.edu/~leon/stats/utest.html.

Micrococcal Nuclease Digestion and Southern Blot—Undifferentiated or RA-treated mESCs were harvested and counted (Beckman Coulter particle counter). Chromatin was isolated as previously described [31] from three different samples of each experimental condition consisting of 3.3×10^6 cells. Each sample was treated with 1/40 unit of micrococcal nuclease enzyme (Sigma, St. Louis, http://www.sigmaaldrich.com) for 2, 5, or

10 minutes at 37°C, and stopped with 1 mM EGTA. Samples were centrifuged for 10 minutes at 10K RPM, and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen). Equal amounts of DNA were resolved on 1% agarose gel, and Southern blots were performed using ^{32}P-labeled probes specific for either the Nanog or Oct4 promoter.

Chromatin Immunoprecipitation and Quantitative PCR

Chromatin immunoprecipitation (ChIP) was performed as previously described [32] with minor modifications. Approximately 1×10^8 mESCs were chemically cross-linked with 1% formaldehyde solution for 10 minutes at room temperature with gentle agitation and subsequently quenched with 0.125M glycine. Cells were rinsed twice with 1x phosphate buffered saline, harvested, flash frozen, and stored at −80°C prior to use. Cells were resuspended, lysed, and sonicated to solubilize and shear cross-linked DNA. The resulting whole cell extract was incubated overnight at 4°C with 100 μl Dynal Protein G magnetic beads preincubated with 10 μg of the appropriate antibody for at least 3 hours. Antibodies used for ChIP in this study are α-trimethyl-Histone3 Lys27 (Upstate; 07-449), α-BAF155 (Santa Cruz; sc10756x), and α-Oct4 (Santa Cruz; sc8628x). Beads were washed five times with RIPA buffer and once with TE containing 50 mM NaCl, and complexes were eluted from beads in elution buffer by heating at 65°C and shaking in a Thermomixer. Reverse cross-linking was then performed overnight at 65°C. Whole cell extract DNA (reserved from sonication) was concurrently treated for cross-link reversal. Immunoprecipitated and whole cell extract DNA were treated with RNaseA, proteinase K and purified using the Qiagen polymerase chain reaction (PCR) purification kit.

Quantitative PCR (qPCR) analyses were performed using the StepOne Plus Real-Time PCR System and Fast SYBR Green Master Mix (Applied Biosystems) following manufacturers' protocol. No PCR products were observed in the absence of template. Fold enrichment was determined by the 2-ΔCT method as described in [33], normalized to the level observed in a beads-only control. The primers used in ChIP-qPCR are as follows: Nanog promoter region 1: AAACCAAAGCATGGACCAAC and GACCTTGCTGCCAAAGTCTC, region 2: ACCTACCCTTTA AATCTATCGCCT and CTTCCTCAGAACTAGGCAAACTGT, region 3: CCGAAGAACCCAGTAAATTAGG and AGTGATAA GGACACCCGCTT, region 4: TAGTCTGAAATAGAGATCC GGGAC and AGGTTGAGAGAAATGCTAACTGCT; Oct4 promoter region 1: GGAACTGGGTGTGGGGAGGTTGTA and AGCAGATTAAGGAAGGGCTAGGACGAGAG; region 2: TGCTCTGGGCTTTTTGAGGCTGTGTGATT and TGGCGGAA AGACACTAAGGAGACGGGATT; region 3: AGCAACTGGTTTGTGAGGTGTCCGGTGAC and CTCCCCAATCCCACCCTCTAGCCTTGAC.

Results

An RNAi Screen for Modifiers of Nanog Expression in Mouse ESCs

We first engineered a murine Nanog-GFP ESC line (NG4) (supplemental online Figs. S1 and S2). The NG4 line reports endogenous Nanog promoter activity in undifferentiated mESCs, in NG4-derived primordial germ cells, and also during ESC differentiation where

GFP levels drop commensurate with endogenous Nanog mRNA and protein levels (supplemental online Fig. S1; Fig. 1C) [12, 18]. We compiled a shRNA "pilot" library targeting 312 gene-products, each with at least two hairpins. The shRNA pilot set was enriched for gene targets involved in chromatin regulation, early developmental signal transduction, and transcription (supplemental online Table S1). Individual shRNAs were introduced into NG4 cells, and GFP levels were measured after treatment with 2 μM retinoic acid (RA) (Fig. 1A). RA treatment strongly attenuates Nanog expression and promotes neural differentiation in ESCs [8, 34]. We chose to perform the screen in RA because this should reveal gene-products that act to promote, as well as inhibit, a differentiation process. During normal growth conditions, 85–90% of NG4 cells are GFP positive, whereas after RA treatment this number is reduced to 30–50% (depending on cell density). Control experiments targeting GFP or Oct4 demonstrated that, with effective shRNAs, silencing can be observed in >90% of the cells (Fig. 1B–1D) [29]. We decided to operationally designate a gene-product a Nanog "activator" when at least two hairpins targeting the gene resulted in decreased GFP expression in more cells than the best Nanog-shRNA, as well as decreased levels of endogenous Nanog protein. On the other hand, gene-products where targeting with multiple shRNAs resulted in maintenance of GFP levels above the level seen for the strongest RA-receptor alpha (RARα) shRNA, as well as increased levels of endogenous Nanog, were designated as Nanog "repressors". Figure 1E shows the complete results of the screen. Confirming the validity of our screening approach, many previously and more recently identified molecules required for ESC self-renewal are found in the "activator" category. These include gene-products involved in the Lif-Jak-Stat3, Bmp-Smad4, and Wnt-Ctnnb1 signaling pathways, as well as the transcription factors, Esrrb, Oct4, Sall4, Sox2, and Rif1 [8–10, 35–37]. In the "repressor" category (Table 1), we find a direct, physiological repressor of Nanog, Tcf3, negative regulators of Stat3, Ctnnb1, and Smad4 signaling, and Raf1, which has known roles in mESC differentiation [23]. In this set of genes are also four core members of the Brg1/Brm-associated SWI/SNF chromatin remodeling complex, Baf190/ Brg1, Baf155/Smarcc1, Baf57/Smarce1, and Baf47/Smarcb1. Importantly, these hits were confirmed in secondary reporter assays and in Western blot analysis (Fig. 1F and 1G). The latter results demonstrate that these genes are required for RA-dependent down-regulation of Oct4, Nanog, and Sox2.

Functional Validation of Smarcc1/Baf155 and Smarce1/Baf57

In eukaryotes, the SWI/SNF chromatin remodeling complex plays key roles in a variety of gene regulatory phenomena, ranging from mating type switching in yeast to neural development in mammals. Our results suggested SWI/SNF mediated repression may play a key role in down-regulating Nanog and other components of the pluripotency network upon RA-induced differentiation. We chose to further explore its function in repressing self-renewal gene expression. We first validated multiple RNAi triggers targeting the top two scoring SWI/SNF subunits, Smarcc1/Baf155 and Smarce1/Baf57. Figure 2A shows that multiple Smarcc1 shRNAs elicit the same effect on Nanog expression. Interestingly, knockdown of Smarcc1 resulted in attenuation of Smarce1 expression and vice versa. This result is consistent with the observation that uncomplexed SWI/SNF proteins are unstable and subject to proteasome-mediated degradation [38, 39]. To further examine the phenotype of Smarcc1, we carried out a hygromycin resistance (hygroR) assay using NG4 cells, which

express GFP and hygroR gene from the Nanog promoter. In this assay, RA and hygromycin are administered simultaneously, resulting in differentiation and hygro sensitivity in control cells (Fig. 2B). Knockdown of Smarcc1, however, resulted in hygroR similar to non-treated controls, demonstrating that Smarcc1 knockdown de-represses the Nanog promoter while being growth permissive. The latter observation was supported by other measures, including dye retention, cytoplasmic reductive potential, and total protein accumulation, all of which were similar to control cells (data not shown). This was not true for all PBAF subunits; knockdown of Brg1 or Actl6 knockdown resulted in observable growth defects (data not shown).

Alkaline phosphatase (AP) staining, which is associated with pluripotency, was also performed for RA-treated mESCs. Knockdown of Smarcc1 or Smarce1 allowed cells to retain AP activity upon RA-treatment (Fig. 2C; not shown). Taken together, these results confirm that Smarcc1 knockdown prevents down-regulation of self-renewal gene expression upon RA treatment and does not diminish proliferative capacity within the confines of this assay.

We next sought to examine whether other SWI/SNF subunits not represented in our initial screen set would also share similar functions during RA treatment in ESCs. Mammalian cells contain at least three distinct SWI/SNF chromatin remodeling complexes: BAF-A, BAF-B, and PBAF. All three complexes share eight common subunits, including Brg1/ Baf190/Smarca4 (or Brm/Baf190), Baf170/Smarcc2, Baf155/ Smarcc1, Baf60a/Smarcd1, or Baf60b/Smarcd2, Baf57/ Smarce1, Baf53a/Actl6a, actin, and Baf47/Smarcb1 [40]. The SWI/SNF complexes are distinguishable by the presence of unique subunits. BAF-A and BAF-B each contain one additional subunit, Baf250a/Arid1a or Baf250b/Arid1b, respectively, and either a Brg1 or Brm catalytic core. PBAF harbors two additional subunits, Baf200/Arid2 and Baf180/Pbrm1, and only the Brg1 catalytic core [40–42]. All three complexes exhibit chromatin remodeling activity in vitro [43]. Recently, Baf250a and Baf250b were implicated in ESC self-renewal and embryoid body (EB)-derived cardiomyocyte and adipocyte differentiation [44, 45]. We rescreened all 15 known SWI/SNF subunits using independently designed RNAi triggers (Fig. 2D). Consistent with our initial screen, introduction of siRNAs targeting Baf190/Brg1, Baf155/Smarcc1, Baf57/ Smarce1, and Baf47/Smarcb1 into NG4 cells resulted in higher Nanog promoter activity during ESC differentiation. Baf200/Arid2, a unique subunit of PBAF, showed a similar trend.

siRNAs targeting other subunits had little or no effect on Nanog expression. On the basis of control experiments with non-targeting siRNAs, changes of <20% in GFP expression are unlikely to be significant (Fig. 2D; not shown). These results support a role for only select SWI/SNF subunits in Nanog promoter repression during ESC differentiation and implicate the PBAF SWI/SNF complex in repression of self-renewal gene expression in ESCs.

Smarcc1/Baf155 and Smarce1/Baf57 Are Generally Required for mESC Differentiation

Since SWI/SNF is known to be a cofactor for numerous transcription factors, including the RA receptor [46], we next assayed Smarcc1 and Smarce1 knockdown using non-RA forms of mESC differentiation. Figure 3A shows GFP expression levels in NG4 cells using an N2B27-mediated neural differentiation procedure, which promotes the formation of Sox1+

neuroprogenitors [36]. After 4 days in N2B27 media, Nanog promoter activity remained high in Smarcc1 and Smarce1 knockdown condition whereas shRNAs targeting Esrrb and GFP showed the opposite effects, demonstrating that the effect on Nanog expression can be observed independently of RA treatment.

We next assayed the Smarcc1 knockdown phenotype by experimentally reducing the levels of indirect or direct activators of Nanog expression, including Stat3, Esrrb, and Oct4. Experimental reduction of any of these factors causes differentiation [8, 9, 14, 15, 35]. For Stat3 and Esrrb assays, we carried out combinatorial RNAi experiments in NG4 cells. Targeting Stat3, a transducer of Lif/Lifr signaling by shRNAs results in enhanced down-regulation of the Nanog reporter (Fig. 1E and Fig. 3B). Conversely, knockdown of Socs3, a negative regulator of Stat3 activation, increases Nanog expression upon RA treatment (Fig. 1E; Table 1). Since the effect on Nanog expression of Socs3 knockdown should be mediated by Stat3 activation, simultaneous knockdown of Stat3 should effectively block this phenotype. This is indeed the case, as shown in Figure 3B. Combined knockdown of Socs3 and Stat3 produces the same result as Stat3 knockdown alone. In contrast, combined knockdown of Smarcc1 and Stat3 suppresses the effect of Stat3 (Fig. 3B). The same set of experiments was carried out targeting Esrrb, a key mediator of ESC self-renewal, pluripotency, and Nanog expression [8, 9]. The results were identical to the Stat3 experiment; Smarcc1 knockdown could overcome the effects of loss of Esrrb expression, whereas Socs3 knockdown could not (Fig. 3C). Knockdown data for Esrrb, Socs3, and Stat3 is available in supplemental online Figure S3. Control combinatorial experiments using two highly potent shRNAs in NG4 cells demonstrate little if any dilution of gene knockdown in our conditions (supplemental online Fig. S3D).

We next extended this assay to examine loss of Oct4 expression using a doxycylin (Dox)-repressible Oct4 mESC line. Oct4 is a direct and potent activator of the Nanog promoter [47]. In Oct4 deficient ESCs, expression of Nanog and other self-renewal genes is quickly attenuated as cells adopt a trophectoderm-like fate [15, 16]. Figure 3 shows that, in the absence of Oct4, Smarcc1 knockdown can delay or reverse the loss of AP activity. By contrast, knockdown of Esrrb had the opposite effect, exacerbating loss of Oct4 expression. Figure 3F, which displays Nanog and Oct4 expression patterns in the same series of experiments, indicates that knockdown of Smarcc1 or Smarce1 also delays or reverses loss of expression of Nanog when Oct4 is absent. Similar results were seen in NG4 cells using combined knockdown of Oct4 and Smarcc1 (data not shown). Importantly, knockdown of Smarcc1 or Smarce1 does not noticeably affect the expression of Nanog or Oct4 in undifferentiated (i.e., Dox-) cells.

We conclude from these experiments that knockdown of Smarcc1 blocks or delays self-renewal gene repression in response to any differentiation stimulus, including the experimental removal of key Nanog transcriptional activators. Consistent with this notion, induction of lineage-specific genes was also blocked by Smarcc1 knockdown during embryoid-body differentiation (Fig. 3G).

Smarcc1/Baf155 is Required for Global and Local Changes in Chromatin Structure during RA-Induced Differentiation of mESCs

Given that SWI/SNF functions to affect changes in chromatin structure in eukaryotes, we pursued the hypothesis that Smarcc1-dependent changes in chromatin structure are driving mESC differentiation and that, in the absence of this activity, self-renewal genes could not be properly repressed. To test this notion, we asked whether epigenetic changes associated with ESC differentiation were affected by Smarcc1 knockdown. To this end, we first investigated changes in histone H3K9me3 foci formation in RA-treated mESCs. This histone mark is required for the recruitment of heterochromatin protein one (HP1) to chromatin for the establishment and maintenance of heterochromatin [48, 49] and is highly enriched at sites of heterochromatin in nuclei. RA-treated mESC nuclei yielded increased numbers of H3K9me3 foci (which overlap with formation of intense DAPI “blobs”), consistent with the notion that chromosomes become increasingly “heterochroma-tinized” during lineage formation [50]. Knockdown of Smarcc1, however, significantly reduced foci formation, relative to GFP (shown) and Esrrb control knockdown (Fig. 4A and 4B; supplemental online Fig. S4) (Mann-Whitney U test; $p < .0001$). Other quantifiable staining parameters remained unaltered, including the nuclear morphology (i.e., nuclei area) and foci intensity and size (data not shown). These results suggest that Smarcc1 is required for changes in global hetero-chromatin patterning during ESC differentiation.

We next investigated Smarcc1-dependent changes in chromatin structure at the Nanog and Oct4 promoters. Micrococcal nuclease (MNase) Southern blot assays were performed for both promoters. In these assays, relative changes in chromatin structure are revealed through changes in MNase digestion patterns. Compact chromatin blocks enzymatic processing, resulting in higher molecular weight bands, while “open” chromatin promotes digestion, increasing the production of lower molecular weight bands and nucleosome laddering. Figure 4C shows MNase-Southern blot results for the Nanog and Oct4 promoters during RA treatment in the presence or absence of Smarcc1 knockdown. In the control lanes, it is clear that RA treatment causes chromatin compaction of these loci (RA- compared to RA+ GFP shRNA) (Fig. 4C); RA-treated chromatin requires prolonged enzyme treatment to produce a high-mass-shifted nucleosome ladder (also see supplemental online Fig. S5). In contrast, knockdown of Smarcc1 produces low-mass-shifted nucleosome laddering at the 5-minute time point and a single mononucleosomal band at 10 minutes, indicative of an “open” chromatin structure. Importantly, the input material for this assay was controlled so that equal numbers of nuclei were added to each MNase reaction, and the results were confirmed in two independent experiments. Further examination of the EtBr stained gel used for MNase-Southern blot (Fig. 4C) revealed the persistence of low molecular weight species of DNA in RA-treated cells harboring knockdown of Baf155. This indicates that a large fraction of the mESC genome, which includes Nanog and Oct4 loci, fails to undergo RA-induced compaction in the absence of Baf155 activity.

To determine whether the effects could be direct, Smarcc1 chromatin binding studies were carried out at the Nanog and Oct4 promoters (Fig. 5A–5D). Smarcc1 showed enrichment similar to Oct4 at both of these promoters in the presence or absence of RA. We conclude

that Smarcc1 is bound to both promoters during RA differentiation and that Smarcc1 is required for RA-driven changes in chromatin structure at these and other loci.

We further investigated another epigenetic mark associated with gene repression at the Nanog and Oct4 loci, histone H3K27me3. This mark is deposited by the Polycomb group complex (PcG) and is associated with transcription repression during development. RA treatment led to dramatic increases in H3K27me3 at both the Nanog and Oct4 promoters (Fig. 5E). However, H3K27me3 deposition could be partially reversed by Smarcc1 knockdown. Thus, Smarcc1 depletion interferes with PcG-dependent patterning during mESC differentiation. Since PcG and SWI/SNF are not known to interact, this result likely indicates that Smarcc1-dependent changes in chromatin structure affect PcG activity. Supporting this notion, H3K27me3 is not associated with Nanog promoter repression during in vitro trophectoderm lineage formation (supplemental online Fig. S6), yet knockdown of Smarcc1 or Smarce1 is nonetheless sufficient to block down-regulation of Nanog expression (Fig. 3F). Taken together, these results demonstrate that Smarcc1 is required for the occurrence of multiple epigenetic events as mESCs differentiate and that, in their absence, mESCs are unable to properly repress self-renewal gene expression and commit to specific lineages.

Discussion

The homeodomain transcription factor Nanog plays a central role in maintaining the pluripotency of ESCs and is exclusively expressed in pluripotent cells, that is, ESCs or primordial germ cells [12, 18]. To gain further insight into ESC pluripotency networks, we carried out a loss-of-function RNAi screen in a Nanog-GFP ESC reporter line. We identified several novel activators and repressors of ESC self-renewal gene expression. Chief among them were core members of the SWI/SNF complex. In eukaryotes, the SWI/SNF complex uses the energy from ATP hydrolysis to alter chromatin structure and transcriptional regulation by affecting interactions between chromatin and chromatin-bound nucleosomes [51, 52]. SWI/SNF can “open” or “close” chromatin to transcription factors, basal transcription machinery, and histone modifying enzymes [51, 52]. SWI/SNF function has been linked to diverse gene regulatory phenomena in eukaryotes, ranging from mating type switching in yeast to neural development in mammals [51–53]. We uncovered new roles for SWI/SNF in differentiating ESCs in repressing self-renewal genes expression and, more broadly, in affecting chromatin compaction and heterochromatin formation as ESCs become lineage restricted (Fig. 5F). In all available forms of ESC differentiation, including RA treatment, neural (non-RA) (Fig. 3A), removal of Nanog activators (e.g., Stat3 and Esrrb) (Fig. 3B and 3C), trophectodermal (Fig. 3D–3F) and EB culture (Fig. 3G), knockdown of specific Brg1 and Brg-associated subunits resulted in retention of Nanog promoter activity and, where tested, Nanog protein levels (Figs. 1G and 3F). Given SWI/ SNF’s ability to act as a transcriptional co-activator or co-repressor in mammals, two hypotheses seemed plausible. First, SWI/SNF activates differentiation-specific gene products, which, in turn, repress Nanog expression (e.g., RA-dependent transcription), or second, SWI/SNF itself directly represses Nanog and other self-renewal gene expression. However, since knockdown of Smarcc1/Baf155 or Smarce1/Baf57 could reverse or delay the loss of Nanog expression in the absence of key self-renewal activators, we wondered whether these phenotypes were

the result of loss of changes in chromatin structure that normally occur during ESC differentiation, including condensation of heterochromatin into distinct foci [50]. Our results are consistent with a role for Smarcc1 in global and local reorganization of chromatin. Smarcc1 knockdown blocked global histone H3K9me3 foci formation (Fig. 4A and 4B), which is associated with the formation of heterochromatin, and chromatin compaction at the Nanog and Oct4 promoters (Fig. 4C), which normally occur during differentiation.

However, two recent studies characterizing two SWI/SNF subunits in mESCs, Baf250a and Baf250b, at first glance, are at odds with the notion that SWI/SNF is a repressor in ESCs [44, 45]. These subunits are uniquely found in two SWI/SNF subcomplexes, BAF-A and BAF-B, respectively, and represent two of the three distinct SWI/SNF complexes found in mammals (the other being PBAF) [40, 54]. All three complexes share common subunits, including Brg1/Baf190 (or Brm/Baf190), Baf170, Baf155, Baf60a, Baf57, Baf53a, actin, and Baf47. PBAF differs from BAF-A and BAF-B in containing two additional subunits, Baf200/Arid2 and Baf180/Pbrm1 [40, 41, 54]. In mESCs, deletion of Baf250a compromises un-differentiated growth, affecting expression of Oct4 and Sox2 (but apparently not Nanog), and blocks the formation of ESC-derived adipocytes and cardiomyocytes [45]. Similarly, deletion of Baf250b in ESCs results in defects in self-renewal, accompanied by reduced levels of Nanog and Oct4, and premature differentiation [44]. On the one hand, ESCs treated with Brg1 siRNAs show a marked reduction in their proliferation and neural differentiation ability, which is in agreement with our observation [55]. However, our knockdown of Smarcc1 did not diminish the proliferative capacity of mESCs in our assays, possibly reflecting differences in level of knockdown, assay, and/or subunit utilization. Two additional studies found that acute depletion of Brg1, on the other hand, results in an initial up-regulation of pluripotency genes in blastocyst and ESCs, including Nanog and Oct4, followed by a secondary defect in self-renewal and pluripotency [26, 27]. In one of these studies, Crabtree and colleagues concluded from chromatin immunoprecipitation and gene expression analyses that, paradoxically, Brg1/ Smarca4 containing esBAF acts both in opposition to and in coordination with the ESC core circuitry (Oct4/Sox2/Nanog) [56]. As such, esBAF acts as a repressor to refine and maintain correct levels of ESC-specific genes, similar to what is proposed for Tcf3 [23, 24], and in coordination with Oct4/Sox2 as a repressor to prevent expression of differentiation-associated genes. In our scheme, Baf250a and Baf250b did not score as repressors of Nanog expression, while Brg1 and other Baf-associated factors did. Our results suggest that these differences reflect underlying differences in biological functions among SWI/SNF complexes in mESCs as cells differentiate. In particular, our results support roles for SWI/SNF in global conformational changes in chromatin during differentiation, which permit the collapse of the self-renewal circuit and the establishment of heterochromatic regions characteristic of somatic cells. However, this raises several questions. How does such a global conformational change occur? What are the specific triggers? How is SWI/SNF regulated and localized to specific regions of chromatin? Can all phenotypes be explained by SWI/SNF-dependent activities (e.g., nucleosome eviction, sliding, etc.)? These questions await further experimentation. In summary, we propose that SWI/SNF acts as an important and general coordinator of changes in ESC fate and perhaps as an integrator of several distinct modes of epigenetic regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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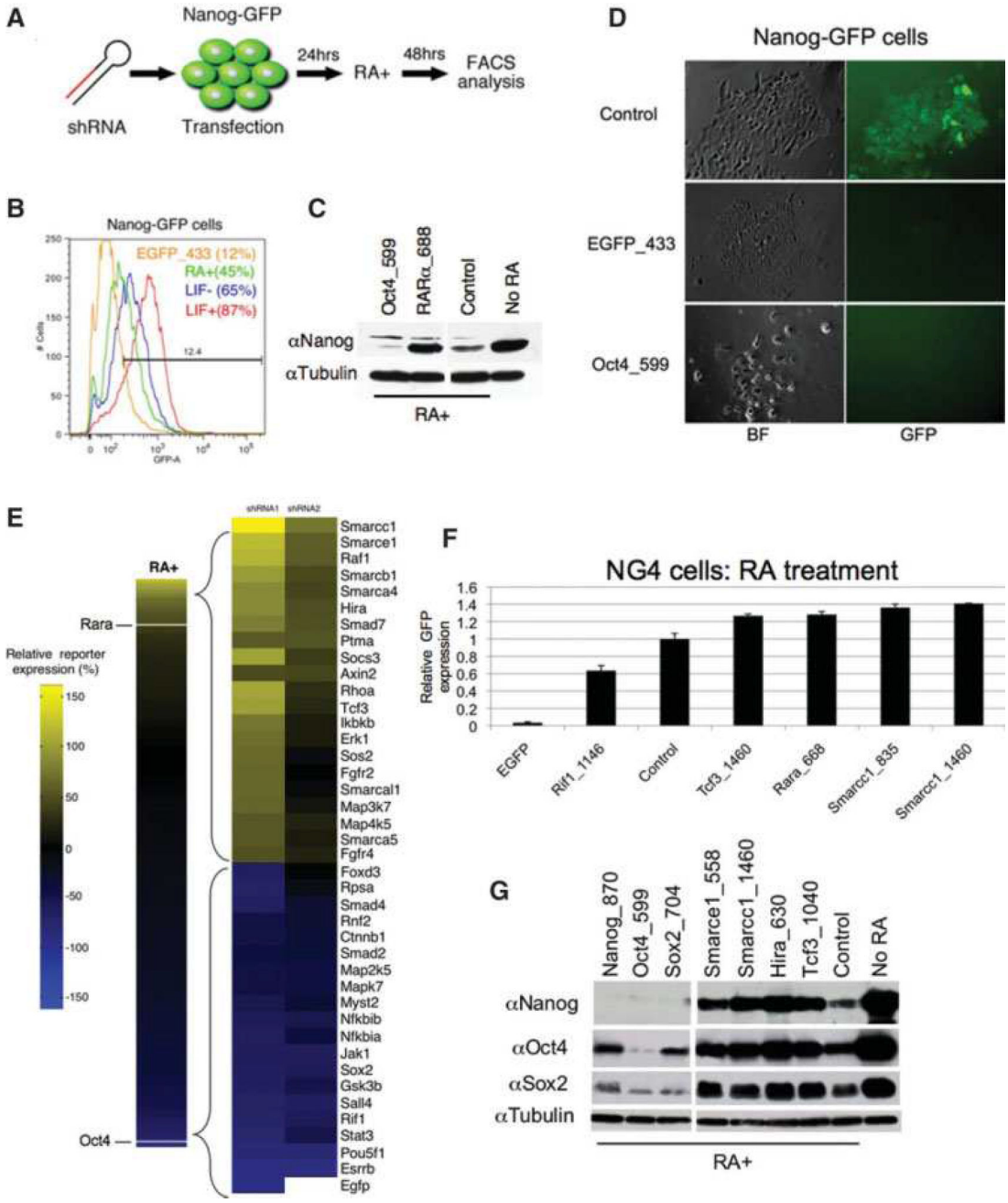


Figure 1. An RNAi screen for modifiers of Nanog expression in mouse embryonic stem cells

(**A**) Outline of the screening procedure. (**B**) FACS profiles of NG4 cells in self-renewal conditions (LIF+), after 48 hours of LIF withdrawal, after 48 hours of treatment with 2 μM RA, or 48 hours post-treatment with an shRNA targeting GFP. (**C**) Western blots for Nanog expression using positive and negative control shRNAs during RA treatment. (**D**) A representative experiment using GFP and Oct4 shRNAs in the screening procedure (100x magnification). (**E**) Total screen results from 2 μM RA treated (RA+) NG4 cells. 40 genes are shown on the right and are ranked by the performance of two independent shRNAs and whether they scored above the RARα for "repressors" or Nanog for the "activators". (**F**)

Examples of select screen positives using the screen assay in NG4 cells ($n = 3$). (**G**) Western blot analysis of self-renewal gene markers Nanog, Oct4, and Sox2 after shRNA triggers against the indicated genes in wt CCE mESCs. Abbreviations: EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; NG4, Nanog-GFP ESC line; RA, retinoic acid; RARα, retinoic acid receptor alpha.

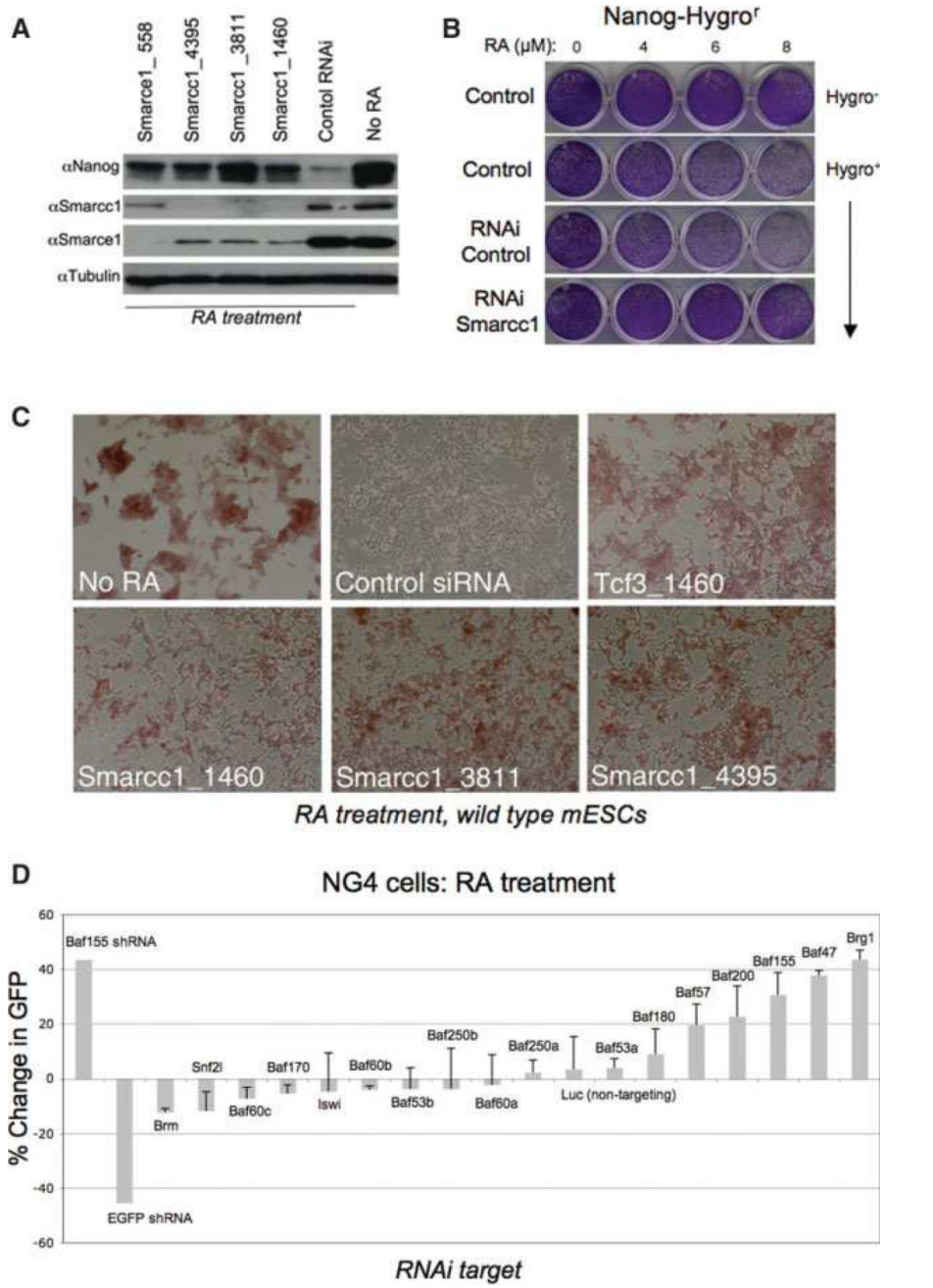


Figure 2. Confirmation of the phenotypic effects of Smarcc1/Baf155 knockdown

(**A**) Nanog, Smarcc1/Baf155, and Smarce1/Baf57 protein levels after knockdown of Smarcc1, Smarce1, or scrambled RNAi control 48 hours post-RA treatment. (**B**) A mouse embryonic stem cell (mESC) self-renewal assay utilizing the hygromycin resistance of NG4 cells. NG4 cells, which express a green fluorescent protein (GFP)–hygomycin fusion gene from the Nanog promoter, were treated with hygromycin (250 μg/ml) and various concentrations of RA in combination with knockdown of Baf155. Cells were stained with crystal violet 4 days post treatment to assess relative outgrowth. RA-induced down-regulation of the Nanog reporter results in hygromycin sensitivity in the control cells. (**C**)

Alkaline phosphatase staining of wt CCE mESCs with knockdown of Smarcc1/Baf155 and Tcf3 during RA treatment. (**D**) Functional specificity of SWI/SNF subunits in mESCs. siRNAs targeting 15 known subunits of mammalian SWI/SNF were introduced into NG4 cells [40, 54]. One day post-transfection cells were treated with 2 μM RA and assayed 48 hours later for GFP expression. Controls include shRNAs targeting Smarcc1/Baf155 from the initial screen and GFP and a non-targeting Lucifer-ase siRNA. Experimental values are the average of three independent siRNA transfections. Abbreviations: GFP, green fluorescent protein; NG4, Nanog-GFP ESC line; RA, retinoic acid.

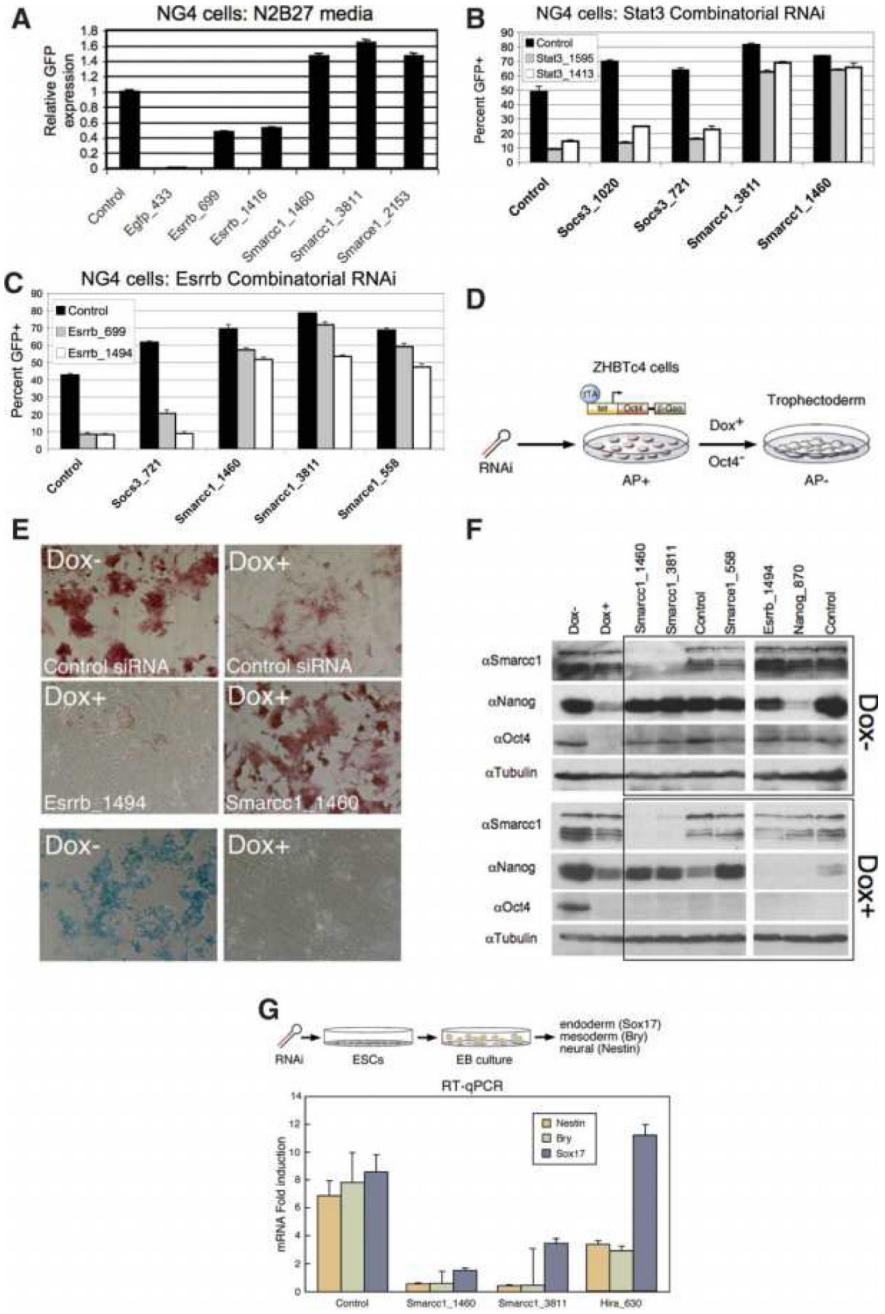


Figure 3. Smarcc1/Baf155 and Smarce1/Baf57 are generally required for down-regulating Nanog during mouse embryonic stem cell (mESC) differentiation

(**A**) Non-retinoic acid (non-RA) induced neural differentiation of NG4 cells using N2B27 media. NG4 cells were carried through the screening procedure in Figure 1A except that N2B27 was substituted for RA [36]. (**B–C**) Differentiation tests in NG4 cells using knockdown of Stat3 (**B**) or Esrrb (**C**) to down-regulate Nanog expression. NG4 were co-transfected with 15 pmoles of control, Socs3, Smarcc1, or Smarce1 in combination with 15 pmoles of hairpins targeting Stat3 or Esrrb. Figures show relative green fluorescent protein (GFP) expression after RA treatment. Similar results were seen with Jak1 (not shown). (**D–F**) Differentiation tests using loss of Oct4 expression to down-regulate Nanog expression.

(**D**) Experiments were carried out in an mESC line with doxycylin (Dox)-repressible Oct4 expression (ZHBTc4 cells; [15]). 24 hours post-transfection cells were treated with 1 μM of Dox for 48 hours. (**E**) Alkaline phosphatase staining (upper panels) of ZHBTc4 cells treated with Dox (Dox+) in combination with control siRNA, Esrrb or Smarcc1 shRNAs. Similar results were seen with other control shRNAs targeting GFP and Agouti and two additional Smarcc1 shRNAs (not shown). The bottom panels show a parallel, control experiment showing LacZ staining, which reports Oct4 expression, with and without Dox treatment. (**F**) Western blot analysis of ZHBTc4 cells treated with various RNAi triggers in self-renewing conditions (Dox-) and after 48 hours of Dox treatment (72 hours post-transfection). Note that Nanog expression was not noticeably affected by Smarce1 or Smarcc1 knockdown in the Dox- condition. (**G**) Quantitative PCR analysis of 4-day old embryoid bodies treated with control, two Smarcc1, and one Hira shRNA. The data is normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Abbreviations: Dox, doxycylin; EB, embryoid body; ESCs, embryonic stem cells; GFP, green fluorescent protein; NG4, Nanog-GFP ESC line; RT-qPCR, reverse transcription–quantitative polymerase chain reaction.

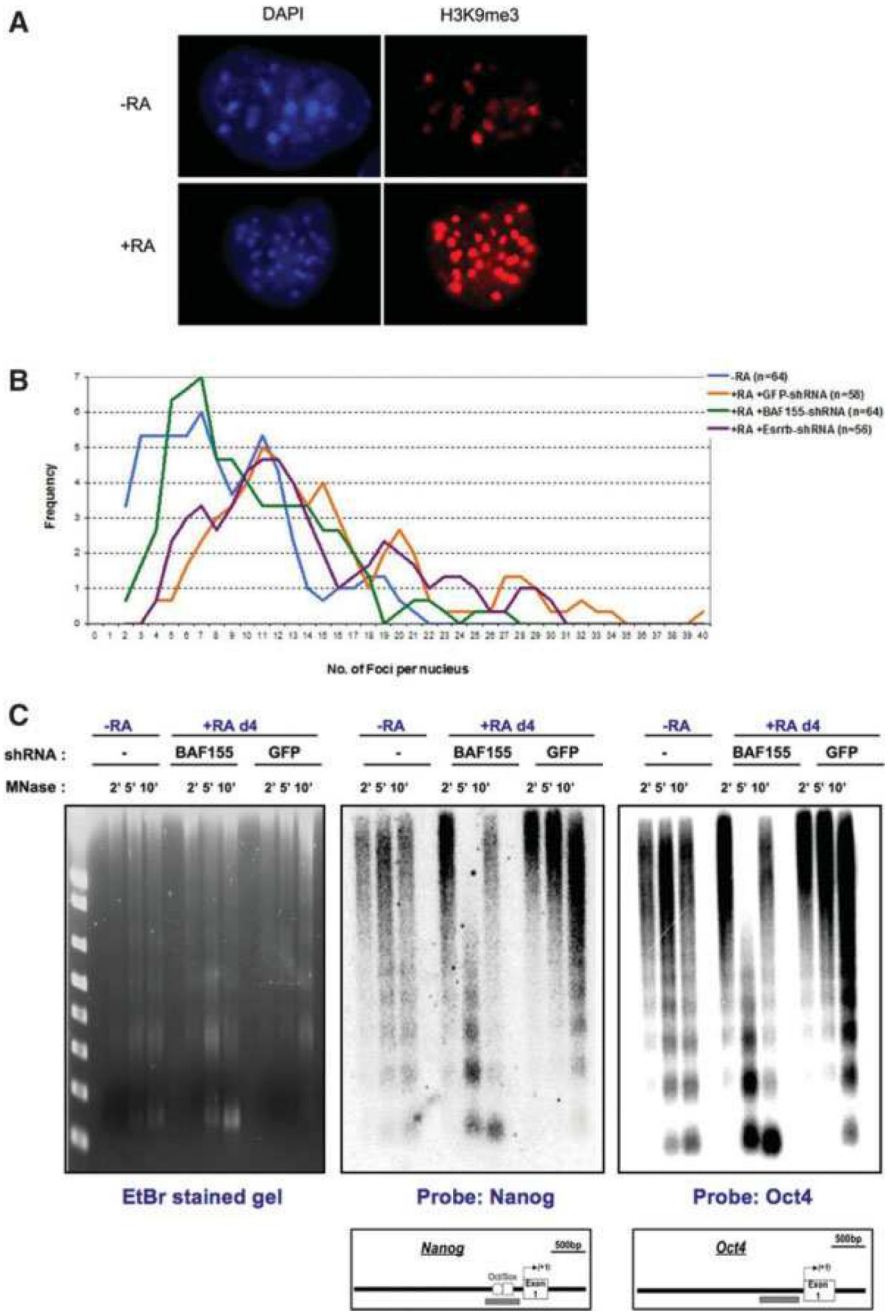
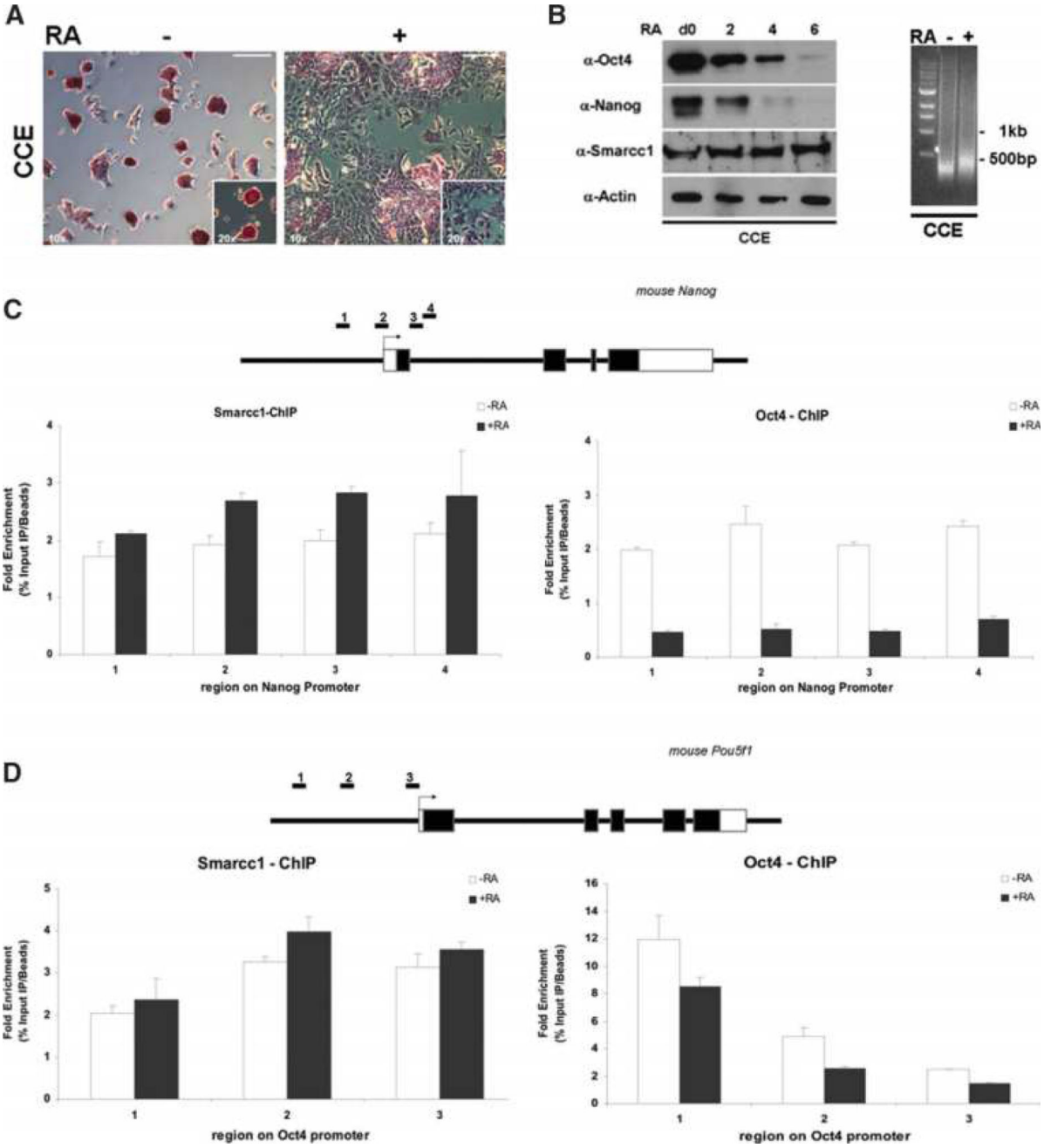


Figure 4. Smarcc1/Baf155 is required for global and local changes in chromatin structure during RA-induced differentiation of mouse embryonic stem cells (mESCs)

(**A--B**) Histone H3K9me3 staining in RA-treated wild-type CCE mESCs. RNAi treated cells were stained for H3K9me3 foci formation during differentiation 48 hours post-RA treatment. (**A**) A representative picture of RA-induced changes in H3K9me3 foci frequency. Other examples are shown in supplemental online Figure S4. (**B**) A plot of H3K9me3 foci frequency per nucleus in RA-treated cells with knockdown of Smarccl compared to two control populations (green fluorescent protein (GFP) and Esrrb knockdown). Quantification was carried out using ImageJ software as described in Materials and Methods. (**C**) MNase-

Southern blots of RA-treated CCE mESCs with knockdown of Smarccl or GFP (control). Cells were treated for 4 days with RA post-transfection. Assays were carried out in two independent experiments with similar results. Abbreviations: RA, retinoic acid.



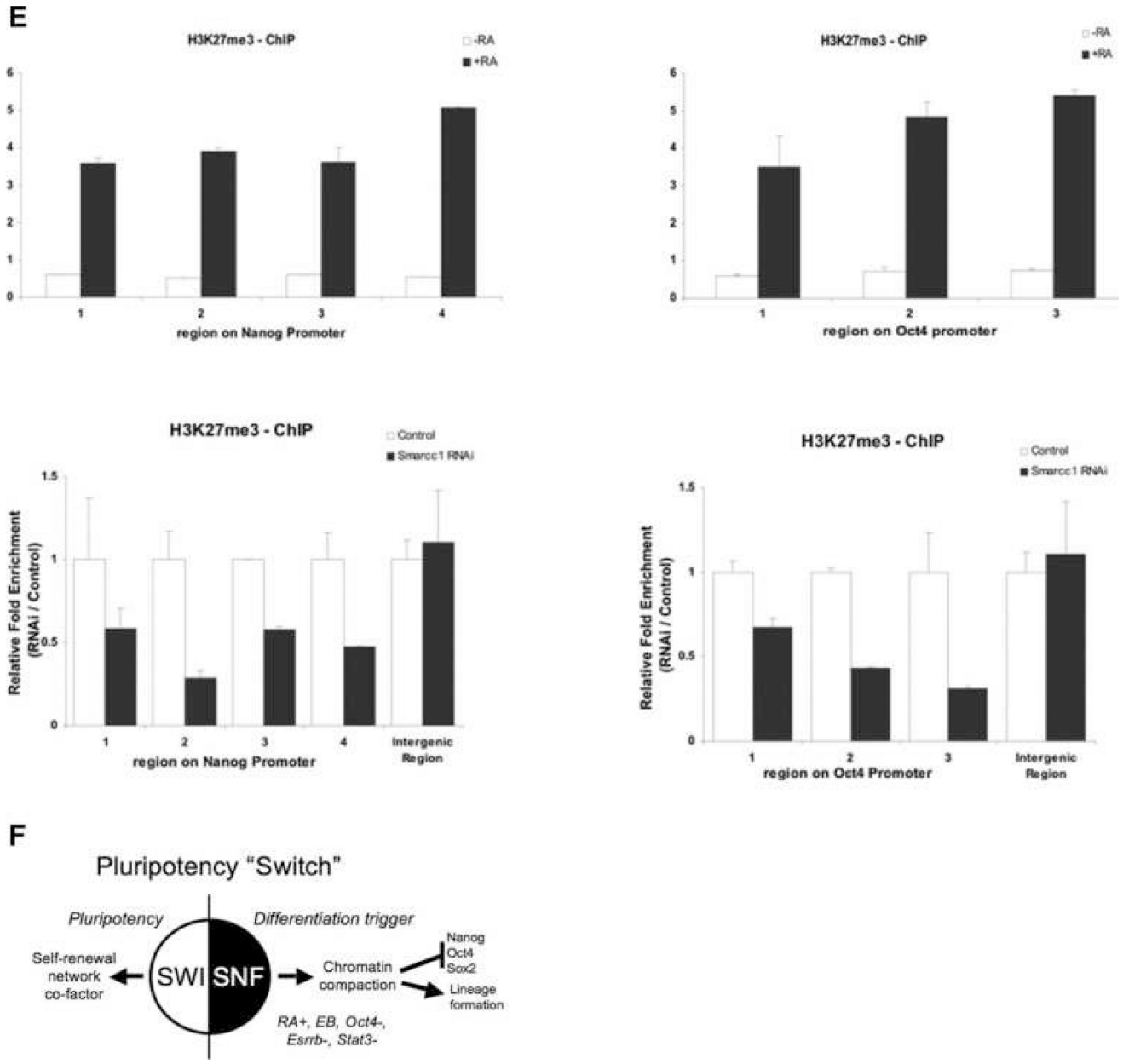


Figure 5. Smarcc1 binds directly to the Nanog and Oct4 loci and affects H3K27me3 marking during RA treatment

(**A-B**) Control experiments for chromatin immunoprecipitation analysis for RA-treated CCE ESCs, showing alkaline phosphatase staining, western blot analysis, and quality of post-sonication DNA fragments. (**C-D**) Enrichment of Smarcc1 and Oct4 proteins at the Nanog and Oct4 promoters in self-renewing and RA-treated mESCs. (**E**) Analysis of H3K27me3 at the Nanog and Oct4/Pou5f1 promoters in self-renewing and RA-treated mouse embryonic stem cells with and without knockdown of Smarcc1. (**F**) A model for SWI/SNF function in ESCs. Abbreviations: CCE, countercurrent centrifugal elutriation; EB, embryoid body; RA, retinoic acid.

Table 1

Negative regulators of Nanog expression identified in this study

Gene	Accession	Description	Function
Axin2	NM_015732	Axin-related protein, 2	Regulation of beta-catenin stability
Hira	NM_010435	HIR histone cell cycle regulation defective homolog A	Histone chaperone; transcriptional activation and repression
Ptma	NM_008972	Prothymosin alpha	Chromatin remodeling and decondensation
Raf1	NM_029780	Serine/threonine-protein kinase	Ras-dependent signal transduction
RhoA	NM_016802	Ras homolog gene family, member A	Regulation of assembly of focal adhesions and actin stress fibers
Smad7	NM_008543	Mothers against decapentaplegic homolog 7	Antagonist of TGF-beta/Smad4 signaling
Smarca4/Brg1	XM_134660	Swi/Snf related, Brg1-ATPase	Chromatin remodeling; transcriptional activation and repression
Smarcb1/Baf47	NM_011418	Swi/Snf related, Brg1-associated	Chromatin remodeling; transcriptional activation and repression
Smarcc1/Baf155	NM_009211	Swi/Snf related, Brg1-associated	Chromatin remodeling; transcriptional activation and repression
Smarce1/Baf57	BC061498.1	Swi/Snf related, Brg1-associated	Chromatin remodeling; transcriptional activation and repression
Socs3	NM_007707	Suppressor of cytokine signaling 3	Stat-induced negative regulator of Jak/Stat pathway
Tcf3	NM_009332	Transcription factor 3	Transcriptional effector of Wnt signaling; repressor of Nanog expression