Reduction of Hox Gene Expression by Histone H1 Depletion

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Abstract

The evolutionarily conserved homeotic (Hox) genes are organized in clusters and expressed collinearly to specify body patterning during embryonic development. Chromatin reorganization and decompaction are intimately connected with Hox gene activation. Linker histone H1 plays a key role in facilitating folding of higher order chromatin structure. Previous studies have shown that deletion of three somatic H1 subtypes together leads to embryonic lethality and that H1c/H1d/H1e triple knockout (TKO) embryonic stem cells (ESCs) display bulk chromatin decompaction. To investigate the potential role of H1 and higher order chromatin folding in the regulation of Hox gene expression, we systematically analyzed the expression of all 39 Hox genes in triple H1 null mouse embryos and ESCs by quantitative RT-PCR. Surprisingly, we find that H1 depletion causes significant reduction in the expression of a broad range of Hox genes in embryos and ESCs. To examine if any of the three H1 subtypes (H1c, H1d and H1e) is responsible for decreased expression of Hox gene in triple-H1 null ESCs, we derived and characterized H1c–/–, H1d–/–, and H1e–/– single-H1 null ESCs. We show that deletion of individual H1 subtypes results in down-regulation of specific Hox genes in ESCs. Finally we demonstrate that, in triple-H1- and single-H1- null ESCs, the levels of H3K4 trimethylation (H3K4me3) and H3K27 trimethylation (H3K27me3) were affected at specific Hox genes with decreased expression. Our data demonstrate that marked reduction in total H1 levels causes significant reduction in both expression and the level of active histone mark H3K4me3 at many Hox genes and that individual H1 subtypes may also contribute to the regulation of specific Hox gene expression. We suggest possible mechanisms for such an unexpected role of histone H1 in Hox gene regulation.

Introduction

The Hox genes, encoding a family of evolutionarily conserved transcription factors that contain a DNA binding homeodomain, play fundamental roles in specifying anterior-posterior body patterning during development and are critical for cell fate determination [1–3]. The expression levels of Hox genes are tightly controlled throughout embryonic development, and aberrant expression and mutation of Hox genes can lead to body malformations and multiple types of malignancies [4,5].

Hox genes are organized into genomic clusters and their physical order within the cluster corresponds to their expression order along the anterior-posterior axis. In mammals, there are 39 Hox genes arranged in four genomic clusters of thirteen paralog groups (A–D) [6], which are thought to derive from tandem duplication of ancestral genes [7,8]. Progressive transition of histone modifications and local chromatin decondensation have been found to associate with sequential expression of Hoxb and Hoxd loci during embryonic development and/or stem cell differentiation [9–13]. Hox gene clusters are spatially compartmentalized and the transition in their 3D structure corresponds with the changes of H3K4me3 and H3K27me3 [14]. The temporal collinearity of the order of Hox gene activation along their physical sequence at genomic loci [15], stepwise transition of chromatin status and spatial configuration [9,14], and the necessity of the cluster organization for full repression of the entire cluster suggest an important role of chromatin structure in regulation of Hox genes [9-13]. However, it remains to be determined whether the change of chromatin structure is a contributing factor or a consequence of Hox gene activation.

Linker histone H1 is the major chromatin structural protein involved in folding of chromatin into high order structure. H1 binds to the nucleosome and the linker DNA between nucleosomes to promote compaction of nucleosome arrays [16,17]. Multiple H1 subtypes exist in mammals, providing additional levels of modulation on chromatin structure and function. Among the 11 mammalian H1 subtypes identified, 5 somatic H1 subtypes (H1a-e) are present in abundance in all dividing and non-dividing cells, whereas the replacement H1 (H1r) and the 4 germ cell specific H1s are expressed in differentiating cells and germ cells, respectively [18]. Depletion of three somatic H1 subtypes (H1c, H1d, and H1e) together results in embryonic lethality at midgestation, demonstrating the necessity of H1 for mammalian development [19]. We have previously shown that H1c, H1d, and
H1c, H1d, and H1e leads to decreased expression of Hox genes in ESCs. These results suggest that the marked reduction of H1 levels and decondensation of bulk chromatin cause repression of many Hox genes in embryos and ESCs, which may be in part mediated through individual H1 subtypes as well as changes in H3K4me3 and H3K27me3.

**Results**

**Loss of H1c, H1d and H1e Leads to Decreased Expression of Hox Genes in Embryos and Embryonic Stem Cells**

To gain a comprehensive view of the effects histone H1 depletion and changes in bulk chromatin on the regulation of Hox gene clusters, we designed a full set of quantitative reverse-transcription PCR assays (qRT-PCR) to measure the expression levels of all 39 murine Hox genes across the 4 Hox gene clusters in H1 TKO embryos. H1c/H1d/H1e triple heterozygotes were intercrossed to obtain H1 TKO and wild-type (WT) littermate embryos. Most of the H1 TKO embryos display growth retardation and various defects at E9.5 [19]. To minimize the secondary effects caused by broad defects of H1 TKO embryos, we chose to analyze Hox gene expression at E8.5 when H1 TKO embryos with comparable size to WT embryos can be recovered. We selected two littermate pairs of WT and H1 TKO embryos at E8.5, and examined the expression patterns of all 39 Hox genes using the highly sensitive qRT-PCR assays. As expected, most Hox genes were expressed in E8.5 embryos, except the most posterior genes within each cluster (Figure 1). However, surprisingly, many Hox genes were expressed at reduced levels in H1 TKO embryos, including Hoxa2, Hoxa3, Hoxa5, Hoxa6, Hoxa9, Hoxa14, Hoxc5, Hoxc6, Hoxc8, Hoxc9, Hoxc10, Hoxd3, and Hoxd8 (Figure 1). This effect is especially prominent in Hoxa and Hoxc clusters, in which nearly all of the expressed genes were reduced 3-fold or more (Figure 1A). Interestingly, we did not find increased expression among any of the Hox genes (Figure 1B), and none of the Hoxb genes were affected in H1 TKO embryos in comparison with WT embryos.

The reduction of expression of many Hox genes may cause the growth retardation often observed in H1 TKO embryos at E9.5. However, it remained a formal possibility that the decreased expression of Hox genes in H1 TKO embryos was a result of the slight growth retardation presented in the KO embryos, although the H1 TKO embryos used for this analysis were indistinguishable from their WT and heterozygous littermate controls in size and developmental stage. In order to analyze the effects of H1 on a homogeneous cell population, we gauged the effects of H1 depletion on Hox gene expression in H1 TKO ESCs. Hox genes are repressed by polycomb repressive complexes (PRCs) in ESCs [21–26]. Loss of components of either PRC1 or PRC2 in ESCs leads to upregulation of Hox genes, presumably due to respective loss of chromatin compaction and H3K27 trimethylation activity [13,22,27]. We have shown previously that H1 TKO ESCs have decondensed local chromatin and reduced levels of H3K27me3 in bulk chromatin [28]. It is thus possible that the decreased growth retardation observed in H1 TKO embryos at E9.5 may be due to decondensation of bulk chromatin in ESCs (Figure S1A). Consistent with the finding that pluripotent ESCs possess a hyperactive transcriptome [28], we detected expression of 21 Hox genes, albeit at low levels, in either or both of WT and H1 TKO ESCs. These genes include Hoxa1, Hoxa2, Hoxa4, Hoxa7, Hoxa9, Hoxa10, Hoxb2, Hoxb4, Hoxb5, Hoxb8, Hoxb9, Hoxb13, Hoxc4, Hoxc5, Hoxc8, Hoxc9, Hoxc10, Hoxc13, Hoxd1, Hoxd11, and Hoxd13 (Figure 2). Unexpectedly, no increased expression in any of the Hox genes was found in H1 TKO ESCs. Instead, the expression levels of 6 Hox genes, Hoxa1, Hoxb5, Hoxb8, Hoxb13, Hoxc13, and Hoxd13, were reduced, with an average of 2–3 fold less in H1 TKO ESCs compared with WT (Figure 2A). Other Hox genes did not show consistent changes in expression by loss of H1c, H1d and H1e in ESCs (Figure 2B).

**Specific Regulation of Hox Genes in ESCs by Individual H1 Subtypes**

To assess the effects of each of the three deleted somatic H1 subtypes in H1 TKO (H1c, H1d, and H1e) on Hox gene expression in ESCs, we established ESCs that are null for only one of these three H1 subtypes, H1c−/−; H1d−/−; and H1e−/− mice develop normally and are fertile [29]. Male and female mice homozygous for each single-H1 deletion were bred, H1c−/−; H1d−/−; and H1e−/− blastocysts were harvested from pregnant female mice at 3.5 day post coitum and their respective single-H1 knockout (KO) ESCs were derived from outgrowth of blastocysts. As shown in metaphase chromosome spreads, the single-H1 KO ESCs had normal karyotypes with 40 chromosomes (Figure S1A) and showed colony morphology typical of undifferentiated ESCs when cultured under conditions promoting self-renewal of ESCs (Figure S1B). They expressed high levels of pluripotency factor OCT4, which is absent in differentiated cells, such as mouse embryonic fibroblasts (MEF) (Figure S1C). These single-H1 KO ESCs also had comparable growth rate to WT ESCs (data not shown). Upon differentiation, the single-H1 KO ESCs were able to form embryoid bodies (EB) with characteristic cystic structures and differentiated cell morphologies (Figure S1C). As expected, these ESCs displayed decreased levels of OCT4 (Figure 3A), and increased expression of many differentiation markers, such asAFP, Gata4, T (Brachyury), and FLTI, compared with ESCs (Figure S1C). In addition, teratoma formation analysis indicated that the single-H1 KO ESCs formed typical teratomas containing cells differentiated into all three germ layers after injection into immunodeficient mice (data not shown). These data indicate that any one of these three somatic H1 subtypes is dispensable for self-renewal and differentiation of ESCs.

We next analyzed the total H1 levels and composition of H1 subtypes in these single-H1 KO ESCs. HPLC and mass spectrometry analyses of histone extracts from these cells confirmed the lack of the deleted H1 subtype in the respective H1c−/−, H1d−/−, and H1e−/− ESCs (Figure 3A). As described previously and shown here [30,31], quantification of the peaks of...
each H1 subtype and H2B allows calculation of the H1 to nucleosome ratio (H1/nuc). Such analysis showed that, except for H1e in H1d-KO ESCs, the absolute levels of the remaining H1 subtypes were largely unchanged in single-H1 null ESCs (Figure 3B), indicating that there was little increase or compensation in the levels of the remaining H1s for the lost H1. As expected, undifferentiated ESCs express negligible amount of H10 (Figure 3A), an H1 subtype enriched in differentiating and non-dividing cells [32,33]. Although relative proportions of H1 subtypes were altered by single-H1 deletion (Figure 3C), the total H1/nuc ratios of H1c/2, H1d/2, and H1e/2 ESCs were comparable with respective values of 0.38, 0.35, and 0.35 (Figure 3B). These ratios were about 25% lower than that of WT ESCs (0.45), but about 50% higher than that of H1 TKO ESCs (0.25) [20]. These single-H1 KO ESCs provide ideal cell resources to ascertain if the effects present in H1 TKO ESCs were caused by any one of the lost H1 subtypes or by the marked reduction in total H1 levels in TKO cells.

Dynamic Changes of H3K4me3 and H3K27me3 at Affected Hox Genes in H1 TKO ESCs

Trithorax group (TrxG) and polycomb group (PcG) proteins are known to regulate the expression of Hox genes [34,35]. TrxG mediates H3K4 tri-methylation (H3K4me3), corresponding to transcriptional activation [36,37], whereas PcG directs H3K27 tri-methylation (H3K27me3), correlating with transcriptional repression [22,23,39]. In ESCs, many developmental genes display both H3K4me3 and H3K27me3 marks, a “bivalent” chromatin signature for genes poised for expression and important for maintenance of ESC pluripotency [21,40].
To investigate whether H1 depletion has an impact on bivalent chromatin marks on the 6 Hox genes (Hoxa1, Hoxb5, Hoxb8, Hoxb13, Hoxc13 and Hoxd13) affected in H1 TKO ESCs, we performed quantitative chromatin immunoprecipitation (qChIP) analysis on the promoter regions of these genes as well as two Hox genes (Hoxb4 and Hoxd11) whose expression levels were not altered by triple-H1 deletion. As expected, most Hox genes analyzed displayed the bivalent marks in WT ESCs, with higher levels of H3K4me3 and H3K27me3 compared with Hoxa3 and Tcf4 (Figure 5A&C), which have been shown to harbor minimum levels of respective histone marks [40]. The levels of H3K4me3 were decreased significantly at all six Hox genes affected in H1 TKO ESCs (Figure 5A), but not at Hoxb4 or Hoxd11 loci, suggesting that H1 depletion did not lead to a general reduction of H3K4me3 throughout the Hox gene clusters. The changes in H3K4me3 level at the promoters of the six Hox genes correlated with the reduction of gene expression in H1 TKO ESCs, indicating that the effects of H1 depletion on Hox genes may be mediated through regulating the establishment and/or maintenance of specific H3K4me3 patterns. Increased levels of H3K27me3 were observed at 4 of the 6 Hox genes affected in H1 TKO ESCs (Hoxa1, Hoxb5, Hoxb13, and Hoxd13) (Figure 5C), suggesting that an increase in the H3K27me3 level may also contribute to the reduced expression of these genes. In contrast, H3K36me3, which is enriched at gene bodies of active genes [41], and H3K9me3, which marks heterochromatin and associated with gene repression [42], remained unchanged at all sites after triple H1 deletion (Figure 5B&D), indicating that the effects of marked H1 reduction on H3K4me3 and H3K27me3 (to a less extent) are rather specific. qChIP analysis in single-KO ESCs indicated that H3K4me3 was decreased significantly at the promoters of the Hox genes with reduced expression in the respective H1 KO ESCs, but not at unaffected genes, such as Hoxd11 (Figure S2A). The level of H3K4me3 was not affected by single-H1 deletion at those genes which displayed reduced expression only in H1 TKO ESCs, such as Hoxb5 (Figure S2A). The increase of H3K27me3 occupancy was more restricted, detected only at Hoxa1 promoter in H1c- and H1d- KO ESCs with 2–3 fold over WT (Figure S2B). Taken together, our results demonstrate that H1 depletion leads to dynamic changes of the H3K4me3 and H3K27me3 marks, which may regulate Hox gene expression.

**Discussion**

Hox genes encode a large family of transcription factors crucial for body patterning and positioning along the anterior-posterior axis during animal development [1,43]. Multiple mechanisms have been shown to regulate the spatial and temporal collinearity of Hox genes, such as the antagonism between PcG and TrxG proteins [34,35], local chromatin condensation and reorganization [10,11,13], spatial configuration or compartmentalization [14], targeting of miRNAs and long non-coding RNAs (lncRNAs) [44,45]. Chromatin conformation and compaction appear to be key mediators for regulating the expression of Hox gene clusters [10,11,13,14], however, whether changes in chromatin structure have a direct impact on Hox gene expression remains undetermined.

In this study, we have taken advantage of a number of mutants, null in one or several major somatic H1 subtypes, with different levels of reduction in total H1 proteins, to investigate the role of H1, a key component in promoting chromatin compaction, in regulating Hox gene clusters in mouse embryos and ESCs. We find that depletion of three H1 subtypes leads to the transcriptional
reduction of a group of Hox genes in embryos and ESCs, and that the reduced expression levels correlate with dynamic changes in H3K4me3 and H3K27me3 marks. This is in contrast to the deletion of PRC1 or PRC2 repressive chromatin complexes, which causes upregulation of specific Hox genes in embryos [46–48] or ESCs [13,22,24].

We first systematically analyzed the impacts of H1 depletion on expression levels of all 39 Hox genes in mouse embryos. Consistent with previous findings [9], the posterior genes are not detected by qRT-PCR assays in E8.5 embryos. The 13 affected genes include many paralogous Hox gene members (Figure 1B), suggesting a broad effect of H1 on regulation of Hox genes. Hoxa2, expressed in hindbrain and crucial for trigeminal system development [49,50], is drastically repressed in H1 TKO embryos. The remaining 12 of the 13 Hox genes with reduced expression in H1 TKO embryos are located within paralogous genes Hox3–10, a region important for axial morphology and patterning [1,51–53]. H1 TKO embryos have significant reduction in total H1 levels and die during midgestation [19]. H1 depletion in vivo causes local reductions in chromatin compaction [19,20]. The finding that all affected Hox genes are down-regulated in H1 TKO embryos is surprising because chromatin decompaction and progressive changes in 3D chromatin architecture coincide with activation of Hox genes during embryonic development [10–14] and thus one may expect that H1 depletion would result in up-regulation of certain Hox genes. We believe that the down-regulation of Hox genes is a direct effect due to H1 depletion, and contributes to, rather than merely reflects, the growth retardation observed in a fraction of H1 TKO embryos at a later stage [19]. The E8.5 H1 TKO embryos analyzed in this study did not exhibit obvious phenotypic difference compared with WT littermates. It is noteworthy that H1 depletion in embryos did not lead to changes in expression of any of the Hox genes on the entire Hoxb cluster, which harbors a large intergenic repeat-rich region with a different 3D chromatin structure compared with other Hox clusters [14]. Furthermore, similar to our findings from analyzing H1 TKO embryos, H1 depletion in ESCs does not lead to increased expression in any of the Hox genes, but causes further reduction in the expression of 6 Hox genes. The less prominent effects of H1 depletion on ESCs could be due to the following reasons: 1) ESCs have no or minimum expression of most Hox genes; 2) embryos consist of a more heterogeneous cell population which are likely to have very different bulk and/or local chromatin structure at Hox gene clusters compared with the undifferentiated ESCs. Indeed,
embryos at midgestation have a H1/nuc of 0.74 [19], suggesting a more compact chromatin than ESCs with a H1/nuc of 0.45 [20]; and 3) triple-H1 deletion reduces H1/nuc by 0.34 (from 0.74 to 0.40) in embryos, a larger reduction in total H1 levels than the 0.20 (from 0.45 to 0.25) in ESCs [19,20].

Importantly, we find that the levels of H3K4me3, a chromatin mark catalyzed by TrxG proteins, are decreased at promoters of all affected Hox genes, corresponding to the reduction in gene expression levels of these Hox genes in H1 TKO ESCs. Likewise, the correlation of changes in H3K4me3 and Hox gene expression extends to the single-H1 KO ESCs, suggesting that individual H1 subtypes may also contribute to epigenetic regulation of Hox genes. The effects of triple-H1 deletion on H3K27me3 are more limited, with mild increase at 4 of the 6 affected genes. We speculate that loss of H1 may lead to changes in occupancy of H3K4me3 methyltransferases/demethylases, and/or affect binding of polycomb complex components to the Hox genes [54], resulting in alterations in the histone H3K4 and H3K27 trimethyl marks. It is especially interesting to note that JARID proteins contain an AT-rich interacting domain (Arid) [55,56] that preferentially binds to AT rich tracts [57] and the matrix attachment region (MAR) [58], a region that is involved in the regulation of Hox genes [59] and has a high affinity for H1 binding [60]. However, the levels of JARID1A and JARID1B, two H3K4me2/3 demethylases, do not appear to differ significantly in cellular protein amounts or at affected Hox genes in H1 TKO ESCs compared with WT (Cao, Zhang and Fan, unpublished observations). Similarly, H3K4 methyltransferase MLL1 [36] does not display consistent changes by H1 depletion in ESCs (Cao, Zhang and Fan, unpublished observations). Whether any other H3K4me3 methyltransferase(s)/demethylase(s) is responsible for H1 regulated H3K4me3 at Hox genes in ESCs remains to be determined. We also cannot exclude additional possible regulatory mechanisms mediated through changes in other epigenetic events upon H1 depletion. For instance, nucleosome positioning is thought to impact DNA accessibility and transcription [61], and H1 depletion leads to a reduction in nucleosome repeat length of bulk chromatin and at specific loci [19,20]. Nucleosomes are

Figure 4. The expression profiles of Hox genes in single-H1 KO ESCs. Relative expression of Hoxa1, Hoxb8, and Hoxc13 in H1c−/− (A), H1d−/− (B), and H1e−/− (C) ESCs were shown. *, P<0.05, **: P<0.01, ***: P<0.001.
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found to be positioned at Hox gene clusters, preferentially at 3' of the expressed Hox genes [62], thus the expression of Hox genes may be impaired by altered nucleosome positioning in H1 TKO embryos and ESCs. Alternatively, DNA methylation may be affected at Hox gene clusters by H1 depletion, which has been shown to affect specific DNA methylation patterns at specific imprinted genes and other loci [20,63–65]. Furthermore, the distance between enhancers or regulatory regions for Hox clusters and individual Hox genes [66–68] may be altered by H1 loss, which in turn reduces Hox gene expression.

In order to determine if any of the three deleted H1 subtypes is responsible for the reduction of Hox genes identified in H1 TKO ESCs, we derived single-H1 KO ESCs that are null for H1c, or H1d, or H1e. Surprisingly, unlike adult tissues of the single-H1 knockout mice [29], which display no changes in the total H1 levels, single-H1 KO ESCs established in this study exhibit a moderate reduction in the total H1 levels, and a lack of significant compensation for the deleted H1s by the remaining H1 subtypes. Interestingly, the analysis of the 6 Hox genes whose expression levels were significantly reduced in H1 TKO ESCs shows that loss of H1c or H1d has similar effects on Hoxa1, Hoxb8, and Hoxd13 as triple-H1 deletions. On the other hand, 5 of these 6 Hox genes show no expression change in H1c−/− ESCs (Figure 4C). This differential role of the individual H1 subtypes in activating expression of specific genes is reminiscent of the effects of loss of H1a on the expression of non-variegating transgenes in mice [69] and the activation of MMTV promoter by overexpression of H1b [70]. Hoxb5, Hoxb13 and Hoxd13 are not changed in single-H1 null ESCs, suggesting that the expression reduction of these genes in H1 TKO ESCs may be due to additive effects of deficiency of all three H1 subtypes. It is interesting to note that the levels of H3K4me3 are differentially affected at several Hox genes, suggesting potential roles of individual H1 subtypes in contributing to the patterns of this histone mark at specific Hox genes.

Taken together, the results in this study establish a novel link between histone H1 and Hox gene regulation. Furthermore, the reduction of Hox gene expression by marked H1 depletion correlates with dynamic patterns of H3K4me3 and H3K27me3 marks. The single-H1 KO ESCs established in this study should be useful cell resources for studying specificity of the individual H1 subtypes in regulating gene expression and epigenetic events.

Materials and Methods

Establishment of Mouse Single-H1 KO ESCs and Formation of Embryoid Bodies

Mouse ESCs deficient in histone H1c, or H1d, or H1e were derived from outgrowth of the respective H1c−/−, H1d−/−, and H1e−/− blastocysts (E3.5) as described previously [20]. Two ESC lines were established for each single KO. Genotyping analysis of WT and KO alleles of H1c, H1d, and H1e loci was carried out as reported [19]. Animal breeding and experimental procedures were approved by Georgia Tech Institutional Animal Care and Use
Committee. Embryoid bodies were formed by seeding 1x10^6 ESCs in a 10-cm ultra-low attachment culture dish (Corning) and cultured for 10 days in media containing Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) with 15% fetal bovine serum (Gemiini), 0.1 mM MEM Non-essential amino acids (Life Technologies), 55 μM 2-mercaptoethanol (Life Technologies) and 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies).

RNA Extraction and Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNAs from ESCs were extracted with Trizol reagent (Life Technologies) according to the manufacturers’ instructions. Total RNAs from embryos were prepared using Allprep DNA/RNA Micro kit (Qiagen). Reverse transcription was carried out using a SuperScript III First-Strand cDNA Synthesis kit (Life Technologies). cDNAs were subsequently analyzed with real-time quantitative PCR (qPCR) using iQ SYBR Green Supermix (Bio-Rad) with a MyIQ Single Color real-time PCR Detection System (Bio-Rad). Hox gene specific primers used for qRT-PCR are listed in Table 1.

Statistical Analysis

Statistical analyses and P-values were calculated by the Student t two-tailed test. A P-value of less than 0.05 was considered to be statistically significant.

Preparation and HPLC/MS Analysis of Histones

Total histones were extracted from ES cells as described previously [30,31]. Briefly, the cells were washed with PBS and harvested. The cell pellet was resuspended in Sucrose Buffer (0.3 M Sucrose, 15 mM NaCl, 10 mM HEPES [pH 7.9], 2 mM EDTA, 0.5 mM PMSE, protease inhibitor) with 0.5% NP-40 and homogenized with a dounce homogenizer (Wheaton). 0.2 N H2SO4 was used to extract histones from chromatin pellet. HPLC and mass spectrometry analysis of histone proteins were carried out as described previously [30,31,65]. Approximately 50 μg histone proteins were injected to a C18 reverse phase column (Vydac) on an Aktaapurifier UPC 900 instrument (GE Healthcare). The effluent was monitored at 214 nm (A214), and the profiles of soluble chromatin were collected at a 60x objective on an Olympus Fluorescence Microscope.

Quantitative Chromatin Immunoprecipitation (qChIP)

qChIP assays were performed as described previously [20] with modifications. The following antibodies were used: anti-H3K4me3 (Millipore 07–473), anti-H3K9me3 (Abcam 8896), anti-H3K27me3 (Millipore 07–449), anti-H3K36me3 (Abcam 9050), anti-JARID1A (Abcam 65769), anti-JARID1B (Abcam 50958), anti-MLL1 (Bethyl Lab A500–086A) and rabbit IgG (Millipore 12–370). Briefly, crosslinked chromatin was sheared by sonication. Pre-blocked Protein G Dynabeads (Life Technologies) were incubated with the antibody and 40 μg of soluble chromatin overnight in 4°C, and subsequently washed with Washing Buffer (50 mM HEPES pH 7.6, 1 mM EDTA pH 8.0, 500 mM LiCl, 0.7% Sodium Deoxycholate, 1% NP-40). Immunoprecipitated protein-DNA complexes were eluted and reverse-crosslinked at 65°C, and DNA was purified with a Qiagen DNA Isolation Kit. The amount of each specific immunoprecipitated DNA fragment was determined by real-time PCR. All samples were analyzed in triplicate in two independent experiments. The percentage of input was calculated by dividing the amount of each specific DNA fragment in the immunoprecipitates by the amount of input DNA.
Table 2. Primers for qChIP analysis.

| Name               | Forward                  | Reverse                 |
|--------------------|--------------------------|-------------------------|
| Homeobox A1        | gggaatcatcacagcaccac     | tcctccagtcagctctccttg   |
| Homeobox A1–2     | ggccactacacactcactct     | gaaaaacctccccaaaaggt    |
| Homeobox A3        | aattacctgccctctaatcaaa   | ttatagcgagacccaaatg     |
| Homeobox B4        | atttctatcgggggaatct     | gtttcgaagcgcctctctc     |
| Homeobox B5        | tggagaaggcagctccttcc    | gtcgtaagctgctcttcctc    |
| Homeobox B5–2      | cctcttctctctcttcatcaaa  | gctcttcagagccaaaagcaaa  |
| Homeobox B8        | ctcctgctcagctactcctac   | gaattacgcgtaaagagcga    |
| Homeobox B8–2      | ttaaagcgaacgcttctgctg   | ggagcggagctgctgctgctg   |
| Homeobox B13       | ccctctctctctctcatcaaaa  | gctcttcagagccaaaagcaaa  |
| Homeobox B13–2     | cagggggggtcggaatctgctc  | cgcctccaaagtagcctatcaaa|
| Homeobox C13       | aagcggaacgcttctgctgctg  | gcgccttcagagccaaaagcaaa |
| Homeobox C13–2     | ttcgtgcctgctctctcaaaa   | aattctgcgcttcctctcag    |
| Homeobox D11       | tgaagctcttgagcagctgtca  | ggttggaggagtaggggaaa    |
| Homeobox D11–2     | cctctctctctctctctctctc  | cggccttcagagccaaaagcaaa |
| Homeobox D13       | actgtcgcctgctctctcaaaa  | aattctgcgcttcctctcag    |
| Homeobox D13–2     | gaaaggggtgtcttcctcaca   | tgcgttctctctctcagctgta  |

by the amount of DNA present in input DNA. qChIP primers are listed in Table 2.

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Supporting Information

Figure S1 Characterization of the single-H1 KO ESCs and EBs. (A, B) Karyotypes (A) and phase images (B) of the single-H1 KO ESCs. Scale bar: 50 μm. (C) Characterization of EBs. (i) hematoxylin and eosin staining images of single-H1 KO EBs. Scale bar: 50 μm. (ii) Western blotting analysis of OCT4 in single-H1 KO ESCs and EBs. GAPDH expression levels indicate equal loading of cell lysates. (iii) qRT-PCR analysis of differentiation markers in single-H1 KO ESCs and EBs. (TIF)

Figure S2 qChIP analysis of H3K4me3 in single-H1 KO ESCs. qChIP signals of H3K4me3 (A) and H3K27me3 (B) at indicated Hox genes in single-H1 KO ESCs were normalized to input controls and represented as fold changes over that of WT ESCs. *: P<0.05, **: P<0.01. (TIF)

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Author Contributions

Conceived and designed the experiments: YF. Performed the experiments: YZ ZL MM KC. Analyzed the data: YZ ZL MM KC YF. Wrote the paper: YF KC.

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