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SMARCA4 ATPase mutations disrupt direct eviction of PRC1 from chromatin

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Abstract

Trithorax-group genes and mammalian homologues, including BAF (mSWI/SNF) complexes, have been known for nearly 30 years to oppose Polycomb repressive activity^{1–5}. This opposition underlies the tumor-suppression role of BAF^{3,5–7} and is expected to contribute to neurodevelopmental disorders, as evidenced by frequent driving mutations^{8,9}. However, the mechanisms underlying opposition to Polycomb silencing are poorly understood. Here we report that recurrent disease mutations of BAF subunits induce genome-wide increases in Polycomb complex deposition and activity. We show that point mutations of the Smarca4 (Brg) ATPase domain cause loss of direct binding between BAF and PRC1 that occurs independently of chromatin. Release of this direct interaction occurs via an ATP-dependent mechanism, consistent with a role as a transient intermediate of eviction. Using a new *in vivo* assay, we find that BAF directly evicts Polycomb factors within minutes of its occupancy, together establishing a new mechanism for the widespread opposition underlying development and disease.

Main Text

In vertebrates and invertebrates, Polycomb complexes are associated with chromatin containing repressive marks and silent or low transcriptional states. Polycomb silencing is critical to development^{10–14}, and aberrant repressive activity can promote malignancy in several cancers^{15–18}. BAF complexes oppose Polycomb silencing, and disruption of the balance between BAF and Polycomb underlies oncogenic transformation in two model

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Contributions

B.Z.S. and C.H. designed and conducted the experiments and wrote the paper, performed analyses, and wrote the paper; J.P.C., S.M.G.B., and C.K. performed experiments; W.L.K. performed analyses; K.Z. and G.R.C. designed experiments and wrote the paper.

cancers^{3,5,6}. Furthermore, BAF inactivating mutations have been found in independent screens for driving mutations in renal¹⁹, ovarian²⁰, medulloblastoma²¹, rhabdoid²², colorectal²³, and lung²⁴ tumors, as well as pan-cancer studies^{7,25}, suggesting that disrupted opposition to Polycomb silencing may be a general feature of common cancers. Additionally, a large number of tumors and patients affected by neurologic disorders contain heterozygous mutations of the BAF ATPases Smarca4 or Smarca2²⁶, and CRISPR-Cas9 tiling screens in cancer cell lines indicate that the ATPase domain is the most functionally important domain of Smarca4²⁷. Thus we hypothesized that the Smarca4 ATPase domain plays a central role in the opposition to Polycomb silencing.

To investigate the effects of Smarca4 inactivation, we began by identifying the acute effects of Smarca4 deletion on PRC1 and PRC2 using a conditional knockout line of mouse embryonic stem cells (mESCs). Following conditional deletion of Smarca4, ChIP-seq of Ring1b (a dedicated catalytic subunit of PRC1) revealed reproducible increased PRC1 occupancy at ~34% (1,634 of 4,754) of PRC1 sites over the genome (Figure 1A,E, Figures S1–S2) although Ring1b expression levels did not change (Figure S3). Sites of increased occupancy were distributed across several defined classes²⁸ of Polycomb target genes, as well as genes not typically targeted by Polycomb in wild-type cells (Figure S1).

Developmental regulators were among the most highly enriched gene sets among increased sites (Figure S1). We obtained similar results for ChIP-seq of Suz12, a dedicated subunit of PRC2, with ~36% (1,892 of 5,279) of sites showing increased PRC2 occupancy (Figure 1B,E). We sought to compare the magnitude of this effect to conditional deletion of Arid1a, another essential BAF subunit commonly inactivated in cancer (Figure 1C,D). Conditional deletion of Arid1a similarly caused increased Ring1b and Suz12 peaks, but at substantially fewer loci; these were largely a subset of those that increased following Smarca4 deletion (Figure 1F). Previous ChIP-seq studies of H3K27me3 levels following Smarca4 deletion showed less dramatic effects⁴, an effect we attribute to the slow rate of H3K27me3 accumulation. The high degree of correlation between PRC1 and PRC2 changes induced by BAF subunit deletion across the genome (Figure 1F), indicates that there are characteristic, subunit-dependent changes in Polycomb occupancy following BAF inactivation. Using RNA-seq, we confirmed that genes with increased PRC1/2 following Smarca4 deletion showed reduced expression, indicating that the changes we observed were sufficient to alter gene expression (Figure 1G–I).

In many malignancies, deleterious missense mutations of Smarca4 occur more frequently than truncating mutations (Figure 2A), suggesting that functional inactivation rather than loss of expression is under positive selection in cancer. Smarca4 is a member of the SF2 helicase-like family of ATPases²⁹, and mutation counts in primary tumors and cancer cell lines compiled from The Cancer Genome Atlas (TCGA)³⁰ and The Cancer Cell Line Encyclopedia (CCLE)³¹ show clusters of mutations at or near conserved SF2 motifs, including Walker A (ATP binding), Walker B (hydrolysis), and the intervening linker region that contains the conserved SF2 helicase Motif 1A (substrate binding, Figure 2B)³².

As many cancer mutations of Smarca4 are heterozygous, we sought to investigate whether missense mutations within the ATPase domain resulted in deregulation of Polycomb even when co-expressed with wild-type Smarca4. Using a conditional knockout mESC line⁴, we

rescued Smarca4 deletion with lentiviral transduction of wild-type Smarca4-GFP fusion, and either wild-type or mutant Smarca4 tagged with V5 (“Smarca4-V5”), resulting in endogenous expression levels (Figure S3). This strategy allowed us to observe the effects of mutation in a setting that mimics heterozygous mutation and permitted us to differentiate the two Smarca4 variants in the same line. Based on mutation frequency in cancer and neurodevelopmental disorders⁹, we focused on six mutations to examine: p.Gly784Glu (G784E), p.Lys785Arg (K785R), p.Tyr860His (Y860H), p.Glu861Lys (E861K), p.Glu882Lys (E882K), and p.Arg885His (R885H; residues numbered according to the human RefSeq gene). Of these, K785R has been previously characterized as a bona-fide ATPase-inactivating mutation^{33,34}. These positions represent three conserved regions: Walker A, Walker B, and the intervening linker. Hence, we co-expressed each of these mutants as Smarca4-V5 fusions, alongside wild-type Smarca4-GFP. As a control, we compared these to similarly prepared cells, which expressed wild-type Smarca4-V5 instead of a mutant.

Compared to wild-type, heterozygous expression of each Smarca4 ATPase mutant resulted in increased PRC1 occupancy at specific genes, including at developmental regulators such as the *Fgf11* gene (Figure S4). Among all PRC1 sites over the genome, sites with increased PRC1 occupancy upon either Smarca4 ATPase mutation or deletion were significantly overlapping ($p=1e-30$, Figure S5). Patterns of PRC1 changes were highly correlated and reproducible across all mutants, and uncorrelated with the variation between wild-type replicates (Figure 2C–D). Compared to unchanged sites, sites with increased PRC1 occupancy induced by Smarca4 ATPase mutants were enriched at promoters, transcription start sites, and CpG islands, but depleted at 3′ UTRs and intergenic regions (Figure 2F). To confirm this effect was not unique to mouse cells, we examined genome-wide Ring1b occupancy in the human lung cancer cell line A549, which contains a null mutation of Smarca4; following expression of either wild-type or ATPase-mutant Smarca4, we observed similar genome-wide effects (Figure S6).

We sought to determine if specific genomic marks were predictive of PRC1 occupancy changes upon expression of Smarca4 mutants. Thus, we examined ChIP-seq data from 111 factors previously obtained from wild-type mESCs. Rank ordering revealed that several positive marks, notably H3K4me3, have elevated read densities at sites predisposed to increase PRC1 occupancy in the presence of Smarca4 ATPase mutants (Figure S7). We used Fisher’s linear discriminant^{35,36} to identify the combinations of features that optimally distinguish sites that increase PRC1 occupancy from those that decrease PRC1 occupancy. We found that indeed H3K4me3 and several other features associated with active promoters, including the H3K4 methyltransferase Kmt2b³⁷, the H3K4 demethylase Kdm5a, as well as H3K27ac and Hdac1/2, are enriched from increased sites and depleted from decreased sites. Remarkably, these and other features completely distinguish the two classes of sites along the chromatin landscape (Figure 3A). Lasso multivariate regression³⁸ independently confirmed that H3K4me3 is the mark most positively associated with sites showing increased Ring1b occupancy induced by Smarca4 ATPase mutants (Figure 3B). Indeed, across bivalent sites, Ring1b changes caused by ATPase mutants were much better predicted by the levels of H3K4me3 than H3K27me3 in wild-type cells (Figure S7). Together, our results indicate that CpG-rich bivalent promoters with the highest levels of positive marks,

particularly H3K4me3, are those most sensitive to Smarca4 ATPase mutants. This observation suggests that these two Trithorax-group complexes (BAF and Mll-containing COMPASS complexes) work in concert.

Because PRC1 contributes to recruitment of PRC2³⁹, we reasoned that expression of the mutants could also lead to increased H3K27me3, a mark reflecting PRC2 activity, at sites where PRC1 increased. Therefore, we performed ChIP-seq for H3K27me3 on the same ATPase-mutant cell lines described above. We observed consistent increases in H3K27me3 across the genome (Figure 4A–D). H3K27me3 changes were highly correlated across the set of mutants, and uncorrelated from changes between wild-type replicates (Figure 4C, D). Although all mutants showed correlated changes (including the ATPase-dead mutant K785R, see Figure S8), we focus on E861K as an example. Elevated H3K27me3 marking was frequently located immediately adjacent to sites with increased Ring1b (Figure 4A). We examined all H3K27me3 peaks that occur within ± 3 kbp of a CpG island and found that these sites consistently showed increased Ring1b at the flanking CpG island ~ 2 kbp from the H3K27me3 increased peak (Figure 4E). Furthermore, we classified PRC1 sites into decreased, unchanged, or increased categories, and plotted the mean profiles of both H3K27me3 and H3K4me3 within each category (Figure 4F). At PRC1-increased sites, we observed increased H3K27me3 in regions ~ 2 kbp from Ring1b peaks. This effect was substantially diminished at unchanged sites, and we observed no change in H3K27me3 at sites where Ring1b decreased (Figure 4F). H3K4me3 levels were unaffected, indicating that the effects on H3K27me3 are unique and arise through the effects of Smarca4 mutations on Polycomb activity. Together, our results indicate that disease-associated Smarca4 ATPase mutants permit accumulation of PRC1 at CpG-rich bivalent promoters, as well as increased activity of PRC2, with increased H3K27me3 marking ~ 2 kbp away. The origin of this offset is not currently known, but may reflect the footprints of Polycomb factors, topological changes, nucleosome depletion, or other unknown factors.

To examine the molecular mechanisms that underlie Polycomb opposition, we sought to ascertain whether there was a direct interaction between BAF and PRC1. In earlier mass spectrometry studies, our laboratory observed that Rybp, a subunit of variant PRC1 complexes, co-immunoprecipitated with Smarca4 (Table S1 in ref ⁴⁰), suggesting a direct interaction between BAF and PRC1. We therefore performed immunoprecipitation (IP) experiments (Figure 5A) and found that Smarca4 and Smarcc1 (BAF155), both core dedicated BAF subunits, co-immunoprecipitate with Rybp and Ring1b at low but detectable levels (Figure 5B). Furthermore, this interaction is reciprocal, as IP of the core BAF subunit Smarcb1 (BAF47) shows an interaction with Rybp and Ring1b (Figure 5C). This interaction occurs in the soluble nuclear fraction, and is insensitive to DNase I (Figure 5D), consistent with a direct interaction that does not require mediation through chromatin. Moreover, incubation with ATP induces the release of PRC1 from BAF (Figure 5E), but this release is impaired in the presence of ATP analogs that inhibit hydrolysis (Figure 5F). Taken together, our results indicate a direct interaction between BAF and PRC1 that is regulated by ATP hydrolysis. Similar results were obtained using Cbx7, a dedicated subunit of canonical PRC1 complexes⁴¹ (Figure S9), indicating that Smarca4 performs direct ATP-dependent regulation of both variant and canonical PRC1 complexes.

To examine how Smarca4 ATPase mutations affected this interaction, we selected from each class identified above the mutant with the strongest effect on Ring1b occupancy: G784E (Walker A), E882K (Walker B), and E861K (linker region). We then repeated IPs of Ring1b and Rybp in cells co-expressing both wild-type Smarca4-GFP and either wild-type or mutant Smarca4-V5. Although wild-type and mutant Smarca4 were expressed at near identical levels, we find that ATPase mutants do not efficiently co-IP with Ring1b and Rybp; instead the wild-type protein preferentially co-IPs with these factors (Figure 5G–H). Hence our results confirmed that Smarca4 ATPase activity regulates this direct interaction. The requirement of a functioning ATPase for both binding and release of PRC1 can be rationalized by considering that ATP hydrolysis lowers the energy barrier between the bound state and unbound state, therefore catalyzing both binding and release (Figure S10A). An example of a possible mechanism might involve an ATP-regulated latch (Figure S10B).

Our co-IP results indicated that BAF regulates PRC1 by via direct ATP-dependent interactions. Indeed, direct ATP-dependent interactions have been reported for Snf2-like remodelers for other protein factors, like Mot1 and its substrate TBP^{42,43}. However, it remained uncertain in cells whether the BAF–PRC1 interaction was limited to the soluble nucleoplasm or occurred directly on chromatin. To answer this question and to demonstrate that the interaction was functional, we adapted a strategy for chemical-induced proximity in living cells^{44,45}. We used genome editing to introduce an array of 12 Zinc-finger binding elements upstream of ASCL1, a bivalent gene decorated with PRC1/2 at its CpG-island promoter. By expressing a fusion of FKBP to a Zinc-finger binding domain anchor, and a fusion of the Frb domain of mTOR to the BAF subunit SS18, we were able to use rapamycin to induce recruitment of BAF to the Zinc-finger site (Figure 6A). Following incubation with 30 nM rapamycin, ChIP-qPCR showed recruitment of BAF (Figure 6B) and loss of PRC1/2 (Figure 6C–D). These changes were detectable within 5 min, and steadily continued for up to 60 min. Reduction of PRC1/2 occupancy was specific to the ASCL1 locus and not observed at the bivalent HOXA3 control region (Figure 6D), allowing us to conclude that BAF directly ejects PRC1 on chromatin. Removal of PRC1 may contribute to reduction in PRC2 since PRC1 is thought to lead to PRC2 placement⁴⁶. Recruitment of Lsh1, another Snf2-like remodeler, did not lead to removal of PRC1/2 at the ASCL1 locus, demonstrating that Polycomb eviction is not common to all Snf2-like remodelers (Figure S11). A separate study reported by our laboratory shows that disruption of this direct eviction is a widespread consequence of BAF deregulation in cancer⁴⁵.

We conclude that both binding and release of PRC1 are regulated by ATP-dependent conformational states within BAF complexes. This direct regulation results in the local eviction of PRC1 from chromatin within minutes of BAF recruitment, further supporting a direct role. By failing to evict PRC1 from chromatin, Smarca4 deletion and ATPase mutations permit accumulation of PRC1/2 at CpG-island promoters across the genome, which may contribute to oncogenesis or epigenetic plasticity during tumor development. Furthermore, our results establish that BAF complexes have non-histone substrates like PRC1, which are direct targets of ATPase activity. As a result, deregulation of Polycomb occupancy and activity may be more common in BAF-mutated cancers than has been previously recognized. Eviction of Polycomb factors from chromatin may also play a critical

role in neural development, where neuron-specific nBAF complexes are essential for post-mitotic neuronal function^{47,48}.

Materials and Methods

Culture of animal and human cells

Mouse ES cells were cultured using standard conditions. ES culture media containing Dulbecco's Modified Eagle's Medium (Cat# 10829018; Life Technologies), 15% FBS (Cat# ASM-5007; Applied StemCell), Penicillin-Streptomycin (Cat# 15140122; Life Technologies), Glutamax (Cat# 35050061; Life Technologies), HEPES buffer (Cat# 15630080; Life Technologies), 2-mercaptoethanol (Cat# 21985023; Life Technologies), MEM-NEA (Cat# 11140050; Life Technologies), and LIF supplement⁴⁰, was replaced daily, and ES cells were passaged every 48 hours. For inducible deletions, *Smarca4^{flox/flox}* actin-CreER ES cells⁴ or *Arid1a^{flox/flox}* actin-CreER ES cells, which previously tested negative for mycoplasma contamination using PCR testing, were respectively plated onto irradiated feeder mouse embryonic fibroblasts, treated with 0.8 μ M 4-hydroxytamoxifen (Tam) or ethanol (EtOH) for 48 h, and harvested for further experiments after trypsin dissociation at 72 h. A549 cells were obtained from ATCC (Cat# CCL-185), and directly cultured upon receipt without testing for mycoplasma contamination, using F-12K media supplemented with 10% FBS and Penicillin-Streptomycin.

Chromatin Immunoprecipitation (ChIP) experiments

Cells (5–10 million per chromatin IP) were trypsin dissociated and resuspended in 10 ml fix buffer (50 mM HEPES pH 8.0, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 100 mM NaCl) to which formaldehyde (11% solution in fix buffer) was added and incubated at room temperature for 12 minutes. The fixing was quenched with 0.125 M glycine and incubated on ice for 5 minutes, centrifuged for 5 minutes at 1200g, and pellets were washed once on PBS, then resuspended in Rinse Buffer 1 (50 mM HEPES pH 8.0, 140 mM NaCl, 1 mM EDTA pH 8.0, 10% glycerol, 0.5% NP-40, 0.25% Triton-X100) and incubated for 10 minutes on ice. After centrifugation at 1200g for 5 minutes, nuclei were resuspended in Rinse Buffer 2 (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 200 mM NaCl) and centrifuged for 5 minutes at 1200g. Two subsequent rinses in shearing buffer (0.1% SDS, 1 mM EDTA pH 8.0, 10 mM Tris pH 8.0) were performed without resuspending the pellet, and finally the pellet was resuspended in 990 μ l of shearing buffer and transferred to a 1 ml Covaris tube for 12 minutes of sonication using a Covaris focused ultrasonicator at 5% duty cycle, intensity 4, 140 PIP, and 200 cycles per burst. Sonicated material was spun at 10,000g for 5 minutes, and the supernatant was diluted with 1/4 volume of 5x IP buffer (250 mM HEPES, 1.5 M NaCl, 5 mM EDTA pH 8.0, 5% Triton-X100, 0.5% DOC, 0.5% SDS) and used directly for subsequent immunoprecipitation.

Preparation of nuclear extracts

Cells were dissociated with trypsin and washed with PBS. Cells were resuspended in Buffer A (25 mM HEPES (pH 7.6), 5 mM MgCl₂, 25 mM KCl, 0.05 mM EDTA, 10% glycerol, 0.1% NP-40) supplemented with 1 mM DTT and complete protease inhibitor cocktail (Roche), and incubated on ice for 7 minutes. After centrifugation (1000g), nuclei were

resuspended in Buffer C (10 mM HEPES (pH 7.6), 3 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, 10% glycerol) with 1 mM DTT and protease inhibitor cocktail (Roche), and ammonium sulfate was added to a final concentration of 300 mM. Nuclei were lysed at 4 °C with overhead rotation for 30 minutes, and soluble proteins were isolated from nuclear extracts via ultracentrifugation at 100,000 rpm for 15 minutes. The supernatant was incubated on ice for 20 minutes with 0.3 mg/ml of ammonium sulfate then subject to ultracentrifugation at 100,000 rpm for 15 min. The supernatant was discarded, and precipitated protein was used for immunoprecipitation and Western blotting.

Immunoprecipitation

For individual immunoprecipitation reactions, protein precipitate derived from nuclear extracts was resuspended in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40) supplemented with 1 mM DTT, and protease inhibitor cocktail (Roche), with or without 10 mM ATP, AMPPNP, or ATP γ S. After Bradford assay, protein concentration was adjusted to 300 ug protein lysate in 250 ul IP buffer and incubated with 3 ug antibody (see Table S2) 12–16 h at 4°C. Antibodies were immobilized with subsequent 4-hour incubation with Protein A Dynabeads (ThermoFisher) and washed three times with 1 ml IP buffer at room temperature, then resuspended in 12 ul 2x loading buffer (LDS, ThermoFisher) with 2-mercaptoethanol for subsequent Western blot analysis. For experiments with DNase, 2 ug plasmid reporter with or without 10 U of DNase I (Roche) was added to the 300 ul reaction volume. All antibodies used in this study are described in Table S2.

ChIP-seq library preparation

All libraries were independently prepared from separately cultured samples in duplicate, according to previously described protocols^{51–53}. Size selection was performed by extracting 200–400 bp DNA fragments on a 2% agarose E-gel (Invitrogen) before PCR amplification, then extracted using RNeasy MinElute cleanup kits (Qiagen). PCR amplification was performed using 14 cycles, and the resulting DNA quantified by Qubit fluorometric quantitation. Sequencing was performed using single-end reads on the Illumina HiSeq2000 sequencer.

Processing of ChIP-seq data

Analysis of high-throughput sequencing data was not performed blindly, but the following techniques were applied uniformly to all datasets. Single-end ChIP seq reads were processed by mapping to the mm9 reference mouse genome using Bowtie 1.1.1⁵⁴, rejecting reads that contain more than a single mismatch. Duplicate reads were discarded, leaving only unique reads. For all analyses, peak calling was performed by MACS 1.4.2⁵⁵ by comparing to input samples for each cell type.

All peaks from control and treatment datasets within ± 1 kbp were merged, and peaks below a threshold of 30 RPM (in at least one dataset) were discarded to remove low-quality peak calls. For each dataset, the total number of reads overlapping each of the resulting peaks was compared for differential peak calling. Differential peak calls were made using DESeq2⁵⁶, using the summed number of reads at all sites above the 95th-percentile as size factors to avoid the influence of background ChIP-seq reads. DESeq2 accounts for individual site

variances across all replicates to make differential peak calls. Log2 fold changes were calculated by the default use of maximum *a posteriori* estimation using a zero-mean normal prior (Tikhonov-Ridge regularization). FDR-corrected P-values were calculated using the Benjamini-Hochberg procedure. Differential calls were made by requiring fold changes of >1.5-fold in either direction and FDR-corrected $p < 0.10$. RPM values in genome tracks are the mean values across both replicates from each condition. Calculation of mean genome track densities was performed using bwtool⁵⁷ and browser tracks were prepared using Gviz⁵⁸. Mean density profiles were computed using bwtool by calculating the mean basepair coverage across all replicates for a given condition, then calculating the mean coverage across all sites using a 6-kbp window. Overlap of peaks was performed using Bedtools⁵⁹.

RNA-seq library preparation

RNA was prepared by QIAzol extraction, followed by poly(dT) capture. mRNA was converted to DNA using previously described protocols⁵³, then size selected for 200–400 bp DNA fragments on a 2% agarose E-gel (Invitrogen). Following PCR amplification (14 cycles) and Qubit fluorometric quantitation, sequencing was performed using single-end reads on the Illumina HiSeq2000 sequencer.

Processing of RNA-seq data

Reads within coding regions were counted using htseq⁶⁰, with gene definitions obtained from the refGene table of RefSeq genes in the UCSC Table Browser. The table of all counts was processed using DESeq2, using default parameters. Log2 fold changes were calculated using maximum *a posteriori* estimation using a zero-mean normal prior (Tikhonov-Ridge regularization). FDR-corrected P-values were calculated using the Benjamini-Hochberg procedure. Differential calls were made by requiring fold changes of >1.5-fold in either direction and FDR-corrected $p < 0.10$.

Public datasets analyzed in this study

Read densities were obtained from publicly available as NCBI GEO datasets (Table S1). For each of these chromatin feature datasets, read fragments were extended to 200 bp from the 3' end, and basepair coverage was determined using Bedtools⁵⁹.

Multivariate analyses of chromatin factors/marks

For rank ordering chromatin features, iterative bootstrap resampling of 100 samples was used to obtain the 99% confidence interval for the mean. Fisher's linear discriminant was used to assay combinations of features within qualitative site classes (increased, decreased, unchanged), and Lasso regression was performed using the 'glmnet' package³⁸ in R to assay the importance of each chromatin feature directly to the observed fold change at that site. For both analyses, the number of ChIP-seq reads within ± 3 kb of peak centers was summed for each chromatin feature.

Genome annotation enrichment

Enrichment for overlap with genomic annotations was performed by comparing how frequently each peak fell into basic genomic annotations, separately determined for each

class of differential peak call described above. Enriched GO terms and log enrichment of genomic annotation overlap was calculated for each class of site using HOMER⁶¹.

Residue mutation frequencies and conservation scores

Mutation frequencies were obtained by assembling all non-silent coding mutations to SMARCA4 from TCGA and CCLE. Mutation frequency values are presented using kernel density estimation to obtain an estimate of the true underlying mutation frequency. Residue conservation scores were obtained by generating and performing multiple sequence alignment on 7,033 RefSeq and GenBank sequences with homology to amino acid residues 750–900 of SMARCA4, using FRpred⁶². This alignment was used to generate a residue conservation score based on Shannon Entropy⁶³, using a three-residue averaging window⁶⁴.

Functional impact of mutations in TCGA datasets

Mutations present in TCGA data were analyzed for functional impact and assigned to one of four categories (truncating, deleterious missense, benign, or unknown) using PolyPhen2 scores⁶⁵, generated by Oncotator⁶⁶. Truncating mutations include frameshift and nonsense mutations and may lead to nonsense-mediated decay; all other mutations are missense mutations that affect coding sequence but are otherwise expected to be fully expressed.

Densitometry and analysis

All densitometry of Western blot bands was performed by calculating integrated band intensities with LiCor ImageStudio 5.2.5. To compare enrichment of Smarca4-GFP and Smarca4-V5, the intensities of each band following co-immunoprecipitation was measured and compared to the input lane. Log2 enrichment ratios arising from the co-IP were calculated using the following expression: $\log_2(V5/GFP) - \log_2[V5(input)/GFP(input)]$.

Generation of mouse CIP-ASCL1 ES cells

Genome engineering of mouse ES cells was performed using the CRISPR-Cas9 protocol previously described⁶⁷. Briefly, a repair template plasmid containing the modified ASCL1 locus was generated with 1-kbp flanking homology arms. Two distinct DNA binding sequence arrays were added 278 bp upstream of the TSS of ASCL1, 12x ZFHD1 elements (TAA TGA TGG GCG) and 5x Gal4 elements (CGG AGT ACT GTC CTC CGA G). A nuclear EGFP was inserted at the ATG of exon 1 of ASCL1 to differentiate the edited allele. Two guide RNA sequences (sg1: AAT AAA CAG GCC GCG CGC TCG GG, sg2: TGC CGG GCC AAA CTG TCG CGG GG) were cloned into the Cas9-2A-Puro plasmid from Feng Zhang.

Chemical Induction of Proximity (CIP) on chromatin

The CIP assay was performed as previously described⁴⁴. CIP-ASCL1 mESCs were transduced with lentivirus expressing ZFHD1 fused directly to the FKBP anchor and SS18 fused to two tandem repeats of Frb. Proximity was induced by addition of rapamycin at 30 nM (final concentration) for 0, 5, 15, or 60 min before harvesting for ChIP-qPCR.

Quantitation of chromatin factors by ChIP-qPCR

Chromatin immunoprecipitation (ChIP) was performed as described previously⁴⁴, and quantitative PCR samples were prepared using the SensiFAST SYBR Lo-Rox Kit (Bioline, Cat# BIO-94020), according to the manufacturer's instructions. Analysis of qPCR samples was performed on a QuantStudio 6 Flex system (Life Technologies). Antibodies used for ChIP are provided in Table S2. Primers used are provided in Table S3.

Statistics

All differential calls for genomic datasets (ChIP-seq and RNA-seq) were made using DESeq2, where P-values are calculated using the Wald test, as described in the DESeq2 documentation⁵⁶. FDR-corrected P-values were calculated using the Benjamini-Hochberg procedure. For presentation in heat maps, RNA-seq and ChIP-seq data were grouped using k-means clustering into k=3 groups. Analysis of correlation was performed using the Pearson correlation test. All Student's t-tests were performed as two-sided tests. Fisher's exact tests were performed as two-sided tests. Two-sample Kolmogorov-Smirnov tests were performed as two-sided tests. To validate the effects of Smarca4 disruptions using ChIP-qPCR data, qPCR enrichment values were compared for each primer set across antibodies and Smarca4 conditions using one-way ANOVA as a two-sided test. All of the above statistical tests were performed using R. The hypergeometric test for overrepresentation was performed as a one-sided test using HOMER⁶¹.

Lasso multivariate regression

Lasso multivariate regression³⁸ was used to relate the fold-change in Ring1b occupancy induced by Smarca4 ATPase mutants ($\hat{f}_c$) to a linear combination of 111 individual chromatin features:

$$\log_2 \hat{f}_c = \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_n x_n$$

At each Ring1b peak, the number of reads within ± 3 kbp from the center of the peak was summed for each of 111 features, using data from previously published datasets. The summed read count at each site was $\log_{10}$ transformed, and scaled to unit variance across all sites (x_i). Lasso regression was performed using the "glmnet" R package⁶⁸, with the default mixing penalty parameter $\alpha=1$. Values for the restricted parameter

$$\lambda = \sum_i \beta_i$$

were obtained for each Smarca4 mutant by 10-fold cross-validation, with the minimal value selected that provided the lowest mean cross-validated error.

Data availability

The datasets generated in this study have been deposited in the NCBI GEO repository (GSE88968).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This paper is dedicated to the memory of Joseph P. Calarco, a great friend and passionate scientist. We apologize to our colleagues whose work we could not cite due to space constraints. We thank Gangqing Hu, Wenfei Jin, Emma Chory, James Bradner, and Diana Hargreaves for helpful discussions, and Erik Miller for sharing curated GEO datasets. Arid1a conditional deletion cells were a gift from Diana Hargreaves (Salk Institute). All libraries were sequenced by the DNA Sequencing and Genomics Core facility of NHLBI. Analysis was performed using the Stanford BioX3 cluster, supported by NIH S10 Shared Instrumentation Grant 1S10RR02664701. This work was also supported by the SFARI Foundation (G.R.C.), NIH grants R37NS046789 (G.R.C.) and R01CA163915 (G.R.C.), and Division of Intramural Research, NHLBI/NIH (K.Z.). G.R.C. is an HHMI Investigator. S.M.G.B. is supported by a Swiss National Science Foundation (SNSF) postdoctoral fellowship. C.H. is supported by NCI career transition award K99CA187565. The datasets generated in this study have been deposited in the NCBI GEO repository (GSE88968).

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Fig. 1. Characteristic accumulation of PRC1 and PRC2 following BAF subunit deletion
(a) Genome-wide increases of PRC1 occupancy by Ring1b ChIP-seq in conditional Smarca4 knockout mESCs. Each point on the plot represents an individual Ring1b peak. Sites that are increased (orange), decrease (blue), or remain unchanged (gray) are labeled using the criteria described in the Methods section. **(b)** Genome-wide increases of PRC2 occupancy by Suz12 ChIP-seq in conditional Smarca4 knockout mESCs. **(c)** Ring1b ChIP-seq in conditional Arid1a knockout mESCs. **(d)** Suz12 ChIP-seq in conditional Arid1a knockout mESCs. **(e)** Example track of PRC1/2 upon knockout of BAF subunits, aligned to histone mark tracks from the mouse ENCODE project⁴⁹. Additional tracks and analysis are presented as Supplemental Figures. **(f)** Pearson correlation heatmap of genome-wide PRC1/2 fold changes following Smarca4 or Arid1a knockout (top left); heatmap of all peaks with altered Polycomb occupancy. PRC1 and PRC2 are highly coupled, and changes arising from Arid1a knockout are a subset of changes arising from knockout of Smarca4 (right). **(g)** Comparison of RNA-seq data from wild-type and conditional Smarca4 knockout mESCs.

Altered gene expression upon Smarca4 knockout is consistent across replicates. Comparison of changes of gene expression to the changes of **(h)** Ring1b peaks, or **(i)** Suz12 peaks found at TSSs. Following knockout of Smarca4, the expression levels of genes are negatively correlated with changes of Ring1b and Suz12 over their TSSs. Correlation and P values obtained from Pearson's product moment correlation test.

Fig. 2. Mutations of the Smarca4 ATPase domain induce increased PRC1 occupancy at CpG-rich promoters

(a) The proportion of BAF-subunit mutation types varies continuously from truncating mutations to deleterious missense mutations. Smarca4 mutations are frequently deleterious missense mutations, rather than truncating mutations. (b) Mutated positions (black) of the Smarca4 ATPase domain summed across primary tumors and cancer cell lines overlap with conserved sequence motifs. Residue mutation frequency (blue) coincides with residue conservation scores (gray). (c) Heatmap of all fold changes of Ring1b peaks between wild-type and ATPase mutant Smarca4. ATPase mutants predominantly induce Ring1b increases. (d) Fold changes of Ring1b occupancy are correlated between Smarca4 ATPase mutants. Pearson correlation values of genome-wide fold changes are presented as a heatmap. (e) Observed changes between wild-type and Smarca4 mutants are consistent across independent replicates. (f) Heatmap of overlap enrichment of sites where Smarca4 mutants

induce increased Ring1b occupancy based on genomic annotation. Values reflect enrichment of increased sites compared to unchanged sites.

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Fig. 3. Chromatin features define sites predisposed to Polycomb increases upon expression of Smarca4 ATPase mutants

(a) Fisher's linear discriminant shows that combinations of chromatin features define response to Smarca4 ATPase mutants. Increased and decreased sites have distinct combinations of features, with increased sites having elevated H3K4me3 and other positive factors/marks. **(b)** Regression weights relating each of the above chromatin features to the fold change of Ring1b at a given site obtained via Lasso multivariate regression, presented as a heatmap. The consistent positive regression weights for H3K4me3, its methylase Kmt2b, and demethylase Jarid1a, show that high levels of these marks in wild-type cells are associated with increased Polycomb occupancy upon expression of Smarca4 ATPase mutants.

Fig. 4. Smarca4 ATPase mutations result in increased H3K27me3 levels near sites of Ring1b increases

(a) Example genome tracks of Ring1b increases with adjacent H3K27me3 increases caused by E861K Smarca4. (b) Genome-wide changes of H3K27me3 peaks between wild-type and E861K Smarca4. Sites that are increased (orange), decrease (blue), or remain unchanged (gray) are labeled using the criteria described in the Methods section. (c) Heatmap of global H3K27me3 changes between cells expressing wild-type and mutant Smarca4. (d) Pearson correlation heatmap of H3K27me3 changes between all mutants and wild-type Smarca4. (e) H3K27me3 peaks occur adjacent to CpG islands; sites that increase H3K27me3 levels upon expression of mutant Smarca4 have increased Ring1b occupancy over the adjacent CpG island. (f) Meta-gene plots of H3K27me3 and H3K4me3 marking levels classified by Ring1b status [decreased (N=66 sites), increased (N=716 sites), or unchanged (N=3,078 sites) in E861K compared to wild-type Smarca4]. Sites with increased Ring1b have increased H3K27me3 marking ~2 kb away; however, sites show no change in the levels of H3K4me3.

Fig. 5. The ATPase of Smarca4 directly regulates the PRC1 complex

(a) Workflow for immunoprecipitation (IP) experiments. Nuclear protein was isolated for analysis from the soluble portion of nuclear lysates. (b) Co-IP of BAF components with PRC1 subunits (N=3; two cell-culture replicates). (c) Reciprocal co-IP of PRC1 components with BAF subunits. Co-IP of Ring1b, Rybp, and Smarcc1 was observed with antibodies directed against the dedicated BAF subunit Smarcb1 (N=3; two cell-culture replicates). (d) BAF binding of PRC1 is unaffected by the addition of DNase I, consistent with a direct interaction which does not require mediation through chromatin. See Figure S3F regarding the lack of staining of some factors in the input samples in (c) and (d). (e) Interaction of Smarca4 and Smarcc1 with PRC1 subunit Rybp is disrupted by the addition of 10 mM ATP (N=5; three cell-culture replicates). (f) Release of PRC1 is inefficient in the presence of ATP analogs that inhibit hydrolysis. ATP leads to reduced co-IP of BAF and PRC1 compared to AMPPNP [$t(2)=4.5$, $p=0.046$] and ATP γ S [$t(2)=6.2$, $p=0.025$; two-sample t-tests]. As observed in other ATPases⁵⁰, ATP γ S is weakly hydrolyzed, leading to partial release. Error bars are SEM (N=3; two cell-culture replicates). (g) Interaction of BAF and PRC1 is disrupted by ATPase mutants of Smarca4 (N=3; two cell-culture replicates). (h)

Densitometry of individual replicates in (g). Mutant Smarca4 shows reduced interaction with PRC1.

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Fig. 6. Chemical-induced proximity of BAF causes rapid loss of Polycomb occupancy on intact chromatin

(a) Schematic representation of chemical-induced recruitment experiments in live cells. Details are provided in main text. ZF, Zinc finger; FKBP, FK506 binding protein; Frb, FKBP-rapamycin binding domain of mTOR. (b) ChIP-qPCR enrichment profile of BAF subunit Smarcc1 following addition of rapamycin. (c) ChIP-qPCR profile of PRC1 subunit Ring1b following addition of rapamycin. (d) Time course for loss of Ring1b and Suz12 at recruitment site following addition of rapamycin. Differential loss occurs at the recruitment site, confirming that Polycomb occupancy is locally and not globally reduced, consistent with eviction activity on chromatin. For all subfigures, data shown are the means of N=3 cell-culture replicates; all error bars are SEM.