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Graphical Abstract

Highlights
- Alterations in MNase footprints reveal functional interactions among gene regulators
- esBAF and Mbd3/NuRD regulate the occupancy of multiple regulatory proteins in ESCs
- esBAF is required for Klf4 binding in ESCs
- esBAF functions in subnucleosome maturation through regulation of H2A.Z occupancy

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In Brief
Functional connections among chromatin regulators and factors that control gene-regulatory networks are often elusive. Hainer and Fazzio use nuclease footprinting in embryonic stem cells to uncover functions of two chromatin-remodeling complexes in regulatory factor binding and intra-nucleosome architecture.

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Regulation of Nucleosome Architecture and Factor Binding Revealed by Nuclease Footprinting of the ESC Genome

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SUMMARY

Functional interactions between gene regulatory factors and chromatin architecture have been difficult to directly assess. Here, we use micrococcal nuclease (MNase) footprinting to probe the functions of two chromatin-remodeling complexes. By simultaneously quantifying alterations in small MNase footprints over the binding sites of 30 regulatory factors in mouse embryonic stem cells (ESCs), we provide evidence that esBAF and Mbd3/NuRD modulate the binding of several regulatory proteins. In addition, we find that nucleosome occupancy is reduced at specific loci in favor of subnucleosomes upon depletion of esBAF, including sites of histone H2A.Z localization. Consistent with these data, we demonstrate that esBAF is required for normal H2A.Z localization in ESCs, suggesting esBAF either stabilizes H2A.Z-containing nucleosomes or promotes subnucleosome to nucleosome conversion by facilitating H2A.Z deposition. Therefore, integrative examination of MNase footprints reveals insights into nucleosome dynamics and functional interactions between chromatin structure and key gene-regulatory factors.

INTRODUCTION

In eukaryotes, genomic DNA is packaged with proteins to form chromatin: a repeating array of nucleosomes that each contain 147 bp of DNA wrapped around an octamer of histone proteins composed of a tetramer of H3 and H4 and two H2A and H2B heterodimers (Luger et al., 1997). In some cases, these canonical histone proteins can be replaced with histone variants (such as H2A.Z or H3.3), which contain high sequence similarity to their canonical counterparts but have somewhat specialized functions in vivo. Regulation of access to factor binding sites through alteration of nucleosome occupancy or positioning is an important mechanism shared among eukaryotes (Almer and Hörz, 1986; Boeger et al., 2003). As a result, most eukaryotic regulatory regions are found within nucleosome-depleted regions, which permit binding of regulatory factors and transcription machinery (Mavrich et al., 2008a; Weiner et al., 2010; Yuan et al., 2005).

Nucleosome-remodeling factors reposition, deposit, or remove nucleosomes at regulatory regions by altering histone-DNA contacts (Bartholomew, 2014; Racki and Narlikar, 2008). esBAF (Brg1-associated factor) is an embryonic stem cell (ESC)-specific nucleosome-remodeling complex that mainly activates transcription of genes and silences transcription near enhancers (Hainer et al., 2015; Ho et al., 2009a, 2009b, 2011) and is necessary for ESC self-renewal and pluripotency (Fazzio et al., 2008; Ho et al., 2009a; Kidder et al., 2009; Schaniel et al., 2009). The Mbd3/NuRD (nucleosome remodeling and deacetylase) complex creates repressive chromatin structure and is required for normal ESC differentiation (Denslow and Wade, 2007; Kaji et al., 2006, 2007; Yildirim et al., 2011). Interestingly, esBAF and Mbd3/NuRD antagonistically regulate many overlapping gene targets, resulting in moderate levels of expression (Yildirim et al., 2011).

Although nucleosome positioning and occupancy have been examined in multiple systems (Carone et al., 2014; Li et al., 2012; Mavrich et al., 2008b; Schones et al., 2008; Valouev et al., 2011), very little is known about the regulation of subnucleosomes—histone-DNA structures that lack some components of the histone octamer. Hexasomes (one H3/H4 tetramer and one H2A/H2B dimer) and half-nucleosomes (either an H3/H4 tetramer or half an H3/H4 tetramer and one H2A/H2B dimer) have been observed in vivo (Rhee et al., 2014). However, the conditions under which subnucleosomes form, the mechanisms underlying their assembly, and the roles of nucleosome-remodeling factors in regulating interchange of subnucleosome and nucleosome structures are largely unknown.

Here, we take an integrative approach to survey the functions of two chromatin-remodeling complexes with key roles in ESC pluripotency, utilizing micrococcal nuclease (MNase) footprinting to reveal nucleosome footprints (135–165 bp), subnucleosome footprints (100–130 bp), and footprints of regulatory factors (≤80 bp), as previously described (Carone et al., 2014; Henikoff et al., 2011; Kent et al., 2011). Using this method, we analyzed the chromatin structure of ESCs depleted of important factors to determine their roles in controlling nucleosome and subnucleosome architecture, as well as regulatory factor occupancy. We provide evidence that esBAF and Mbd3/NuRD modulate the binding of several regulatory factors, and we...
Figure 1. Klf4 Binding Is esBAF-Dependent

(A) Log2 fold change of small read footprints (left: Mbd3/EGFP KD; right: Smarca4/EGFP KD) spanning 200 bp directly over binding peaks sorted by either Mbd3 (left) or Brg1 (right) occupancy.

(B) Aggregation plot of normalized small reads (% 80 bp) and nucleosome size reads (135–165 bp) of MNase-seq upon EGFP, Mbd3, or Smarca4 KD over Klf4 binding sites. Klf4 binding sites were called from GEO: GSE11431 (Chen et al., 2008). Asterisks (* and **) indicate p values (<0.05; <0.01), reflecting statistical significance of changes in MNase footprints over the relative to EGFP KD, colored as indicated in key.

(C) Histone H3 ChIP-seq in EGFP, Mbd3, or Smarca4 KD ESCs shown as normalized occupancy aggregated over Klf4 binding sites.

(D) Klf4 ChIP

(E) EGFP KD

(F) Cyto

(legend continued on next page)
RESULTS AND DISCUSSION

Alterations in Footprinting of Multiple Regulatory Proteins upon Loss of esBAF or Mbd3/NuRD Function

Previously, we used deep sequencing of DNA footprints protected from MNase digestion to map nucleosomes in ESCs depleted of esBAF and Mbd3/NuRD (Hainer et al., 2015). To test the roles of these complexes in regulation of transcription factor and chromatin regulatory factor binding, we focused on the information provided by the small reads (<80 bp) obtained from these MNase-seq experiments. Proof-of-concept analyses have been performed in yeast and mammalian cells, showing that peaks of small MNase footprints correspond to binding sites for factors determined by independent methods, such as ChIP-seq (Carone et al., 2014; Henikoff et al., 2011; Kent et al., 2011). Therefore, we plotted the average abundance of small read footprints (<80 bp) from EGFP, Mbd3, and Smarca4 KD ESCs that mapped to 32 distinct genomic regions: the experimentally determined binding sites of 30 key components of the ESC gene regulatory network (including Brg1 itself, as a positive control), annotated transcription start sites (TSSs), and a random selection of nucleosome-bound regions as a negative control (Figure S1A).

We performed several analyses to assess data quality. First, we quantified changes in small read footprints directly over the factor binding sites and used available ChIP-seq data for Mbd3 and Brg1 to distinguish changes in factor occupancy directly due to loss of Mbd3/NuRD or esBAF function (Figure 1A). For both esBAF and Mbd3/NuRD, we observed minimal alterations in foot-printing at factor binding sites at which Brg1 and Mbd3 were not highly enriched (Figure 1A). As a positive control, small read footprints were dramatically changed at the empirically determined binding sites of Brg1 upon Smarca4 KD. Finally, as a negative control, we observed no changes in small reads over a random sampling of nucleosomes, demonstrating alterations in esBAF and Mbd3/NuRD are confined to specific regions of the genome.

Smarca4 KD resulted in a substantial reduction in small read accumulation at several sites (Figure 1A; Data S1A), consistent with the function of esBAF to create open chromatin structure to facilitate binding of regulatory factors and the general transcription machinery (Ho et al., 2009a, 2009b, 2011; Novershtern and Hanna, 2011; Yildirim et al., 2011). Whereas KD of Mbd3 resulted in subtle changes in MNase footprinting at most sites relative to Smarca4 KD, we observed a strong increase in peaks of small reads at p300 binding sites in Mbd3 KD cells, consistent with the antagonistic roles of Mbd3/NuRD and p300 in enhancer function (Pasin et al., 2010; Reynolds et al., 2012). Therefore, alterations in small read profiles from MNase-seq data suggest that both esBAF and Mbd3/NuRD are important regulators of transcription factor and chromatin regulatory factor binding.

esBAF Is Required for Klf4 Binding

Although alterations in small read profiles at transcription factor binding sites imply altered binding of transcription factors themselves, these changes could alternatively result from altered binding of cofactors that co-occupy the same binding sites or loss of esBAF or Mbd3/NuRD footprints when these factors are knocked down. To distinguish between these possibilities, we tested one functional interaction by an independent method. One of the factors that appeared most strongly affected by Smarca4 KD was Klf4—small MNase footprints over Klf4 binding sites were strongly reduced upon Smarca4 KD, whereas Mbd3 KD had a very modest increase (Figures 1A and 1B). Klf4 plays a critical role in maintenance of the ESC gene-regulatory network (Kim et al., 2012; Schuh et al., 1986; Takahashi and Yamanaka, 2006), and Klf4 binding sites are highly bound by Brg1, consistent with the possibility that esBAF promotes Klf4 binding (Figure 1A).

When we examined the nucleosome size (135–165 bp) MNase footprints over Klf4 binding sites, we observed a small increase in nucleosome footprints upon Smarca4 KD (Figure 1B), suggesting that esBAF may promote Klf4 binding in part by clearing its binding sites of nucleosomes. To test this prediction, we performed ChIP-seq for histone H3 and Klf4 in EGFP, Mbd3, and Smarca4 KD ESCs. Consistent with our MNase footprinting data, we observed increased histone H3 occupancy over Klf4 binding sites upon Smarca4 KD and decreased histone H3 occupancy upon Mbd3 KD (Figure 1C). These data are consistent with changes we observed in nucleosome size footprints at Klf4 binding sites and demonstrate that esBAF is required to maintain open chromatin structure over these regions. Furthermore,
ChIP-seq of Klf4 showed a dramatic reduction in Klf4 occupancy over Klf4 binding sites in Smarca4 KD cells and a slight increase in Mbd3 KD cells (Figures 1D and 1E), demonstrating that alterations in small read abundance over Klf4 binding sites upon Smarca4 and Mbd3 KD directly reflect alterations in Klf4 binding. Depletion of Brg1 does not result in reduced levels or altered intracellular localization of Klf4, ruling out these potential indirect effects on Klf4 binding (Figure 1F).

We conclude that esBAF functions directly to promote Klf4 occupancy by maintaining open chromatin structure over Klf4 binding sites. These findings confirm that changes in small read profiles from MNase-seq experiments can uncover alterations in factor occupancies when mapped over experimentally determined peaks from ChIP-seq data sets. Future studies following up additional functional interactions predicted by these data should provide additional insights into the ESC gene-regulatory network.

esBAF and Mbd3/NuRD Regulate Factor Binding by Modulating Nucleosome Occupancy over Factor Binding Sites

We previously found that esBAF activates expression of many genes by reducing promoter-proximal nucleosome occupancy and facilitating binding of RNA polymerase II (RNAPII), whereas Mbd3/NuRD acts oppositely (Hainer et al., 2015; Yildirim et al., 2011). To test whether these complexes modulate binding of regulatory proteins by similar mechanisms, we examined the effect of Smarca4 or Mbd3 KD on the abundance of nucleosome footprints at regulatory factor binding sites (many of which are far from promoters). Because nucleosome occupancy often inhibits binding of regulatory proteins, we plotted the changes in small read footprints versus nucleosome footprints in Mbd3 (Figure 2A) or Smarca4 (Figure 2B) KD cells, relative to control, for all 30 sets of binding sites.

Similar to promoters, Mbd3 KD resulted in decreased and Smarca4 KD resulted in increased average abundance of nucleosome footprints at the binding sites of most factors (Figures 2A and 2B; compare points above and below horizontal lines). Also consistent with the requirement of most regulatory proteins for a nucleosome-free binding site, changes in the abundance of small read footprints anti-correlated with changes in nucleosome size footprints (Figures 2A and 2B). These data indicate that esBAF promotes factor binding by creating open chromatin structure and Mbd3/NuRD inhibits factor binding by creating a closed chromatin environment, consistent with the known biological functions of these factors (Ho et al., 2011; Novershtern and Hanna, 2011; Reynolds et al., 2012; Yildirim et al., 2011).

Although nucleosome footprints negatively correlate with small read footprints overall, there are exceptions at several
locations (compare Data S1A and S1B), suggesting that nucleosome occupancy does not inhibit the binding of some factors (Figures 2A and 2B). At many of these sites, there are clear peaks of nucleosome size footprints centered on factor binding sites (Data S1B), consistent with this model. Importantly, the presence of nucleosomes over several of these sites is predicted by their functions. PRC2 binds and methylates histone H3K27 within nucleosomes (Margueron and Reinberg, 2011; Simon and Kingston, 2009), consistent with the co-localization of its Suz12, Ezh2, and Mtf2 subunits (Zhang et al., 2011) with nucleosome footprints. Similarly, Pwp1 (a WD40-repeat-containing protein) occupies regions marked with H4K20me3 and regulates H4K20 methylation at some sites (Shen et al., 2015) and Ring1b (an E3 ubiquitin ligase within PRC1 complexes) mono-ubiquitinates H2AK119 (Wang et al., 2004). Furthermore, Lsd1 is a histone H3 lysine demethylase (Shi et al., 2004). Finally, Esrrb has been shown to bind within regions occupied by nucleosomes (Teif et al., 2012), and NcoA3 interacts with Esrrb, co-occupying some locations on chromatin (Percharde et al., 2012).

To validate these findings, we analyzed our histone H3 ChIP-seq data in control, Mbd3, and Smarca4 KD cells over a subset of factor binding sites (Figure 2C). As positive controls, we found that sites of H2A.Z incorporation have peaks of histone H3 occupancy over factor binding sites found to have nucleosome size MNase footprints, demonstrating that these sites are indeed occupied by nucleosomes (Figure 2C).

For one factor examined, the role of nucleosome architecture in regulating factor binding has not been addressed. MafK is a leucine zipper transcription factor that, to our knowledge, has not been shown to bind nucleosomes. Here, we found a peak of nucleosome size reads over MafK sites, and our histone H3 ChIP-seq data support these findings, suggesting that MafK binds DNA within nucleosome-occupied regions. Together, these data confirm that, although most regulatory factors bind to nucleosome-depleted regions of the genome, some do not. In addition, these data suggest that differential affinities of factors for nucleosome-bound DNA must be taken into account in studies examining their biochemical functions and roles within gene-regulatory networks.

esBAF Regulates Nucleosome-Subnucleosome Interconversion at Specific Sites

To globally address whether and how Mbd3/NuRD and esBAF regulate the composition of nucleosomes in ESCs, we compared nucleosome footprints (135–165 bp; Data S1B) to intermediate size footprints (100–130 bp; Data S1C) over the same factor sites. The intermediate size fragments could result from either large non-histone protein complexes or non-standard nucleosomes (i.e., hexasomes or half-nucleosomes). Consistent with the latter possibility, the profiles of nucleosome and intermediate size footprints (hereafter, subnucleosomes) were strongly positively correlated in both Mbd3 and Smarca4 KD cells (Figures 3A and 3B).

Interestingly, although subnucleosomes and nucleosomes were strongly correlated at all regions examined upon Mbd3 KD (Figure 3A), KD of Smarca4 resulted in alterations to subnucleosome footprints that were uncoupled from alterations in nucleosome location (Figure 2C). Consistent with our MNase footprinting data, we observed a strong peak of histone H3 occupancy over factor binding sites found to have nucleosome size MNase footprints, demonstrating that these sites are indeed occupied by nucleosomes (Figure 2C).
nucleosome footprints at some sites (Figure 3B). At four locations where Smarca4 KD results in either decreased (Ezh2, Ring1B, and H2A.Z sites) or had no effect on (Lsd1 sites) nucleosome occupancy, subnucleosome footprints are increased (Figure 3C). Importantly, histone H3 occupancy measured by ChIP-seq (which likely cannot discriminate between nucleosomes and subnucleosomes) is increased over Lsd1, Ezh2, Ring1B, and H2A.Z binding sites upon Smarca4 KD (Figures 2C and S3), confirming that subnucleosome footprints at these sites reflect the presence of histones. Taken together, these data show that Smarca4 KD results in higher subnucleosome occupancy and reduced nucleosome occupancy at a subset of genomic locations, suggesting esBAF is necessary for either maturation of subnucleosomes to nucleosomes or prevention of nucleosome disassembly at these sites.

**Brg1 Is Required for Normal H2A.Z Localization**

Whereas the mechanisms underlying subnucleosome-nucleosome interconversion are unknown, prior reports suggest that hexasomes are composed of an H3/H4 tetramer and a single H2A/H2B dimer (Rhee et al., 2014; Weintraub et al., 1975). These findings suggest that regulation of H2A/H2B (or H2A variant/H2B) dimer incorporation could be responsible for subnucleosome maturation. Due to our observation that subnucleosomes were enriched at H2A.Z sites upon Smarca4 KD, we hypothesized that one role of esBAF in subnucleosome regulation could be through regulation of H2A.Z occupancy or incorporation at specific locations throughout the genome.

In mammals, H2A.Z is incorporated into nucleosomes by p400 and SRCAP via exchange of H2A/H2B dimers for H2A.Z/H2B dimers (Cai et al., 2005; Park et al., 2010; Ruhl et al., 2006; Wong et al., 2007), and these nucleosomes are enriched near specific genomic features, including enhancers and promoters (Mavrich et al., 2008b; Zilberman et al., 2008). H2A.Z nucleosomes play key roles in gene regulation, although the effect of H2A.Z incorporation on nucleosome stability and dynamics and their specific effects on transcription by RNAPII are controversial (Abbott et al., 2001; Jin and Felsenfeld, 2007; Park et al., 2004; Suto et al., 2000; Thambirajah et al., 2006; Thakar et al., 2010; Zhang et al., 2005).

We considered the possibility that esBAF regulates H2A.Z occupancy at enhancers and promoters either directly or indirectly. To test this possibility, we performed histone H2A.Z ChIP-seq in EGFP, Mbd3, and Smarca4 KD cells. In EGFP KD cells, H2A.Z localization was similar to that observed previously in ESCs, confirming the specificity of our data sets (Figures 4A and S4A). We found that Smarca4 KD led to decreased H2A.Z occupancy when one examines either all H2A.Z binding sites or TSSs in particular (Figures 4A and 4B). Although Smarca4 KD also resulted in decreased H2A.Z occupancy at Lsd1 binding sites (Figure S4B), it had no effect at Pwp1 sites (Figure 4C) and modestly increased H2A.Z occupancy at MaF sites (Figure S4B), demonstrating that esBAF is required for H2A.Z occupancy at some, but not all, of its locations throughout the genome. Although we found no evidence that depletion of Smarca4 alters p400 occupancy (Data S1A), Smarca4 KD also...
resulted in increased subnucleosome footprinting over p400 binding sites (Data S1C), consistent with the role of esBAF in regulation of H2A.Z localization.

Taken together, these data suggest that esBAF is required for H2A.Z occupancy at some locations. Upon Smarca4 KD, H2A.Z is strongly depleted, and subnucleosomes partially replace nucleosomes at several regions where H2A.Z is normally enriched. Whether esBAF promotes H2A.Z incorporation by facilitating the functions of SWR1 family complexes, either directly or indirectly, or is required for the stability of H2A.Z containing nucleosomes remains unresolved (Figures 4D and 4E).

Conclusions
Chromatin-remodeling enzymes have been examined for their roles in regulation of nucleosome architecture in many cell types. However, their effects on intra-nucleosome architecture, as well as their roles in regulation of DNA-binding proteins, are not fully understood. Here, we showed that use of a single footprinting method, MNase-seq, combined with available factor occupancy data, uncovers dynamic regulation of factor binding and subnucleosome structures that can be confirmed by traditional approaches. We focused on two antagonistically functioning chromatin regulators, esBAF and Mbd3/NuRD, to gain a deeper understanding of their roles in modulating ESC chromatin architecture. However, this method should be broadly applicable as a screen for functional interactions between chromatin regulators and the gene-regulatory network in any organism/cell type where transcription-factor-binding data are available.

EXPERIMENTAL PROCEDURES

Cell Culture
E14 mouse ESCs were cultured as previously described (Chen et al., 2013). RNAi-mediated KD was performed with endoribonuclease-III-digested siRNAs (esiRNAs) as previously described (Fazzio et al., 2008) using Lipofectamine 2000 (Invitrogen). KDs were performed for 48 hr.

gQ-PCR
RNA was isolated using TRIzol reagent (Invitrogen) and used in a cDNA synthesis reaction with random hexamers (Promega). cDNA was used as a template in qPCR reactions using a FAST SYBR mix (KAPA Biosystems) on an Eppendorf Realplex with the core transcriptional network in embryonic stem cells. Cell Reports 13, 2681–2687.

MNase-Seq Analysis
We re-analyzed MNase-seq footprinting data for ESCs depleted of the indicated factors that were previously published (Hainer et al., 2015). See the Supplemental Experimental Procedures for details.

ChIP-Seq
ChIP experiments and single-end libraries of ChIP-enriched DNA were prepared as previously described (Chen et al., 2013). See the Supplemental Experimental Procedures for details.

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SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.071.

AUTHOR CONTRIBUTIONS
S.J.H. and T.G.F. designed all experiments. S.J.H. carried out all experiments and performed analyses of genomic data. S.J.H. and T.G.F. wrote the manuscript.

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