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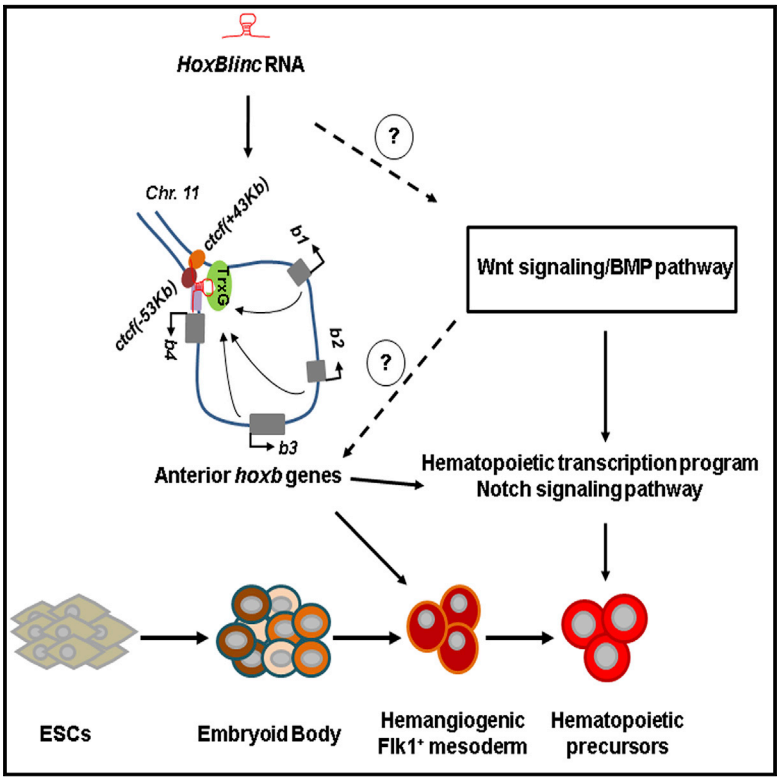
Cell Reports

HoxBlinc RNA Recruits Set1/MLL Complexes to Activate *Hox* Gene Expression Patterns and Mesoderm Lineage Development

Graphical Abstract



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In Brief

Hoxb genes play a critical role in mesoderm and hematopoietic development. Deng et al. find that *HoxBlinc* RNA specifies hemangiogenic/cardiogenic mesoderm and promotes hematopoietic development by upregulating *hoxb* gene expression. *HoxBlinc* acts as a regulator of chromatin loop structure by guiding Setd1a/MLL1 to *hoxb* genes to control lineage-specific transcription.

Highlights

- *HoxBlinc* RNA is required for mesoderm specification and *hoxb* gene activation
- *HoxBlinc* RNA specifies Flk1^{+} mesoderm toward cardiac/hematopoietic lineages
- *HoxBlinc* activates the Notch signaling and hematopoietic transcription networks
- Recruitment of Set1/MLL by *HoxBlinc* controls chromatin dynamics and *hoxb* expression

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HoxBlinc RNA Recruits Set1/MLL Complexes to Activate *Hox* Gene Expression Patterns and Mesoderm Lineage Development

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SUMMARY

Trithorax proteins and long-intergenic noncoding RNAs are critical regulators of embryonic stem cell pluripotency; however, how they cooperatively regulate germ layer mesoderm specification remains elusive. We report here that *HoxBlinc* RNA first specifies Flk1$^+$ mesoderm and then promotes hematopoietic differentiation through regulation of *hoxb* pathways. *HoxBlinc* binds to the *hoxb* genes, recruits Setd1a/MLL1 complexes, and mediates long-range chromatin interactions to activate transcription of the *hoxb* genes. Depletion of *HoxBlinc* by shRNA-mediated knockdown or CRISPR-Cas9-mediated genetic deletion inhibits expression of *hoxb* genes and other factors regulating cardiac/hematopoietic differentiation. Reduced *hoxb* expression is accompanied by decreased recruitment of Set1/MLL1 and H3K4me3 modification, as well as by reduced chromatin loop formation. Re-expression of *hoxb2–b4* genes in *HoxBlinc*-depleted embryoid bodies rescues Flk1$^+$ precursors that undergo hematopoietic differentiation. Thus, *HoxBlinc* plays an important role in controlling *hoxb* transcription networks that mediate specification of mesoderm-derived Flk1$^+$ precursors and differentiation of Flk1$^+$ cells into hematopoietic lineages.

INTRODUCTION

The *homeobox-containing Hox* genes are critical for body patterning and lineage determination during embryogenesis (Chambeyron et al., 2005; Pindyurin and van Steensel, 2012). In mammals, *Hox* genes are clustered in four chromosomes and reveal coordinated expression pattern during development. They are regulated by a combination of long-intergenic noncoding RNAs (lincRNAs) and trithorax (TrxG)/polycomb (PcG) complexes (Brock et al., 2009; Deng et al., 2013; Rinn et al., 2007; Soshnikova and Duboule, 2009). For example, *HOTAIR* suppresses transcription of the *HoxD* locus in trans by targeting the polycomb-repressive complex 2 (PRC2) and the H3K4 demethylase LSD1 to this locus (Tsai et al., 2010). In the *HoxA* locus, two lincRNAs, *HOTTIP* and *Mistra*, control transcription of *HoxA* genes by recruiting MLL H3K4 histone methyltransferase (HMT) complexes (Bertani et al., 2015; Wang et al., 2011). Recent studies revealed that dozens of ESC-expressed lincRNAs maintain the ESC pluripotency by acting as regulatory circuitries of ESC gene expression programs (Guttman et al., 2011). The fact that lincRNAs are promiscuously associated with PRC2 suggests that many lincRNAs may play a dominant role in maintaining general repressive chromatin states (Davidovich et al., 2013; Khalil et al., 2009). Despite these findings, the role of lincRNAs in the regulation of chromatin dynamics and activation of *Hox* gene expression patterns during lineage differentiation remains poorly understood.

During embryonic development, several signaling pathways specify mesoderm and hematopoietic fates of ESCs (Blank et al., 2008; Lengerke et al., 2008; Trompouki et al., 2011). Coordination of these pathways induces mesoderm patterning and specification by activating *Cdx*/*Hox* genes (Lengerke et al., 2008). Collinear activation of *HoxB* genes causes epiblast precursors to migrate into the primitive streak for mesoderm specification (Iimura and Pourquié, 2006). Both cardiogenic and hemangiogenic progenitors are developed from mesoderm-derived Flk1$^+$ cells (Chan et al., 2013; Liu et al., 2012).

The temporal expression of *hoxb* genes is regulated by dynamic chromatin reorganization in nuclei (Chambeyron and Bickmore, 2004). Upon retinoic acid (RA)-induced ESC differentiation, *hoxb* genes sequentially loop out of repressive chromosome territories

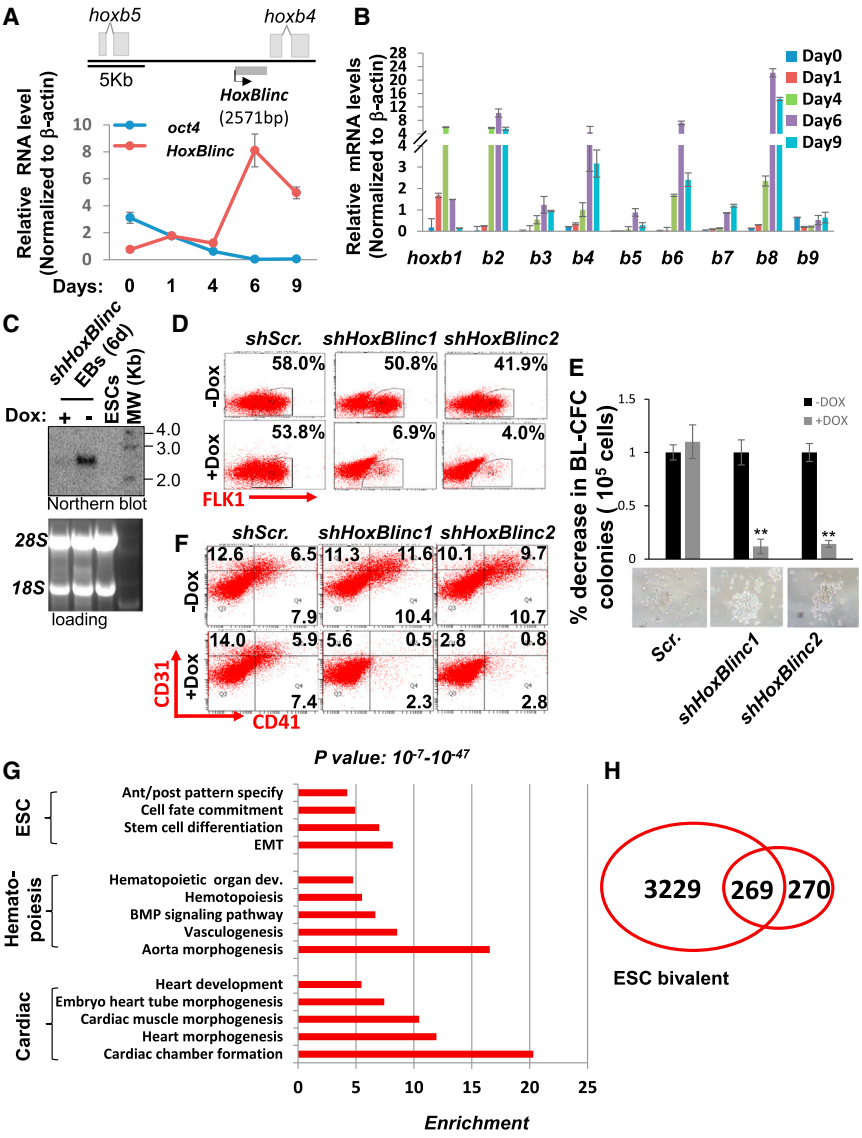

Figure 1. *HoxBlinc* Specifies Flk1$^+$ Mesodermal Cells

(A) Expression of *HoxBlinc* negatively correlates with *Oct4* expression during EB hematopoietic differentiation.

(B) Expression of *hoxb* genes was gradually induced upon EB differentiation.

(C) Northern blot analysis of *HoxBlinc* RNA was performed in ESCs and day 6 EBs with or without Dox-induced *HoxBlinc* KD.

(D) FACS analysis of Flk1$^+$ cells in induced *HoxBlinc* KD and control EBs. KD was induced at the day 2 epiblast stage, and FACS analysis was carried out at day 4 of EB differentiation.

(E) BL-CFC potential of EBs with or without Dox-induced *HoxBlinc* KD at day 2 epiblast stage.

(F) FACS analysis of CD31$^+$/CD41$^+$ endothelial/hematopoietic cells differentiated from the induced *HoxBlinc* KD at day 2 epiblast stage and control blast colonies.

(G) RNA-seq analysis of induced *HoxBlinc* KD (+Dox) and control EBs (−Dox) at epiblast stage (day 2). RNA was isolated from day 6 differentiated EBs. *HoxBlinc*-RNA-regulated genes were annotated by Gene Ontology (GO) analysis.

(H) Overlap between *HoxBlinc*-RNA-activated genes and total ESC bivalent genes.

(CTs) for expression whereas silent genes remain located within dense CTs (Chambeyron et al., 2005). Recent studies revealed that the anterior *HoxB* genes, *B2*, *B3*, *B5*, and *B6*, are dependent on MLL1 for activation (Liu et al., 2011). In contrast, *HoxB4* is regulated by the Setd1a complex (Deng et al., 2013). The mechanisms by which specific Set1/MLL proteins are targeted to specific *HoxB* gene loci to introduce active H3K4 methylation patterns, coordinate three-dimensional (3D) chromatin domains, and initiate differentiation of particular cell lineages remain elusive.

Here, we discovered a *hoxb* locus-associated lincRNA, termed *HoxBlinc,* that regulates *hoxb* gene transcription by modulating local chromatin alterations. Inhibition of *HoxBlinc* resulted in a block of early cell lineage commitment by perturbing specification of mesoderm-derived Flk1$^+$ precursors and by subsequently inhibiting hematopoietic differentiation of Flk1-expressed cells. Furthermore, *HoxBlinc* RNA recruits Setd1a/MLL1 complexes and facilitates the organization of a specific 3D chromatin architecture that activates the anterior *hoxb* genes, resulting in cardiogenic/hemogenic mesoderm differentiation.

RESULTS

The Expression of *HoxBlinc* Is Positively Correlated with Anterior *hoxb* Gene Transcription Upon EB Differentiation

hoxb genes play a critical role in hematopoietic development (Abramovich and Humphries, 2005). We identified a 2.57-Kb noncoding region upstream of the *hoxb4* gene that is actively transcribed upon differentiation of R1/E ESCs into EBs (Figure 1A). Expression of this transcript positively correlates with a gradual increase in the expression of anterior *hoxb* genes (Figures 1A and 1B), suggesting a potential role of this lincRNA in *hoxb* gene activation. We defined this lincRNA as *HoxBlinc* because it is located at the *hoxb* locus and co-expressed with *hoxb* genes. We further employed 5′- and 3′-RACE-PCR using cDNA prepared from day 6 differentiated EBs to clone the full-length *HoxBlinc*, which is 2,571 nucleotides long and lacks introns (Figure S1A). Its transcription start site (TSS) is located 2,638 bp upstream of the TSS of the *hoxb4* gene on mouse chromosome 11 (Figure 1A).

HoxBlinc RNA Regulates Key Genes Required for Early Lineage Commitment

To delineate the role of *HoxBlinc* in embryonic development, we created two Tet-ON-Dox-inducible *HoxBlinc* shRNA ESC clones

that allowed specific knockdown (KD) of *HoxBlinc* at two distinct stages of EB differentiation, at the epiblast cell stage at day 2 and Flk1$^+$ mesodermal stage at day 3.5. Northern blotting and RT-PCR analysis demonstrated that the 2.57-Kb *HoxBlinc* transcript is silenced in ESCs and induced upon EB differentiation (Figures 1C and S1B). KD of *HoxBlinc* at the ESC or EB (day 2) stages affected neither pluripotency and expression of stemness genes, e.g., OSKM (Figures S1C and S1D), nor cell-cycle progression upon EB differentiation (Figure S1E). The transcript levels of *HoxBlinc RNA*, anterior *hoxb* genes, and mesodermal/endodermal marker genes were significantly inhibited in differentiated EBs (Figures 1C, S1F, and S1G). During differentiation, murine ESCs differentiate into Flk1$^+$ cells capable of generating a blast colony consisting of hematopoietic and endothelial cells (Kennedy et al., 1997) at day 3. KD of *HoxBlinc* using two different inducible shRNAs at the epiblast stage (day 2) resulted in a significant reduction of Flk1$^+$ cells from an average of 49.2% to average of 6.9% (Figure 1D shows the result of one experiment; Figure S1H shows the average of three independent experiments). Furthermore, we found that KD of *HoxBlinc* at the epiblast stage blocked formation of blast colonies (Figure 1E) and CD31$^+$ endothelium cells (Figure 1F).

RNA-seq analysis was carried out to elucidate the role of *HoxBlinc* RNA in EB differentiation by comparing differentiated day 6 EBs that express or silence *HoxBlinc* at an early stage. A total of 539 genes showed a more than 3-fold decrease in mRNA levels whereas only 51 genes increased expression upon *HoxBlinc* KD (Tables S1 and S2), suggesting that *HoxBlinc* RNA mainly functions as a transcriptional activator during EB differentiation. Among the affected genes, cdx2 and *hoxb2–4* genes were decreased by more than 3-fold (Tables S1 and S2). Gene Ontology (GO) analysis revealed that the pathways specifically affected by *HoxBlinc* KD are those involved in cell fate commitment, epithelial to mesenchymal transition, and anterior/posterior patterning, which are linked to *Hox* gene function in early embryogenesis, as well as hematopoietic and cardiac development (Figure 1G). Furthermore, the pathways that regulate hemangiogenic/cardiogenic mesoderm differentiation such as BMP/Wnt signaling and aorta morphogenesis are also dysregulated upon *HoxBlinc* KD (Figure 1G). Genes encoding for early developmental regulators are often associated with bivalent chromatin marks (H3K4me3 and H3K27me3) and are in a poised state that maintains lineage commitment programs. The comparison of bivalent marks with gene expression changes showed that 50% of *HoxBlinc*-downregulated genes (269 genes) are bivalently marked in ESCs (Figure 1H). Interestingly, genes encoding *brachyury* (*T*) or the H3K9 HMT *suv39h1* are among those that are highly upregulated upon KD (Figure 2A). Thus, *HoxBlinc* RNA plays a critical role in coordinating anterior *hoxb* gene expression during ESC commitment and differentiation toward hematopoietic lineages.

HoxBlinc Regulates Specification of Mesoderm Toward Hematopoietic Lineages

The Flk1$^+$ cells possess both hematopoietic and cardiac potentials and can be committed to either lineage (Ema et al., 2003; Kattman et al., 2006). The data in Figure 1G suggest that *HoxBlinc* RNA is required for hematopoietic cell fate specification. Further analysis of the RNA-seq data revealed that expression of transcription factors (TFs) or markers critical for the early onset of hematopoiesis, such as TAL1, LMO2, Lyl1, Meis1, Fli1, CD34, and βH1, decreased by more than 3-fold upon *HoxBlinc* KD (Figures 2A and 2C). The NOTCH-signaling pathway plays a pivotal role in the development of hematopoietic stem and progenitor cells (HS/PCs) (Bigas and Espinosa, 2012). Ablation of *HoxBlinc* RNA led to a significant decrease in genes encoding components of the NOTCH pathway (Figure 2A, right). Importantly, GO analysis revealed that hematopoiesis and hematopoietic organ development are regulated by *HoxBlinc* RNA (Figure 1G).

Loss of *HoxBlinc* RNA led to a strong decrease in *hoxb1-b6* gene expression and that of hematopoietic TF and marker genes (Figures 2B, 2C, and S2A). Anterior *hoxb* genes are critical regulators of early hematopoiesis (Björnsson et al., 2003; Magnusson et al., 2007). Depletion of *HoxBlinc* did not decrease expression of the posterior *hoxb9* and *hoxa* genes (Figures 2B and S2B). During EB differentiation, Flk1$^+$ hemangiogenic mesoderm continues to form blast colonies and differentiate into CD41$^+$/c-Kit$^+$ HS/PCs at day 4 and beyond (Figure 2D; Chung et al., 2002; Kennedy et al., 1997). Although KD of *HoxBlinc* at the Flk1$^+$ mesoderm stage reduced the CD41$^+$/c-Kit$^+$ HS/PCs from an average of 9.03% to 2.88% (Figures S2C and S2D), the effects on hematopoiesis could result from the fact that *HoxBlinc* KD reduced Flk1$^+$ cells (Figures 1D and S1H). To ensure that *HoxBlinc* not only acts on Flk1$^+$ mesoderm formation but also specifies differentiation of Flk1$^+$ cells toward hematopoietic lineages, we FACS sorted hemangiogenic Flk1$^+$ cells and followed the blast culture. KD of *HoxBlinc* using two different inducible shRNAs in purified Flk1$^+$ cells resulted in a significant reduction of CD41$^+$ HS/PCs by 56.7% (Figure 2E). In contrast, expression of the scrambled shRNA had no effect (Figure 2E). Furthermore, KD of *HoxBlinc* blocked the differentiation of CD41$^+$ HS/PCs into various hematopoietic lineages including GM, GEMM, Ery-D, and macrophages (Figure 2F). Accompanied with inhibition of hematopoiesis by *HoxBlinc* loss, anterior *hoxb* genes and genes encoding hematopoietic-specific TFs were also downregulated (Figures 2G and 2H). Thus, *HoxBlinc* RNA is also a crucial regulator of early hematopoiesis, perhaps by regulating anterior *hoxb* gene transcription and expression of other important hematopoietic TFs.

HoxBlinc also regulates anterior *HOXB* genes in human hematopoietic cells, which is supported by our observation that *HoxBlinc* RNA and anterior *HOXB* genes are highly expressed in human erythroleukemia K562 cells. KD of *HoxBlinc* decreased anterior *HOXB* gene expression by downregulating H3K4me3 enrichment at the promoter regions (Figures S2E–S2G). In addition, loss of *HoxBlinc* inhibited K562 cell proliferation (Figure S2H).

HoxBlinc Modulates Cardiac Differentiation by Regulating *hoxb* Gene Transcription and Flk1$^+$ Mesoderm Specification

The cardiogenic mesoderm is also derived from Flk1$^+$ cells. Four important TFs, Isl1, Nkx2.5, Mef2c, and Gata4, are critically involved in the differentiation of cardiac mesoderm (Chan et al., 2013; Olson, 2006; Watanabe and Buckingham, 2010). RNA-seq data revealed that KD of *HoxBlinc* resulted in a more

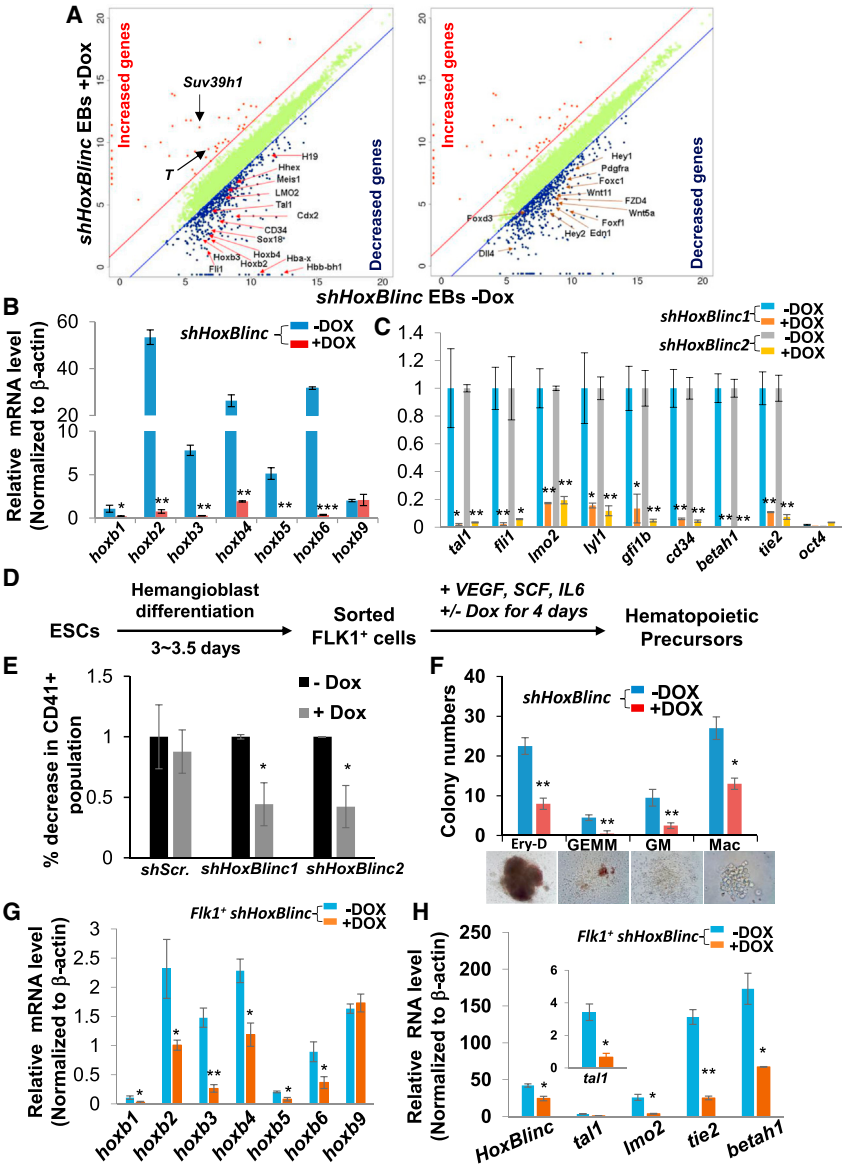

Figure 2. *HoxBlinc* Controls Hematopoietic Differentiation by Regulating *hoxb* Genes and Hematopoietic Transcription Programs

(A) Scatterplots showing that expression levels of TFs and those encoding genes required for hematopoiesis (left) or the NOTCH pathway (right). Activated genes are shown in red, repressed genes in blue (only genes are shown that alter expression by more than 3-fold upon *HoxBlinc* KD).

(B) qRT-PCR analysis of *hoxb* gene expression comparing induced *HoxBlinc* KD (+Dox) and control EBs (−Dox) at day 6.

(C) qRT-PCR analysis of expression of genes encoding key hematopoietic TFs and markers in induced *HoxBlinc* KD (day 3 Flk1$^+$ stage) and control EBs collected at day 6.

(D) Schematic representation of hematopoietic differentiation from the purified FLK1$^+$ cells following hemangioblast development.

(E) FACS analysis of CD41 expression upon hematopoietic differentiation of sorted FLK1$^+$ cells, which were then induced for *HoxBlinc* KD and cultured in blast culture media.

(F) CFC analysis of definitive hematopoietic colonies (Ery-D, GEMM, GM, and Mac) in induced *HoxBlinc* KD and control EB-derived cells.

(G) qRT-PCR analysis of *hoxb* gene expression upon hematopoietic differentiation of sorted FLK1$^+$ cells that were then induced for *HoxBlinc* KD and cultured in blast culture media.

(H) Analysis of the expression of hematopoietic-specific TFs and markers during hematopoietic differentiation of sorted FLK1$^+$ cells with and without *HoxBlinc* KD cultured in blast culture media. Data are presented as mean ± SD from three or four independent experiments; *$p < 0.05$; **$p < 0.01$ by Student's t test.

than 3-fold decrease in expression of these important regulators (Figures 3A and 3B). Consistently, expression of other TFs important for heart development such as Hand1, Hand2, and Tbx5 was also drastically decreased (Figures 3A and 3B).

Next, we examined whether *HoxBlinc* is important for the formation of cardiogenic mesoderm and cardiac lineage specification. KD of *HoxBlinc* led to a strong reduction in the formation of Flk1$^+$/PDGFRα^+ cardiogenic mesoderm cells (Figure 3C). The number of Flk1$^+$/PDGFRα^+ cells was reduced from an average of 32.75% without Dox to an average of 12.9% with Dox induction (Figures 3C and S3A). The cardiomyocytic markers cTnT and actc1 are also inhibited by the *HoxBlinc* KD (Figure 3D). Depletion of *HoxBlinc* prevented formation of contracting cardiomyocytes that exhibited spontaneous beating (Movies S1 and S2). Furthermore, when we silenced *HoxBlinc* in CGR8 MHC-GFP ESCs, which harbor a GFP gene driven by the cardiac lineage tracer *MHC*α chain promoter (Singh et al., 2007), we observed a decrease in GFP$^+$ EBs from 33% in WT cells to only 7% upon Dox-induced *HoxBlinc* KD (Figures 3E and S3B). The reduction in the number of GFP-expressing cells was accompanied by reduced expression of the *cTnT* and *actc1* genes (Figure S3C). To distinguish whether the effect is due to blocking of Flk1 mesoderm specification or later cardiac differentiation, we FACS sorted hemangiogenic Flk1$^+$ cells, induced *HoxBlinc* KD, and followed cardiomyocytic differentiation. Depletion of *HoxBlinc* using two different inducible shRNAs in purified Flk1$^+$ cells did not affect the ability of Flk1$^+$ cells to differentiate and express cardiomyocytic markers cTnT and actc1 (Figures S3D and S3E), indicating that *HoxBlinc* regulates cardiomyocytic differentiation by modulating *hoxb* gene expression and Flk1$^+$ mesoderm specification.

Re-expression of *hoxb2–4* Genes Rescues Hematopoietic Differentiation in *HoxBlinc*-Deficient ESCs

We further addressed whether *HoxBlinc*-RNA-mediated anterior *hoxb* gene activation is required for the formation of

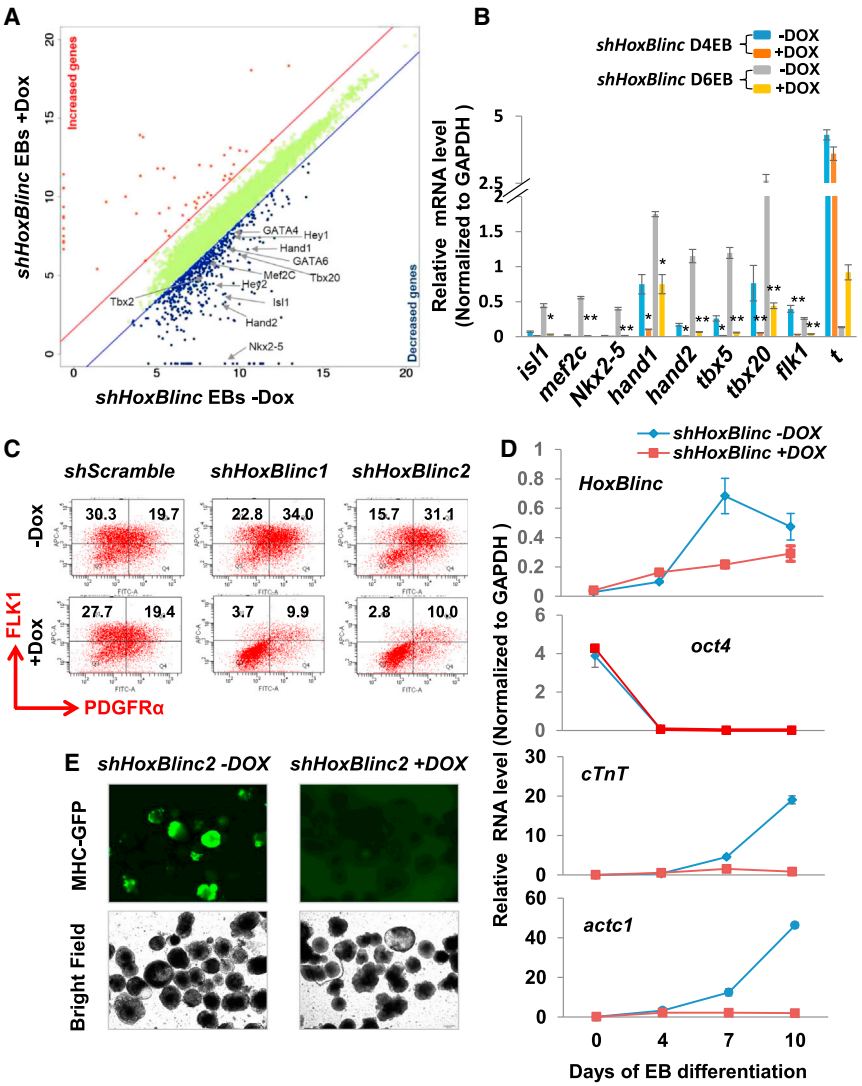

Figure 3. KD of *HoxBlinc* Inhibits *hoxb* Gene Expression and Cardiogenic Mesoderm Formation

(A) Scatterplot showing expression levels of genes encoding TFs and markers important for heart development increased (red) or decreased (blue) by more than 3-fold upon *HoxBlinc* KD.

(B) qRT-PCR analysis of expression of genes encoding TFs and markers important for heart development in induced *HoxBlinc* KD (at the epiblast stage) and control EBs (−Dox) collected at day 4 and day 6. Data are presented as mean ± SD from three or four independent experiments; *$p < 0.05$; **$p < 0.01$.

(C) FACS analysis of Flk1$^+$/PDGFRα$^+$ cells in induced *HoxBlinc* KD (at the epiblast stage) and control EBs collected at day 4.

(D) qRT-PCR analysis of the expression of *cTnT* and *actc1* in *HoxBlinc* KD and control EBs collected at days 0, 4, 7, and 10.

(E) Green fluorescence visualization of the expression of the cardiomyocyte marker, MHC-GFP, in induced *HoxBlinc* KD (at the epiblast stage) and control EBs (−Dox) at day 12.

Flk1$^+$ hemangiogenic mesoderm and lineage specification. We re-expressed the *hoxb2–b4* genes, which play an important role in hematopoietic development (Björnsson et al., 2003; Krumlauf, 1994; Magnusson et al., 2007) in the *HoxBlinc* KD ESCs. Expression of *hoxb2–b4* in the *HoxBlinc* KD cells (Figure 4A) partially rescued Flk1$^+$ precursors at day 4 on average (three experiments) from 4.7% in *HoxBlinc* KD cells to 22.03% in rescued cells (Figures 4B and 4C, top) and c-Kit$^+$/CD41$^+$ HS/PCs at day 6 on average (three experiments) from 2.85% in KD cells to 4.85% in rescued cells (Figures 4B and 4C, bottom). Importantly, expression of *hoxb2–b4* genes did not affect the KD efficiency of *HoxBlinc* RNA (Figure 4A). Furthermore, expression of *hoxb2–b4* genes in the *HoxBlinc* KD cells partially rescued hemangioblast colonies (Figure 4D) and differentiation into various hematopoietic colonies including GM, GEMM, and Ery-D (Figure 4E). To further confirm the role of *HoxBlinc* in Flk1 mesoderm specification, we expressed full-length *HoxBlinc* in WT ESCs and allowed them to differentiate. Expression of *HoxBlinc* enhanced the Flk1$^+$ mesodermal cell population at day 4 of EBs (Figure S3F).

Hematopoiesis is a coordinated process that requires the action of many hematopoietic TFs and signaling pathways. We reasoned that expression of *hoxb2–b4* genes in the *HoxBlinc* KD cells might reactivate key hematopoietic TFs and markers inhibited by the *HoxBlinc* KD. To address this, we analyzed the expression levels of *tal1*, *lmo2*. *fli1*, *cdx2*, and *βh1* in the WT control, *HoxBlinc* KD, and rescued cells. Re-expression of *hoxb2–b4* led to a significant increase in expression levels of these hematopoietic TFs and markers compared to the *HoxBlinc* KD cells (Figure 4F). Furthermore, re-expression of *hoxb2–b4* also rescued the expression of genes encoding components of the NOTCH pathway (Figure 4G) consistent with the role of the NOTCH pathway in HSC function and hematopoietic development (Benveniste et al., 2014). Thus, these data revealed a molecular pathway by which *HoxBlinc* regulates hematopoietic transcription networks and the NOTCH signaling by controlling transcription of the anterior *hoxb* genes during early hematopoiesis.

HoxBlinc Directly Recruits Setd1a/MLL1 HMTs to Activate Transcription

Next, we sought to understand the mechanism(s) by which *HoxBlinc* regulates *hoxb* genes. We performed chromatin immunoprecipitation (ChIP) analysis to examine whether *HoxBlinc* KD affects chromatin structure in the *hoxb* cluster. *HoxBlinc* KD led to a broad decrease in H3K4me3 across *hoxb1–b6* genes, but not in the posterior *hoxb9* gene (Figure 5A), indicating that *HoxBlinc* is required for maintaining H3K4me3 levels at the

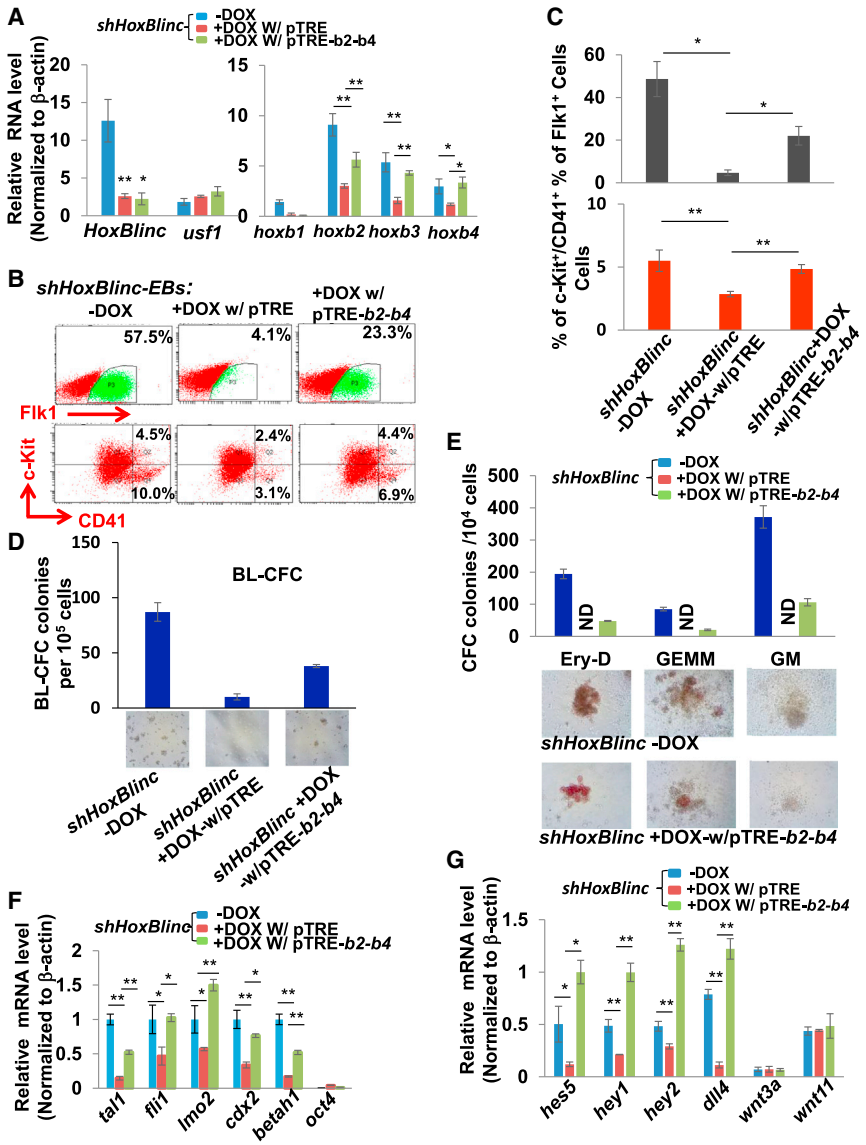

Figure 4. Re-expression of Anterior *hoxb2–b4* Genes Rescues Flk1$^+$ Mesoderm and HS/PCs in the *HoxBlinc* KD EBs

(A) qRT-PCR analysis showing expression levels of *HoxBlinc* (left) and *hoxb2–b4* (right) genes in control or induced *HoxBlinc* KD EBs rescued with the vector or constructs expressing *hoxb2–b4* genes.

(B) FACS analysis of Flk1$^+$ cells (top) and CD41$^+$/c-Kit$^+$ HS/PCs (bottom) in control and induced *HoxBlinc* KD EBs rescued with vector or constructs expressing the *HoxB2–B4* genes.

(C) Percentage of Flk1$^+$ cells (top) and CD41$^+$/c-Kit$^+$ HS/PCs (bottom) in control and rescued EBs.

(D) BL-CFC potential of EBs in control or induced *HoxBlinc* KD EBs rescued with the vector or constructs expressing *hoxb2–b4*.

(E) Secondary hematopoietic CFC assays of definitive hematopoietic colonies (Ery-D, GEMM, and GM) in induced *HoxBlinc* KD and rescued EBs-derived blast colony cells.

(F and G) qRT-PCR analysis showing expression levels of TFs and markers required for hematopoiesis (F) or genes encoding NOTCH pathway in control or rescued EBs (G). Data are presented as mean ± SD from three or four independent experiments; *$p < 0.05$; **$p < 0.01$ by Student's t test.

transcriptionally active *hoxb* genes. The Set1/MLL complexes maintain H3K4 methylation patterns at the *Hox* gene loci and play important roles in hematopoiesis (Deng et al., 2013; Ernst et al., 2004; Liu et al., 2011). We examined the association of *HoxBlinc* with Set1/MLL complexes using biotin-labeled *HoxBlinc* RNA to pull down Setd1a protein from day 6 differentiated EB nuclear extracts (NEs) (Figure 5B). Only purified biotinylated *HoxBlinc* RNA, but not λRNA, *gapdh* RNA, or antisense *HoxBlinc* RNA, retrieved Setd1a from differentiated EB NEs, and the interaction was mediated by a 3′ segment of the *HoxBlinc* RNA (Figure 5B). Furthermore, only Setd1a and MLL1 specifically interacted with *HoxBlinc* RNA, but not other core components of the Set1/MLL complexes, such as ASH2L and RBBP5, or LSD1 (Figure S4A). RNA immunoprecipitation experiments using day 3 EB (before Flk1 formation) or day 6 EB (after Flk1 formation) NEs confirmed that antibodies against Setd1a and MLL1 specifically pulled down endogenous *HoxBlinc* RNA at both Flk1 specification and hematopoietic cell stages (Figure 5C). Both Setd1a and MLL1 contain a highly conserved catalytic SET domain, which interacts with the N-terminal tails of histone H3 and other chromatin regulators (Katsani et al., 2001; Rozenblatt-Rosen et al., 1998). We tested whether *HoxBlinc* associates with both H3K4 HMT complexes by interacting with the SET domain. We purified GST-fusion proteins containing SET domains of Setd1a or MLL1 and incubated them with in-vitro-transcribed *HoxBlinc* RNA or a control *usf1* RNA. Compared to the GST control, the SET domains of both Setd1a and MLL1 specifically interacted with *HoxBlinc*, but not with *usf1* RNA (Figure 5D). The results confirm that *HoxBlinc* RNA directly interacts with the SET domains of the Set1/MLL complexes.

To ascertain whether *HoxBlinc* RNA activates transcription through recruiting the Set1/MLL complexes, we employed a *BoxB*-tethering reporter system in which *BoxB-HoxBlinc* RNA binds to λN fused to the GAL4-DNA-binding domain to regulate a UAS-driven luciferase reporter gene (Figure 5E; Wang et al., 2011). The *BoxB-HoxBlinc* specifically stimulated transcription of the luciferase gene when compared to the *BoxB* alone control (Figure 5E). Strikingly, KD of ASH2L, a component required for the enzymatic activities of the Set1/MLL1 complexes, specifically abolished the *BoxB-HoxBlinc*-RNA-mediated transactivation of the reporter gene (Figure 5E). These data indicate that *HoxBlinc* functionally recruits Set1/MLL1 complexes to activate transcription.

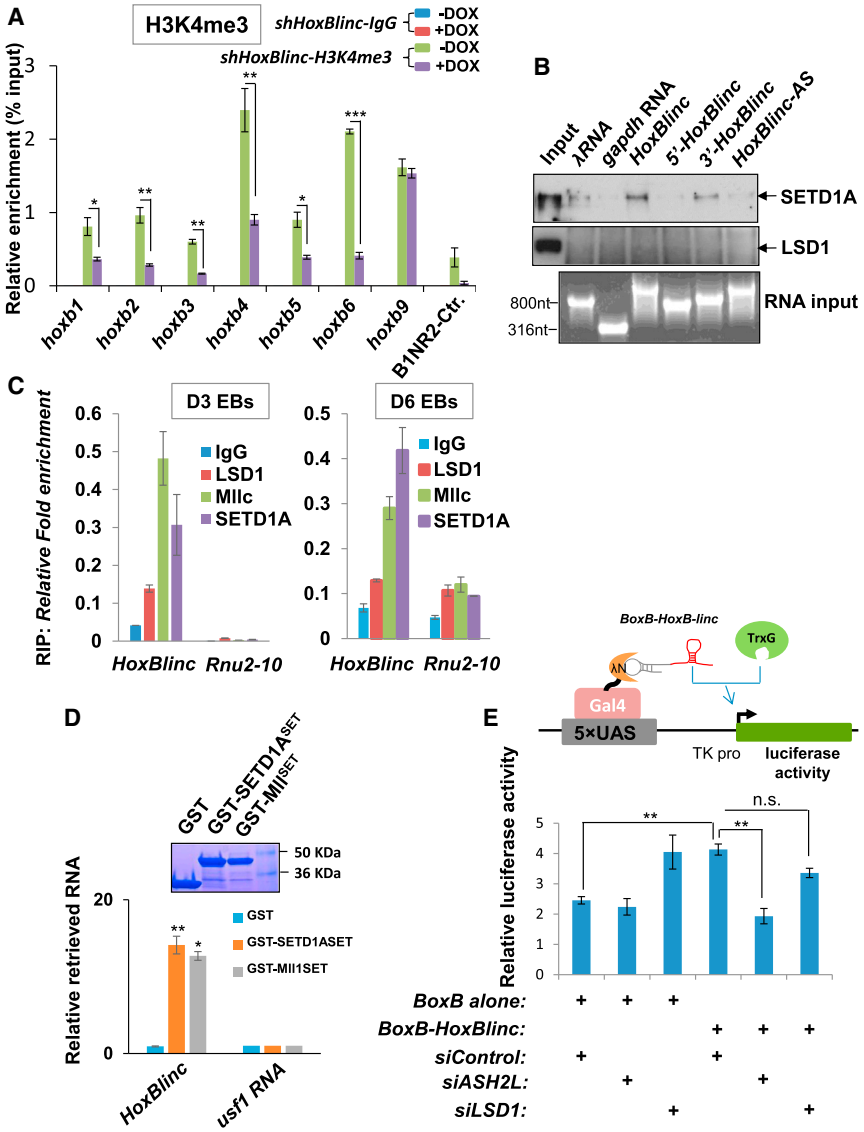

Figure 5. *HoxBlinc* Interacts with the Set1/MLL1 Complexes to Activate Target Genes

(A) ChIP analysis of H3K4me3 enrichment at promoters of *hoxb* genes in induced *HoxBlinc* KD and control EBs.

(B) Biotinylated *HoxBlinc* RNA and a 3′ domain of *HoxBlinc* RNA specifically interact with Setd1a in 6d EB NEs.

(C) RIP showing that both Setd1a and MLL1 are associated with *HoxBlinc* before FLK1 specification (d3 EBs) and after FLK1 specification (d6 EBs).

(D) *HoxBlinc* directly interacts with the SET domain of Setd1a and MLL1 proteins shown by incubating GST-Setd1a^{SET} or GST-MLL1^{SET} with in-vitro-transcribed RNAs.

(E) *HoxBlinc* activates luciferase reporter gene transcription by recruiting TrxG HMT complexes. (Top) Diagram of the *BoxB*-tethering reporter system in which GAL4-fused λN recruits *BoxB-HoxBlinc* fusion RNA-associated TrxG complexes to a UAS-driven luciferase reporter construct is shown. (Bottom) Luciferase activity was analyzed 48 hr posttransfection with the indicated constructs. Data are presented as mean ± SD from three or four independent experiments; *p < 0.05; **p < 0.01 by Student's t test.

HoxBlinc RNA Binds to the *hoxb* Locus and Is Dependent on Set1/MLL Complexes for Transcriptional Regulation and Lineage Differentiation

Next, we tested whether *HoxBlinc* directly binds to the *hoxb* locus. We carried out chromatin isolation by RNA purification (ChIRP) analysis (Chu et al., 2011) using biotinylated oligonucleotide probes tiling *HoxBlinc* RNA, which specifically retrieved *HoxBlinc* RNA from chromatin prepared from day 3 and day 6 EBs comparing to control *lacZ* probes (Figure 6A). Furthermore, *HoxBlinc* RNA specifically interacted with the promoters of *hoxb1–b6* genes, but not with the posterior *hoxb9* promoter and other control regions at both Flk1 specification and hematopoietic cell stages (Figure 6B).

Consistent with the RIP and ChIRP data showing that *HoxBlinc* RNA binds to the promoters of anterior *hoxb* genes and recruits Set1/MLL1 complexes (Figures 5 and 6B), ChIP assays revealed that loss of *HoxBlinc* specifically decreased recruitment of Setd1a/MLL1 complexes to promoters of the *hoxb1–b6* genes, but not to the posterior *hoxb9* gene or other control regions at both Flk1 specification and hematopoietic cell stages (Figures 6C, 6D, and S4B–S4D). The reduced recruitment of Set1/MLL1 correlated with a decrease in H3K4me3 levels at the same sites (Figure 5A).

We further asked whether recruitment of the Set1/MLL1 complexes is necessary for *HoxBlinc*-mediated anterior *hoxb* gene activation. To test this possibility, we generated Dox-inducible *setd1a* and *mll1* KD ESCs and analyzed the effects on the coordinated expression of *HoxBlinc* RNA and anterior *hoxb* genes upon EB differentiation (Figure S5A). Although neither *setd1a* loss nor *mll1* KD affected *HoxBlinc* expression (Figure S5B), loss of either *setd1a* or *mll1* impaired expression of anterior *hoxb* genes, but not expression of the posterior *hoxb9* gene (Figures S5C and S5D). Interestingly, KD of *mll1* did not interfere with transcription of the *hoxb4* gene (Figure S5C), which is consistent with previous reports showing that Setd1a, but not MLL, is required for promoter activation of the *hoxb4* gene (Deng et al., 2013; Liu et al., 2011).

KD of *setd1a* or *mll1* resulted in reduced formation of Flk1$^+$ hemangiogenic cells (Figures S5E and S5F) and CD41$^+$/c-Kit$^+$ HS/PCs (Figures S5G and S5H). Notably, the reduction in the numbers of CD41$^+$ and c-Kit$^+$/CD41$^+$ cells was more pronounced in *setd1a* KD cells compared to *mll1* KD cells (Figures S5G and S5H), which may be attributed to the ability of Setd1a to regulate the *hoxb4* gene in contrast to MLL1 (Deng et al.,

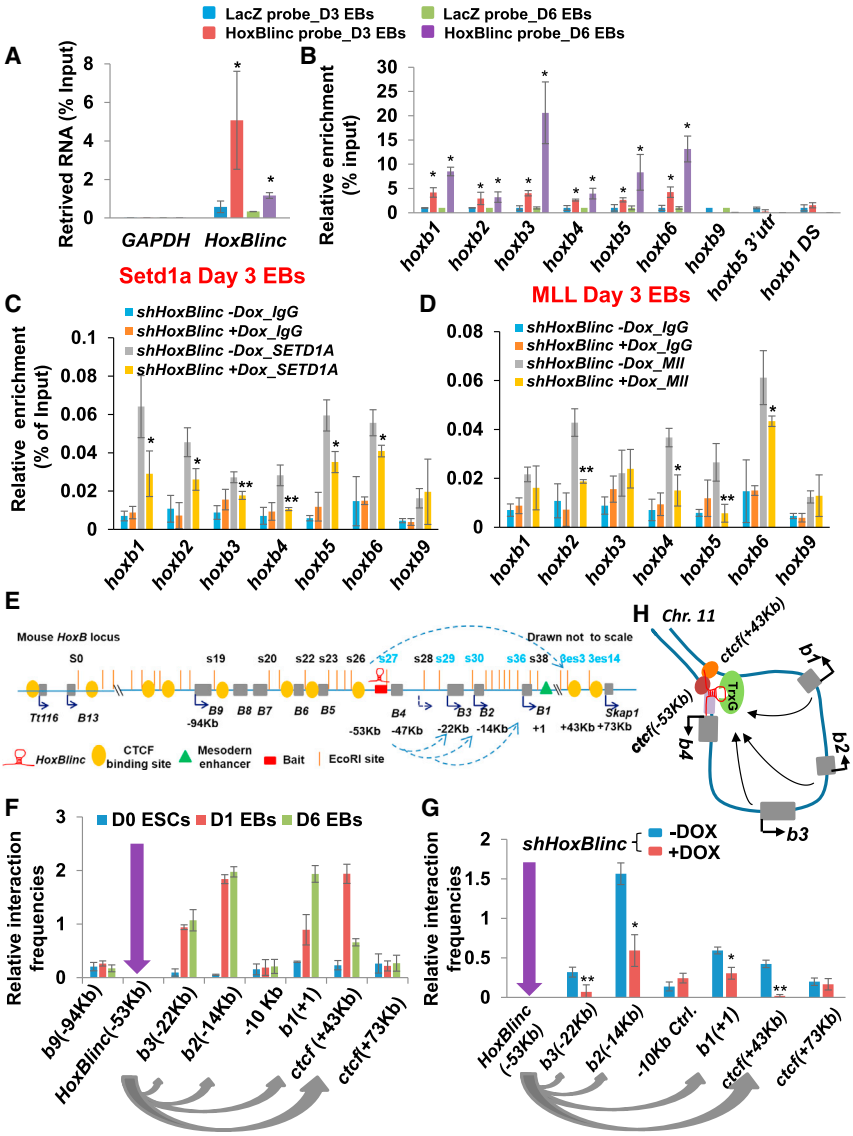

Figure 6. *HoxBlinc* Binds to Anterior *hoxb* Genes and Regulates Chromatin Structure Alterations at the Anterior *hoxb* Locus

(A) qRT-PCR analysis of RNA retrieved by the complementary *HoxBlinc*-tiling probes and *LacZ* probes in day 3 and day 6 EBs.

(B) ChIRP analysis of the *HoxBlinc* RNA enrichment at the *HoxB* locus in day 3 and day 6 EBs.

(C and D) ChIP analysis showing binding of Setd1a (C) and MLL1 (D) at the promoters of *hoxb* genes in *HoxBlinc* KD and control d3 EBs prior to the Flk1 specification.

(E) Schematic diagram representing the cluster organization of the *hoxb* genes.

(F) 3C analysis showing interactions between the *HoxBlinc* locus and the anterior *hoxb* genes in ESCs and differentiated EBs at days 1 and 6.

(G) 3C analysis showing interactions between the *HoxBlinc* locus and the anterior *hoxb* genes in *HoxBlinc* KD and control day 6 EBs.

(H) Model depicting how *HoxBlinc* organizes the anterior *hoxb* genes into a CTCF-mediated inducible active chromatin domain that facilitates long-range interactions between the *HoxBlinc* locus and the anterior *hoxb1–b3* genes. Data are presented as mean ± SD from three independent experiments; *p < 0.05; **p < 0.01 by Student's t test.

2013; Oshima et al., 2011). Nevertheless, these data suggest that Setd1a and MLL1 mediate *HoxBlinc* RNA function in specifying mesoderm development and subsequent hematopoietic differentiation.

HoxBlinc-Mediated Chromatin Looping Remodels the hoxb Gene Cluster

Previous studies have shown that chromatin looping establishes transcriptionally active domains in the *hoxb* locus (Chambeyron et al., 2005). We therefore tested whether *HoxBlinc* is required for mediating proximity between the *hoxb* genes and the *HoxBlinc* locus using chromosome conformation capture (3C) (Figure 6E). All of the EcoRI sites in the *hoxb* locus were efficiently digested (Figure S6A). *HoxBlinc* KD did not change recruitment of H3K4me1 and p300 enhancer marks to known *hoxb* regulatory elements (Figures S6B–S6D). Interestingly, the *HoxBlinc* locus was brought into close proximity to the *hoxb1*, *b2*, and *b3* genes (Figures 6E and 6F) during differentiation of EBs, which correlated positively with the activation of these genes (Figure 1B). The *HoxBlinc* locus also interacted with a +43-Kb element containing a *ctcf* site (*hoxb1* TSS was set as +1), which may serve as a boundary to insulate the anterior genes from interfering with other genetic elements in the locus (Figures 6F and 6H).

Furthermore, KD of *HoxBlinc* significantly decreased looped interactions between the *HoxBlinc* locus and individual *hoxb1*, *b2*, and *b3* genes (Figure 6G). The differentiation-induced CTCF-mediated active chromatin loop between the −53-Kb *ctcf* site and the downstream +43-Kb *ctcf* site was also disrupted by *HoxBlinc* KD (Figures 6E and 6G), indicating that *HoxBlinc* KD leads to an impairment of the formation of an active chromatin domain at the anterior *hoxb* locus (Figure 6G). Thus, *HoxBlinc* RNA is an important regulator for the establishment and maintenance of a 3D active chromatin domain that supports expression of the anterior *hoxb* genes during EB differentiation (Figure 6H).

Functional Analysis of the Role of *HoxBlinc* in Mesoderm Differentiation by CRISPR-Cas9-Mediated Genome Editing

Many lncRNAs are expressed at very low levels, and traditional RNAi-mediated KD may lead to subtle or inconsistent changes in gene expression. To provide genetic evidence for a role of *HoxBlinc* in mesoderm specification, we deleted the *HoxBlinc*

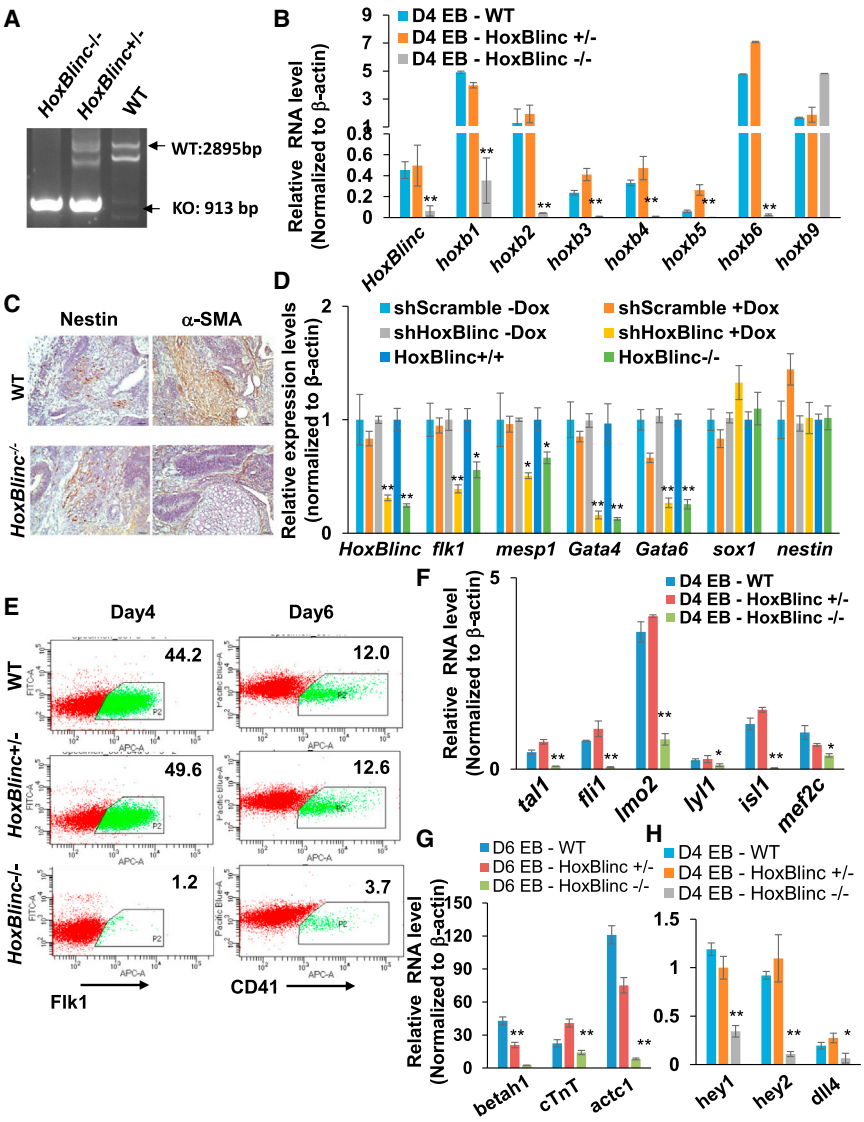

Figure 7. Deletion of the *HoxBlinc* Locus by CRISPR-Cas9 Genome Editing Inhibits Flk1$^+$ Mesoderm Differentiation

(A) PCR-based genotyping analysis of the *HoxBlinc* genomic KO.

(B) qRT-PCR analysis of *HoxBlinc* and *hoxb* gene expression upon CRISPR-Cas9-mediated deletion at day 4 EBs.

(C) Immunohistochemistry staining of ectodermal marker nestin and mesodermal marker α-SMA comparing WT and *HoxBlinc* KO teratomas.

(D) qRT-PCR gene expression analysis of three germ layers in teratomas derived from the control, *HoxBlinc* KD, and CRISPR-Cas9-mediated homozygous *HoxBlinc* KO.

(E) FACS analysis of Flk1$^+$ cells (left) and CD41$^+$ HS/PCs (right) in control and *HoxBlinc* KO EBs.

(F–H) qRT-PCR analysis of the expression levels of TFs (F), markers (G), and Notch-signaling pathway (H) required for hematopoietic/cardiac differentiation upon CRISPR-Cas9-mediated deletion. Data are presented as mean ± SD from three independent experiments; *p < 0.05; **p < 0.01 by Student's t test.

locus in ESCs using the CRISPR-Cas9 genome-editing system (Figure 7A). Targeting of Cas9 to the *HoxBlinc* locus caused a 1,982-bp deletion that encompassed the promoter and the 5′ region of *HoxBlinc* (Figures S7A, S7B, and 7A) and led to a complete elimination of *HoxBlinc* RNA expression (Figures 7B and S7C). Deletion of *HoxBlinc* did not affect pluripotency of ESCs (Figure S1C, bottom). However, *HoxBlinc* loss impaired in vivo mesoderm formation (Figures 7C and S7E) and the expression of mesoderm markers (Figure 7D). However, expression of endoderm markers *gata4* and *gata6*, which are also required for cardiac mesoderm differentiation, was also affected (Figure 7D). Consistent with the shRNA-mediated KD, deletion of the *HoxBlinc* locus severely impaired specification of Flk1$^+$ hemangiogenic mesoderm cells and formation of CD41$^+$ HS/PCs (Figure 7E). Furthermore, the inhibition of mesoderm specification was accompanied by a strong decrease in anterior *hoxb* gene transcription in vitro and in vivo (Figures 7B, S7C, and S7F). As a result, expression of components of the NOTCH pathway, TFs, and markers critical for hematopoietic and cardiac differentiation was inhibited by the deletion of the *HoxBlinc* locus (Figures 7F–7H and S7D). Thus, the CRISPR-Cas9-mediated deletion approach confirmed that *HoxBlinc* RNA regulates mesoderm specification by controlling expression of the anterior *hoxb* genes.

DISCUSSION

Although lincRNAs play important roles in a variety of biological processes, such as innate immunity, heart development, and maintenance of ESC pluripotency (Guttman et al., 2009; Hu et al., 2013; Klattenhoff et al., 2013; Loewer et al., 2010), it remains unclear how lincRNAs counteract ESC self-renewal and promote lineage commitment and differentiation. We demonstrate that *HoxBlinc* RNA specifies hemangiogenic/cardiogenic mesoderm and promotes development of HS/PCs by regulating Wnt/NOTCH signaling and *Hox* pathways. Blocking of these pathways has two distinct and sequential effects. It first inhibits mesoderm differentiation and then blocks mesoderm specification to blood cells (Lengerke et al., 2008). We show that *HoxBlinc* RNA serves as a regulator of the *hoxb* chromatin loop domain, which subsequently attracts the positive epigenetic regulators Setd1a and/or MLL1 complexes to the anterior *hoxb* genes in order to control both Flk1$^+$ cell specification and differentiation of Flk1 mesoderm into hematopoietic lineages. The detailed dissection of the transcriptional and signaling pathways affected by *HoxBlinc* RNA and how these pathways control early Flk1$^+$ mesoderm and lineage

differentiation has important clinical ramifications for understanding hematological/cardiovascular diseases.

Several *HoxBlinc* RNA targets and/or partners have been implicated in the regulation of hematopoietic and cardiac stem cell function. Among them, Notch signaling is a key pathway critical for both hematopoietic and cardiac development (Beneniste et al., 2014; Niessen and Karsan, 2008; Oh et al., 2013). The RNA-seq analysis of *HoxBlinc* KD EBs revealed that Notch signaling is regulated by *HoxBlinc* RNA (Figure 2A). Furthermore, *hoxb* gene rescue experiments demonstrated that *hoxb2–b4* genes at least partially mediate Notch pathway regulation by *HoxBlinc* during mesoderm specification and hematopoietic differentiation (Figure 4G). Thus, consistent with its role in HSC function, Notch signaling may play a critical role in the *HoxBlinc*-mediated specification of mesoderm to hematopoietic lineages.

The anterior *HoxB* genes are critical for the specification of early hematopoietic cell fates and for HSC function (Lawrence et al., 1996). Both Setd1a and MLL1 have been implicated in early hematopoiesis through modulating *Hox* gene transcription (Deng et al., 2013; Ernst et al., 2004; Liu et al., 2011). In particular, Setd1a regulates *hoxb4* transcription by controlling promoter H3K4me3 during hematopoietic differentiation (Deng et al., 2013). Interestingly, a genetic screen in zebrafish identified Set1 as an important epigenetic regulator of hematopoiesis (Huang et al., 2013). The role of Set1/MLL complexes in mesoderm specification toward the blood lineage is consistent with the role of *HoxBlinc* in specifying hematopoietic cell fates by controlling *hoxb* genes. This notion is supported by the observation that ectopic *hoxb2–b4* gene expression rescued the defects in the hematopoietic transcription program and in hematopoietic differentiation observed in the *HoxBlinc* KD EBs (Figure 4). Thus, anterior *hoxb* genes are the key transcription targets and mediators of *HoxBlinc* RNA action during early hematopoietic cell fate decisions.

Several cardiovascular differentiation pathways are impaired by loss of *HoxBlinc* (Figure 1G). Both hematopoietic and cardiovascular lineages originate from mesoderm-derived Flk1$^+$ multipotent progenitors (Ema et al., 2003; Kattman et al., 2006), formation of which requires *HoxBlinc* and its cofactors Set1/MLL (Figures 1, 5, and S5). It was recently reported that a lateral mesoderm-specific lncRNA *Fendrr* also recruits TrxG complexes to specify the fate of lateral mesoderm derivatives for heart development in mice (Grote et al., 2013), indicating the importance of TrxG complexes in specification of Flk1$^+$ mesoderm. In addition, dynamic expression of *hoxb* genes also plays an important role in heart development (Nolte et al., 2013). Exogenous expression of the *hoxb2–b4* genes rescues only about 39.45% of the Flk1$^+$ mesoderm population, but not the cardiac lineage, suggesting that *HoxBlinc* primarily regulates hemangiogenic/cardiogenic mesoderm specification, but not cardiac differentiation, by coordinating anterior *hoxb* gene expression.

Chromatin looping plays a central role in regulating long-range transcription activation, chromatin insulation, and imprinted gene expression (Handoko et al., 2011; Li et al., 2012; Sanyal et al., 2012; Wallace and Felsenfeld, 2007). Although active histone modifications such as H3 acetylation and H3K4 methylations are implicated in the communication between genes and distal *cis*-regulatory elements (Andreu-Vieyra et al., 2010; Patel et al., 2014), it was suggested that sequence-specific TFs initiate contacts between regulatory elements (Deng et al., 2012; Song et al., 2007). Our data revealed that *HoxBlinc* RNA also directs chromatin loop formation (Figure 6H). *HoxBlinc*-mediated chromatin loops may have several important functions in maintaining locus-specific H3K4me3 and *Hox* gene activation. First, the looping could aid in distributing *HoxBlinc* RNA and epigenetic regulators to anterior *hoxb* genes. Second, looping between two *ctcf* sites that flank *hoxb1* and *hoxb4* genes establishes a differentiation-specific active chromatin domain and separates the active anterior and silent posterior chromatin domains. Finally, looping brings the *HoxBlinc* locus in close proximity with anterior *hoxb* genes for transcription activation by controlling local chromatin structure (Figure 6). Obviously, *Hox* clusters are compound genomic loci that contain many regulatory DNA elements. Regulation of the *Hox* gene loci involves complicated processes, which may include formation of various chromatin loops during different development stages. Although our study did not rule out the possibility that other regulatory elements in the locus may also be required for anterior *hoxb* activation, we demonstrated that the interactions between the *HoxBlinc* locus and the anterior *hoxb* promoters are mediated by *HoxBlinc* RNA. Thus, our data reveal important and general mechanisms by which *HoxBlinc* RNA modulates local chromatin structure to control lineage specification and differentiation during development.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are provided in the Supplemental Experimental Procedures. All experiments were performed according to the guidelines of the institutional animal care and use committee of The University of Florida.

Generation of Inducible KD and CRISPR-Cas9 KO Cell Lines, In Vitro Differentiation, and Teratoma Assays

Murine ESC lines, R1/E (ATCC SCRC-1036), and CGR8 MHC-GFP were maintained as previously described (Deng et al., 2013). The differentiation of hematopoietic/cardiac cells, BL-CFC, and colony-forming cell (CFC) assays of ESCs were performed as previously described (Choi et al., 1998; Kennedy et al., 1997; Lancrin et al., 2009; Shen and Qu, 2008; Sroczynska et al., 2009) with minor modifications. The teratoma assay was performed as previously described (Nelakanti et al., 2015). For FACS analysis, single-cell suspensions obtained from differentiated EBs were subjected to FACS analysis using antibodies against FLK-1 APC or CD117 PE (c-Kit) and CD41 FITC (BD Biosciences). Data analysis was performed using a BD LSRII system (BD Biosciences).

Northern Blot and qRT-PCR

Total RNA was prepared by using the RNeasy mini isolation kit according to the manufacturer's instruction (QIAGEN). Two micrograms of RNA was reverse transcribed using ProtoScript II Reverse Transcriptase (New England Biolabs). cDNA was analyzed by real-time PCR (qRT-PCR) using the SYBR Green PCR master mix and the Bio-Rad CFX96 system (Bio-Rad). Primer and probe sequences are listed in the Supplemental Information (Table S3).

RNA Sequencing and Data Analysis

Paired-end RNA-seq was performed by Otogenetics according to standard protocols. The sequence reads have been deposited in the NCBI Short Read Archive (GSE74894). Data analysis was provided in the Supplemental Experimental Procedures.

Generation of Biotinylated RNA, Pull-Down Assay, and RIP

Biotinylated *HoxBlinc* and control RNAs were generated using the AmpliScribe T7-Flash Transcription Kit (Epicenter) according to the manufacturer's instructions. Pull-down assay and RIP were performed as described previously (Wang et al., 2011).

BoxB-Tethering Reporter Assay

2×10^5 K562 cells were seeded into 24-well plates 12 hr prior to transfections. Cells were transfected with plasmids encoding a luciferase gene under the control of five tandem GAL4 UAS sites, GAL4-λN (the 22-amino-acid RNA-binding domain of the lambda bacteriophage anti-terminator protein N), *BoxB* (containing five repeats of the λN-specific 19-nucleotide-binding site), or *BoxB* fused to full-length *HoxBlinc* or siRNAs specifically targeting ASH2L or LSD1. Transfected cells were collected and analyzed by luciferase reporter gene activities 48 hr after transfection using a luciferase assay kit (Promega).

ChIP, ChIRP, and 3C Assays

ChIP assays were performed as described previously (Li et al., 2011). The ChIRP assay was performed according to a protocol described in a previous study (Chu et al., 2011) with some modifications. Antibodies, probes, and primers are listed in the Supplemental Information.

3C assay was performed as previously described (Andreu-Vieyra et al., 2010; Patel et al., 2014) with minor modifications. In brief, chromatin from 2×10^6 cross-linked cells were digested with 800 U of EcoRI overnight and subjected to ligation in a 6-ml volume at 16°C for 3 days followed by 60 min at room temperature. The 3C-ligated DNA was purified and amplified using PCR, and the products were cloned into pGEM T easy vector (Promega) for sequencing. Primers are listed in the Supplemental Information.

ACCESSION NUMBERS

The accession number for the sequence reads reported in this paper is NCBI SRA: GSE74894.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, three tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.007.

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