RING1 proteins contribute to early proximal-distal specification of the forelimb bud by restricting Meis2 expression

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ABSTRACT
Polycomb group (PcG) proteins play a pivotal role in silencing developmental genes and help to maintain various stem and precursor cells and regulate their differentiation. PcG factors also regulate dynamic and complex regional specification, particularly in mammals, but this activity is mechanistically not well understood. In this study, we focused on proximal-distal (PD) patterning of the mouse forelimb bud to elucidate how PcG factors contribute to a regional specification process that depends on developmental signals. Depletion of the RING1 proteins RING1A (RING1) and RING1B (RNFK2), which are essential components of Polycomb repressive complex 1 (PRC1), led to severe defects in forelimb formation along the PD axis. We show that preferential defects in early distal specification in Ring1A/B-deficient forelimb buds accompany failures in the repression of proximal signal circuitry bound by RING1B, including Meis1/2, and the activation of distal signal circuitry in the prospective distal region. Additional deletion of Meis2 induced partial restoration of the distal gene expression and limb formation seen in the Ring1A/B-deficient mice, suggesting a crucial role for RING1-dependent repression of Meis2 and likely also Meis1 for distal specification. We suggest that the RING1-MEIS1/2 axis is regulated by early PD signals and contributes to the initiation or maintenance of the distal signal circuitry.

KEY WORDS: Meis, Mouse limb development, Polycomb, Proximal-distal specification, RING1

INTRODUCTION
Polycomb group (PcG) genes were originally identified as encoding regulators of Hox genes in Drosophila and are evolutionarily conserved among metazoa (reviewed by Lanzuolo and Orlando, 2012; Simon and Kingston, 2009, 2013). PcG proteins form at least two multicellular complexes: Polycomb repressive complex 1 (PRC1) and PRC2. PRC1 and PRC2 cooperate to mediate transcriptional repression of target genes via histone modifications and chromatin compaction. PRC2 contains the EZH1 or EZH2 catalytic subunits that mediate histone H3 lysine 27 trimethylation (H3K27me3), while PRC1 utilizes its RING1A (RING1) and RING1B (RNFK2) subunits to catalyze histone H2A monoubiquitylation at lysine 119 (H2AK119ub1). PRC1 also targets chromatin compaction by SAM domain polymerization of PHC1 and PHC2, a mammalian PH ortholog, via H3K27me3 recognition by chromodomain proteins such as CBX7 and CBX2 (Cao et al., 2002; Endoh et al., 2012; Isono et al., 2013; Kuzmichev et al., 2002). Recent studies identified a variant PRC1 complex containing RYBP (Ring1 and YY1 binding protein)/YAF (YY1-associated factor), RING1A/B and a distinct PCGF subunit (Gao et al., 2012; Tavares et al., 2012), and demonstrated that H2AK119ub1 mediated by this variant PRC1 leads to the recruitment of PRC2 and placement of H3K27me3 to initiate Polycomb repression (Blackledge et al., 2014). PcG factors are therefore expected to use diverse mechanisms for both binding and silencing of their target genes.

Studies of PcG-deficient embryonic stem cells (ESCs) showed that PcG factors contribute to the maintenance of the ESC undifferentiated state by repressing the expression of developmental genes (Boyer et al., 2006; Endoh et al., 2008; Pasini et al., 2007). Similar PcG functions have been reported in pluripotent hematopoietic stem cells, which give rise to all the blood cell lineages (Caless et al., 2008; Oguro et al., 2010). These observations support the prevailing view that PcG factors maintain epigenetic memory of gene silencing, even following cell division. On the other hand, recent studies have linked enhancer activity with the regulation of PcG-mediated gene repression. For example, a distal enhancer was shown to interfere with binding of PcG factors at the human α-globin Cpg island promoter during gene activation (Lynch et al., 2012). Similarly, activation of the PcG-bound Meis2 promoter during mouse midbrain development was accompanied by association with an active enhancer, leading to eviction of PcG factors from the promoter region (Kondo et al., 2014). These observations suggest that PcG factors are not simply a memory module for gene silencing but also play important roles in regulating gene expression by regulating promoter/ enhancer interactions in response to developmental signals. However, how PcG factors contribute to morphogenetic processes, particularly during mammalian development, is poorly understood, mainly because of the complexity and dynamic nature of these events.

Limb patterning is a dynamic and complex morphogenetic process during which proliferation, regionalization and differentiation take place in a highly coordinated manner as a result of the spatiotemporally regulated expression of diverse morphogenetic signaling molecules and transcription factors (reviewed by Tabin and Wolpert, 2007; Towers and Tickle, 2009a,b; Zeller et al., 2009). Proximal-distal (PD) patterning of limb mesenchyme is coupled with limb bud outgrowth and depends on diffusible signals that define the prospective proximal and distal domains. Previous studies have suggested that the opposing activities of retinoic acid (RA) emanating from the flanking trunk mesenchyme and fibroblast growth factors (FGFs) derived from the apical ectodermal ridge (AER) contribute to
early PD limb bud specification (Cooper et al., 2011; Mariani et al., 2008; Mercader et al., 2000; Rosello-Diez et al., 2011). However, this model is still controversial, mainly because the contribution of endogenous RA has yet to be formally proven (Cunningham et al., 2011, 2013). Upon elongation of forelimb buds, interplay between the AER and underlying mesenchymal cells mediated by diffusible signals, which is interfered with by excess RA signaling, is proposed to be activated and/or stabilized to mediate development of the distal domain.

Recent studies revealed the participation of PcG factors in the PD patterning process. Limb-specific depletion of EZH2 in mice leads to skeletal defects along the PD and anterior-posterior (AP) axes (Wyngaarden et al., 2011). In zebrafish, Ring1B-mediated gene regulation has been shown to help sustain FGF signaling during pectoral fin development (van der Velden et al., 2012). However, this differential outcome is thought to be due to lower Cre activity in the hindlimb bud in Prx1-Cre deleter mice (Logan et al., 2002), we designed this study to focus on the role of RING1 proteins during forelimb development.

To test the effects of complete RING1 protein removal and Ring1A/B gene dosage on limb skeletal patterning, we generated Prx1-Cre;Ring1A+/−, Ring1B+/− (Ring1A+/−;Ring1B+/−;Ring1A-BKO) in addition to the Ring1A/B-dKO mice. Indeed, forelimb defects observed in the Ring1B-KO were exaggerated by additional deletion of Ring1A in a gene dosage-dependent manner (Fig. 1AB, Table 1). In Ring1A+/−; Ring1B-KO, there was a more severe shortening of the forelimbs, accompanied by various morphological alterations that were not seen in the Ring1B-KO, such as poor articulation of each skeletal element, decreased digit number, fusion of radius and ulna, shortening of the presumptive stylopod, and hypoplastic scapula. Ring1A/B-dKO fetuses possessed only rudimentary forelimbs and hindlimbs. We found only two cartilaginous elements, the origins of which could not be identified, in mesomelic forelimbs of the Ring1A/B-dKO. These results indicate that PRC1 plays a crucial role in limb skeletal patterning along the PD and AP axes, in agreement with an important role for PRC2 in this process (Wyngaarden et al., 2011). In the remainder of this study, we focused on how RING1 proteins contribute to PD forelimb patterning.

RING1 activity is required for distal specification of the forelimb bud

Previous studies indicated that early PD specification of forelimb buds occurs by E10.5, a conclusion based on differential expression of genes such as Meis1/2 and Hoxa13 between proximal and distal domains (Mariani et al., 2008; Mercader et al., 2009; Yashiro et al., 2004; Zeller et al., 2009). We investigated morphological and molecular properties of the Ring1A/B-dKO forelimb buds at E10.5. By visual inspection, their outgrowth was indistinguishable in Ring1A/B-dKO and Ring1A+/− (Ring1A−dKO) control embryos until E10.5, but then became progressively more affected at later stages. Consistent with normal forelimb bud morphology, we did not observe significant differences in the frequency of apoptotic or proliferative cells in Ring1A/B-dKO forelimb buds at E10.5 compared with the control (Fig. S2A).

We went on to investigate the expression of genes that demarcate proximal and distal regions of forelimb buds at E10.5 by microarray analysis (Fig. 1C). We first selected genes exhibiting at least 2-fold biased expression levels between proximal and distal domains (hereafter termed proximal and distal genes, respectively) in the control (Ring1A+/−;Ring1B+/−; designated RING1B+/−; in Fig. 1D) forelimb buds. We found that average expression levels of 2104 proximal and 2667 distal genes were significantly higher in proximal and distal regions, respectively (RING1B+, Fig. 1D). Next, we tested the mean expression level of these genes in the Ring1A/B-dKO (designated RING1B− in Fig. 1D). Interestingly, we found these differences in gene expression levels were

RESULTS
RING1 loss affects forelimb skeletal patterning along the PD and AP axes

We first tested RING1B depletion efficiency by IF analysis and observed its depletion in a majority of mesenchymal cells but not in ectodermal cells, including the AER, in Prx1-Cre;Ring1A−/−; Ring1B+/− (Ring1A/B-dKO) forelimb buds at E10.5 (Fig. S1D). We then investigated Prx1-Cre;Ring1B+/− (Ring1B-KO) mice because Ring1B-deficient limb phenotypes have not been reported. Visual inspection of limb development at E17.5 revealed considerable shortening of the forelimbs, whereas the hindlimbs were less severely affected (Fig. 1A). Skeletal preparation showed that RING1B loss significantly affected the elongation of radius and ulna and to a lesser extentibia and fibula (Fig. 1B, Table 1). Since this differential outcome is thought to be due to lower Cre activity in the hindlimb bud in Prx1-Cre deleter mice (Logan et al., 2002), we designed this study to focus on the role of RING1 proteins during forelimb development.

We went on to investigate the expression of genes that demarcate proximal and distal regions of forelimb buds at E10.5 by microarray analysis (Fig. 1C). We first selected genes exhibiting at least 2-fold biased expression levels between proximal and distal domains (hereafter termed proximal and distal genes, respectively) in the control (Ring1A+/−;Ring1B+/−; designated RING1B+/−; in Fig. 1D) forelimb buds. We found that average expression levels of 2104 proximal and 2667 distal genes were significantly higher in proximal and distal regions, respectively (RING1B+, Fig. 1D). Next, we tested the mean expression level of these genes in the Ring1A/B-dKO (designated RING1B− in Fig. 1D). Interestingly, we found these differences in gene expression levels were
significantly blunted in both proximal and distal regions of the forelimb buds (RING1B−, Fig. 1D). RING1 depletion thus induces defects in the early specification of both prospective proximal and distal domains. However, gene expression differences were more obviously blunted in the distal region ($P=1.85 \times 10^{-9}$) than in the proximal region ($P=1.44 \times 10^{-83}$). This difference suggests that gene regulation in the distal region is more dependent on RING1 activity than in the proximal region.

To further examine this tendency in genes known to be involved in limb development and/or limb morphogenesis, we...
selected 112 genes based on Gene Ontology (GO) and analyzed their expression (Fig. S2B). These limb-related genes included 31 distal and 13 proximal genes (Fig. S2B). As revealed by clustering analysis, limb-related distal genes tended to be variably affected from no major changes (3/15) to moderate shortening of both zeugopod and stylopod and a slight change in the proximal or distal region (Fig. S3A), it might affect the gene expression in the AER was considerably downregulated in the distal region of Ring1A/B-dKO forelimb buds (Fig. S2C). Consistent with this trend, the gene expression profile in the distal region of the Ring1A/B-dKO was more closely related to the proximal region than the distal region of the control based on the correlation coefficient (Fig. S2C,D). These results suggest that acquisition of distal identity in the forelimb bud is more dependent than proximal identity on RING1 activity.

We validated these results by ISH analysis (Fig. 1E, Fig. S2E), first testing Fgf8, Cyp26b1, Shh and Greml1, which exhibit distally restricted expression and are known to contribute to distal development. Consistent with the microarray data, we found that the expression of these genes was considerably reduced in Ring1A/B-dKO forelimb buds. By contrast, expression of Pitx2 was distally expanded (Fig. 1E). Expression of Rabr (Niederreiter et al., 2002) also showed a major difference, although not by ISH but by RT-qPCR analysis of the distal forelimb bud (Fig. S2E). We also observed a considerable downregulation of Hoxa11 but only a slight change in Hoxa11 in the Ring1A/B-dKO (Fig. 1E, Fig. S2E), even though Hox genes are known as the classical PcG targets. We further examined Meis2/1, which are known proximal marker genes (Merkader et al., 1999, 2000) but are not categorized as limb-related genes by GO, and found that their expression was distally expanded in the Ring1A/B-dKO forelimb buds (Fig. 1E). RT-qPCR analysis confirmed activation of Meis1/2 expression in the distal region of these embryos, reaching levels observed in the control proximal region (Fig. 1E). We therefore suggest a role of RING1 proteins in establishing a coordinated gene expression pattern that demarcates the distal region of the forelimb bud by E10.5. Importantly, Fgf8 expression in the AER was considerably downregulated irrespective of mesenchyme-specific deletion of Ring1B (Fig. S1D). Together with downregulation of Cyp26b1, Shh and Greml1 in the Ring1A/B-dKO, RING1 proteins in the mesenchyme are suggested to contribute to establishing or maintaining signaling circuitry that mediates interactions between the AER and underlying mesenchyme and the subsequent specification of the prospective distal domain of forelimb buds.

**RING1 activity is correlated with regionalizing signals of forelimb buds**

To explore how RING1 activity contributes to early distal specification of forelimb buds, we next addressed whether its activity is linked to early PD specification signals. Since signaling circuitry involving the AER and underlying mesenchyme has been reported to be inhibited by high levels of RA, which is thought to mimic an exaggerated version of proximal signals (Probst et al., 2011), we investigated whether RING1 activity could be influenced by excess RA signaling. We thus examined whether transient increases in RA signaling at E10.5 could modify the limb defects in Ring1B mutant mice.

All-trans RA (50 mg/kg body weight) was orally administered once to pregnant Ring1B+/ females that had been mated with Prx1-Cre;Ring1B+/− males. A single administration of RA at E10.5 

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**Table 1. Summary of forelimb skeletal deformities observed in Ring1A/B mutant and/or excess RA-treated mice**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>RA treatment</th>
<th>Scapula</th>
<th>Stylopod</th>
<th>Zeugopod</th>
<th>Digit number</th>
<th>Severity</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring1B+/− or Ring1B−−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1B+/−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1B−−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>6/5 (40)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1B+/−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>6/5 (40)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1A+/−:Ring1B+/−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1A+/−:Ring1B+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>6/3 (37.5)</td>
</tr>
<tr>
<td>Prx1-Cre;Cyp26b1−−:Ring1B+/−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>6/3 (37.5)</td>
</tr>
<tr>
<td>Prx1-Cre;Ring1A+/−:Ring1B+/−,Meis2+/−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>6/6 (100)</td>
</tr>
</tbody>
</table>

The presence of morphological abnormalities in scapula, stylopod and zeugopod and changes in digit number are indicated by +, and their absence by −. After observation of skeletal preparations, we scored the severity of the forelimb phenotypes from 0 to 5, according to the number of + scores, except for the Prx1-Cre; Ring1A+/−:Ring1B+/− and Prx1-Cre;Ring1A+/−:Ring1B+/−,Meis2+/− mice. Because of extensive alterations in Prx1-Cre;Ring1A+/−:Ring1B+/− mice, we could not accurately determine the origin of each skeletal element and thus scored the phenotypes as 5. Furthermore, this phenotype was partially restored in Prx1-Cre; Ring1A+/−:Ring1B+/−,Meis2+/− mice and we thus scored it as 5−.
Regulation of Meis1/2 by RING1 and PD specification signals

We next aimed to identify a group of genes concurrently regulated by RING1 and early PD signals. Such genes should be bound and repressed by RING1B but be induced by excess RA in the distal region of the forelimb bud. We used ChIP assays combined with microarray technology (ChIP-on-chip analysis) to identify genes bound by RING1B and marked by H3K27me3 in E10.5 forelimb buds. We identified 2085 genes bound by RING1B, and these are a subset of 5010 H3K27me3-positive genes (Fig. S3B, Table S1).

RING1B targets in forelimb buds significantly overlapped with those in ESCs (Endoh et al., 2008). RING1B-bound genes were significantly derepressed in the distal region of forelimb buds in Ring1A/B-dKO at E10.5 (Fig. 3A,B).

We next identified genes activated by excess RA in distal forelimb buds. We treated wild-type embryos at E10.25 with a single oral administration of all-trans RA (100 mg/kg) to pregnant females and performed transcriptome analysis on the distal region of the forelimb buds at E10.5. We found that proximal genes were significantly upregulated by excess RA (Fig. 3A,B). We identified 70 genes that are bound by RING1B, activated by RING1 loss or excess RA, and are normally repressed in the distal region, 27 of which encode transcription factors (Fig. 3C).

To investigate whether RING1B preferentially binds to these genes in the distal region of the forelimb buds, we performed ChIP-seq analysis using E11.5 proximal and distal regions. In the distal region, we found the most biased RING1B binding around transcription start sites (TSSs) of the Meis1 gene, with Meis2 as a second hit (Fig. S3C,D). At both Meis1 and Meis2, we observed intense RING1B binding around the TSS and the 3′ region, which we designated as a RING1B binding site (RBS) in our previous study (Kondo et al., 2014), and more RING1B accumulation in the distal than in the proximal region (Fig. 3D, Fig. S3E). We further confirmed skewed RING1B binding in the distal domain at the TSS and RBS by ChIP-qPCR analysis (Fig. 3E, Fig. S3F). Consistent with the RING1B binding, we observed enrichment of H3K27me3 at both TSS and RBS of Meis1/2 (Fig. 3E, Fig. S3F) and close TSS/RBS contact at Meis2 in the distal region (Fig. 3G).

We then investigated whether H3K27me3 accumulation at the TSS and the TSS/RBS association at Meis2 depend on RING1 activity and found significant reduction of H3K27me3 and dissociation of TSS and RBS in the distal region of Ring1A/B-dKO forelimb buds (Fig. 3F-H). By contrast, no obvious change was seen for H3K4me3 accumulation at these genomic regions in the distal region of the Ring1A/B-dKO (Fig. 3F).

Together with the expression of Meis1 and Meis2 in the distal region of Ring1A/B-dKO forelimb buds at E10.5 (Fig. 1E), these observations indicate that RING1 proteins directly contribute to the repression of both genes in the distal region of the forelimb bud. Notably, both Meis1 and Meis2 were shown to be activated by excess RA in the distal region of forelimb buds (Mercader et al., 2000; Yashiro et al., 2004) (N.Y.-K., unpublished data), and overexpression of Meis1 or Meis2 has been reported to impair the expression of distal genes and distal limb development (Capdevila et al., 1999; Mercader et al., 1999, 2009). We therefore hypothesized that RING1-mediated repression of Meis1 and/or Meis2 could be an important step in the early specification of the distal region of the forelimb bud.

RING1-dependent repression of Meis2 is involved in early distal specification of forelimb buds

To test the above hypothesis, we examined whether additional mutation of Meis2 could restore the forelimb defects resulting from RING1 deficiency. Since a constitutive mutant allele of Meis2 exhibits haploinsufficiency and the mice do not survive the perinatal period (T.K., unpublished data), we generated a conditional allele (Meis2fl/fl) (Fig. S4). We first examined the forelimb skeletal phenotypes of Prx1-Cre;Meis2fl/fl (Meis2-KO) fetuses at E17.5 and found no major changes, as reported for Meis1-deficient mice (Fig. 4A) (Azcoitia et al., 2005; Hisa et al., 2004).

We then generated Prx1-Cre;Ring1A−/−;Ring1Bfl/fl;Meis2fl/fl (Ring1A/B;Meis2-tKO) mice and observed considerable
Fig. 3. RING1 activity and PD specification signals converge at Meis genes. (A) Venn diagram representation for the overlap among proximal genes that are normally repressed by RING1B but can be activated by RA signals in the distal region of the forelimb bud. We investigated genes that are bound by RING1B in the distal region (RING1B target), derepressed in the distal region of Ring1A/B dKO, activated by excess RA (+RA WT) and preferentially expressed in the proximal region (proximal gene) at E10.5 and tested their overlap. The number of genes in each category is indicated. (B) A numerical representation for the correlations between respective gene groups. Odds ratios for overlaps of respective gene groups are shown in the red panels and P-values are shown in the blue panels. (C) Heat map representation for expression changes of 27 transcription factor genes included among 70 proximal genes that are bound by RING1B, derepressed in Ring1A/B-dKO and activated by excess RA signals. The left column shows expression differences between proximal and distal regions in the control (Ring1A-KO) forelimb buds. The middle column shows the expression changes in the distal region of Ring1A/B-dKO. The right column shows the expression changes caused by excess RA in the distal region of wild-type forelimb buds. (D) RING1B binding at the Meis2 locus in E11.5 proximal and distal forelimb buds revealed by ChIP-seq analysis. Right-hand panels show the binding around the TSS. Note the intense RING1B enrichment around the TSS. (E) Intense accumulation of RING1B and H3K27me3 around the TSS and RBS of the Meis2 locus in the distal region of forelimb buds at E10.5 as revealed by ChIP-qPCR analysis. Binding of RING1B and H3K27me3 at sites P-I and P-II, which are both located around the TSS, intronic region and RBS were compared between proximal and distal regions of E10.5 forelimb buds. Error bars indicate the s.e.m. of three biological replicates. (F) RING1-dependent promoter/RBS association at Meis2 in the distal region of E10.5 forelimb buds as revealed by DNA-FISH analysis. The subnuclear localization of promoter (green) and RBS (red) regions was examined in the proximal and distal regions of control (Ring1A-KO) and Ring1A/B-dKO forelimb buds at E10.5. Scale bar: 1 µm. (H) Box plot representation for the DNA-FISH analysis. The box plots show the interprobe distances (µm) in each tissue. Over 200 loci were observed in this experiment. **P<10−300, *P=2.1×10−15, Student’s t-test.
RING1-dependent repression of including development by repressing the expression of proximal genes, RING1 activity preferentially contributes to distal forelimb outgrowing forelimb bud. Partial phenotypic correction of PD specification. During distal specification, antagonistic features functionally correlated with signaling pathways that contribute to important process for proper distal specification (Fig. 4C). RING1-dependent repression of Meis genes is an important process for proper distal specification and/or maintenance of the SHH-GREM1-AER-FGF loop in the prospective distal region. The RING1 function is probably repressed or activated by a proximal or distal signal, respectively. Scale bars: 1 mm in A; 100 µm in B.

DISCUSSION

In this study, we reveal a role for RING1 proteins in regulating the early PD regionalization of forelimb buds that occurs at ∼E10.5. RING1 activity preferentially contributes to distal forelimb development by repressing the expression of proximal genes, including Meis1/2, in the prospective distal domain of the outgrowing forelimb bud. Partial phenotypic correction of Ring1A/B-deficiency by additional deletion of Meis2 suggests that RING1-dependent repression of Meis2, and likely also Meis1, is an important process for proper distal specification (Fig. 4C).

This study further suggests that the RING1-MEIS1/2 axis is functionally correlated with signaling pathways that contribute to PD specification. During distal specification, antagonistic features of early proximal and distal signals emanating from trunk mesenchyme and AER, respectively, are reported to help establish the SHH-GREM1-AER-FGF loop in the prospective distal region of elongating forelimb buds that subsequently facilitates distal development (Probst et al., 2011). Our study shows that RING1-dependent repression of Meis genes is an important process for proper distal specification and/or maintenance of the SHH-GREM1-AER-FGF loop in the prospective distal region. The RING1 function is probably repressed or activated by a proximal or distal signal, respectively. Scale bars: 1 mm in A; 100 µm in B.
unpublished data). Importantly, this finding further suggests the involvement of other classes of RING1 targets that are likely to act at an earlier stage than Meis2 repression. These possibilities require experimental elucidation in the future.

Our observations imply that robust and stable repression of target genes is not the sole activity of PcG factors; they also mediate inducible repression under the regulation of morphogenetic signals. Notably, PcG factors have been reported to be linked with RA, FGFs, SHH and MAP kinase pathways (Schwermann et al., 2009; Shi et al., 2014; van der Velden et al., 2012; Wu et al., 2013; Yokobayashi et al., 2013). By such interactions with signaling pathways, PcG factors may contribute to the compartmentalization of outgrowing or enlarging fetal tissues by regulating the expression of developmental genes. In addition, the skeletal phenotypes of the Ring1A/B-dKO forelimb may not simply be due to the dysregulation of PD patterning but might also involve defects in AP patterning, skeletogenesis and/or chondrogenesis as reported previously (Schwarz et al., 2014; van der Velden et al., 2013; Wyngaarden et al., 2011). Ring1 proteins could help to coordinate and integrate various morphogenetic signals to accomplish forelimb patterning. However, the underlying mechanisms by which PcG factors are linked to signaling pathways remain to be elucidated. Identification in this study of Meis1/2 as common targets of Ring1 and PD specification signals provides a good entry point for further study to elucidate the molecular mechanisms underlying the signal-dependent regulation of PcG activity.

This and previous studies suggest a role for PcG factors during induced repression and activation of Meis genes in the forelimb bud and midbrain, respectively (Kondo et al., 2014). Ring1-associated PcG factors thus play a role in both the stable repression and inducible expression of their target genes. Consistent with the functional diversity of PcG factors during development, recent biochemical studies suggest that Ring1 proteins are components of several different PRC1-related complexes that can be discriminated by their biochemical properties, as represented by PCGF factors that interact with Ring1B (Blackledge et al., 2014; Gao et al., 2012). We thus suspect that the use of different PRC1-related complexes could at least partly account for the functional diversity of PcG factors during development. Indeed, our preliminary analysis of Pcgf3 knockout fetuses revealed zeugopod shortening similar to that of the Ring1B-KO, whereas Pcgf2 (Meli18) or Pcgf4 (Bmi1) mutants, or even their compound mutants, did not have any zeugopod defects (N.Y.-K. and H.K., unpublished data) (Akasaka et al., 2001). We thus suspect that different PRC1-related complexes play different roles at each phase of the developmental regulation of Meis1/2 expression. This model and its applicability to other target genes are under investigation.

**MATERIALS AND METHODS**

**Animals**

Prx1-Cre, Ring1A−/−, Ring1B−/− and Cyp26b1−/− mouse lines were described previously (Cales et al., 2008; del Mar Lorente et al., 2000; Logan et al., 2002; Yashiro et al., 2004). Conditional deletion of Ring1B was achieved by crossing Prx1-Cre;Ring1B−/− males with Ring1B−/− females. For the production of Ring1A-B-dKO embryos, Prx1-Cre;Ring1A−/−; Ring1B−/− males were mated with Ring1A−/−;Ring1B−/− females and Ring1A−/−;Ring1B−/− or Ring1A−/−;Ring1B−/− embryos were used as controls. The Meis2−/− mouse is described in Fig. S4. All animal experiments were carried out according to institutional guidelines for the care and use of laboratory animals of the RIKEN, Yokohama Institute, Japan.

**Skeletal preparation and whole-mount in situ hybridization (ISH)**

Skeletons of fetal and newborn mice were stained with Alizarin Red and Alcian Blue as described previously (Parr and McMahon, 1995). Whole-mount ISH was performed according to Wilkinson and Nieto (1993). The number of biological replicates is shown in figures.

**Immunostaining and apoptosis detection**

E10.5 embryos were subject to immunostaining and apoptosis detection using the ApopTag In Situ Detection Kit (Chemicon) as described in the supplementary Materials and Methods.

**Retinoic acid (RA) treatment and sampling**

RA treatment was performed as previously described (Yashiro et al., 2004). Fetuses obtained by crossing Ring1B−/− females with Prx1-Cre;Ring1B−/− males received all-trans RA at a dose of 50 mg/kg body weight at E10.5 by maternal oral administration, and they were sampled at E17.5. Control pregnant females were administered only sesame oil.

**Chromatin immunoprecipitation (ChIP) analysis**

Forelimb buds at E10.5 and E11.5 from wild-type embryos, Ring1A−/− or Prx1-Cre;Ring1A−/−;Ring1B−/− were used for ChIP-on-chip, ChIP-seq and ChIP-qPCR. Forelimb buds were fixed in PBS for 20 min at room temperature, washed three times with cold PBS containing protease inhibitors and stored at −80°C before use. ChIP was performed as described previously (Endoh et al., 2012). For each immunoprecipitation, 100 μl anti-RING1B (Atsuta et al., 2001), 4 μg anti-RING1B (39663, Active Motif), 3 μg anti-H3K27me3 (07-449, Millipore) and 3 μg anti-H3K4me3 (07-473, Millipore) were used.

**ChIP-on-chip and ChIP-seq**

Purified immunoprecipitated and whole-cell extract (input) DNAs were amplified using the double-round T7 RNA polymerase-based amplification method (van Bakel et al., 2008). Labeled DNA samples were hybridized to the Mouse Promoter ChIP-on-chip 244 K Microarray (G4955A#14716, 14717, Agilent Technologies), washed and scanned according to the mammalian ChIP-on-chip protocol (Agilent Technologies). ChIP-seq was performed as described previously (Kondo et al., 2014).

**Microarray**

Forelimb buds at E10.5 from each embryo were dissected into proximal and distal portions. Total RNA was extracted using the RNeasy Micro Kit (Qiagen) following the manufacturer’s protocol. cDNA was synthesized using the GeneChip 3′ IVT Express Kit (Affymetrix) or Ovation RNA Amplification System V2 (Nugen) according to the manufacturers’ protocols. Labeled samples were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) to assess and compare the overall gene expression profiles.

**Data analysis**

Fold enrichment of ChIP-chip signals between input and immunoprecipitated DNA was calculated using an in-house program on probes that had a significantly different signal level from background (P<10−7). Whole intensities were normalized using rank consistency filtering and binding regions were determined as previously reported (Endoh et al., 2012). Sequences retrieved in ChIP-seq experiments were aligned on the mouse genome (mm9) using the Bowtie 2 program (Johns Hopkins University) and accumulated reads were normalized using total mapped reads and counted using our in-house program. Genes were represented by the mapped normalized counts around the TSS (−4 kb to +4 kb).

Microarray signals were converted into numeric values with statistical calls using the MAS5.0 algorithm provided as R packages. When multiple probes were assigned to a single gene, expression changes between two samples were indicated as the geometric mean of all probes for a gene.

GO analysis was performed using the annotation database in Gene Ontology (http://geneontology.org/). Enrichment of genes having a specific GO term was counted and statistically evaluated using a hypergeometric distribution.
Reverse transcription
RNA extraction and cDNA synthesis were performed using the RNeasy Micro Kit (Qiagen) and SuperScript VILO (Invitrogen), respectively, according to the manufacturers’ protocols.

Quantitative real-time PCR (RT-qPCR)
RT-qPCR was performed on an Mx3005P system (Agilent Technologies) using the Brilliant SYBR Green QPCR Master Mix (Agilent Technologies). RT-qPCR and ChIP-qPCR were carried out with two and three biological replicates, respectively. Primer sequences are listed in Table S2.

DNA-fluorescent in situ hybridization (FISH) analysis
FISH analyses on sections were carried out according to a protocol described previously (Kondo et al., 2014). Mouse embryos were fixed with 4% paraformaldehyde (PFA), embedded in paraffin and sectioned at 5 μm thickness. After deparaffinization, sections were soaked in HistoVT One solution (Nakalai Tesque, 06380-05) in glass vessel, and the glass vessel containing sections was placed in boiling water for 20 min. Immediately following this treatment, sections were washed with distilled water to remove detergents and used for FISH.

Accession numbers
Microarray and ChIP-on-chip data are available from the NCBI Gene Expression Omnibus repository under accession number GSE70077. The ChIP-on-chip data for H3K27me3 and RING1B in ESCs and ChIP-seq data for RING1B are from GSE38650 (ESCs, H3K27me3, RING1B) and GSE48464 (limb buds, RING1B).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
N.Y.-K. and H.K. designed the experiments; N.Y.-K. and T.K. performed the experiments; T.A.E. and O.O. performed sequencing and data analyses; Y.K., K.K. and M.V. generated compound mutants; N.Y.-K., T.A.E. and H.K. wrote the manuscript.

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Supplementary information
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