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BAF Complex Association with Enhancers and H3K14 Acetylation in Mouse
Embryonic Stem Cells

A Thesis submitted in partial satisfaction of the requirements for the degree Master of
Science

in

Biology

by

Audrey Young-Jin Kim

Committee in charge:

Professor Bing Ren, Chair
Professor James Kadonaga, Co-Chair
Professor Tracy Johnson

2010

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Co-Chair

Chair

University of California, San Diego

2010

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ABSTRACT OF THE THESIS

BAF Complex Association with Enhancers and H3K14 Acetylation in Mouse Embryonic Stem Cells

by

Audrey Young-Jin Kim

Master of Science in Biology

University of California, San Diego, 2010

Professor Bing Ren, Chair
Professor James Kadonaga, Co-Chair

Enhancers are regulatory elements that increase transcription activity of genes. The general mechanism of enhancers is well known, but the role of chromatin structure in the mechanism is unclear. We wanted to study the nature of chromatin modifications and chromatin remodeling at enhancers. We determined that Brg1, the catalytic subunit of the human SWI/SNF chromatin remodeling complex, associates with enhancers by chromatin immunoprecipitation with massive parallel sequencing (ChIP-seq). We then observed that the subunits, BAF45a and BAF45d, of Brg1 Associated Factors (BAF complex) specifically bind to acetylated lysine 14 of histone H3 (H3K14ac) via their PHD domains by peptide pulldown assays. We confirmed that this acetylation mark is found at enhancers by ChIP-seq. Elucidating the relationship between the co-activator and the histone modification can help us better understand the role of chromatin structure in enhancer function.

INTRODUCTION

Gene expression is regulated by interactions between transcription factors and regulatory DNA elements. Classes of regulatory elements include promoters, insulators, and enhancers (1). Enhancers are DNA sequences that increase transcription activity of a target promoter. They can be located either upstream or downstream from their target genes, and can activate transcription independent of their orientation (1). The classical model of enhancer function is called “activation by recruitment.” In this model, specific sequences of DNA in an enhancer are bound by transcription factors. These transcription factors can then interact with the general transcription machinery or they can recruit additional proteins, termed co-activators. Ultimately this sequence of events serves to recruit, stabilize or activate RNA polymerase II at the enhancer’s target promoter (2). Despite the extensive research into the mechanism of enhancers, certain components of enhancer biology remain unclear. For instance, in vivo, DNA is wrapped around nucleosomes and interacts with numerous DNA binding proteins in a structure called “chromatin.” The role of chromatin structure in enhancer function remains to be elucidated.

It has been recently observed that enhancers may have a unique chromatin structure (3). Enhancers have high levels of H3K4me1 and low levels of H3K4me3 (3). This signature has been shown to accurately predict areas of the genome with enhancer activity (4). Furthermore, these H3K4me1 predicted enhancers correlate with cell type specific patterns of gene expression, suggesting that H3K4me1 enhancers may be critical for regulating gene expression (4).

In this study, I worked with a graduate student in Dr. Bing Ren’s lab, Jesse Dixon, to determine if other chromatin modifying enzymes and histone modification

are associated with enhancers. We focused on Brg1, the catalytic ATPase of mammalian SWI/SNF complex, also known as the BAF complex. BAF complex is involved in remodeling chromatin to open up the structure for gene transcription. Brg1 in this complex is known to interact with enhancers on a single loci basis. Our goal was to determine if Brg1 associates globally with H3K4me1 predicted enhancers as well as to identify if these co-activators interact with histone modifications at enhancers.

Using chromatin immunoprecipitation with massive parallel sequencing (ChIP-seq), we found that Brg1 localized throughout the genome of predicted enhancers. By peptide pulldown assays, we were able to find that the subunits of Brg-Associated Factors (BAF complex) specifically bind to acetylated marks on histone H3 via PHD domains, previously characterized as only a methyl lysine binding domain. We also found that this acetylation mark is found at enhancers, expanding the repertoire of enhancer associated histone modifications and suggesting a possible mechanism for enhancer associated histone modifications in the regulation of the BAF complex.

METHODS

Chromatin Immunoprecipitation with Massively Parallel Sequencing (ChIP-seq)

Dynabeads M-280 Sheep anti-Mouse IgG (Invitrogen, Cat. No. 112-02D) or Dynabeads M-280 Sheep anti-Rabbit IgG (Invitrogen, Cat. No. 112-04D) were washed 3 times with 5mg/ml BSA (Sigma-Aldrich, Cat. No. A7906) in PBS. 2.5 µg (for qPCR) or 5 µg (for sequencing) of antibodies were added to the beads in BSA/PBS and incubated for 5 hours at 4°C: H3K4me1 (Abcam, Cat. No. ab8895-50, Lot# 766300), H3K4me3 (Millipore, Cat. No. 04-746, Lot# NG1643014), FLAG M2 (Sigma-Aldrich, Cat. No. F1804, Lot# 088K6018), β-Actin AC15 (Sigma-Aldrich, Cat. No. A1978, Lot# 118K4827), β-Actin AC74 (Sigma-Aldrich, Cat. No. A2228, Lot# 128K4813), Actin (Abcam, Cat. No. ab8226, Lot# 699688), Actin C2 (Santa Cruz, Cat. No. sc-8432, Lot# G2607), Actin C4 (Santa Cruz, Cat. No. sc-47778, Lot# J2207), H3K14ac (Millipore, Cat. No. 07-353, Lot# DAM1548623), Rabbit Control IgG (Abcam, Cat. No. ab46540-1, Lot#820425), and Mouse Control IgG (Abcam, Cat. No. ab18413, Lot# 862061). The antibody-bound beads were washed 3 times with BSA/PBS and incubated with 25 µg of chromatin and a master mix of 1% Triton X-100, 0.1% DOC, 1x Complete (Roche, Cat. No. 05056489001), and 1x TE, at 4°C overnight on a rotator. The samples were washed 8 times with RIPA Buffer (50mM Hepes-KOH, pH8.0, 1mM EDTA, 1% NP-40, 0.7% DOC, 0.5M LiCl, 1x Complete) and once with 1xTE. The samples were eluted with Elution Buffer (10mM Tris-HCl, pH8.0, 1mM EDTA, 1% SDS), incubated at 65°C for 20 minutes in a Thermomixer (Eppendorf), and reverse crosslinked at 65°C overnight with input chromatin. Proteinase K mix (1xTE, 0.6 µg/µl Glycogen from Roche (Cat. No. 10901393001), and 0.93 µg/µl Proteinase K from Fermentas (Cat. No. EN0531)). DNA was precipitate by phenol

extraction using Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich, Cat. No. 77617) and Phase Lock Gel tubes (5 Prime, Cat. No. 2302820). 200mM NaCl and 100% ethanol were added to the samples, which were then incubated at -80°C for 30 minutes and spun at 14000 rpm for 15 minutes at 4°C. The pellets were washed with 70% ethanol, air-dried to resuspend with 1µg of RNase A in 1xTE, and incubated for an hour at 37°C. The samples were purified using QIAquick PCR Purification Kit (Qiagen, Cat. No. 28104) and stored at -20°C.

Chromatin-Immunoprecipitated DNA's ends were modified with End-it DNA End-repair Kit (Epicentre Technologies, Cat. No. ER0720). Adapters were ligated to the ends of the DNA fragments by using Quick Ligation Kit (NEB. Cat. No. M2200L) and Adapter Oligo mix from Illumina's Kit. The ligation products were purified by 8% polyacrylamide gel electrophoresis. DNA fragments ranging from 200bp to 400bp were selected and extracted from the gel in EB buffer (Qiagen). The selected fragments were amplified with PCR Primer 1.1 and 2.1 from Illumina's Kit and Phusion Hot Start High-Fidelity DNA Polymerase (NEB. Cat. No. F0540L). The fragments were then purified with MinElute PCR Purification Kit (Qiagen, Cat. No. 28004). DNA fragments from 200bp to 400bp were selected again by 8% polyacrylamide gel electrophoresis. The selected fragments were eluted in EB buffer (Qiagen) and precipitated with sodium acetate, glycogen, and 100% ethanol. The resulting DNA was quantified with Qubit by the manufacturer's protocol and was diluted to 10nM for sequencing.

Cloning of Recombinant DNA coding for GST-BAF Fusion Proteins

mRNA of mouse embryonic stem cells was extracted with TRIzol Reagent (Invitrogen, Cat. No. 15596-018) following the manufacturer's protocol. cDNA of the

extracted RNA was synthesized using SuperScriptIII First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, Cat. No.11752-050). The cDNA of each subunit, Dpf2, Phf10, Neud4, SmarcC1, and SmarcC2 was then amplified using Platinum Pfx DNA Polymerase (Invitrogen, Cat. No. 11708-013). The following primers were used in cDNA amplification: forward primer of the first PHD domain of Dpf2 (GATCGAATTCTTGGCCCTGCCTAACAACTAC), reverse primer of the first PHD domain of Dpf2 (GATCCTCGAGTTAGCAGAGGTTGCAGCACTTGCAT), forward primer of the second PHD domain of Dpf2 (GATCGAATTCTGAATGCAAGTGCTGCAACCTCT), reverse primer of the second PHD domain of Dpf2 (GACTCCTCGAGTTACAGCAGATCCAGACACAGGT), forward primer of the first PHD domain of Neud4 (GATCGAATTCATCCCCAATGGCTACTGTGACT), reverse primer of the first PHD domain of Neud4 (GATCCTCGAGTTAGCCACACAGGCTGCAGGACTT), forward primer of the second PHD domain of Neud4 (GATCGAATTCCAAGTCCTGCAGCCTGTGT), reverse primer of the second PHD domain of Neud4 (GATCCTCGAGTTAAGCAGAGGCCTTTTCCTTCA), forward primer of the first PHD domain of Phf10 (GATCGAATTCCCAAAGGTCATTCCAAATGC), reverse primer of the first PHD domain of Phf10 (GATCCTCGAGTTAGTGGGGCTGTCCACATATAA), forward primer of the second PHD domain of Phf10 (GATCGAATTCCCATGGCAGTGTATGGAATG), reverse primer of the second PHD domain of Phf10 (GATCCTCGAGTTAAGCTCGCTGACAACAGTCAC), forward primer of the chromo domain of SmarcC1 (GATCGAATTCCAAGAGGATGAGGAGTGGCTG), reverse primer of the chromo domain of SmarcC1 (GATCCTCGAGTTAATGAACCTTCCAGGGCTTTT), forward

primer of the chromo domain of SmarcC2 (GATCGAATTCGTGTATCCTGTCCCAGGGAACC), reverse primer of the chromo domain of SmarcC2 (GATCCTCGAGTTAATGGACCTTCCTCGGTTTCT). The cDNAs and vector pGEX were digested with EcoRV and XhoI and ligated together. The resulting plasmids were selected and confirmed by sequencing.

Expression of GST-BAF Fusion Proteins in E. Coli

Each plasmid was transformed into DH5 α strain of E. Coli. One of the colonies picked from the plate was grown in LB media with ampicillin (100 μ g/ml). The cells multiplied until reaching approximately 0.4 O.D. and were induced with 400 μ M IPTG overnight. The culture was spun down at 2500rpm for 20 minutes at 4 $^{\circ}$ C. The pellet was washed with 0.1M HEMG buffer (25mM Hepes-KOH, pH8, 0.1mM EDTA, 12.mM MgCl₂, 20% Glycerol, 100mM KCl, 0.1% NP-40, 5 μ M DTT, 50 μ M PMSF, and 1x Complete from Roche (Cat. No. 05056489001)). The cells were lysed by French Press or using B-PER Reagent (Pierce, Cat. No. 78248). The lysates from using French Press were spun down either at 7000rpm for 20 minutes at 4 $^{\circ}$ C and those from using B-PER reagent at 15000xg for 5 minutes at room temperature. The supernatants (solubilized proteins) of the lysates were saved for purification.

Purification of GST-BAF Fusion Proteins

Glutathione Sepharose 4B (GE Healthcare, Cat. No. 17-0756-05) beads were washed with 0.1M HEMG buffer and with Buffer D containing 100mM KCl, 0.05% NP-40, 5 μ M DTT, and Protease Inhibitors before elution. The target proteins were eluted with a buffer containing 0.1M Buffer D, 0.05% NP-40, 5 μ M DTT, Protease Inhibitors,

and 10mM Reduced Glutathione. The purified proteins normalized by comparing the concentration to that of the positive control, BPTF.

Peptide Pulldown Assays

Dynabeads MyOne Streptavidin T1 (Invitrogen, Cat. No. 656.02) were washed with Peptide Binding Buffer (5mM Tris-HCl, pH8.0, 1M NaCl, 0.5mM EDTA, and Protease Inhibitors) 3 times. 2.5 µg of peptides (H3 unmodified, H3K4me1, H3K4me2, H3K4me3, H3K9me3, H3K9ac, H3K14ac, H4 poly acetylated; all from either Millipore or Abgent) were incubated with the washed beads for 15 minutes at room temperature on a rotator. No peptides were added to the negative control. The samples were then washed with Peptide Binding Buffer for 3 times again. They were then washed once with Nuclear Extract Binding Buffer (20mM Tris-HCl, pH8.0, 150mM NaCl, 0.2% Triton X-100, and Protease Inhibitors). GST purified protein, added to Nuclear Extract Binding Buffer up to 1ml, was incubated with the peptide-bound beads for 4 hours at 4°C on a rotator. The samples were washed 8 times with Wash Buffer 1 (20mM Tris-HCl, pH8.0, 300mM NaCl, 0.2% Triton X-100, and Protease Inhibitors) and once with Wash Buffer 2 (10mM Tris-HCl, pH8.0, 20mM NaCl, and Protease Inhibitors). The peptide-bound proteins were eluted in 25µl of Laemilli Sample Buffer (BioRad, Cat. No.161-0737). The samples were run on 4-15% gradient Ready Gel Tris-HCl Gel (BioRad, Cat. No. 161-1104 or 161-1122) in Running Buffer (1xTris/Glycine/SDS). The gel was either stained with Instant Blue–One Step Coomassie Stain (Expedeon, Cat. No. ISB1L).

RESULTS

BAF complex is localized to Enhancers in Mouse Embryonic Stem Cells

To determine if Brg1 associates with enhancers, we mapped its binding sites in mouse embryonic stem cells (mESC) by Chromatin Immunoprecipitation with Massively Parallel Sequencing (ChIP-seq). We used a mESC line where the gene encoding Brg1 (SMARCA4) had C-terminal FLAG tag at the endogenous locus. By the algorithm previously used to predict enhancer sites based on high signal intensity at H3K4me1 sites and low signal intensity of H3K4me3 (3), we predicted enhancers in these mESCs. We then generated a heatmap with a 10 kb window around the enhancers (5kb upstream and downstream of predicted enhancers) as shown in Figure 1. As a control, p300 was also mapped by ChIP-seq to confirm the identity of the predicted enhancers because it was used in our lab's original characterization of enhancer chromatin signatures. As Figure 1 shows, Brg1 clearly associates with predicted enhancers. Of the 24,650 sites of the predicted enhancers, 12.5% were bound by Brg1 and 22.9% were bound by p300. Of the 7476 binding sites of Brg1, 41.3% overlapped with the predicted enhancer sites. Of the 10,019 binding sites of p300, 33.5% overlapped with the predicted enhancer sites. The result of our ChIP-seq data demonstrates that these predicted enhancers sequences are bound by multiple co-activators. The strong association between Brg1 and H3K4me1 raises questions as to whether Brg1 or its associated factors in the BAF complex interact with H3K4me1 via methylation binding domain-containing components of BAF complex.

Expression and Purification of GST-BAF Fusion Proteins

From the ChIP-seq data, we hypothesized that the methyl-binding domain containing subunits of the BAF complex might physically interact with H3K4me1. To test this hypothesis, we chose to focus subunits associated with Brg1 in mESC according to previously published mass-spectrometry data characterizing the “esBAF complex” (5). These subunits contain well-known methyl lysine recognition domains - the PHD domains - as shown in Table 1. cDNA of PHD domains or chromo domains of the BAF components, BAF45d, BAF45a, BAF45b, BAF155, and BAF170, were synthesized from mRNA from mESC. BAF45a, b, and d all contain two tandem PHD domains, whereas BAF155 and BAF170 each contain a single chromodomain. Both PHD domains of BAF45a and d were cloned together. Each of the two PHD domains of BAF45b was cloned separately. The single chromo domains of BAF155 and BAF170 were cloned. These clones were expressed in *E. Coli*. as GST fusion proteins, which were then purified using glutathione beads. Each fusion protein was verified by its size and was highly pure as shown in Figure 2. Protein concentrations were estimated and normalized after polyacrylamide gel electrophoresis for use in future biochemical experiments.

Interaction of BAF Complex Subunits with Histone Modification

A peptide pulldown assay was used to test BAF proteins' potential interaction with H3K4me1. The assay consists of chemically synthesized peptides designed to resemble the N-terminus tail of different histones with specific residues modified to mimic lysine modification. The purified proteins were incubated with these modified peptides to detect a direct, specific interaction between the proteins and H3K4me1. None of the fusion proteins specifically bound to H3K4me1. Chromo domain of BAF155 and BAF170 especially did not interact with any of the methylated histone

peptides (Figure 3). As controls, a panel of different histone modifications, H3K9me3, H3K9ac, H3K14ac, and poly-acetylation of histone H4 was added to the assays. Strikingly, BAF45d and BAF45a bound specifically to H3K14ac. In addition, BAF45a bound to H3K9me3 to some extent (Figure 4).

Presence of H3K14ac at Enhancers

The above results raise the possibility that the BAF complex may interact with enhancers *in vivo* via H3K14ac. A prediction of the hypothesis is that H3K14ac should also be found at enhancers. To test this prediction, we performed ChIP-seq for H3K14ac in human IMR90 fibroblasts. We also mapped the location of H3K4me1 and H3K4me3 by ChIP-seq to predict enhancers using our previously mentioned enhancer prediction algorithm. H3K27ac was used as a control as we had previously shown that this modification is also present at enhancers (Figure 5). The signaling patterns between H3K27ac, H3K4me1, and H3K14ac indeed mimic each other, confirming that H3K14ac is present at enhancers *in vivo*.

DISCUSSION

We have demonstrated by ChIP-seq that Brg1 is localized to H3K4me1. However, our biochemical assays, with purified subunits of BAF complex, suggested that none of the methyl lysine binding domains bound specifically to H3K4me1. Surprisingly, the PHD domains of BAF45a and BAF45d bound to acetylation on lysine 14 of histone H3. ChIP-seq with H3K14ac in IMR90 cells suggested that this histone modification is also found at enhancers. From these results, it is possible that BAF complex is recruited to enhancers by interacting with H3K14ac (Figure 6). More evidence is needed to confirm the proposed model. For instance, ChIP-seq with BAF45d or BAF45a can confirm that the subunit co-localizes with H3K14ac *in vivo*. It can provide more convincing evidence that the whole subunit can localize to enhancers via its PHD domains. The effect on localization of BAF complex to enhancers can be observed by knocking down BAF45a or BAF45d followed by ChIP-seq of Brg1. The absence of the subunit can affect the binding of BAF complex to the predicted enhancers.

Our results also suggested that the PHD domains of BAF45a bound to H3K9me3, and those of BAF45d bound to unmodified H3 peptide. To which type of regulatory elements these subunits also bind via H3K9me3 or unmodified histone H3 is still unclear. It is possible that BAF45d's binding to H3K14ac can be interfered by the methylations on H3K4 (6). Nonetheless, this association with repressive or inactive chromatin modifications suggests that the BAF complex could have additional roles in regulating gene expression outside of enhancers. Indeed, previous studies have suggested that BAF complex can both serve to activate for repress target gene expression (7).

Another interesting implication of our data is that we see the PHD domains of BAF45a and BAF45d binding to an acetylated lysine. PHD and chromo domains are known to bind to methylated lysines, but our peptide pulldown assays showed that they can also bind to acetylated lysines. Recently, it has been shown that Dpf3 (BAF45c), a related subunit of BAF complex expressed largely in muscle cells, can also bind to the same acetylated lysine, H3K14ac (6). This evidence lends support to our data showing that PHD domains from BAF complex proteins interact with H3K14ac.

This project evidenced the global localization of Brg1 and identified the subunits, BAF45a and BAF45d, which interact with the predicted enhancers. The information obtained from observing the relationship between enhancers and BAF complex can provide a better understanding of chromatin structure at enhancers.

APPENDIX

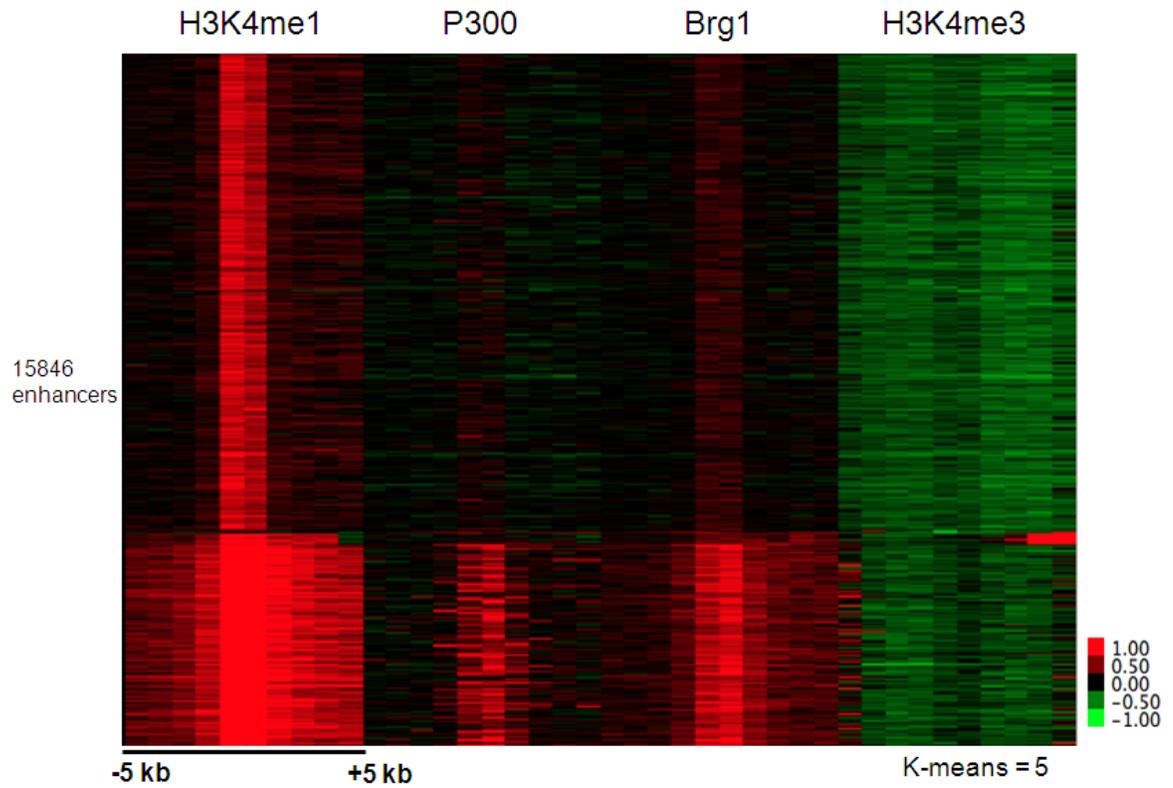


Figure 1: Brg1 Co-localizes With Predicted Enhancers.

Heatmap of ChIP-seq data at predicted enhancers. Enhancers were predicted using a previously defined algorithm to identify regions with high H3K4me1 and low H3K4me3. Each map covered 5kb upstream and downstream of enhancers. ChIP-seq of p300 was used as a positive control for an enhancer-bound co-activator. Brg1 shows localization to predicted enhancers, overlapping with both H3K4me1 and p300.

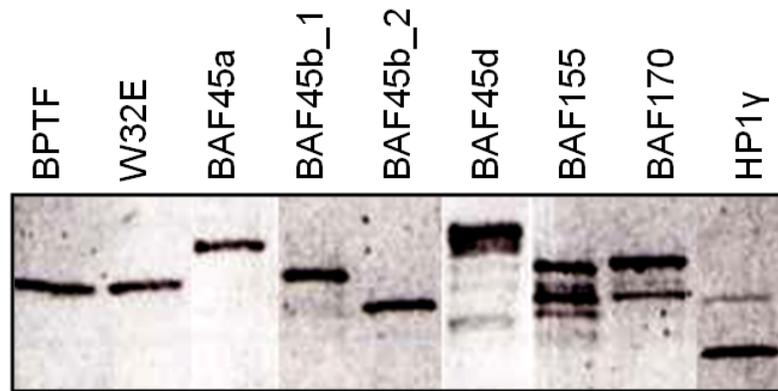


Figure 2: Purification of GST Tag Proteins.

Methyl binding domains, PHD, and chromodomains in BAF45a, BAF45b, BAF45d, BAF155, and BAF170 were tagged with GST and purified. The concentrations of the purified proteins were estimated and normalized to the concentration of the PHD domain of BPTF. BAF45b_1 and 2 are two separate PHD domains. BAF45a and BAF45d each had two PHD domains in tandem. BAF155 and BAF170 each had one chromodomain.

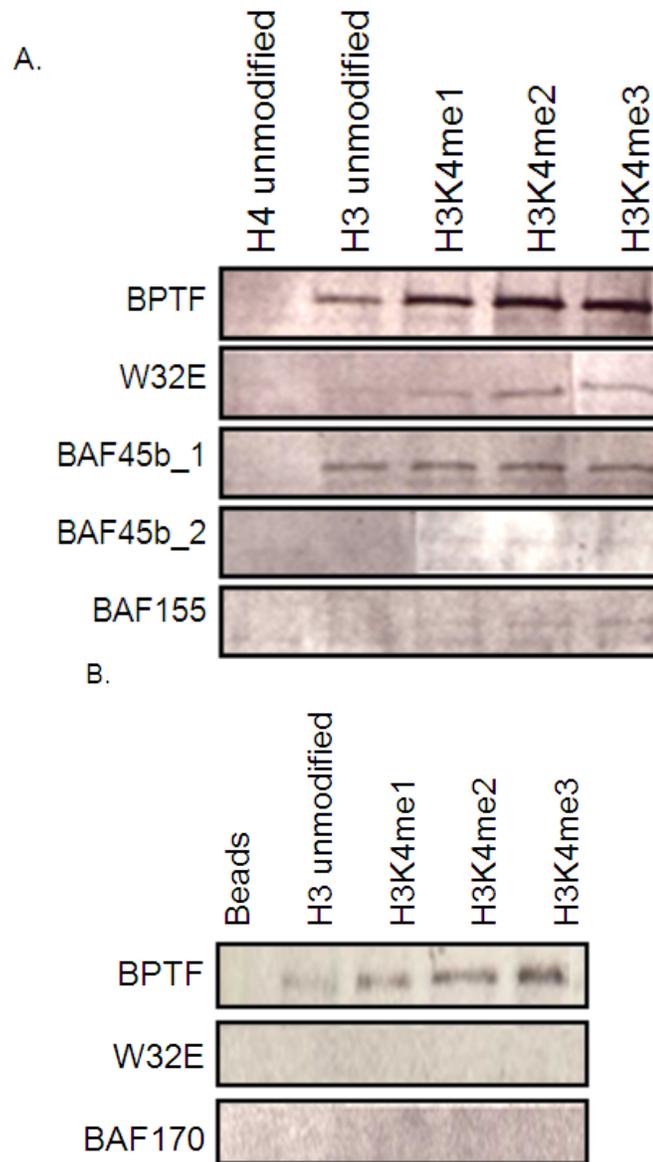


Figure 3: First PHD domain of BAF45b Bind to Methylated Lysines Nonspecifically; Second PHD domain of BAF45b and Chromodomains of BAF155 and BAF170 Do Not Bind to Methylated Lysines.

A and B. A peptide pull-down assay followed by polyacrylamide gel electrophoresis: BPTF was a positive control for binding of PHD domains to methylated lysines. BPTF bound most strongly to tri-methylated lysine residue as expected. W32E is a mutated form of BPTF, that also serves a negative control. BAF45b_1 contained the first PHD domain in BAF45b. It bound to all three of the methylated lysines and to unmethylated lysine nonspecifically. The second domain of BAF45b, or BAF45b_2, and chromodomains of BAF155 and BAF170 did not bind to any of the methylated lysines. H4 unmodified peptide and the magnetic beads were used as negative controls for the assays to confirm that H3 peptides were specific and that nothing else was bound to the beads besides the designed peptides.

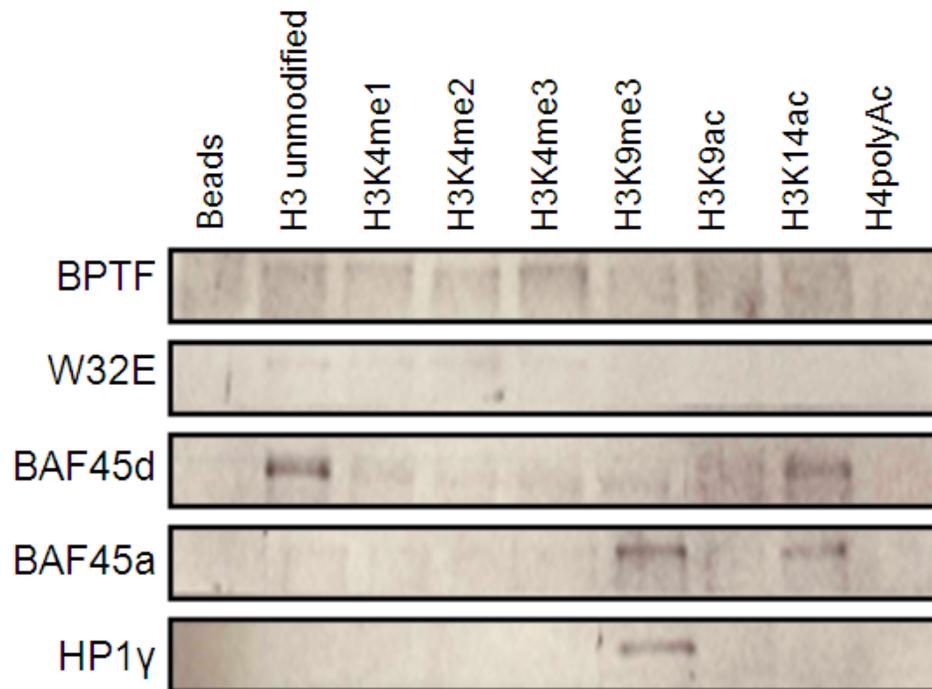


Figure 4: PHD domains of BAF45a and BAF45d Recognizes H3K14ac.

A peptide pull-down assay followed by polyacrylamide gel electrophoresis and coomassie staining: BPTF was a positive control to confirm that PHD domain binds to methylated lysines. BPTF bound most strongly to tri-methylated lysine residue as expected. W32E is a mutated version of BPTF that serves as a negative control to confirm the specificity of BPTF. PHD domains of BAF45d bound to unmodified histone H3 peptide and H3K14ac peptide. PHD domains of BAF45a bound to H3K14ac and H3K9me3. HP1γ serves as a positive control for binding to H3K9me3.

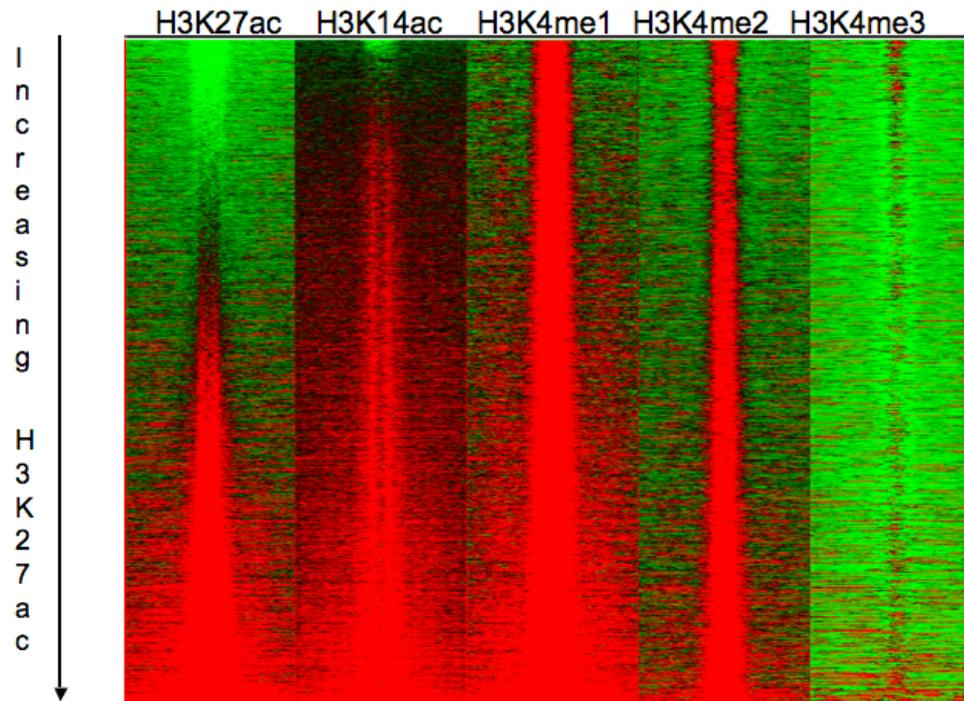


Figure 5: H3K14ac Is Present at Predicted Enhancers.

Heatmaps of ChIP-seq from IMR90 fibroblasts for histone modifications at enhancers.

Enhancers were predicted using a previously described algorithm that identifies regions with high H3K4me1 and low H3K4me3. Each map covered 5kb upstream and downstream of enhancers. H3K27ac was used as a positive control, as this acetylation mark has previously known to localize to enhancers. H3K14ac seemed to be localized at the predicted enhancers where there are high levels (red) of H3K4me1 and low levels (green) of H3K4me3.

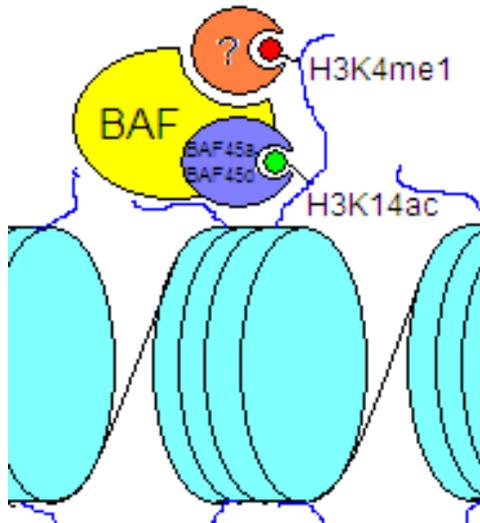


Figure 6: Model for Interaction between BAF Complex and Enhancers

Brg1 within the BAF complex localizes to H3K4me1 sites, but none of the subunits with methyl binding domains specifically bind to H3K4me1. BAF45a and BAF45d, however, directly interact with H3K14ac. The hypothesis is that, while binding to H3K14ac, BAF complex may interact with another co-activator that binds specifically to H3K4me1 on a target enhancer sequence.

Table 1: Candidate Proteins from BAF Complex in Mouse Embryonic Stem Cells

Name	Domain	Scheme
BAF45d	PHD	
BAF45a	PHD	
BAF45b	PHD	
BAF155	Chromo	
BAF170	Chromo	

Notes:

BAF complex components that co-immunoprecipitate with Brg1 in mESCs that have methyl lysine recognition domains. Domain structure of proteins were predicted using the SMART Database and the domain architecture is recreated here.

Each pink square represents a low compositional complexity region determined by SEG program; each blue rectangle represents a zinc finger domain; each navy blue pentagon represents a PHD domain; each purple triangle represents a chromo domain, each gray rectangle represents a SWIRM domain from Pfam database; each dark pink pentagon represents a SANT domain; each green rectangle represents a coiled coil region determined by Colis2 program

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