Brg1 Regulates Hoxa9-Mediated Proliferation and Gene Expression in Acute Myeloid Leukemia

Undergraduate Honors Thesis

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ABSTRACT:

The current molecular study of leukemia frequently finds connections to mixed lineage leukemia (MLL) protein and its various downstream targets. In cases of acute myeloid leukemia, one of the most significant of these target genes is HOXA9. Homeobox proteins have long been associated with development and HOXA9 has been observed to play a role in hematopoiesis. Overexpression of HOXA9, most often with MEIS1, has been found in a large proportion of AML cases and is strongly connected to a severe disease phenotype. The current model describes a complex of HOXA9-MEIS1-PBX2 interacting with numerous other transcription factors to control gene expression in AML. Research has only begun to understand the complex dynamics regulating HOXA9 genomic binding and transcription control.

Our lab has previously characterized Hoxa9 binding in murine hematopoietic cells.[1] In a high-throughput study, Hoxa9-Meis1 binding sites were identified throughout the genome. The trimeric Hoxa9-Meis1-Pbx2 complex was found to bind at sites enriched with motifs for C/ebp, Runx, Stat, and Ets families of proteins. Hoxa9 loci also showed high levels of H3K4 monomethylation, characteristic of enhancer sequences. These data created the model that Hoxa9 localizes at enhancer sequences of genes involved in leukemia and hematopoietic development. Sites identified included *Cd34*, *Erg*, *Flt3*, *Lmo2*, *Myb*, and *Sox4*.

This study was conducted to help elucidate the molecular mechanism controlling Hoxa9 binding to these enhancers sequences and its subsequent control of transcription. Our results identify the SWI/SNF chromatin remodeling family member Brg1 as crucial to Hoxa9 mediated proliferation. We observed an interaction between human BRG1 and HOXA9 through immunoprecipitation. We then generated a Hoxa9-Meis1 transformed murine hematopoietic cell line bearing an inducible Brg1 shRNA knockdown system. Characterization of cells following knockdown show that proliferation is dependent upon the presence of Brg1. Through ChIP and expression studies, we find that Brg1 controls Hoxa9 target gene expression, but not binding of the trimeric complex. By high-throughput sequencing, we identify a subset of genes that are

controlled by Brg1 in Hoxa9-Meis1 cells that may prove targets of future study into the mechanism controlling Hoxa9 leukemogenesis.

INTRODUCTION:

Acute myeloid leukemia (AML) is a cancer common in older individuals, but not unseen in younger patients. It is defined as a halt in differentiation in the myeloid pathway with accumulation of hematopoietic cells at an immature stage. The disease is heterogeneous, often with multiple subclone populations bearing different abnormalities, creating unusual challenges in treatment.[2] AML remains a condition where the majority of young patients and over 90 percent of adults will die from their disease.[3] With frequent relapse from remission,[4] treatments for AML are moving away from chemotherapy and cytotoxic treatment. More study has been dedicated to molecular abnormalities and mechanistic understanding as a goal for future drug treatment.

The protein HOXA9 displays a strong connection to AML. Upregulation of *HOXA9* has been commonly found to be elevated in clinical AML samples,[5] and such overexpression has been identified as the strongest indicator of poor prognosis in patients.[1,6] Furthermore, leukemia resulting from translocations of the mixed lineage leukemia (MLL) gene often requires the presence of HOXA9 for a disease phenotype.[7] Homeobox (HOX) genes play important roles in development. Many early studies found a role for Hox cluster genes on segmentation and body plan in *Drosophila*,[8] with different expression in segmental zones. The role of these transcription factors (TFs) in body plan determination has been conserved in all animals with bilateral symmetry. This family of genes has also been implicated in a number of other processes involving cell growth, such as wound healing and limb regeneration.[9] A number of these *HOX* genes are involved in regulation of hematopoiesis, including HOXA9. Expression of *Hox* cluster genes was found to be significantly elevated in hematopoietic stem cell populations when compared to total bone marrow, particularly for A cluster genes, and reduced upon differentiation.[10] Additionally, overexpression of *Hoxa9* in mice leads to expansion of the myeloid compartment with reduction in lymphoid differentiation.[11] This result has been reinforced by observing that Hoxa9$^{-/-}$ mice demonstrate compromised hematopoiesis through reduced repopulating ability.

Similarly, HSC Hoxa9$^{-/-}$ cell lines show reduced proliferation in liquid culture.[12] Collectively, HOXA9 expression has been seen to be elevated in early stages of hematopoiesis and to direct cells toward the myeloid pathway.

In conjunction with HOXA9 overexpression in AML, the transcription factor MEIS1 has also been found to be commonly upregulated in this disease. Studies have shown that HOXA9 overexpression almost always occurs with aberrant elevated expression of MEIS1.[13] MLL fusion proteins have been seen to directly upregulate *HOXA9* and *MEIS1* expression as a mechanism of overcoming the differentiation pathway and maintaining a primitive state.[14] More current research has also implicated the role of microRNA in the MLL based upregulation of HOXA9 and MEIS1.[15] A synergistic model has been created linking HOXA9 and MEIS1 as partners in leukemias. Overexpression of either of the *Hoxa9* or *Meis1* genes alone has been seen to be only weakly oncogenic[1] or unable to transform primary bone marrow cells. However, together Hoxa9 and Meis1 are able to transform primary bone marrow cells and create an aggressive AML when injected into mice.[16] It has also been observed that the PBX family of proteins enhances in vitro binding of Hox proteins in vitro. Additionally, in a biological context, it has been observed that Pbx2/3 are highly expressed in myeloid nuclei and that Pbx family proteins, with Meis1, are required for efficient transformation in leukemia.[17] Pbx2 particularly colocalizes with HOXA9 and Meis1 in myeloid leukemia cells. Furthermore, in vitro immunoprecipitation shows complex formation between HOXA9-Meis1-Pbx2 in myeloid leukemia cells.[18] Altogether, this supports a model of human HOXA9-mediated AML facilitated through a trimeric complex of HOXA9, MEIS1, and PBX2.

Our lab has published a characterization of Hoxa9 binding in murine hematopoietic cells.[1] Using high throughput methods of ChIP-Seq, ChIP-chip, and microarray analysis, genomic localization of Hoxa9 and Meis1 was identified and a Hoxa9-Meis1-Pbx2 binding motif was characterized. Specifically, it was seen that the Hoxa9 trimeric complex binds to a number of noncoding elements, especially enhancer elements. Genes found to be controlled by Hoxa9 were involved in a diverse array of processes, including immune response,

inflammation, and cell activation. To further understand the large number of Hoxa9 binding sites, a number of other TFs were found to co-localize with the complex and potentially play a role in binding specificity. Such proteins included Pu.1, C/ebpa, Creb1, and Stat5. Overall, while this study expanded the knowledge of Hoxa9 binding, the mechanism of action for Hoxa9 still remains poorly understood. Hoxa9 was seen to be involved in activation and repression of expression, as well as promoting normal development and cancerous expansion.

With this diversity of binding sites for HOXA9, both in leukemia and normal development, interest has grown in studying the mechanism and regulation of its binding and downstream gene expression. Chromatin remodeling is implicated in expression regulation of almost any gene. The current paradigm classifies all chromatin remodeling into two categories: covalent modification and nucleosome mobilization.[19] The former describes the placement of epigenetic marks onto histones, affecting the structure of chromatin packing and the ability of TFs to bind to DNA. Common examples of histone modifications include methylation, acetylation, and ubiquitylation.[20] Specific marks often are associated with particular functions. For example, H3K27Ac is often associated with elevated expression while H3K27me3 with silencing; additionally, the H3K4me1 modification is commonly found at enhancer sequences with a role in transcriptional machinery recruitment.[19]

Nucleosome mobilization is the second classification of chromatin remodeling. This describes a process by which histones are not covalently modified, but nucleosomes are instead shifted in relation to one another. One of the canonical examples of such a family of proteins is the SWI/SNF chromatin remodeling complex. This complex was the first ATP-dependent remodeling complex described, originally characterized in *S. cerevisiae*.[21] The SWI/SNF family is highly conserved amongst eukaryotes, with the related subclasses in humans being BAF and PBAF. Original studies of BAF complexes showed binding to DNA be sequence-nonspecific, suggesting the need for a sequence-specific TF for its recruitment in the genome.[21] Further characterization of human BAF and PBAF forms has shown the complexes to consist of many varying subunits, often with tissue specificity or even variation within one cell type.[22] However, a conserved core complex remains constant in all humans

forms, consisting of INI1, BAF155, BAF170, and either BRG1 or BRM.[23] BRG1 and BRM are mutually exclusive catalytic ATPase subunits of mammalian BAF complexes that share 75% amino acid identity. No genes have been found to be regulated by both BRG1 and BRM. [24] Knockout mice for both subunits have been characterized. Brm null mice display a phenotype of being overall larger, on average by 15 percent;[25] by contrast, Brg1 null mice are embryonic lethal.[26] However, both proteins appear to be present in BAF complexes during development.[30] It has been observed that Brg1 tends to expressed in adult stages in cell types that undergo frequent replication, while Brm tends to found in cells not commonly proliferating.[31] Interestingly, Brg1 has been seen to act in both oncogenic and tumor suppressor roles. It has been found that Rb mediated cycle arrest, a classical tumor suppressive process, is dependent upon the presence of Brg1 or Brm.[27] Similarly, the SWI/SNF complex has been seen to both condense and open chromosome structure, as well as activate and inhibit transcription.[29] On the other hand, Brg1 acts as an oncogene by binding to CBP and constraining p53 tumor suppressor activity.[28] Altogether, this displays a diverse spectrum of roles for the BAF complex.

Our interest in Brg1 stemmed from an experiment conducted by our lab to identify proteins that collaborate with Hoxa9 at its binding sites.[1] An immunoprecipitation was performed for Hoxa9 and Meis1 (using HA and FLAG antibodies) and the eluate was separated via SDS-PAGE. Bands were excised and analyzed by mass spectrometry. Amongst a number of transcription factors, Brg1 and other SWI/SNF components were identified as likely to physically interact with Hoxa9, potentially at its target genes. In this study, we examined the role of Brg1 in Hoxa9-mediated leukemia. We performed an immunoprecipitation experiment in human cells to find an interaction between BRG1 and HOXA9. We proceeded to create a murine cell line with conditional Brg1 knockdown by transforming TetON murine bone marrow cells with Hoxa9 and Meis1 overexpression transgenes, and stably integrated a tetracycline-inducible small hairpin RNA (shRNA) system. Upon knockdown of Brg1, these Hoxa9-Meis1 (TO-HM) cells demonstrated reduction of proliferation and beginning of differentiation. Further study demonstrated the knockdown of Brg1 results in changes in Hoxa9

target gene expression. Using high-throughput RNA sequencing, we found a number of genes demonstrating significant change from Brg1 knockdown. We conclude that Brg1 and the Baf complex play a significant role in the action of Hoxa9 to transform cells and create leukemia.

MATERIALS AND METHODS

Cell Line Establishment and Culture.

Murine primary bone marrow cells were harvested from 5-fluorouracil-treated female 6- to 8- week old TetON mice at 120 hours post treatment. Cells were grown in Iscove's modified Dulbecco's media (IMDM) with 15% fetal bovine serum (StemCell Technologies) and 1% pencillin/streptomycin. Interleukin-3 (IL-3) and stem cell factor (SCF) were added to the media at concentrations of 10 ng/mL and 100 ng/mL, respectively.

After 24 hours following harvest, cells were spinoculated with murine stem cell virus (MSCV) constructs containing cDNAs for HA-tagged Hoxa9 and FLAG-tagged Meis1. Virus was produced by transfecting (FuGene Transfection Reagent) Plat-E cells with 6 ug of total DNA in a 10 cm dish and concentrated in 4 mL of supernatant IMDM. At 48 hours post-transfection, bone marrow cells were resuspended in viral media and centrifuged for 90 minutes at 3200 rpm with 5 ng/mL polybrene. Cells were resuspended in 1 mL viral media and 3 mL IMDM culture media. A second spin was performed 24 hours later. As Hoxa9 and Meis1 overexpression is required for mouse hematopoietic proliferation, no selection was necessary. Integration of constructs was confirmed by fluorescence microscopy for GFP expressed from the MSCV plasmid. SCF concentration was gradually reduced, and eventually eliminated, as cells proliferated.

After cells began replicating, TO-HM cells were spinoculated with TRMPV plasmids containing shRNA constructs targeting either *Renilla* (nontargeting control) or *Brg1* (courtesy of Chris Vakoc, Cold Spring Harbor Laboratories). The same protocol for viral transduction was used. At 72 hours post-spinoculation, cells were selected using 1 mg/mL Geneticin (Gibco) for one week and then cultured at 0.5 mg/mL.

To induce shRNA expression, doxycycline was added to the culture media to a concentration of 1 ug/mL. Induction of shRNA was confirmed by checking for red fluorescence, corresponding to expression of a dsRed gene placed downstream of the shRNA transcript.

Gel Electrophoresis and Western Blotting

One million cells were pelleted and washed once with Dulbecco's PBS (DPBS) before lysis in 100 uL SDS Sample Buffer (Novex). Fifty thousand cells were loaded per lane in 4-15% Tris-Glycine gels (Novex) and electrophoresis was performed at 125 V for 90 minutes. Sample was transferred to 0.45 um PVDF membranes (Immobilon) at 25 V for 90 minutes. Blots were blocked for 1 hour with 5% milk solution Tris-buffered saline with .05% Tween-20 (TBS-T) and primary antibodies were applied at 1 ug/mL in 4% milk solution TBS-T with overnight incubation. Antibodies used included Brg1 G-7 (Santa Cruz Biotechnology), β-Actin (Sigma Aldrich), and Brm (Abcam). Membranes were washed 5 times with TBS-T before secondary antibodies conjugated to horseradish peroxidase (HRP) were used at 50 ng/mL in 3% milk TBS-T solution for 1 hour at RT. Membranes were washed 5 further times with TBS-T before being exposed to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and imaged using ChemidocXRS.

Protein Coimmunoprecipitation

293 human embryonic kidney (HEK) cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) with 10% FBS. Cells were transfected with 0.6 ug of FLAG-Hoxa9-SBP DNA (FuGene Transfection Agent) in a 10 cm dish. At 48 hours after transfection, cells were harvested by lifting and pelleted. Cell pellet as washed once with DPBS and then lysed in 1 mL ice-cold Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific) containing 10 uL/mL Halt Protease Inhibitor Cocktail and 10 uL/mL Halt Phosphatase Inhibitor Cocktail (Thermo Scientific), with 0.5 mM $MgCl_2$. Lysis was conducted for 30 minutes by rotation at 4°C before Benzonase Nuclease (Sigma Aldrich) was added at 500 U/mL and rotated for another 30 minutes at 4°C. Nuclease digestion was terminated by 5 mM EDTA and cell debris pelleted at 14,000xg for 15 minutes. Supernatant was collected, with 50 uL input added to an equal volume of 2X SDS-PAGE sample loading buffer

with 5% 2-mercaptoethanol and boiled at 95°C for 3 minutes. The remaining supernatant was incubated with 5 ug/sample of Brg1 antibody (Bethyl) overnight at 4C.

Antibody was captured with 30 uL/sample of Dynal beads pre-washed with bovine serum albumin. Beads were collected and washed twice with 1 mL M-PER, discarding supernatants. Beads were then washed three times with 1 mL M-PER with 150 mM NaCl, incubating the second wash for 15 minutes at 4°C with rotation. Supernatants were discarded after each wash, and finally resuspended in 50 uL of SDS sample loading buffer and boiled for 3 minutes at 95C. Supernatant was collected.

All input and elution samples were analyzed by SDS-PAGE using aforementioned protocol. Primary antibodies used were FLAG M2 (Sigma Aldrich) and Brg1 G-7 (SCBT).

Methylcellulose Colony Assay.

Methylcellulose was prepared with 20% IMDM media, and IL3, penicillin/streptomycin, and Geneticin at the same concentration as liquid culture. To 2 mL of methylcellulose preparation, 10,000 TO-HM cells were added, with or without doxycycline (1 ug/mL). Plates were incubated for 144 hours before replating 10,000 cells in a new 2 mL of methylcellulose. Colonies were counted 120 hours post replating.

Cytocentrifugation.

At each time point, 100,000 TO-HM cells were resuspended in 0.5 mL DPBS. Suspension was placed in EZ Cytofunnel (Fisher Scientific) containing a polylysine-coated microscope slide (Shandon) and spun for 5 minutes at 700 rpm with medium acceleration (Shandon Cytospin3 Centrifuge). Slides were allowed to air dry, then Wright-Giesma stained (HEMA 3 Stain Set).

Chromatin Immunoprecipitation-quantitative PCR (ChIP-qPCR).

A total of 30 million cells were cross-linked with disuccinimidylglutarate for 45 minutes at RT and then with paraformaldehyde for 15 min at RT. Following washes, cells were pelleted and snap-frozen in dry ice. Pellets were lysed in the presence of Complete EDTA-free protease inhibitors (Roche) and PMSF at 100 uM, and sonicated on highest setting for 30 minutes, alternating 30 seconds on and off. DNA fragmentation was assayed by electrophoresis on a 1% agarose gel. Samples were required to have DNA concentrated in the 100 to 400 bp length.

Immunoprecipitation for Hoxa9 was done using anti-HA antibody (Abcam), and for Brg1 using anti-Brg1 (Abcam 110641). Antibodies were compared to rabbit IgG, all at 2.5 ug per immunoprecipitation. After an overnight incubation at 4C with rotation, antibodies were conjugated to Protein G conjugated Dynal Beads. Beads were washed with Low Salt, High Salt, LiCl, and Tris-EDTAbuffers (Upstate/Millipore). Elution was performed using 0.1% SDS and 0.1 M NaHCO3, and samples were reverse cross-linked by incubation at 65C overnight in 0.2M NaCl. Sample DNA was column purified (Qiaquick, QIAGEN).

ChIP DNA elution was assayed using quantitative polymerase chain reaction (qPCR). DNA was quanitified relative to input by use of Taqman probes (7500 PCR System; Applied Biosystems), with primers designed with Primer Express Software 3.0 (Applied Biosystems).

RNA Harvesting and Purification.

TO-HM Renilla and Brg1 3232 cells were cultured with or without the presence of doxycycline (1 ug/mL). After 72 hours of doxycycline treatment, 1 million cells were pelleted and washed once with 1 mL sterile DPBS.

Pellet was lysed in RLT Buffer (QIAGEN) and frozen at -80°C. RNA was DNAse treated (RNase-free DNase Set, QIAGEN) and purified (RNEasy Kit, QIAGEN).

Purity of RNA samples were measured using a NanoDrop Spectrophotometer (Thermo Scientific), screening for samples with 260/230 ratio greater than 2.00 and 260/280 ratio greater than 1.60. Additionally, RNA samples were checked for degradation by separation on 1% agarose gel in TAE buffer, run for 90 minutes at 50V. High quality samples displayed two distinct bands representing undegraded 28S and 18S rRNA.

Quantitative RT-PCR.

RNA was harvested and purified by the above protocol. cDNA was synthesized using the Superscript III First-Strand Synthesis protocol (Invitrogen).

Primers were designed with DNASTAR. DNA was fluorescently labeled with SYBR green and relative quantification was done using comparative DDCt method (7500 PCR System; Applied Biosystems). Expression levels were normalized to β-Actin endogenous control. At least 2 biologic replicates were used in each analysis.

High Throughput RNA Sequencing and Data Analysis.

Total RNA were collected by protocol described previously. The RNA-seq libraries were prepared at the University of Michigan sequencing core using the Intergenx Apollo324 NGS automated library prep system with the Illumina TruSeq RNAseq kit. After library preparation, quality of the samples was checked using the Agilent bioanalyzer and nanodrop. The samples were sequenced on the HiSeq2000 with 50-cycle single-end reads.

All statistical computations were done by Jingya Wang. RNA-seq reads for each library were mapped independently using TopHat version v2.0.3 (http://tophat.cbcb.umd.edu/) against the mouse genome build mm9 using the GTF file downloaded from iGenome (http://tophat.cbcb.umd.edu/igenomes.html). Differential gene expression was calculated by running Cuffdiff 2 (http://cufflinks.cbcb.umd.edu/) on the alignments from TopHat and the UCSC coding genes. Gene set enrichment analysis was performed using GSEA (http://www.broadinstitute.org/gsea/index.jsp) against the Molecular Signatures Database (MSigDB)

RESULTS

BRG1 and HOXA9 interact in vitro.

Our lab previously conducted an immunoprecipitation-mass spectrometry (IP-MS) experiment to identify potential collaborators of Hoxa9.[1] In this screen, a large number of nuclear proteins eluted with Hoxa9 and Meis1 pulldown. Among those, several SWI/SNF family components were identified (Fig. 1A). In addition to Brg1, we identified Baf complex core components Baf155 and Baf170, as well as accessory components Baf250a, Baf60a, and Baf57.

We sought to confirm interactions with collaborators by performing immunoprecipitation. We transfected 293 HEK cells with plasmids expressing full-length human HOXA9 with a FLAG and streptavidin binding protein (SBP) tag, as well as three deletion mutants—D34-61, D62-135, D136-205 (Fig. 1B). Wild type HOXA9 has a length of 272 amino acids, with a C-terminal DNA binding homeodomain (HD), a Pbx binding motif, and a transactivation domain on its N-terminus where the Meis1 interaction motif (MIM) has been identified.[32] Immunoprecipitation of Brg1 resulted in elution of full length Hoxa9-SBP, as well as D34-61 and D136-205 (Fig. 1C). The association appears weak, but the failure of D62-135 to elute with Brg1 pulldown suggests a specific interaction in this deleted region of Hoxa9.

These results suggest that BRG1 and HOXA9 have an association in hematopoietic cells. As the signal of HOXA9-SBP elution was weak and antibody capture was allowed an overnight incubation, facilitating complex formation, these results may suggest that this interaction is indirect. It is possible that the association of BRG1 and HOXA9 is facilitated through a mutually interacting protein. With BAF complex assemblies consisting of many subunits and often being megadaltons in size,[22] it is possible that HOXA9 interacts with a BAF component that is not BRG1. Such a mutual interactor may be one of the other Baf complex proteins identified by our IP-MS experiment. Nonetheless, we have shown in vitro that BRG1 and HOXA9 are able to associate, suggesting that this interaction may occur in vivo as well.

Loss of Brg1 results in growth inhibition in HoxA9-mediated leukemia.

To investigate whether this in vitro association has a functional role, we wished to study the requirement of BRG1 in HOXA9-mediated leukemic transformation. We generated murine model cell lines transformed with Hoxa9 and Meis1 containing an inducible shRNA expression system allowing conditional knockdown of Brg1. Primary bone marrow cells were harvested from TetON mice and retrovirally transduced with HA-tagged Hoxa9 and FLAG-tagged Meis1. Introduction of these two overexpression transgenes created a transformed myeloblastic cell line modeling AML. Cells were subsequently transduced with a TRMPV vector bearing an shRNA expression system under control of a TetON promoter.[33] TetON mice express a reverse tetracycline transactivator (rtTA) that binds a tetracycline response element (TRE), introduced by the vector, in the presence of doxycycline and activates transcription. The shRNA is placed under control of the TRE and is conditionally expressed with doxycycline treatment. Cells were cultured in media with 1 ug/mL doxycycline, inducing expression of shRNA targeting either *Brg1* (sh3232) or *Renilla*, as a nontargeting control shRNA.

Hoxa9-Meis1 immortalized cells showed nearly complete elimination of Brg1 expression after 72 hours of doxycycline treatment (Fig. 2A). Brm levels did not change, as compared to loading control, indicating that cells were not compensating for the loss of Brg1 and restoring Baf complex function. Knockdown of Brg1 expression created mild reduction in growth at 96 hours of doxycycline treatment and significant decrease in growth at 168 hours (Figure 2B). The delay in cellular proliferation reduction corresponded with the delay in complete Brg1 knockdown, showing that Brg1 is required for growth in Hoxa9-Meis1 transformed cells.

To further study the effect of knockdown on Hoxa9-mediated transformation, a methylcellulose colony assay was performed. In this assay, transformed cells are grown in semi-solid media, with the number of colonies formed over time indicating the relative leukemic potential of the cells. There were not significant differences in colony number between Brg1 and Renilla shRNA cells after 144 hours of culture in media with doxycycline. However after replating, cells with Brg1 knockdown produced significantly fewer colonies than

cells with nontargeting Renilla shRNA (Figure 2C). Here we found that growth differences observed in media culture translated to differences in colony formation. Consistent with the result from liquid culture, this colony formation inhibition in semi-solid media further demonstrated the importance of Brg1 in Hoxa9-mediated transformation.

To examine whether cells with Brg1 knockdown underwent differentiation, cytocentrifugation and staining was performed at 168 hours of doxycycline treatment (Figure 3). Cells expressing the nontargeting Renilla shRNA appeared as blasts, with a high nuclear to cytoplasmic ratio and uniformly round nuclei. Once Brg1 expression was knocked down and growth was inhibited, cells began to show signs of myeloid lineage differentiation with increased amount of cytoplasm and cytoplasmic vacuolization. Additionally, some cells showed nuclear segmentation and circularization, characteristic of early differentiation along the myeloid lineage. These results further the idea that Brg1, and hence most likely Baf complex function as well, is required for the halt of differentiation and expansion of proliferation that characterizes cancer.

With the SWI/SNF family being highly conserved amongst eukaryotes and regulating a wide variety of genes, we wished to study the specificity of growth inhibition from Brg1 knockdown to Hoxa9 leukemia. To do so, we created a cell line transformed by the E2A-HLF fusion protein. The E2A gene has been found to be involved in many chromosomal translocations that result in acute lymphoblastic leukemia, including with transcription factor HLF. This fusion protein has been associated with very poor disease outcome, and its expression is sufficient for transformation of lymphoid progenitors.[34] E2A-HLF leukemias have very low expression of Hoxa9 and Meis1, creating a good model system to study Hoxa9 independent transformation. Using the same procedure as described for TO-HM cells, primary bone marrow cells were harvested from TetON mice and spinoculated simultaneously with virus containing an E2A-HLF cDNA expression plasmid and eithera pool of three shRNA TRMPV constructs targeting Brg1 or nontargeting *Renilla* shRNA. After selection, cells were treated with doxycycline and compared. Whole cell lysate at various time points indicated

successful knockdown of Brg1 without Brm upregulation (Fig. 2D). Through 96 and 168 hours of treatment, we see only a small difference in growth between TO-E2A-HLF cells with Brg1 knockdown and those expressing Renilla shRNA (Figure 2E), suggesting that the phenotype of severe growth inhibition from Brg1 knockdown is specific to leukemias with Hoxa9 overexpression and is not a consequence of the diverse role that the Baf complex plays in transcription regulation.

We see here that the Baf complex is required for growth in Hoxa9-Meis1 transformation and may also be needed for maintenance of an undifferentiated state. This role of Brg1 is seen to be specific to Hoxa9 leukemia, indicating that its mechanism may be relevant to leukemia with high expression of Hoxa9.

Brg1 and Hoxa9 co-localize at enhancer sequences and regulate expression.

After establishing the dependency of Hoxa9-Meis1 immortalization on Brg1, we sought to characterize the mechanistic role of Brg1 in Hoxa9 leukemia. To study the DNA binding of Brg1 and effects with its knockdown, chromatin immunoprecipitation (ChIP) was performed. We first sought to confirm that Brg1 localizes at Hoxa9 binding sites throughout the genome. We performed qPCR on Brg1 and HA (for Hoxa9) ChIP DNA at enhancer sequences *Cd34*, *Lmo2*, and *Runx1* , as well as *Nfm*, a negative control gene expressed specifically in neurons[35] (Fig. 4A). As expected, HA ChIP showed Hoxa9 binding at enhancer sequences but not at the neuron specific *Nfm* gene. Brg1 binding was seen at Hoxa9 enhancer binding sites, and was abolished after 72 hours of doxycycline treatment. Hence, the shRNA knockdown reduces Brg1 expression sufficiently to cause significant reduction in its DNA binding. These data lend support to the conclusion from our IP results that Brg1 and the Baf complex associate with Hoxa9 at its binding sites.

We then tested whether Brg1 and the Baf complex played a role in regulating expression of Hoxa9 target genes. We used reverse-transcriptase qPCR (RT-qPCR) to generate cDNA from mRNA to determine

relative expression of gene expression. By examining a panel of known Hoxa9 regulated enhancers over 96 hours of doxycycline treatment, a variety of changes were seen in Hoxa9 target genes with brg1 knockdown. However, it was identified that *Cd34* and *Flt3* both displayed consistent, time-dependent changes in expression (Fig. 4B). Hence, Brg1 and the Baf complex significantly influence the expression of certain Hoxa9 target genes. Interestingly, both genes are typically associated with an undifferentiated state, yet the two genes showed opposite responses to Brg1 knockdown. Cd34 is a marker of undifferentiated hematopoietic stem and progenitor cells[36] and Flt3 is mutated and up-regulated in acute myeloid leukemia.[37] This may suggest that Brg1 and the Baf complex may play different roles at different Hoxa9 regulated sites, consistent with previous studies showing both repressive and activating roles of SWI/SNF complex. Alternatively, it is also possible that cells are able to survive Brg1 knockdown by compensating with an unusual upregulation of Cd34.

We also studied expression of Hoxa9 and Meis1 under Brg1 knockdown to validate that these changes in expression are due to Brg1 binding and regulation of target genes. By performing RT-qPCR for these RNA (Fig. 4C), we found that *Hoxa9* did not show significant change in expression with knockdown. *Meis1* showed a decrease in expression after 24 hours of doxycycline treatment but no further reduction at later timepoints. This repression of *Meis1* was peculiar as the protein was being expressed from our transgene under control of an exogenous MSCV promoter. It seems unlikely that all clones of our TO-HM cells would have integrated the *Meis1* construct into a genomic region significantly affected by Brg1 knockdown. The qPCR primers used to assay expression are not able to distinguish between endogenous and overexpressed *Meis1*. However, as the exogenous Meis1 bears a FLAG tag, it is slightly larger and migrates slightly slower in SDS-PAGE. Our western blotting shows two distinct bands, neither with significant difference in signal between doxycycline treatment and untreated cells. Hence, the change in transcript level does not seem to have a significant change in protein level.

With negligible Hoxa9 expression changes and distinctive target gene changes, we sought to study the mechanism of regulation. We speculated that Brg1 and Baf complex might play a role in remodeling chromatin structure and controlling Hoxa9 localization. However, our ChIP-qPCR experiments (Figure 5) displayed negligible and inconsistent change in Hoxa9 binding over time with Brg1 knockdown. Early changes in Hoxa9 binding may not be connected to the Baf complex, as Brg1 knockdown is minimal until 48 hours. Such fluctuations may be attributed to global DNA structure change due to shRNA expression. A better comparison than untreated Brg1 3232 cells, to eliminate such changes from shRNA expression, would be using ChIP-qPCR data in Renilla shRNA cells. Yet as previous experiments have demonstrated changes in Hoxa9 target gene expression upon Brg1 departure, our data suggest that the Baf complex is controlling expression of target genes rather than Hoxa9 binding. Thus, our results suggest a model where Hoxa9 binds to DNA independent of Brg1 function but subsequent gene expression is regulated by the Baf complex.

Genome-wide changes in expression in Hoxa9-Meis1 leukemia with Brg1 knockdown.

Having seen that Brg1 and the Baf complex binds Hoxa9 at target enhancers and regulate expression of known genes, we sought to understand the genome-wide impact of Brg1 knockdown on expression in a Hoxa9-Meis1 leukemia model. We collected total RNA in both Brg1 and Renilla shRNA TO-HM cells after 72 hours of doxycycline treatment and performed high throughput sequencing. Statistical analysis performed can be found in the Materials and Methods section. We found that Brg1 knockdown, when compared to expression of a nontargeting Renilla shRNA, resulted in differential expression of 695 genes. Genes were filtered by a fold change of at least 2 and a false discovery rate of less than 0.05. Of the 695 total, 482 genes showed decreased expression upon Brg1 removal, suggesting that Brg1 primarily functions in an activating manner in these cells.

Our list of genes was compared to the list of genes known to be regulated by Hoxa9. We first considered the situation that Brg1 and Hoxa9 functioned cooperatively at genes. Hence, we looked at genes that increased in expression under both Brg1 knockdown and Hoxa9 withdrawal, and well as those that decreased expression under both conditions (Fig. 6A). Here we found that 37 genes showed upregulation with both Brg1 knockdown and Hoxa9 withdrawal, and 84 genes were downregulated under both conditions. This would indicate that Brg1 and Hoxa9 regulate expression cooperatively at only 121 genes. We next considered the possibility that Brg1 and Hoxa9 act antagonistically in controlling genes expression. We searched for genes that were upregulated with Brg1 knockdown and downregulated with Hoxa9 withdrawal, and vice versa (Fig. 6B). With this analysis, it was seen that both Brg1 and Hoxa9 regulated the expression of a total of 179 genes in an antagonistic manner. As mentioned previously, the SWI/SNF complex has been evolutionarily conserved and found to play a role at a multitude of genes. Baf complex assemblies can serve as both activators and repressors of expression, even within one cell type. Hence, it is best to consider both cooperative and antagonistic models of expression control when looking for genes regulated by Hoxa9 and Brg1. This produced a combined overlap of 300 genes. Some interesting genes that were found to be regulated cooperatively were *Wnt2* and *Flt3*, both downregulated by Brg1 knockdown and Hoxa9 withdrawal. Additionally, many genes involved in hematopoietic development and activity were regulated antagonistically (*CD14, CD28, CD38, CD80, MPO*).

We next sought to find the role of Brg1 in Hoxa9-Meis1 leukemia model cells by gene set enrichment analysis. Using the GSEA database, we examined oncogenic gene sets (C6), groups of genes commonly dis-regulated in cancer. Interestingly, gene set enrichment was minimal in this database for our Brg1 knockdown data. However, we saw significant enrichment of gene sets in the data from our nontargeting control Renilla shRNA cells. The sets identified here represent those that are normally activated by Brg1 in our TO-HM model cells and show down-regulation upon knock down. Among the gene sets enriched were those enriched by STK33 knockdown in AML model cell lines. In this study, an RNAi screen for proliferation inhibition was

conducted in human cancer cell lines with a KRAS mutation.[38] STK33, a serine/threonine kinase, was identified as synthetic lethal protein in multiple types of cancer. From here, the AML cell lines of NOMO-1 ad SKM-1 were treated with RNAi targeting *STK33* and gene expression was studied. Interestingly, both the genes upregulated and down-regulated from STK33 knockdown were enriched in our analysis (Fig. 7A), once more lending to the notion that Brg1 and Baf complex play a diverse role of functions in our TO-HM cells. Downregulated genes in this set included genes in involved in antiapoptotic activity (*BCL-2, PAWR*), hematopoietic cancers (*AFF3, TCTA, CDA, HOXA10, ALCAM*), and cell cycle regulation (*CDK8, MAPK14*). There were also several relevant genes found in the set of STK33 knockdown upregulated genes. Pathways involved included cell cycle inhibition and senescence (*CDKN1A, CDKN1B*), cell adhesion (*CD9*), apoptosis (*BMF*), metastasis suppression (*MTSS1*), and development (*WNT5A*).

Another gene set enriched in our Renilla shRNA data was the group of genes up-regulated by HOXA9 suppression in MLL-germline leukemias.[39] As these gene sets are down-regulated with Brg1 knockdown, pathways represented here are regulated antagonistically by Hoxa9 and Brg1. Genes identified were involved in a variety of relevant pathways, such as apoptosis (*BAX, GDF15*), hematopoietic cell interaction and survival (*CD84, CD86, MPO*), adhesion (*PECAM1*), DNA damage repair (*GADD45A*), cell cycle control (*CDK8*), and other leukemias (*TAX1BP3*).

Altogether, our gene set enrichment analysis expectedly does not suggest one specific function for Brg1 in leukemia. For instance, we identified both pro- and anti-apoptotic pathways, as well as genes both promoting and inhibiting progression through the cell cycle. It is also important to note that genes identified in each set may not be individually down-regulated by Brg1 knockdown. Overall, as we observe growth inhibition in TO-HM cells, our RNA-seq data could be used to identify one particular interaction or pathway crucial to Hoxa9-Meis1 transformation that is dependent on Brg1 regulation.

DISCUSSION

We have shown that Hoxa9-Meis1 transformed cells specifically require Brg1 to proliferate. Both in our qPCR data and high throughput sequencing results, a number of genes implicated in AML were affected by Brg1 knockdown. Together with our ChIP data showing Brg1 localization at Hoxa9 binding sites, these results suggest that BRG1 and the BAF complex may play a role in human AML disease with overexpression of HOXA9.

We observed that knockdown of Brg1 resulted in alteration in the gene expression of several Hoxa9 target genes. Interestingly, we saw that *Flt3* showed a dose dependent decrease in expression. In normal human development, FLT3 is tyrosine kinase receptor expressed at high levels in short term hematopoietic stem cells and later downregulated in early stages of differentiation.[40] However, nearly all human cases of AML express activated FLT3, often with internal tandem duplication (ITD) mutations. It has been previously characterized that Hoxa9 collaborates with Meis1 and Pbx proteins to regulate transcription of Flt3. [41] Thus, as a Flt3 expression tends to parallel Hoxa9 leukemia, its downregulation is not surprising and appears to be an instance of Brg1 and Hoxa9 working cooperatively to regulate expression.

Unexpectedly, we saw a sharp increase in *Cd34* expression with Brg1 departure. Cd34 is a glycoprotein that has been used as a marker for primitive cells and, in some studies, as an indicator of CML disease severity.[42] The function of Cd34 has not been studied in detail. Yet its frequent appearance in leukemia and in undifferentiated HSCs suggest it may play some kind of function in proliferation. We previously speculated that cells must overexpress *Cd34* in order to survive the Brg1 knockdown. However, it is more likely due to the Baf complex working antagonistically to Hoxa9 at this particular locus. This function would not be unusual as the SWI/SNF family has long been implicated in a wide variety of cellular pathways.

This notion is reinforced by our RNA-seq data and analysis. Of the 300 genes showing overlap in regulation by Hoxa9 and Brg1, there was a significant number demonstrating anatagonistic regulation. Yet our TO-HM cells show a strong decrease in proliferation and morphological signs of differentiation, indicating that

Brg1 is required for maintenance of Hoxa9 transformation. We can reconcile these seemingly contradictory results with the idea that Brg1 and Hoxa9 cooperate at a few genes crucial to proliferation and Hoxa9 transformation. Thus, when Brg1 is knocked down, the transformative effects of these pathways are lost and this has a stronger influence on the differentiation of the TO-HM cell than other pathways where Brg1 and Hoxa9 are antagonistic. Our RNA-seq data identified a total of 121 genes that are cooperatively regulated by Brg1 and Hoxa9 and demonstrate a greater than two-fold expression change with Brg1 knockdown. Future study of the relationship between Hoxa9 and Brg1 may include detailed characterization of these genes. An shRNA screen in HM cells could be conducted, targeting each of these 121 genes individually, and cells that show inhibition of growth can be considered as likely candidates. A detailed study of one such pathway may elucidate a co-regulated pathway necessary for Hoxa9 leukemia.

While we observe these expression changes from Brg1 knockdown, there are still many questions surrounding the mechanism of interaction between Hoxa9 and Brg1. Our data seem to indicate that Brg1 is not required for maintenance of Hoxa9 binding. We witnessed no consistent trend in HA ChIP signal with knockdown, and that the fluctuations in Hoxa9 binding seen may be a downstream consequence of other effects of Brg1 knockdown. Yet, Brg1 ChIP showed that the protein was found at Hoxa9 binding sites and abolished with shRNA expression. Hence, our results create a model of Hoxa9 leukemia where the Hoxa9 trimeric complex is directed to leukemogenic binding sites via mechanism not involving the Baf complex. With its lack of sequence specificity, Brg1 and the Baf complex is recruited either subsequently or completely independently to control expression.

The possibility of another transcription factor involved in the association between Brg1 and the Baf complex is consistent with our IP data. We observed a weak interaction between BRG1 and HOXA9, but could not determine whether this was direct or through a common interactor. A subsequent experiment that can

clarify this would be to perform an IP using bacterially purified proteins, without a eukaryotic cell lysate. This would eliminate the possibility of other proteins being present or complex formation.

Identification of a TF that facilitates the interaction between Hoxa9 and Brg1 may prove to be a viable drug target. The SWI/SNF complex is highly conserved and some kind of Baf assembly is expressed in many human cell types, making it an improbable therapeutic target. However, it may be found that a TF exclusive to hematopoiesis and leukemia is specifically responsible for Baf complex involvement in expression of leukemogenic genes. Such a protein may be able to be controlled by usage of small molecular inhibitors or similar treatments.

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FIGURE LEGENDS

Figure 1: Human BRG1 associates with HOXA9 in vitro. A. SWI/SNF family members identified in the IP-MS experiment previously published (Huang et al 2012). Antibodies targeting HA and FLAG were used to elute Hoxa9 and Meis1, respectively. Pw represents the probability that the peptide identified is specific to the protein matched in the database. Specs denotes the number times the unique peptide was identified and peps denotes the number of unique peptides identified within a specific protein. **B**. Diagrams of all human HOXA9 constructs used. Full length HOXA9 carries a MEIS1 Interaction Motif (MIM), PBX2 binding motif, and DNA binding homeodomain (HD). All constructs contained an N-terminal FLAG tag and C-terminal SBP tag. Deletion constructs lacked amino acid ranges from 34 to 61 (D34-61), 62 to 135 (D62-135), and 136 to 205 (D136-205). **C**. Human HEK cells were transiently transfected with each HOXA9 construct in pcDNA3. Input shows total level of expression of endogenous BRG1 and transfected HOXA9 constructs. BRG1 specific antibody was added to lysate and precipitated. BRG1 IP samples show successful pulldown of BRG1 with co-precipitation of full length HOXA9, D34-61, and D136-205.

Figure 2: Brg1 knockdown specifically inhibits Hoxa9-Meis1proliferation. TetOn Hoxa9-Meis1 (TO-HM) cells were stably transduced with a TRMPV construct bearing either no shRNA (Empty), a nontargerting shRNA (Renilla), or shRNA targeting Brg1 (Brg1 3232). **A**. Whole cell lysate was collected every 24 hours and analyzed by SDS-PAGE and western blotting. Samples were probed with primary antibodies specific to Brg1, Brm, and β-Actin. **B**. Plots of total cell number in liquid culture, displayed for 96 hours and 168 hours of doxycycline treatment or no treatment (Control). **C**. Methylcellulose colony assay for 192 hours of doxycycline treatment. Cells bearing an empty TRMPV (Empty) construct and nontargeting Renilla shRNA both show no change in colony numbers when treated upon doxycycline treatment. Brg1 3232 knockdown cells show significant reduction in colony formation when compared to untreated cells. **D**. TO-E2A-HLF cells transduced with either nontargeting *Renilla* or a pool of Brg1 *shRNA* constructs (shBrg1 Pool). Whole cell lysate was collected at 0,

96, and 168 hours and analyzed by SDS-PAGE and Western Blotting. **E**. Plots of total TO-E2A-HLF cell number in liquid culture, displayed for 96 hours and 168 hours of doxycycline treatment of either Brg1 shRNA Pool or *Renilla*.

Figure 3: Brg1 knockdown leads to increased cellular differentiation of Hoxa9-Meis1 cells. TO-HM cells stably carrying a TRMPV construct with either nontargeting Renilla shRNA or Brg1 targeting (3232) shRNA were treated with doxycycline at 1 ug/mL in liquid culture. Cytocentrifugation was conducted at 168 hours of treatment and slides were stained. TO-HM Renilla cells appear as primitive blasts, with circular nuclei and a high nuclear to cytoplasmic ratio. TO-HM Brg1 3232 knockdown cells show marked change in phenotype. Morphology changes include decrease in nuclear ratio, increased vacuolization, and nuclear circularization.

Figure 4: Loss of Brg1 at Hoxa9 binding sites results in changes of downstream expression. A. ChIP was performed using antibodies specific to Brg1, HA for Hoxa9, and Rabbit IgG as a control. Quantitative PCR (qPCR) was conducted using primers targeting three enhancer sequences that are known Hoxa9 binding sites (*Cd34, Lmo2, Runx1*) and one negative control site (*Nfm*) that shows neuron specific expression. Hoxa9 and Brg1 binding was confirmed at enhancer loci. Upon doxycycline treatment, Brg1 binding at Hoxa9 binding sites drop significantly. **B**. RT-qPCR of cDNA from TO-HM Renilla and Brg1 3232 cells collected at 24, 48, 72, and 96 hours of doxycycline treatment. Expression was measured by qPCR using primers targeting known Hoxa9 enhancer binding sites. Data is shown as a fold change of Brg1 3232 signal in relation to Renilla. A number of genes show significant change in expression, with *Cd34* and *Flt3* highlighted with the strongest trend. **C**. RT-qPCR also measured expression for *Hoxa9, Meis1*, and *Brg1*. *Hoxa9* shows minor change in expression with Brg1 knockdown relative to Renilla control. *Meis1* shows a sharp decrease at 24 hours then negligible change following. Brg1 expression is successfully abolished. Whole cell lysate from TO-HM Brg1 3232 was collected at 24, 48, 72, and 96 hours of doxycycline treatment (+) or no treatment (-).

Figure 5: Loss of Brg1 leads to inconsistent changes in Hoxa9 binding at enhancers. ChIP was performed on TO-HM cells at 24, 48, 72, and 96 hours of doxycycline treatment. Four known Hoxa9 binding sites were studied, *Dnajc10, Cd34, Lmo2, Runx1*, all located at enhancers. HA qPCR signal at each locus showed no significant change of distinct trend in binding of Hoxa9.

Figure 6: High-throughoput RNA sequencing identifies genes co-regulated by Hoxa9 and Brg1. Circles marked as up_shBrg1 represent genes showing increase in expression upon Brg1 knockdown, and down_shBrg1 are genes showing decrease in expression by Brg1 knockdown. Circles marked up_EtOH represent genes from the database showing increase in expression with Hoxa9 removal by 4-hydroxytamoxifen withdrawal, and down_EtOH represent those showing decrease in expression. **A**. Identification of genes regulated cooperatively by Brg1 and Hoxa9. Overlap between up_shBrg1 and up_EtOH are genes repressed by both Brg1 and Hoxa9. Overlap between down_shBrg1 and down_EtOH are genes activated by both proteins. **B**. Identification of genes regulated antagonistically by Hoxa9 and Brg1. Overlap between down_shBrg1 and up_EtOH are genes upregulated by Brg1 and downregulated by Hoxa9. Overlap between up_shBrg1 and down_EtOH are genes repressed by Brg1 and activated by Hoxa9.

Figure 7: Gene set enrichment analysis identifies a number of cancer-related pathways regulated by Brg1. All plots represent gene sets enriched in TO-HM cells expressing nontargeting Renilla shRNA at 72 hours of doxycycline treatment. Gene sets were drawn from the C6 group of the database, representing cellular pathways often dis-regulated in cancer. **A.** Enrichment plots for gene sets down-regulated (STK33_DN) and up-regulated (STK33_UP) by STK33 knockdown in AML cell lines. **B.** Enrichment plot for gene set upregulated by HOXA9 knockdown in AML cells modeling MLL-rearranged leukemia.

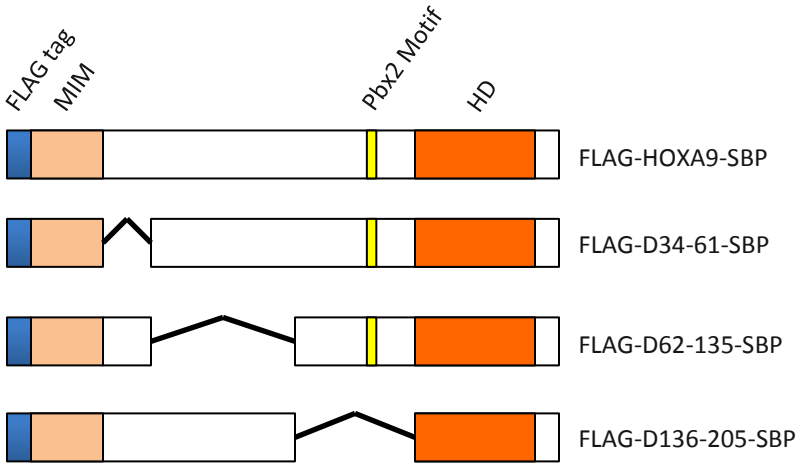
Figure 1: Human BRG1 associates with HOXA9 in vitro.

A

anti-HA (Hoxa9)	Proteins Identified	Immunoprecipitation #1 Pw	Specs	Peps	Immunoprecipitation #2 Pw	Specs	Peps
	Hoxa9 Isoform HoxA-9 of Homeobox protein Hox-A9	1.000	38	9	1.000	120	18
	Meis1 Isoform 1/2 of Homeobox protein Meis1	1.000	5	4	1.000	16	9
	Pbx2 Pre-B-cell leukemia transcription factor 2	1.000	9	3	1.000	20	11
	Pbx3 Isoform PBX3a/b of Pre-B-cell leukemia transcription factor 3				1.000	10	4
	Arid1a AT rich interactive domain 1A (Baf250a)				1.000	7	4
	Smarca4 SWI/SNF related (Brg1)				1.000	19	12
	Smarcc1 Isoform 1 of SWI/SNF complex (Baf155)	1.000	3	2			
	Smarcc2 Isoform 2 of SWI/SNF complex (Baf170)				1.000	20	10
	Smarcd1 SWI/SNF related (Baf60a)				0.996	2	2
	Smarce1 SWI/SNF related (Baf57)	0.970	2	1	1.000	18	8

anti-FLAG (Meis1)	Proteins Identified	Immunoprecipitation #1 Pw	Specs	Peps	Immunoprecipitation #2 Pw	Specs	Peps
	Hoxa9 Isoform HoxA-9 of Homeobox protein Hox-A9	1.000	3	2			
	Meis1 Isoform 1/2 of Homeobox protein Meis1	1.000	141	30	1.000	41	19
	Pbx2 Pre-B-cell leukemia transcription factor 2	1.000	116	33	1.000	31	11
	Pbx3 Isoform PBX3a/b of Pre-B-cell leukemia transcription factor 3	1.000	56	12	1.000	14	6
	Arid1a AT rich interactive domain 1A (Baf250a)						
	Smarca4 SWI/SNF related (Brg1)	1.000	10	7			
	Smarcc2 Isoform 2 of SWI/SNF complex (Baf170)	1.000	6	3			
	Smarcd2 SWI/SNF related (Baf60b)				0.995	3	2
	Smarce1 SWI/SNF related (Baf57)	0.961	2	1			

B



C

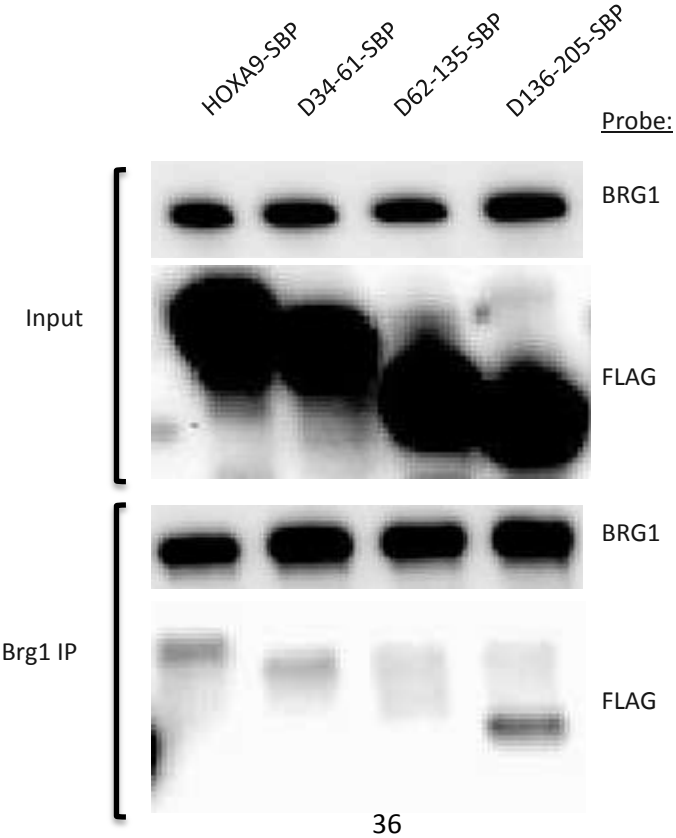


Figure 2: Brg1 knockdown specifically inhibits Hoxa9-Meis1proliferation.

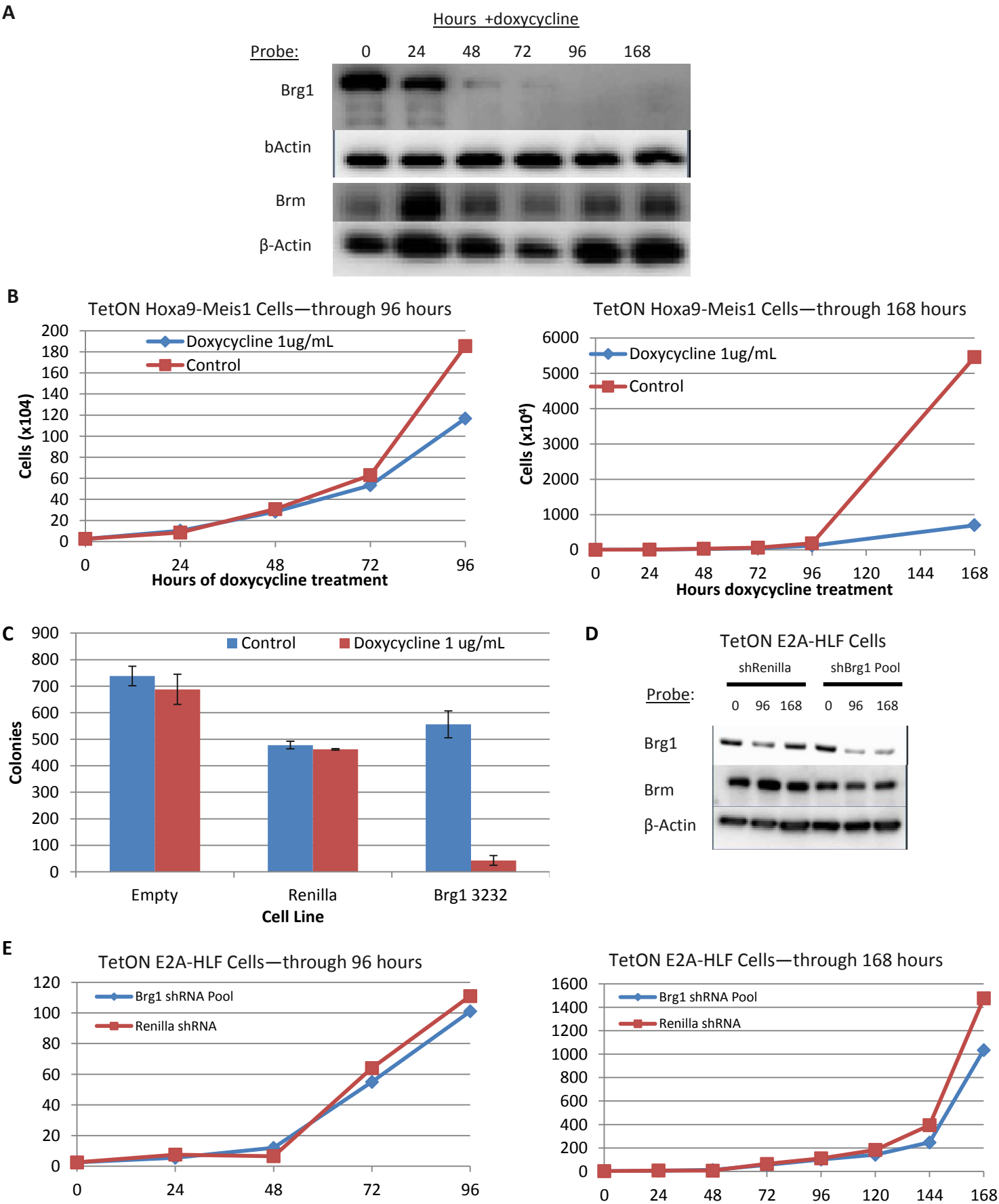
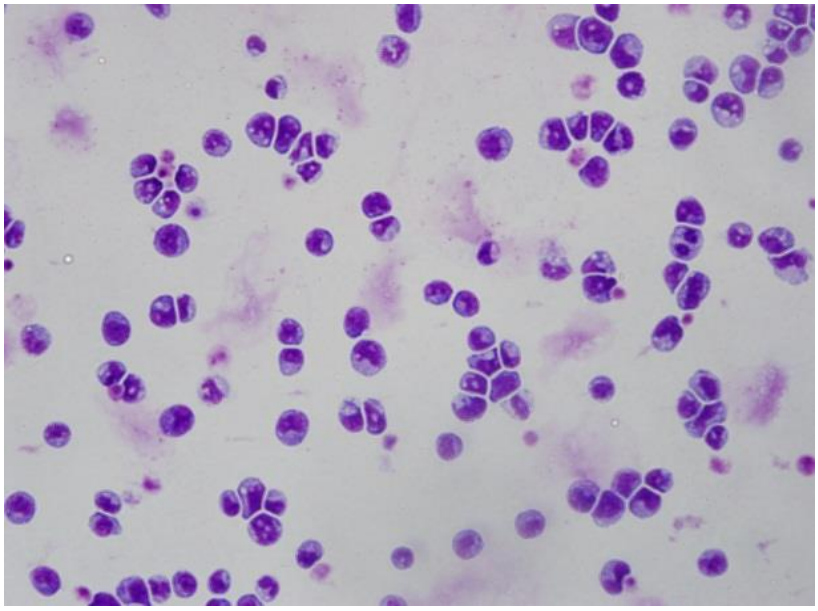


Figure 3: Brg1 knockdown leads to increased cellular differentiation of Hoxa9-Meis1 cells.

TO-HM Renilla 168h +doxycycline



TO-HM Brg1 3232 168h + doxycycline

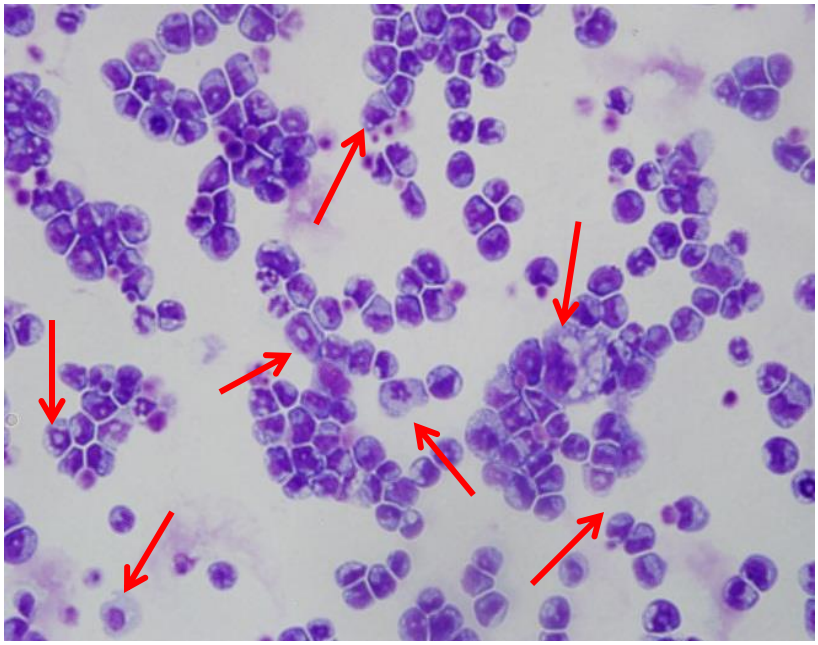


Figure 4: Loss of Brg1 at Hoxa9 binding sites results in changes of downstream expression.

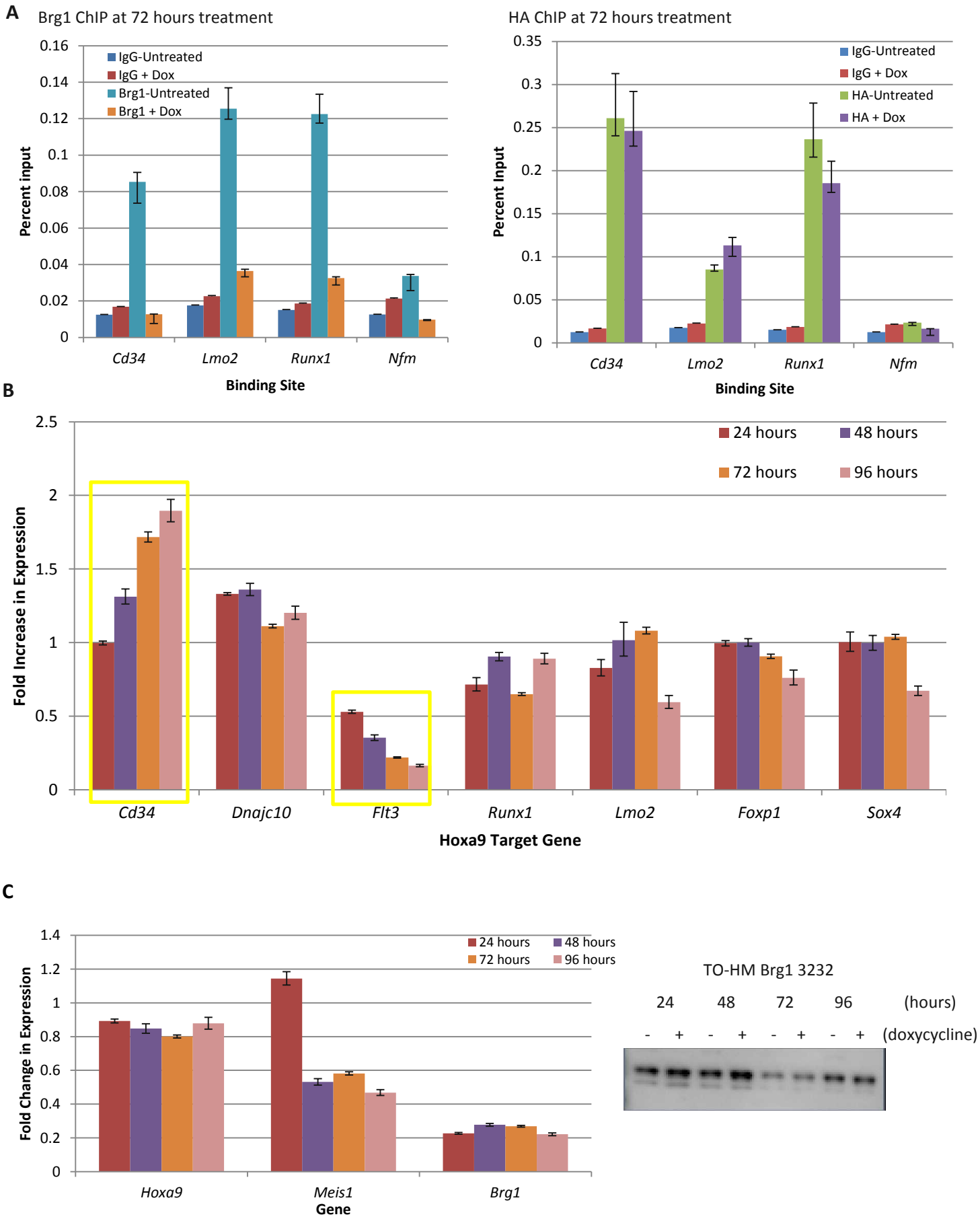


Figure 5: Loss of Brg1 leads to inconsistent changes in Hoxa9 binding at enhancers.

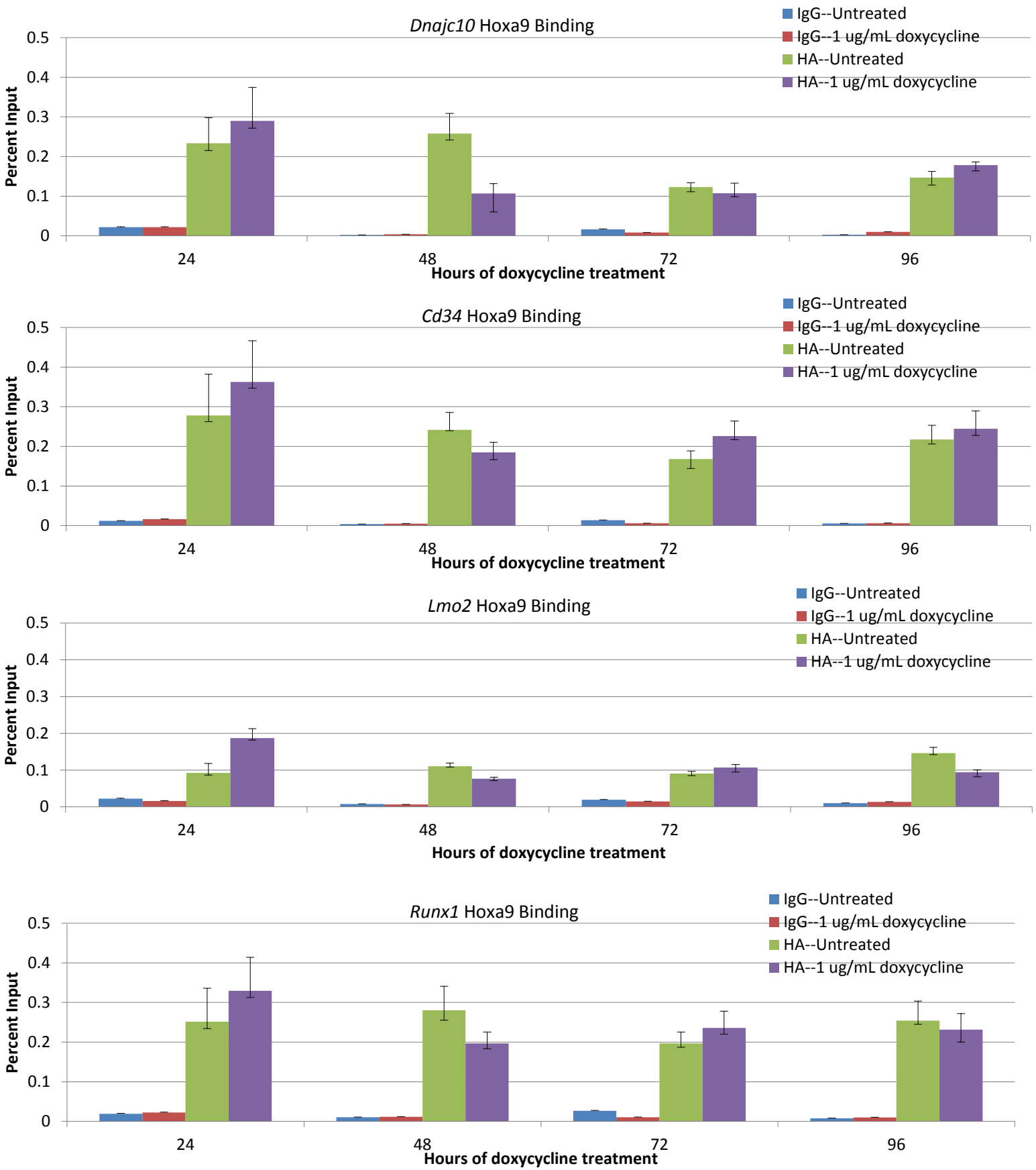


Figure 6: High-throughput RNA sequencing identifies genes co-regulated by Hoxa9 and Brg1.

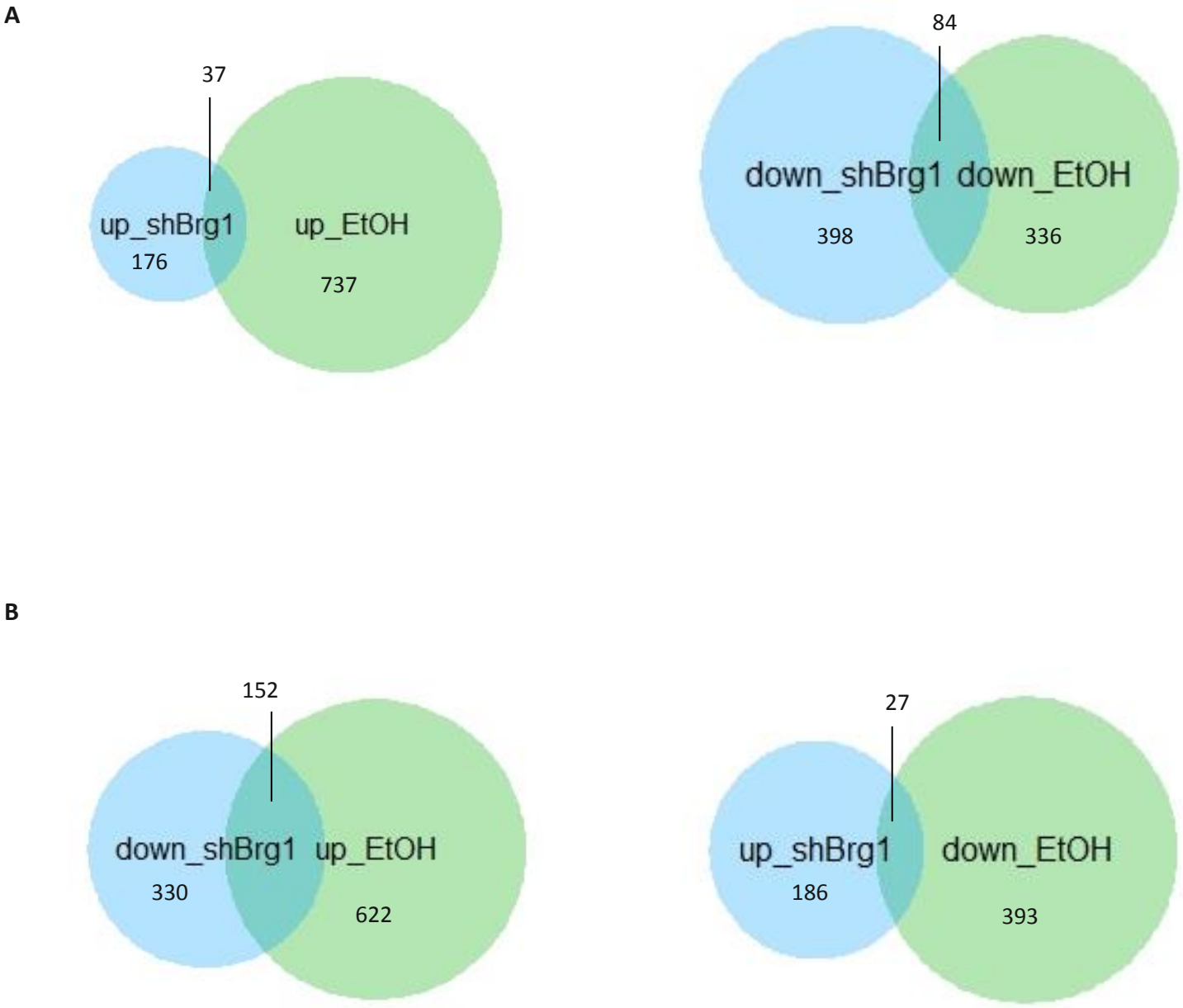
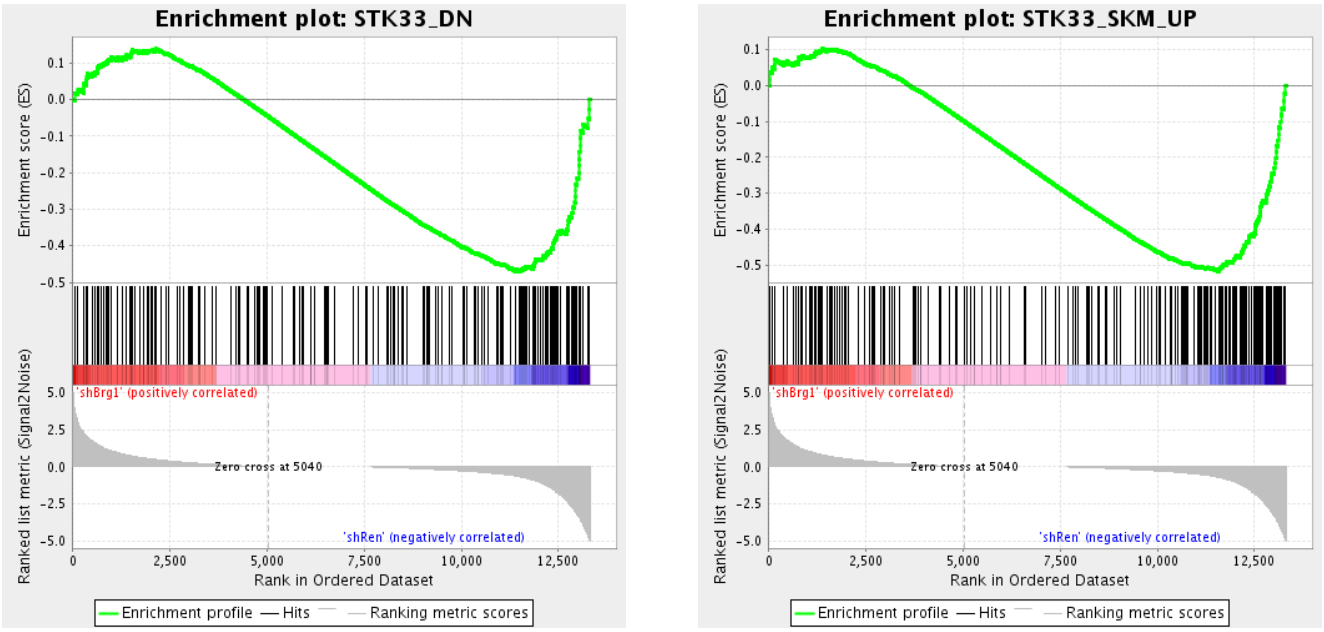


Figure 7: Gene set enrichment analysis identifies a number of cancer-related pathways regulated by Brg1.

A



B