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BRD7, a Novel PBAF-specific SWI/SNF Subunit, Is Required for Target Gene Activation and Repression in Embryonic Stem Cells*[S]

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Matthias D. Kaeser[‡], Aaron Aslanian[§], Meng-Qiu Dong[§], John R. Yates III[§], and Beverly M. Emerson[‡1]

From [‡]Regulatory Biology, The Salk Institute for Biological Studies and the [§]Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

The composition of chromatin-remodeling complexes dictates how these enzymes control transcriptional programs and cellular identity. In the present study we investigated the composition of SWI/SNF complexes in embryonic stem cells (ESCs). In contrast to differentiated cells, ESCs have a biased incorporation of certain paralogous SWI/SNF subunits with low levels of BRM, BAF170, and ARID1B. Upon differentiation, the expression of these subunits increases, resulting in a higher diversity of compositionally distinct SWI/SNF enzymes. We also identified BRD7 as a novel component of the Polybromo-associated BRG1-associated factor (PBAF) complex in both ESCs and differentiated cells. Using short hairpin RNA-mediated depletion of BRG1, we showed that SWI/SNF can function as both a repressor and an activator in pluripotent cells, regulating expression of developmental modifiers and signaling components such as Nodal, ADAMTS1, BMI-1, CRABP1, and thyroid releasing hormone. Knockdown studies of PBAF-specific BRD7 and of a signature subunit within the BAF complex, ARID1A, showed that these two subcomplexes affect SWI/SNF target genes differentially, in some cases even antagonistically. This may be due to their different biochemical properties. Finally we examined the role of SWI/SNF in regulating its target genes during differentiation. We found that SWI/SNF affects recruitment of components of the preinitiation complex in a promoter-specific manner to modulate transcription positively or negatively. Taken together, our results provide insight into the function of compositionally diverse SWI/SNF enzymes that underlie their inherent gene-specific mode of action.

Chromatin plays a key role in the regulation of tissue-specific gene expression during development and differentiation (1). Embryonic stem cells (ESCs)[2] possess a distinctive global chromatin structure that is characterized by hyperdynamic architectural proteins (2) and bivalent domains (3), ultimately resulting in elevated global transcription compared with differentiated cells (4). This chromatin structure is dictated by stem cell-specific transcription factors, chromatin architecture, and epigenetic regulation (5) and is a prerequisite for self-renewal and the capacity to differentiate into the three germ layers (6).

Important determinants of this unique genomic plasticity are ATP-dependent chromatin-remodeling complexes. These multisubunit enzymes catalyze non-covalent eviction, restructuring or repositioning of nucleosomes to modulate the accessibility of transcription factors and other regulatory proteins to chromosomal DNA (7). Multiple distinct families of chromatin-remodeling complexes exist, some of which have been implicated in developmental processes (8, 9). For example, genomic disruption of specific chromatin-remodeling components results in early embryonic lethality (10–14). Other remodeling modules are required to maintain the balance between ESC self-renewal and differentiation (15–17).

Specific components of chromatin remodelers confer specialized activities and selective gene targeting to distinct complexes. For example, the evolutionarily conserved SWI/SNF family exists in several subgroups and is implicated in regulating cellular processes such as transcription, differentiation, development, and tumorigenesis (8, 18, 19). Each subgroup contains one of two highly homologous ATPases, BRG1 or BRM, and a variable composition of BRG1-associated factors (BAFs) (20, 21). BRG1- and BRM-SWI/SNF complexes have very different functions as shown by gene knock-out experiments in which loss of BRG1, but not BRM, is embryonic lethal (11, 22). BAF subunits that constitute "core" SWI/SNF complexes include BAFs 170, 155, 60, 57, 53, and 47 (SNF5/INI1) and actin (20, 21). Additionally "specificity" subunits like ARID2 (AT-rich interactive domain 2, BAF200) and Polybromo (BAF180) distinguish Polybromo-associated BAF (PBAF) from BAF complexes, which are specified by ARID1

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

[1] To whom correspondence should be addressed: The Salk Inst. for Biological Studies, 10010 North Torrey Pines Rd., La Jolla, CA 92037. Fax: 858-535-8194; E-mail: emerson@salk.edu.

[2] The abbreviations used are: ESC, embryonic stem cell; RA, retinoic acid; BAF, BRG1-associated factor; PBAF, Polybromo-associated BAF; MudPIT, multidimensional protein identification technique; shRNA, short hairpin RNA; RNAi, RNA interference; ES, embryonic stem; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; TF, transcription factor; POLII, RNA polymerase II; IP, immunoprecipitation; MS, mass spectrometry; BCL7, B-cell leukemia protein 7; BRD7, bromodomain-containing protein 7; TRH, thyroid releasing hormone; PIC, preinitiation complex.

(BAF250) (23). Orthologous "signature" subunits also have been shown to confer specialized activities to SWI/SNF complexes in *Drosophila* (24). Interestingly some subunits exist as paralogues that are differentially expressed in a tissue-restricted manner, such as BAF60A, -B, and -C, and impart specific functions to SWI/SNF (18). Interestingly incorporation of distinct, mutually exclusive paralogues of the ARID1 protein family into SWI/SNF complexes determines whether SWI/SNF functions as a corepressor (ARID1A) or coactivator (ARID1B) of cell cycle control genes (25).

Given the diverse nature of SWI/SNF enzymes and the requirement for some, but not all, of its subunits in embryonic stem cells, we investigated which complexes exist in pluripotent cells. Our results indicate that several BAF subunits form a core that is contained in the majority of SWI/SNF enzymes. Surprisingly in ESCs specific paralogues predominate in SWI/SNF complexes, and incorporation of related proteins is restricted by transcriptional repression of their genes. This indicates a reduced diversity of SWI/SNF complexes in pluripotent cells that is reversed upon differentiation, probably reflecting the need to regulate more intricate transcriptional programs. Our functional analysis of BRG1 in pluripotent cells revealed that SWI/SNF can both repress and activate target genes. We also identified novel stoichiometric components of SWI/SNF complexes, among them the PBAF-specific bromodomain-containing protein 7 (BRD7) protein. Using an RNAi-based approach for BRD7 and ARID1A, we showed that both BAF and PBAF complexes can play important roles in gene-specific repression and activation. Overall our results add new insights into how the composition of SWI/SNF complexes imposes transcriptional regulation on individual target genes.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—293T and R1 mouse ESCs were obtained from ATCC. ES cells were cultivated on feeder cells according to ATCC guidelines. R201 cells, R218 cells, and their derivatives were maintained on gelatinized tissue culture dishes in Dulbecco's modified Eagle's medium supplied with 15% fetal bovine serum and leukemia-inhibitory factor. For retinoic acid (RA) differentiation, leukemia-inhibitory factor was omitted, and medium was supplied with 1 μM all-*trans*-retinoic acid and changed daily.

Lentiviral Production and Infection—Lentiviral expression vectors are based on pWPT-GFP, which contain an SV40-puro cassette. All transgenes are expressed by an EF1α promoter. Detailed maps are available upon request. Lentiviral shRNAs were constructed as described previously (26) for BRG1 and the control hairpin targeting GLUT4 (27) or purchased from Sigma (ARID1A, BRD7, and scrambled hairpin control). The targeted sequence in BRG1 is AAGCACCAGGAGTACCTCAAC. Lentiviral particles were produced and concentrated as described previously (28). To establish stably expressing cell lines, 10^5 ESCs were infected at low multiplicity and selected with puromycin (0.25 mg/liter) for 7 days. Cell lines R201 and R218 were derived by infection of R1 with viruses expressing Nanog-HA and Nanog-MYC, respectively, and single cell-cloned.

Antibodies, Protein Purification, and ATPase Assay—For immunodetection or ChIP, the following antibodies were used: BRG1 (G7), BAF155 (H76), BAF170 (H116), HA probe (F9), TFIIB (C-18), TFIID (N-12), and RNA polymerase II (POLII) (H-224) from Santa Cruz Biotechnology; BAF47 (612110) and BAF60 (611728) from BD Transduction Laboratories; FLAG probe (M2) from Sigma; BRM (ab15597) from Abcam; and BRD7 (51009-2-AP) from Proteintech. Antibodies recognizing ARID2, BAF57, BAF53, and J1 were generous gifts from Dr. Weidong Wang; ARID1A was from Dr. Elisabeth Moran. For protein purification and co-IPs, cells were lysed in DBP-IP (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 140 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM $ZnCl_2$, 1 mM dithiothreitol, protease inhibitors). After disruption by brief sonication, chromatin proteins were extracted by increasing NaCl to 420 mM. After pelleting insoluble material, protein complexes were purified using M2 FLAG affinity matrix (Sigma). Immunocomplexes were washed six times with DBP-IP containing 420 mM NaCl and 20 μg/ml ethidium bromide and eluted in BC0.1 buffer (20 mM HEPES, pH 7.9, 2 mM EDTA, 20% glycerol, 100 mM KCl) and a 0.5 mg/ml concentration of the corresponding peptide. ATPase assays were essentially performed as described previously (29) with the following modifications: 200 ng of purified SWI/SNF were incubated with trace amounts of ^{32}P-labeled γ-ATP and incubated at 37 °C. DNA stimulation was performed with 20 ng of genomic DNA. Different phosphate species were separated by thin layer chromatography and quantified using a PhosphorImager.

Multidimensional Protein Identification Technique (MudPIT) Analysis—Trichloroacetic acid-precipitated IP samples were resuspended in 8 M urea, 100 mM Tris, pH 8.5; reduced; alkylated; diluted to 2 M urea, 1 mM $CaCl_2$, 100 mM Tris, pH 8.5; and digested with trypsin. The tryptic digests were supplemented with formic acid to 5% and analyzed using the anion and cation exchange MudPIT method as described previously (30). Briefly the samples were loaded onto a 250-μm-inner diameter column with a Kasil frit containing a 2.5-cm reverse phase section packed with 5-μm, 125-Å Aqua C_{18} resin (Phenomenex, Torrance, CA) and a 2.5-cm anion and cation exchange section proximal to the frit. The anion and cation exchange section was packed with a 1:2 mixture of strong cation exchange resin (Partisphere 5-μm SCX resin from Whatman) and anion exchange resin (PolyWAX LP from PolyLC Inc., Columbia, MD). After desalting, this biphasic column was connected to a 10-cm-long, 100-μm-inner diameter analytical reverse phase column made of 3-μm, 125-Å Aqua C^{18} resin (Phenomenex). MS analysis was performed on a linear trap quadrupole mass spectrometer (ThermoFisher Scientific) using a three-step MudPIT method with salt pulses at 0, 40, and 100% buffer C. Each full MS scan was followed by five MS/MS scans. The MS/MS spectra were searched with SEQUEST against a mouse International Protein Index protein data base using a 3-atomic mass unit mass tolerance. The search results were filtered with a modified version of DTASelect (31) with a 5% false positive cutoff at the spectrum level, requiring peptides to be half- or fully tryptic and a minimum of two peptides per protein identification. The false positive rate for protein identification is 2% or lower. SWI/SNF subunits were absent in the control sample (Fig. 1, no tagged subunit) except BRG1 (three peptides) and BAF53A (two peptides). Mowse

protein scores are derived from peptide scores as a non-probabilistic basis for ranking protein hits. Peptide score is $-10 \times \log(p)$ where p is the probability that the observed match is a random event. Individual peptide scores >27 indicate identity or extensive homology ($p < 0.05$).

Chromatin IP—ChIP was performed as described previously (32) with buffers described by Upstate with the following modifications: $\sim 40 \times 10^6$ cross-linked cells were resuspended in 2 ml of SDS lysis buffer and sonicated 4×8 s, power 4. Soluble complexes were diluted in 3 volumes of ChIP dilution buffer, and lysate corresponding to $\sim 10^7$ cells was incubated with 2 μg of antibody (HA, TFIIB, TFIID, or POLII) or 2 μl of J1 prebound on 20 μl of Dynal protein G beads. After two washes in high salt wash buffer, two washes in LiCl wash buffer, and two washes in high salt wash buffer, complexes were decross-linked at 65 °C for 6 h. DNA was precipitated using 10 μg of yeast tRNA and 10 μg of glycogen.

RNA Extraction and Quantitative PCR—Total RNA was extracted using TRIzol (Invitrogen). Reverse transcription was performed with 0.5 μg of total RNA, random hexamers, and SuperScript III polymerase (Invitrogen). Quantitative PCR was performed on a Stratagene Mx3005P system using SYBR Green (Applied Biosystems). The error bars shown represent duplicate measurements from independent biological duplicates. Primer sequences are listed in supplemental materials.

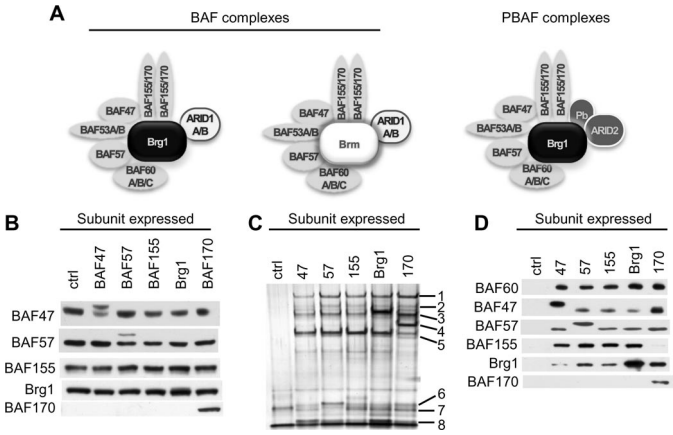

FIGURE 1. **SWI/SNF composition in embryonic stem cells.** *A*, schematic diagram of known SWI/SNF complexes. Signature subunits for BAF complexes are the mutually exclusive ARID1A and ARID1B. BAF complexes can contain either ATPase, BRG1 or BRM. PBAF complexes are characterized by the presence of ARID2 and Polybromo and are restricted to BRG1. *B*, protein levels of ectopically expressed SWI/SNF subunits. R201 cells were infected with lentiviruses expressing FLAG-tagged proteins as indicated. Western blots were performed on whole cell lysates. *C*, composition of purified complexes. Using FLAG affinity purification, SWI/SNF was isolated from R201 expressing the indicated subunits, resolved by SDS-PAGE, and silver-stained. Identification of indicated subunits was performed by Western blot and mass spectroscopy. *1*, ARID1A; *2*, ARID2, Polybromo; *3*, BRG1; *4*, BAF170; *5*, BAF155; *6*, BAF60; *7*, BAF57; *8*, BAF53. *D*, Western blot analysis of purified complexes. SWI/SNF quantities were adjusted to contain similar BAF60 levels. *ctrl*, control; *Pb*, Polybromo.

RESULTS

Composition of SWI/SNF in Pluripotent ES Cells—Initially we investigated which forms of the SWI/SNF multisubunit complex exist in ESCs. We used a strategy in which epitope-tagged subunits are virally integrated into the ESC genome. Affinity purification is then accomplished in a simple one-step procedure, resulting in native multisubunit protein preparations of high yield and purity (33). We expressed several well characterized subunits to increase our ability to purify most of the compositionally distinct forms of SWI/SNF that may be present in ESCs. BAF47, BAF57, BAF155, BAF170, and BRG1 are all core subunits that associate with both BAF and PBAF complexes (Fig. 1*A*). To purify SWI/SNF from substantial amounts of homogeneously undifferentiated ESCs, we created the cell lines R201 and R218. Both were derived from the R1 ESC line by infection with lentiviruses expressing Nanog-HA and Nanog-MYC, respectively. Both cell lines exhibit morphological features similar to pluripotent ES cells and differentiate upon leukemia-inhibitory factor withdrawal or RA treatment (supplemental Fig. 1). We also compared gene expression in parental R1 and R218 cells in response to RA and found them to be very similar albeit with a slower *Oct4* mRNA decrease in R218 cells. Additionally the R218 line was capable of differentiating into spontaneously beating cardiomyocytes with efficiency similar to that reported previously (34) (data not shown).

SWI/SNF in Embryonic Stem Cells Is Composed of a Limited Subset of Components—To prepare purified SWI/SNF complexes, R201 cells were transduced with lentiviruses expressing a C-terminal FLAG-tagged cDNA of *Baf47*, *Baf57*, *Baf155*, *Baf170*, or *Brg1*. Except for BAF170, ectopic expression of SWI/SNF subunits did not lead to an overall increase in protein levels (Fig. 1*B*). To examine SWI/SNF composition in an unbiased manner, we purified sufficient material to analyze by silver staining and MudPIT. As shown in Fig. 1*C*, anti-FLAG eluates from ESCs expressing individual FLAG-tagged BAFs 47, 57, 155, or 170 or BRG1 contained a similar set of proteins, which resembles the subunit pattern observed in the initial SWI/SNF purifications (35). Using Western blotting, mass spectroscopy on the individual bands, and differences in migration upon expression of the FLAG-tagged subunits (supplemental Fig. 2), we co-localized the known SWI/SNF subunits ARID1A, ARID2, BRG1, BAF170, BAF155, BAF60, BAF57, and BAF53 with the indicated bands.

The Majority of SWI/SNF Complexes Contain the Core Subunits BAFs 47, 57, and 155 and BRG1—We then examined by Western blotting whether any subunits were preferentially assembled into particular complexes by unique associations with other BAF proteins. We observed that the bait for the purification was in general slightly overrepresented (Fig. 1*D*). However, with the exception of the BAF170 sample, the preparations showed a remarkably similar ratio between the other examined core components. This is consistent with the notion that the majority of purified complexes contain all four subunits: BAFs 47, 57, and 155 and BRG1. BAF170 was below detection in all samples except where it was ectopically expressed, suggesting that it is rare in ESCs and that the lentiviral expression increased its abundance above normal, endogenous levels. As expected, its ectopic expression and incorporation in SWI/SNF complexes resulted in the replacement of one or both molecules of its paralogue BAF155, explaining why BAF155 levels are lower in complexes purified through BAF170. Because of the overexpression of BAF170, we did not analyze this sample any further.

TABLE 1

Biased paralogue usage in ES cells

SWI/SNF was purified as indicated in Fig. 1*C* and analyzed by MudPIT mass spectroscopy. Listed are the numbers of individual peptides that could be unequivocally assigned to a particular subunit. Among different paralogous subunits, in particular the BRG1/BRM, BAF155/BAF170, and ARID1A/ARID1B show a strongly biased incorporation into ES cell complexes, whereas they are similarly represented in SWI/SNF complexes from HeLa cells. Differences in peptide recovery between individual samples also reflect variations in the total amount of complex subjected to mass spectroscopy. PB, Polybromo.

	HeLa BAF47	ESC			
		BRG1	BAF47	BAF57	BAF155
BAF47	18	23	21	21	15
BRG1	34	76	48	45	50
BRM	34	1	1	2	2
BAF155	44	67	52	62	37
BAF170	60	12	14	10	5
BAF60A	19	34	30	32	27
BAF60B	17	10	9	15	11
BAF60C	2	5	3	3	2
BAF53A	25	38	15	25	18
BAF57	22	17	21	28	17
PB	51	39	37	31	25
ARID1A	48	61	49	64	50
ARID1B	36	11	14	15	12
ARID2	30	18	24	14	16

Several Paralogous Subunits Are Overrepresented by Specific Forms—A comparison of the samples by MudPIT revealed unique peptides of 14 previously documented SWI/SNF components in the four subunit-specific ESC and HeLa purifications (Table 1). The control sample only contained a total of five peptides from two different subunits (see "Experimental Procedures"). Because certain groups of paralogous subunits (BRG1/BRM, BAF155/BAF170, and ARID1A/ARID1B) share substantial sequence homology (>50% in each group), we only included peptides in our analysis that could be unambiguously attributed to one specific polypeptide. Because BRM and BRG1 are mutually exclusive subunits (21), the single unique BRM peptide in our BRG1 purification reflects the expected false positive identification rate of 2% or lower. We expected roughly similar amounts of unambiguous peptides when comparing the paralogous subunits within their groups as observed in our analysis of SWI/SNF from HeLa cells. However, we found that in ESCs specific paralogous subunits were preferentially incorporated. In the case of BRG1/BRM, our results suggest that the underrepresented protein BRM might exist in negligible quantities in ESCs in agreement with its low expression during early development (36). This is also the case for the postmitotic neuron-specific BAF53B of which we could not find any unambiguous peptides (37). Also in contrast to HeLa cells, BAF170 and ARID1B had considerably fewer unambiguous peptides in ESCs than their counterparts BAF155 and ARID1A, implying that these subunits are less abundant. ARID1B-containing BAF enzymes were shown to interact with transcriptional activators as opposed to complexes with ARID1A that associate with transcriptional repressors (25), suggesting that in ESCs SWI/SNF might be compositionally better suited for a repressive role.

Differentiation Increases Incorporation of Previously Underrepresented SWI/SNF Components—Because we observed a surprisingly biased usage of paralogous SWI/SNF subunits in pluripotent cells, we examined the changes in SWI/SNF composition during ESC differentiation. We used RA for 2 and 6 days to differentiate pluripotent stem cells into restricted

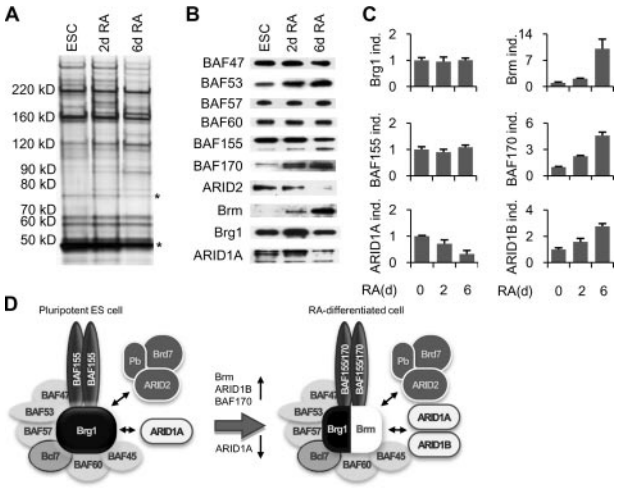

FIGURE 2. **SWI/SNF compositional changes during RA differentiation.** *A*, complex composition of purified complexes. SWI/SNF was isolated from R201 cells expressing BAF47-FLAG (*ESC*) and R1 ESCs with BAF47-FLAG (*2d RA* and *6d RA*) induced for differentiation as indicated. Purified complexes were resolved by SDS-PAGE and silver-stained. *Asterisks* designate nonspecific bands (see also Fig. 1*C*). *B*, changes in SWI/SNF subunits during differentiation. Complexes described in *A* were subjected to Western blotting. *C*, transcriptional changes for SWI/SNF subunits. RNA was isolated during an RA differentiation time course at the indicated time points, and expression levels of indicated subunits were measured by reverse transcription-quantitative PCR and normalized using β-actin. *D*, schematic representation of compositional changes in SWI/SNF triggered by RA differentiation. 6-day retinoic acid treatment leads to increased expression of BRM, ARID1B, and BAF170 but to a decrease of ARID1A. *Pb*, Polybromo; *ind.*, induction; *d*, days. The data presented are the mean values + S.E. from two independent experiments.

descendants. SWI/SNF complexes were purified from differentiating cells using a tagged version of BAF47 as described previously (33) and compared with a preparation from undifferentiated cells (Fig. 2*A*). To identify subunit-specific changes, we performed Western blot analyses (Fig. 2*B*). Each SWI/SNF preparation was standardized to give approximately the same levels of the core components BAF47 and BAF57. Throughout the course of differentiation, we observed considerable induction of some subunits, such as BAF53, BAF170, and BRM. Conversely ARID2 and ARID1A decreased by day 6 of differentiation. BAF155 exhibited a decrease in the immunoreactive 155-kDa band but an increase in the signal at 120 kDa. Mass spectroscopy of this silver-stainable band at 120 kDa identified 18 individual peptides of BAF155, supporting the notion of a splice variant, specific cleavage, or degradation product of BAF155, which may represent the previously observed BAF110 (21).

RA-mediated Differentiation Increases ARID1B at the Expense of ARID1A—Several distinct mechanisms could be responsible for the changes in SWI/SNF composition, such as differences in subunit transcription, protein stability, or variations in subunit incorporation rates. It was shown previously that differentiation leads to changes in cellular protein levels that are very similar to the variations we observed in SWI/SNF composition (17). We therefore tested whether differences in subunit transcription might explain the changes in SWI/SNF composition by measuring their individual RNA expression levels (Fig. 2*C*). We observed little change, if any, in expression of *Baf155* and *Brg1*, whereas considerable increases in *Brm* and *Baf170* transcripts were apparent. This corroborated our measurements of protein levels and the indications obtained from

TABLE 2

Differentiation increases usage of previously underrepresented paralogues

SWI/SNF was purified as indicated in Fig. 2 from pluripotent cells or cells differentiated for the indicated time with RA using heterologous BAF47-FLAG expression. Listed are the numbers of individual peptides that could be unequivocally assigned to a particular subunit. Differences in peptide recovery between individual samples also reflect variations in the total amount of complex subjected to mass spectroscopy. PB, Polybromo.

	ESC	Day 2	Day 6
BAF47	44	37	46
BRG1	60	68	87
BRM	2	1	20
BAF155	98	123	108
BAF170	21	55	76
BAF60A	41	38	39
BAF60B	12	21	30
BAF60C	3	7	31
BAF53A	21	29	33
BAF57	26	28	32
PB	92	75	75
ARID1A	58	49	66
ARID1B	7	19	41
ARID2	45	37	22

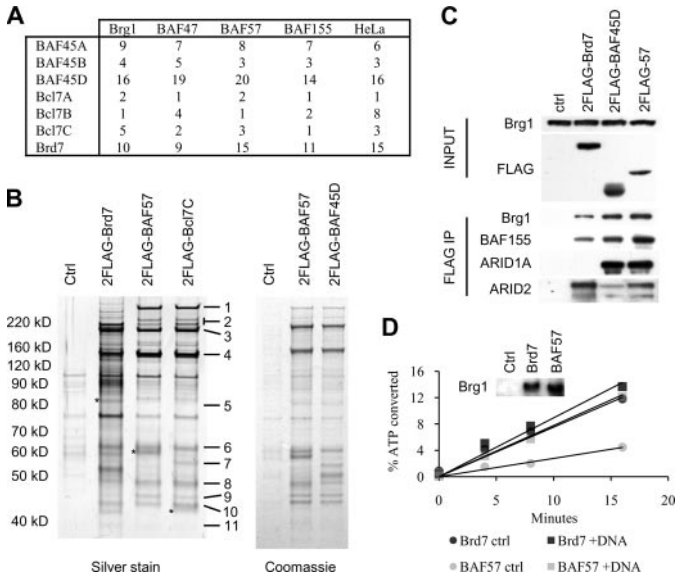

FIGURE 3. **Identification of novel SWI/SNF subunits.** *A*, polypeptides consistently found in MudPIT spectroscopy. SWI/SNF was purified as indicated in Fig. 1*C*. Listed are the numbers of individual peptides that could be unequivocally assigned to a particular polypeptide. Differences in peptide recovery between individual samples also reflect variations in total amount of complex subjected to mass spectroscopy. *B*, putative new subunits co-purify with SWI/SNF. Associated complexes were purified from R218 cells expressing 2FLAG-tagged proteins as indicated, resolved by SDS-PAGE, and stained by silver or Coomassie Blue as indicated. Indicated bands were excised and identified by mass spectroscopy according to highest Mowse score (in parentheses). *1*, ARID1A (1274); *2*, ARID2 (654); Polybromo (1233); *3*, BRG1 (2131); *4*, BAF155 (1751); *5*, BRD7 (273); *6*, BAF60A (797); *7*, BAF57 (959); *8*, DPF2 (531); BAF53 (353); *9*, BAF47 (519); *10*, β-actin (719); *11*, BCL7C (347). *Asterisks* indicate the tagged subunit of each preparation. *C*, BRD7 is a PBAF-specific subunit. Lysate and FLAG-immunoprecipitated complexes were resolved by SDS-PAGE and subjected to Western blotting using the indicated antibodies. *D*, ESC PBAF exhibits different ATP hydrolysis activities than total ESC SWI/SNF. Purified complexes from pluripotent cells were incubated in the presence or absence of DNA. ATPase activity was assessed by calculating the percentage of hydrolyzed ^{32}P-labeled phosphate after separation by thin layer chromatography. The *inset* contains a Western blot for BRG1 of both ATPase reactions. *ctrl*, control.

MudPIT analysis of these samples (Table 2). We also detected a reduction in *Arid1a* message, confirming the decrease observed in Western blotting by 6 days of differentiation. Interestingly *Arid1b* exhibits an opposing phenotype revealed by the increase in both transcript and unique peptides in mass spectroscopy. Unfortunately we could not confirm this by Western blotting because we lacked antibodies that recognized ARID1B under these conditions. The compositional changes we observed by different approaches (and suggested by MudPIT for ARID1B) are summarized in Fig. 2*D*.

B-cell Leukemia Protein 7 (BCL7) Family Members Are Novel SWI/SNF Subunits—Our MudPIT analyses of complexes from ESCs and HeLa cells consistently identified unique peptides from a protein family and another single protein that were not previously recognized as SWI/SNF subunits (Fig. 3*A*). These peptides are found in all individual SWI/SNF preparations independent of the subunit used for purification. The family represented by the BCL7 A/B/C proteins is of unknown function except for its involvement in chromosomal translocations in Burkitt lymphoma (38). We also found peptides from BRD7, which was shown to interact with IRF-2 (39) and acetylated histones (40). One additional group, represented by the proteins BAF45A/B/D, has been well characterized by Crabtree and co-workers (41). To confirm the incorporation of these proteins into SWI/SNF complexes in ESCs, we affinity-purified lentiviral expressed 2FLAG-tagged versions of these proteins and compared the interacting proteins with SWI/SNF from a 2FLAG-BAF57 purification (Fig. 3*B*). Both BAF45D and BCL7C preparations showed a remarkably similar interacting protein pattern compared with BAF57 as judged by Coomassie and silver staining. The only obvious differences between individual purifications correspond to shifted bands in the molecular weight range where the differentially tagged proteins are expected. We conclude that both BAF45D and BCL7C co-purify with SWI/SNF in a stoichiometry similar to that of BAF57, establishing these proteins as *bona fide* subunits of SWI/SNF.

BRD7 Is a Novel PBAF Subunit and Defines Complexes with Distinct Biochemical Properties—Compared with BAF57, BRD7 exhibits a similar, albeit slightly different protein interaction pattern (Fig. 3*B*). The absence of a 250-kDa band and the comparatively overrepresented 200-kDa band suggested that the BRD7-containing complexes lacked ARID1A/B proteins but had close to stoichiometric amounts of ARID2. This suggests that BRD7 is a potential PBAF-specific subunit. We tested this hypothesis by Western blot analysis (Fig. 3*C*). Both BRG1 and ARID2 co-precipitated with tagged versions of BAF57, BRD7, and BAF45D, underscoring the incorporation of all three baits into SWI/SNF complexes. In contrast, ARID1A can only be detected in association with BAF57 and BAF45D, confirming the selective incorporation of BRD7 into PBAF complexes.

Different SWI/SNF subunits contribute specific functionalities to the enzymatic complex. We tested whether BRD7-containing PBAF complexes possessed the same biochemical properties as the mixture of SWI/SNF complexes containing both BAF and PBAF. To this end, we assayed both 2FLAG-BRD7- and 2FLAG-BAF57-purified complexes in an ATP hydrolysis assay. Both preparations contained similar amounts of the catalytic subunit BRG1, the only ATPase present in ESCs (Fig. 3*D*, *inset*). As expected, the total mixture of SWI/SNF exhibited a basal ATPase activity that was stimulated by the addition of

DNA (Fig. 3*D*, *light* and *dark gray circles*, respectively). Surprisingly BRD7-purified PBAF complexes had a substantially higher basal activity that was comparable to the DNA-stimulated activity of total SWI/SNF. This activity, however, was not further stimulated by the addition of DNA. This indicates that compositionally distinct subcomplexes have different enzymatic properties, which may impart specialized functions to BAF and PBAF and facilitate their ability to differentially regulate individual target genes.

SWI/SNF Functions as Either an Activator or a Repressor—To define the transcriptional functions of SWI/SNF in pluripotent ESCs and identify its target genes, we performed a BRG1 depletion experiment coupled to a transcriptome survey. R1 ESCs were infected in duplicate with shRNA targeting BRG1 or a non-expressed control, and RNA was harvested. Microarray analysis revealed changes of >2-fold in a total of 104 individual genes of which 88 transcripts were induced by the RNAi and expression of 16 genes was reduced. This suggests that SWI/SNF can act both as an activator and a repressor in pluripotent cells (supplemental Table 1). Interestingly when analyzing the gene ontology annotation of all target genes, we found that in the SWI/SNF-repressed group, angiogenic modulators were overrepresented (p value below 10^{-4} as annotated below).

Antagonism between BAF and PBAF Subcomplexes—Next we asked whether regulation of these BRG1-responsive genes relied on BAF, PBAF, or both subcomplexes. To this end, we infected R218 cells with shRNA targeting either the BAF signature subunit ARID1A, the PBAF-specific subunit BRD7, or the only ATPase present in ESCs, BRG1. All shRNAs reduced the protein level of their target significantly (Fig. 4*A*) with a decrease in target RNA to 60 (BRG1), 80 (ARID1A), and 80% (BRD7) (data not shown).

To identify complex-specific regulation, we tested a subset of 12 identified target genes after infection with the shRNA-lentiviruses (Fig. 4*B*). The expression of four of these genes was decreased by RNAi targeting BRG1, suggesting that SWI/SNF activates their transcription: *Mm.34431* (a conserved gene with unknown function), *Crabp1* (cellular retinoic acid-binding protein 1), *Trh* (thyroid releasing hormone), and *Nupr1* (nuclear protein 1). By contrast, eight genes were activated by interference with BRG1, suggesting that SWI/SNF represses their expression. These genes included *Eng* (endoglin, angiogenic modulator), *Ia-1* (insulinoma associated-1), *Nodal* (angiogenic modulator), *Rnf128* (ring finger protein 128), *Bmi-1* (polycomb component), *Adamts1* (extracellular protease, angiogenic modulator), *Thbs1* (thrombospondin 1, angiogenic modulator), and *Dub1* (deubiquitinating enzyme 1). Interference with ARID1A affected all 12 SWI/SNF target genes significantly, indicating that BAF complexes are required for SWI/SNF-imposed regulation. RNAi targeting BRD7 revealed a very different situation. Six genes, including *Crabp1*, *Nupr1*, *Ia-1*, *Rnf128*, *Bmi-1*, and *Thbs1*, were deregulated in the absence of the PBAF-specific subunit BRD7, whereas the expression of four genes, *Mm.3443*, *Eng*, *Nodal*, and *Adamts1*, did not change upon loss of BRD7. Interestingly transcription of two genes, *Trh* and *Dub1*, was affected by the absence of BRD7 but in an opposite manner compared with that obtained upon loss of ARID1A.

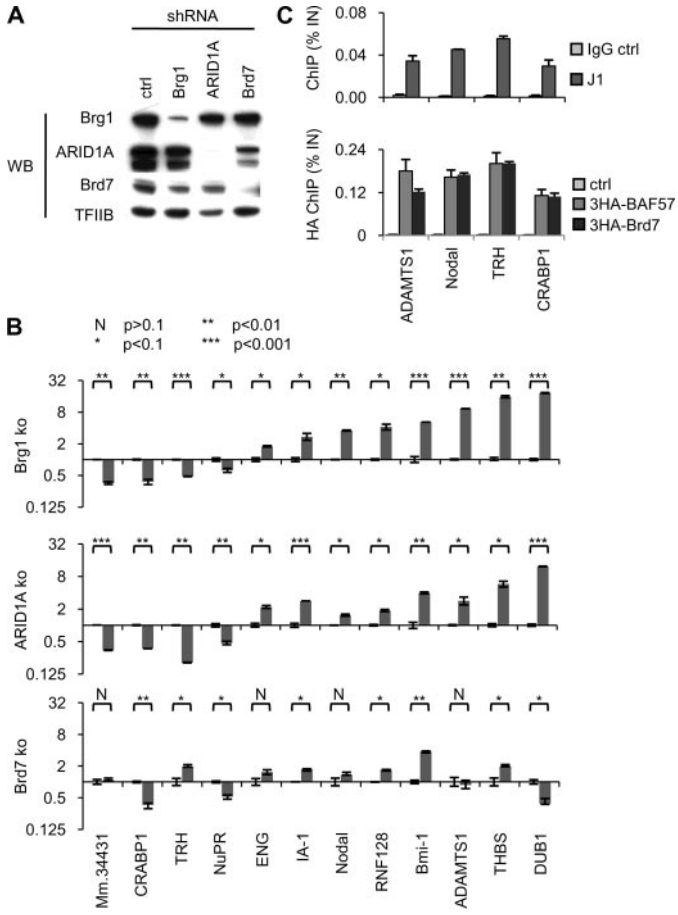

FIGURE 4. **BRD7 regulates a subset of SWI/SNF target genes.** *A*, shRNA-mediated inhibition of specific SWI/SNF subunits. R218 cells were infected with lentiviral shRNA against the indicated subunit or scrambled control. Proteins were extracted and detected by Western blot. *B*, quantification of SWI/SNF target gene expression. RNA was isolated from cells treated with shRNA viruses and quantified by reverse transcription-quantitative PCR. Target gene expression was normalized to β-actin, and induction over a control virus was plotted on a log scale. Indicated p values were calculated using Student's t test (two independent experiments). *C*, ChIP assay to quantify SWI/SNF binding to the promoter region of indicated target genes. For the *first panel*, lysates from cross-linked R218 cells were precipitated using antibodies directed against BRG1 (J1) or a non-expressed epitope (*IgG ctrl*). For the *second panel*, ChIP using an antibody directed to the HA epitope was used to precipitate from lysates of cells stably expressing the indicated HA-tagged subunit. Precipitated DNA is expressed as percentage of input DNA. *WB*, Western blot; *ctrl*, control; *ko*, knock-out; *IN*, input. The data presented are the mean values + S.E. from two independent experiments.

Next we measured association of the PBAF complex with the genomic loci containing differently regulated groups of target genes (Fig. 4*C*). Our ChIP experiments in ESCs localized BRG1 binding near the transcriptional start site of all four tested target genes, *Adamts1*, *Nodal*, *Trh*, and *Crabp1*, as indicated by the more than 15-fold increase in DNA precipitated by the BRG1-specific antibody J1 compared with a control antibody (IgG). Comparing HA precipitates from ESCs infected with a control virus or with HA-tagged BAF57 or BRD7, respectively, we observed an increase of greater than 70-fold on all target genes, emphasizing the presence of both subunits. Taken together, these results suggest that BAF and PBAF subcomplexes can antagonize the expression of specific target genes possibly because of their intrinsically different biochemical properties.

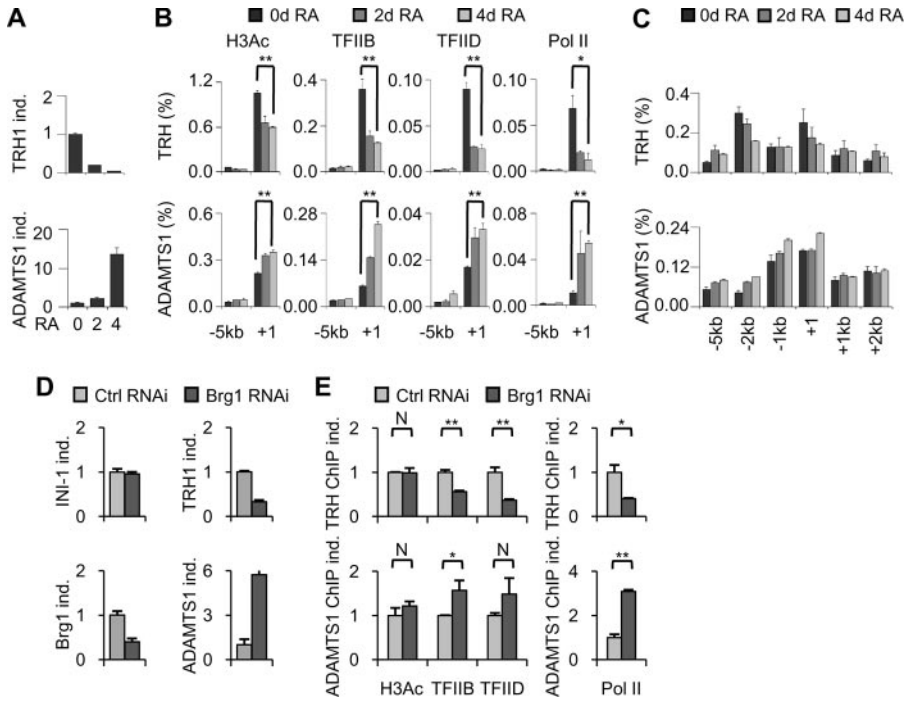

FIGURE 5. **SWI/SNF and differentiation dictate expression by regulating PIC occupancy.** *A*, quantification of target gene expression. RNA was extracted from R218 cells differentiated with RA for the indicated days. mRNA levels were measured by quantitative reverse transcription-PCR and normalized using β-actin. *B*, ChIP assay to quantify changes in occupancy at the genomic locus of the target genes. Antibodies directed against the indicated proteins/modifications were used to precipitate from lysates of differentiated cross-linked cells. Precipitated DNA is expressed as percentage of input DNA. *N*, $p > 0.15$; *, $p < 0.15$; **, $p < 0.05$. *C*, genomic localization of SWI/SNF by ChIP. R218 cells expressing 3HA-BAF57 were differentiated for the indicated time, and ChIP was performed. Primer sets used for amplification were spread over 7 kb as indicated. Precipitated DNA is expressed as percentage of input DNA. *D*, quantification of target gene expression. RNA was extracted from R218 cells infected with lentiviral shRNAs targeting BRG1 or control (GLUT4) for 96 h. mRNA levels were measured by quantitative reverse transcription-PCR and normalized using β-actin. *E*, ChIP assay to quantify changes in occupancy at the genomic locus of the target genes *Trh* and *Adamts1*. Antibodies directed against the indicated proteins/modifications were used to precipitate from lysates of cross-linked cells infected as in *A*. Occupancy is expressed as -fold change over control shRNA. For *p* values, see *B*. *Ctrl*, control; *d*, days; *ind.*, induction. The data presented are the mean values + S.E. from two independent experiments.

SWI/SNF Target Gene Regulation throughout Differentiation—To examine whether differentiation affects SWI/SNF-mediated regulation of target genes, we analyzed the chromatin status of two target genes throughout RA-induced differentiation (Fig. 5). We chose one SWI/SNF-activated gene, *Trh*, and one SWI/SNF-repressed gene, *Adamts1*, because of the extent and consistency of changes in their transcript levels throughout differentiation and BRG1 interference. TRH is a secreted hormone that participates in the cascade resulting in the release of thyroid hormones. *Trh* expression is reported to be modulated by several signaling pathways, including cAMP-response element-binding protein and signal transducers and activators of transcription (42), epidermal growth factor (43), and thyroid hormone. ADAMTS1 is an extracellular protease involved in heart development very recently shown to be repressed by BRG1 (44) and speculated to be regulated by E2Fs, Sma and MAD-related proteins (SMADs), or the T-cell factor pathway. Retinoic acid differentiation of ESCs resulted in considerable changes in the expression profiles of both genes. *Trh* was strongly repressed, whereas expression of *Adamts1* was substantially increased (Fig. 5*A*). To correlate any alterations in chromatin structure and transcription factor occupancy at these promoters with differentiation-induced changes in transcription, we performed ChIP analyses for acetylated histone H3; components of the preinitiation complex (PIC), TFIIB and TFIID; and POLII (Fig. 5*B*). On the *Trh* gene, we observed a general decrease of histone acetylation and a loss of both TFIIB and TFIID binding. Because this was accompanied by a significant reduction of total POLII, we conclude that retinoic acid differentiation led to transcriptional shutdown of the *Trh* gene in part through loss of stable PIC formation. The opposite was true for *Adamts1* where differentiation led to a considerable increase in binding of PIC components and POLII accompanied by a slight increase in H3 acetylation. In contrast to the events at the proximal promoter, we saw little if any occupancy of these components at a control site 5 kb upstream of the transcriptional start sites of either gene.

SWI/SNF Can Regulate Target Genes by Generating Accessibility to the PIC—Next we analyzed how differentiation affects binding of SWI/SNF to the *Trh* and *Adamts1* promoters. To address this, we performed ChIP for the SWI/SNF subunit BAF57 in an ESC line expressing 3HA-BAF57 (Fig. 5*C*). Spatially resolved analysis revealed that SWI/SNF has two occupancy peaks in the *Trh* promoter at −2 kb and near the transcription start site. Differentiation led to a decrease in SWI/SNF occupancy in both sites, closely resembling the decrease of PIC components TFIIB and TFIID. To establish a causal relationship between SWI/SNF binding and PIC occupancy, we performed similar ChIP experiments in a population of cells where knockdown of BRG1 led to a 3-fold decrease in *Trh* expression (Fig. 5*D*). In these cells, decreased occupancy of PIC components TFIIB, TFIID, and POLII was observed at the proximal promoter despite any significant changes in H3 acetylation. We conclude that SWI/SNF regulates *Trh* expression by modulating PIC accessibility to the promoter in ESCs. Together with diminished H3 acetylation, loss of SWI/SNF occupancy is a possible mechanism by which the *Trh* gene is silenced upon differentiation. By contrast, this mechanism does not apply for *Adamts1*. Induction of *Adamts1* expression by either differentiation or BRG1 interference was correlated with increased occupancy of PIC components and POLII (Fig. 5, *B* and *E*), and SWI/SNF remained bound during differentiation (Fig. 5*C*). Taken together, these results under-

score the ability of SWI/SNF to regulate its target genes in a positive or negative manner using very distinct mechanisms.

DISCUSSION

In this study, we investigated different compositional aspects of the SWI/SNF complex in embryonic stem cells. Our results show that, in contrast to differentiated cells, ESCs have comparably low levels of BAF170 and BRM in agreement with other recent reports (17). During mouse development, *Brm* expression is concomitant with the onset of vasculogenesis (36). The mutually exclusive BRM and BRG1 compete for incorporation into the same remodeling complex, and both ATPases can recruit SWI/SNF to their specific target genes through selective interactions with transcription factors (45). Interestingly BRG1 negatively regulates a subset of angiogenic modulators in ESCs. Therefore, the onset of *Brm* expression is likely to impact BRG1 target genes by reducing the overall abundance of BRG1-containing SWI/SNF complexes. This may provide a potential *Brm* expression-dependent mechanism for the onset of vasculogenesis and heart development. However, neither *Brm* nor *Baf170* expression alone in ESCs is sufficient to derepress a subset of target genes including several angiogenic modulators that are repressed by SWI/SNF in ESCs (supplemental Fig. 3).

Selective incorporation of two mutually exclusive signature subunits of the BAF complex, ARID1A and ARID1B, was shown to confer activating *versus* repressive functions of SWI/SNF on cell cycle regulatory genes (25). Our BRG1 interference experiments indicate that in ESCs SWI/SNF has a predominantly repressive role, which correlates with higher incorporation of the repressive ARID1A subunit. Upon differentiation, the increase in ARID1B and decrease in ARID1A suggests that compositionally repressive SWI/SNF is converted into a more transcriptionally activating enzyme. Other studies have not reported an increase in ARID1B (17) upon differentiation. However, Wang and co-workers (17) monitored total ARID1B protein levels, whereas our data were derived from detection of ARID1B incorporated into SWI/SNF complexes by MudPIT mass spectrometry and transcriptional measurements of ARID1B message. Overall we found that the diversity of core and specificity subunits increases upon differentiation, presumably reflecting the need to regulate more intricate transcriptional programs.

Among our main observations is the identification of BRD7 as a new PBAF-specific subunit. We found BRD7 present in purifications from pluripotent ESCs, differentiated ESCs, and HeLa cells, arguing for its incorporation in a variety of cell types. By performing MudPIT mass spectrometry on BRD7-containing complexes, we found five unambiguous peptides of the Polybromo protein (data not shown), suggesting that the PBAF signature module consists of BRD7, ARID2, and Polybromo. We were unable to confirm the presence of Polybromo in BRD7-containing complexes because of the lack of antibodies that recognize the Polybromo protein. BRD7 contains a bromodomain that interacts with acetylated histone H3 (40), potentially providing additional tethering of the PBAF complex to transcriptionally active chromatin.

We also found that BRD7-containing PBAF complexes have very distinct biochemical properties from those of BAF as reflected by differences in the ability of DNA to stimulate their enzymatic activities. We estimate that PBAF complexes represent only a minor fraction of total SWI/SNF in ESCs, which contain predominately BAF complexes, because ARID2 was considerably less abundant in SWI/SNF complexes (purified by tagged BAF57) than ARID1A. It is possible that the basal activity of total SWI/SNF in our ATPase assays was contributed by the high ATPase activity of PBAF. The stimulation observed after addition of DNA would therefore be entirely due to the biochemical properties of BAF. Given the multitude of bromodomains in PBAF complexes, it will be interesting to determine whether PBAF exhibits a greater ATPase activity stimulation by specifically modified histones than BAF.

It is well documented that PBAF and BAF complexes have non-redundant functions, for example, in transactivation of interferon-inducible genes (46). In addition, there is considerable overlap of common target genes as might be expected for genes in which common subunits are responsible for SWI/SNF recruitment. Interestingly in ESCs, BAF appears to be required for the majority of BRG1-dependent transcriptional regulation. By contrast, BRD7/PBAF function was dispensable for a sizeable subset of the genes we examined, although this complex could be readily detected at some of these promoters such as ADAMTS1. A possible explanation is that PBAF may be recruited through a subunit other than BRD7. Overall our RNAi results are consistent with the severity of the knock-out phenotype of respective signature subunits. ARID1A knockout mice (14) arrest very early in development and display deficiencies in early germ layer formation. On the other hand, Polybromo knock-outs (47) die relatively late (embryonic day 12.5–15) probably because of heart defects.

A majority of SWI/SNF target genes were derepressed by RNAi-mediated knockdown of BRG1, indicating that functional SWI/SNFs are involved in repressing their expression. Repression by SWI/SNF is well documented and shown to be required for the repressive function of the RB (Retinoblastoma) protein complex (48, 49) and transcriptional repression of neuronal genes by the repressor element 1-silencing transcription factor (REST) protein (50, 51). SWI/SNF has also been implicated in silencing of methylated genes (52). These studies highlight the ability of SWI/SNF to play critical roles as either a transcriptional coactivator or corepressor. In ESCs, this correlates with the biased incorporation of the repressive ARID1A subunit (25). However, our interference experiments of ARID1A show that ARID1A is not exclusively repressive as it was required for SWI/SNF-mediated activation of a subset of target genes.

Different models have been proposed to explain the function of chromatin-remodeling complexes in the sequence of events that govern transcription. For example, some studies show that promoter activation requires SWI/SNF recruitment before association of the histone acetyltransferase Spt-Ada-Gcn5-acetyltransferase (SAGA) (53) and the PIC (54). In other models, transient recruitment of remodeling complexes to poised promoters allows release of elongating RNA polymerase II (55). Repression by SWI/SNF (and the Rsc complex in yeast) is mediated by establishing an inhibiting nucleosomal distribution (56), recruitment of histone deacetylases (57), or indirectly

stimulating expression of inhibitory non-coding RNAs (58). Taken together, these studies reveal that chromatin remodeling can function at many discrete steps to impact transcriptional regulation. This mechanistic diversity is likely to be dictated by the particular chromatin environment of individual target genes and the specific remodeler. Because histone acetylation can serve as a recruitment platform for chromatin-remodeling complexes, we speculate that H3 acetylation is a contributing factor in selective SWI/SNF recruitment and function on the *Trh* promoter. Such a model could explain the loss of SWI/SNF occupancy throughout differentiation-mediated H3 acetylation decrease and also why BRG1 knockdown leads to lowered transcription without affecting H3 acetylation. From this perspective, it will be very interesting to analyze the basis of selective BAF and PBAF complex recruitment and how they regulate PIC access to the *Trh* core promoter in ESCs. From a broader viewpoint, several components of SWI/SNF have been shown to be necessary for long term survival of the inner cell mass (11–14). Moreover decreased cell numbers are observed after BRG1 depletion suggesting that BRG1 is required for ESC growth (4) perhaps through functions in addition to transcriptional regulation. In this regard, SWI/SNF has been linked to genome organization, recombination, cell cycle progression, and the DNA damage response (8, 18, 59). Future investigations into the potential roles for SWI/SNF in these and other processes should generate a more comprehensive understanding of its contribution to ES cell pluripotency.

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