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Ring1B Compacts Chromatin Structure and Represses Gene Expression Independent of Histone Ubiquitination

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SUMMARY

How polycomb group proteins repress gene expression in vivo is not known. While histone-modifying activities of the polycomb repressive complexes (PRCs) have been studied extensively, in vitro data have suggested a direct activity of the PRC1 complex in compacting chromatin. Here, we investigate higher-order chromatin compaction of polycomb targets in vivo. We show that PRCs are required to maintain a compact chromatin state at Hox loci in embryonic stem cells (ESCs). There is specific decompaction in the absence of PRC2 or PRC1. This is due to a PRC1-like complex, since decompaction occurs in *Ring1B* null cells that still have PRC2-mediated H3K27 methylation. Moreover, we show that the ability of Ring1B to restore a compact chromatin state and to repress Hox gene expression is not dependent on its histone ubiquitination activity. We suggest that Ring1B-mediated chromatin compaction acts to directly limit transcription in vivo.

INTRODUCTION

Transcriptionally inactive chromatin is generally considered to have a compact structure, while active chromatin is open and decondensed. The inference is that compact chromatin structure inhibits gene expression. However, while histone modifications and the proteins that deposit, remove, or bind them are increasingly well understood, mechanisms that control higher-order chromatin structure are poorly characterized.

Proteins implicated in driving higher-order chromatin compaction include variant and linker histones, HP1, and polycomb group (PcG) proteins. Linker histone H1 is thought to be important in forming 30 nm chromatin fibers (Allan et al., 1981; Bates et al., 1981), and its downregulation in *Drosophila* results in misaligned polytene chromatids and dispersed heterochromatin (Lu et al., 2009). In mammals, reducing the stoichiometry of linker histone to nucleosomes by a knockout of 3 of the 6 somatic H1 genes (herein called ΔH1) results in an altered nucleosome repeat length and a widespread decondensation of chromatin fibers, but misexpression of only a few genes (Fan et al., 2005). H1:nucleosome stoichiometry also varies between cell types, with the ratio in pluripotent cells, such as undifferentiated embryonic stem cells (ESCs), being lower than that in differentiated cells.

PcG proteins are key regulators of developmentally regulated loci in flies and mammals and are found in two broad classes of complex. The mammalian polycomb repressive complex 2 (PRC2) complex (Ezh/Suz12/Eed) trimethylates histone H3 at lysine 27 (H3K27me3) (Cao et al., 2002) through the activity of the histone methyltransferases (HMTases) Ezh2 and Ezh1 (Shen et al., 2008). The PRC1 complex can ubiquitinate H2AK119 through the E3 ligase activity of Ring1A/B (de Napoles et al., 2004; Wang et al., 2004a; Buchwald et al., 2006).

Even appreciating that PRCs have histone-modifying activities, it remains unclear how they actually repress gene expression (Simon and Kingston, 2009). In vitro, PRCs can decrease the accessibility of Hox genes to enzymes (Fitzgerald and Bender, 2001), inhibit chromatin remodeling (Francis et al., 2001), and block transcription (King et al., 2002). PRC components can also compact a nucleosomal array in vitro into a form that is refractory to chromatin remodeling (Francis et al., 2004; King et al., 2005; Lo et al., 2009; Margueron et al., 2008). This also occurs on nucleosome templates assembled from tail-less histones (Francis et al., 2004; Margueron et al., 2008) suggesting that this chromatin compaction is independent of histone tail modifications.

There has been little evidence to date to suggest that PRC components might function in vivo to change chromatin packaging in a way that is independent from their histone-modifying activities, and this remains a major gap in our understanding of polycomb function. PRC components are bound at, modify the chromatin of, and repress sets of developmentally regulated genes in human and mouse ESCs (Azuara et al., 2006; Boyer

et al., 2006; Jørgensen et al., 2006; Lee et al., 2006; Stock et al., 2007; Endoh et al., 2008). Among these targets are the Hox loci, encoding key players in early developmental cell fate and cell identity decisions.

We have previously shown that Hox loci visibly decompact and undergo nuclear reorganization as their genes are activated, both during ESC differentiation (Chambeyron and Bickmore, 2004; Morey et al., 2007) and in the embryo at sites of Hox activation (Chambeyron et al., 2005; Morey et al., 2007), consistent with a function for PRCs in maintaining a compact chromatin state at silent loci. Here, we show that there is a specific visible decompaction of chromatin at the murine Hoxb and d loci in ESCs lacking functional PRC1 or 2 complexes. This contrasts with the apparently genome-wide chromatin decompaction in cells deficient in H1. We attribute chromatin compaction activity to PRC1 components, not PRC2, since decompaction is seen in Ring1B mutant cells in which H3K27me3 still blankets the Hox loci. We show that addition of Ring1B can restore in vivo chromatin compaction, and moreover, using a catalytically inactive Ring1B, we present evidence that this is independent of Ring1B's ability to ubiquitinate histone H2A. Finally, we demonstrate that Ring1B-mediated chromatin compaction is functionally significant, as it represses the expression of Hox genes. We suggest that Ring1B, likely through a PRC1-like complex, functions in vivo to maintain gene repression through a change in higher-order chromatin structure, not just by histone modification.

RESULTS

Chromatin Decompaction and H3K27me3 Loss during Differentiation

Unlike most PRC targets, where PRC binding and H3K27me3 are restricted to within ~1 kb of the transcriptional start site (TSS), Hox loci are demarked by broad domains of this PRC2-mediated histone modification (Bernstein et al., 2006; Lee et al., 2006; Bracken et al., 2006; Soshnikova and Duboule, 2009). Such an extensive domain of modification would be compatible with a large-scale change in chromatin structure.

To confirm this, we hybridized micrococcal nuclease (MNase)-digested chromatin from undifferentiated ESCs (input) together with the H3K27me3-modified chromatin immunoprecipitated from this digested chromatin to a custom tiling array encompassing the murine Hoxb and d loci, flanking regions, and control loci. We indeed detect extensive domains of H3K27me3 over Hoxb and d (Figure 1A). At Hoxb, the major domain of modification is a 140 kb region encompassing *Hoxb1-Hoxb9*, with a separate 50 kb modified region over the most 5′ gene, *Hoxb13*. Across Hoxd, a 145 kb domain of H3K27me3 includes *Evx2* 5′ of *Hoxd13* and appears to be bipartite, with subdomains that encompass *Hoxd1-d4* and *Hoxd8-Evx2*.

H3K27me3 stops abruptly outside of Hoxb and d. However, there is a small 16 kb area of H3K27me3 just 3′ of *Hoxb1*, and 5′ there are small blocks of modification within *Tt116*. There is a 10 kb block 5′ of Hoxd at a conserved region between *Evx2* and *Lnp*, but we found no evidence for H3K27me3 modification downstream of *Lnp* over the conserved global control region (GCR), which is responsible for the regulation of 5′ Hoxd gene expression in digits and in the CNS (Spitz et al., 2003). The functional significance of small blocks of H3K27me3 ~300 kb 3′ of Hoxd and beyond *Mtx2* (Figure 1A) is not known.

Within Hox loci, H3K27me3 is enriched at all sequence classes (Figure 1C), though it is the promoters and exons of Hox genes and, surprisingly, other oligonucleotide probes of no known function from within the clusters that have higher levels of modification. Consistent with previous findings, there is a peak of H3K27me3 <2 kb from the TSS of Hox genes (Figure S1) (Boyer et al., 2006; Pan et al., 2007).

Retinoic acid (RA)-directed ESC differentiation activates temporally regulated Hox expression (Papalopulu et al., 1991; Morey et al., 2007). After 3 days of differentiation, the most pronounced decrease in H3K27me3 was over the 3′ genes (*Hoxb1-b5* and *Hoxd1-d8*) (Figures 1A and 1B), consistent with the polarized activation of more 3′ Hox genes at this time point (Morey et al., 2009). Proportionately, the biggest changes occur over the introns, known regulatory regions, and other sequences from within the Hox clusters. The smallest changes were over the exons of Hox genes and CpG islands (Figure 1C). Loss of hybridization signal could formally be due to loss either of the histone modification, or of the histone itself. However, a comparative hybridization of MNase-digested chromatin and MNase-digested naked DNA did not reveal any obvious large-scale loss of nucleosomes from the Hox loci during differentiation (data not shown). Therefore, we conclude that we are detecting the specific loss of H3K27me3 from nucleosomes at Hox loci.

To assay chromatin compaction, we used fluorescence in situ hybridization (FISH) with closely apposed pairs of fosmid probes. Squared interprobe distances (d^2) are generally linearly related to genomic separation (in kb) (van den Engh et al., 1992) and can be used to identify differences in chromatin compaction, either between different regions of the genome in a particular cell type (Yokota et al., 1997) or at specific loci between cells at different stages of differentiation (Chambeyron and Bickmore, 2004). Any change attributable solely to altered nuclear size is assessed by normalizing d^2 by the nuclear radius (r^2). Chromatin decompaction was seen across Hoxb and d loci upon differentiation, but not across the control α-globin locus, which is not a polycomb target but is on the same chromosome (MMU 11) as Hoxb (Garrick et al., 2008) (Figure 2). Chromatin decompaction during differentiation was contained within the domain of H3K27me3, unlike looping out of Hox loci from their chromosome territories, which extends beyond the H3K27me3 domain (Morey et al., 2009).

Specific Chromatin Decompaction at Hox Loci in the Absence of H3K27me3

To determine whether decompaction is a direct consequence of the loss of H3K27me3/PRC2, rather than an indirect effect of differentiation, we analyzed Hox loci in undifferentiated wild-type (WT) ESCs and in the corresponding ESCs that are null for the Eed component of PRC2 (*Eed*$^{-/-}$). Results from two independent *Eed*$^{-/-}$ cell lines (G8.1 and B1.3) (Azuara et al., 2006) were indistinguishable, and H3K27me3 is lacking from these cells (Montgomery et al., 2005; Shen et al., 2008) (Figure 3A).

We found significant chromatin decompaction (d^2/r^2) within (*Hoxb1-b9*) and across (*Hoxb1-Calcoco2*) Hoxb in *Eed*$^{-/-}$