Ezh2 regulates anteroposterior axis specification and proximodistal axis elongation in the developing limb

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SUMMARY
Specification and determination (commitment) of positional identities precede overt pattern formation during development. In the limb bud, it is clear that the anteroposterior axis is specified at a very early stage and is prepatterned by the mutually antagonistic interaction between Gli3 and Hand2. There is also evidence that the proximodistal axis is specified early and determined progressively. Little is known about upstream regulators of these processes or how epigenetic modifiers influence axis formation. Using conditional mutagenesis at different time points, we show that the histone methyltransferase Ezh2 is an upstream regulator of anteroposterior prepattern at an early stage. Mutants exhibit posteriorised limb bud identity. During later limb bud stages, Ezh2 is essential for cell survival and proximodistal segment elongation. Ezh2 maintains the late phase of Hox gene expression and cell transposition experiments suggest that it regulates the plasticity with which cells respond to instructive positional cues.

KEY WORDS: Ezh2, Hox, Polycomb, Axis formation, Limb bud, Plasticity, Mouse

INTRODUCTION
Pattern formation in many embryonic contexts follows the acquisition of regionally distinct positional identities by cells. Specification (initial instruction) of positional identity precedes determination (commitment), which is then followed by differentiation. The orthogonal axes of the limb bud serve as good model systems for understanding these processes.

In recent years substantial progress has been made in understanding the timing and molecular regulation of limb axis formation. With regard to the anteroposterior limb axis, it is clear that specification occurs early (Hamburger, 1938), prior to the establishment of the zone of polarising activity (Chiang et al., 2001; Ros et al., 1996). Early regulators of the anteroposterior axis include the mutually antagonistic genes Gli3, which marks the anterior limb field, and Hand2, which marks the posterior region (Galli et al., 2010; te Welcher et al., 2002). Recently, it was shown that Hox9 genes are required to initiate Hand2 expression in the forelimb (Xu and Wellik, 2011). Anteroposterior pattern elaboration is subsequently governed by feedback loops between the epithelium and the mesenchyme involving multiple downstream signalling pathways, including that of sonic hedgehog (Shh) (Benazet et al., 2009).

The temporal sequence of proximodistal axis specification is perhaps less certain than that of the anteroposterior axis. Early (Barna et al., 2003; Dudley et al., 2002), intercalary (Mercader et al., 2000) and progressive proximal to distal (Summerbell et al., 1973) frameworks have been proposed. Recent data strongly suggest that diffusible proximal and distal cues specify the positional identity of mesenchymal cells while they are plastic (Cooper et al., 2011; Rosello-Diez et al., 2011). The subsequent steps of determination (Wyngaarden and Hopyan, 2008) and differentiation (Saunders, 1948) of the proximodistal axis occur in a progressive, proximal to distal order. Members of the fibroblast growth factor (Fgf) family of genes and retinoic acid are candidate instructors of proximodistal identity (Mariani et al., 2008; Mercader, 2000; Cooper et al., 2011; Rosello-Diez et al., 2011). A prevailing view is that the appropriate elaboration, rather than specification, of proximodistal skeletal elements depends upon homeobox (Hox) gene co-linear expression (Kmita et al., 2005). In particular, paralogous Hox9/Hox10, Hox11 and Hox13 genes are required for full stylopod (Fromental-Ramain et al., 1996a; Wellik and Capeschi, 2003), zeugopod (Davis et al., 1995; Wellik and Capeschi, 2003) and autopod (Fromental-Ramain et al., 1996b) formation, respectively. Genes that regulate the process of determination, however, remain unidentified.

Modification of histone methylation is one of the best candidate mechanisms that might underlie a switch between cell fate plasticity and determination during development. The modification of small chemical groups on histone tails is an efficiently modifiable mechanism that would be advantageous during embryogenesis (Reik, 2007). It is clear that enactors of histone methylation, Polycomb and Trithorax group proteins, are key modifiers of cell identity. Drosophila Polycomb mutants display homeotic transformations (Kim et al., 2006; Lorente et al., 2006) and exhibit increased frequency of the phenomenon of leg-to-wing transdetermination (Klebes et al., 2005; Lee et al., 2005), an indication of cellular plasticity.

Chromatin remodelling by Plzf (Zbtb16 – Mouse Genome Informatics) influences limb pattern in mice (Barna et al., 2000; Barna et al., 2002). Also, regeneration of the zebrafish fin (Stewart et al., 2009) and amphibian limb (Yakushi et al., 2007) is regulated by histone and DNA methylation, respectively. These few studies suggest that epigenetic modifiers might play an underappreciated, substantial role in the regulation of primary limb development.
Polycomb group proteins form two main heterocomplexes: Polycomb repressive complex (PRC) 1 and PRC2 (Kuzmichev et al., 2002). PRC2 acts in part by binding target Polycomb response elements (PREs) at Hox loci via the protein YY1, and methylates associated target histone lysine residues (Kim et al., 2006). Polycomb proteins generally repress, whereas Trithorax proteins maintain, gene expression. However, there are examples in both *Drosophila* and mouse in which Polycomb group members have been shown to maintain gene expression or to enhance Trithorax function (Alasaka et al., 2001; Bracken et al., 2003; Gildea et al., 2000; LaJeunesse and Shearn, 1996).

Enhancer of zeste homolog 2 (Ezh2) is the catalytic subunit of PRC2 and is one of the most evolutionarily conserved polycomb-group members (O’Carroll et al., 2001). The SET (Suvar, Ez, Trithorax) domain of Ezh2 confers PRC2 with histone methylesterase activity with specificity for histones H3K27 and H3K9 (Erhardt et al., 2003; Kuzmichev et al., 2002). These methylation marks are associated with repression of gene expression (Kirmizis et al., 2004). Ezh2-null mouse embryos fail to develop beyond the initiation of gastrulation (O’Carroll et al., 2001) and diverse developmental functions are regulated by Ezh2 (Carette et al., 2004; Erhardt et al., 2003; Etchegaray et al., 2006; Plath et al., 2003; Sher et al., 2003; Su et al., 2003; Su et al., 2005). Given the highly structurally unique function and structure of polycomb elements and the role of E(z) and of Ezh2 in regulating cell identity, we hypothesised that Ezh2 would repress cell fate determination in the mouse limb bud. Our data suggest that the histone methyl esterase activity of Ezh2 is essential for appropriate axis formation in the limb bud. At early stages, Ezh2 acts primarily as a suppressive polycomb gene to regulate specification of the anteroposterior axis. At later stages, Ezh2 is required to permit full elaboration of proximodistal segment lengths.

**MATERIALS AND METHODS**

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed as previously described (Wyngaarden and Hopyan, 2008). Mutant and wild-type littermates were treated identically in the same assay for comparison.

Alcian Blue and Alizarin Red skeletal preparations

Embryonic day (E) 13.5 mouse embryos were eviscerated then fixed in 95% ethanol. They were stained with 0.3 mg/ml Alcian Blue (Alldrich) and cleared with alternating washes of either 30% glycerol, 10% KOH or 20% glycerol. Postnatal day (P) 0, 3-week-old and 7-week-old animals were eviscerated then fixed in 95% ethanol. Cartilage was stained using 0.3 mg/ml Alcian Blue (Alldrich) and bone with 75 µg/ml Alizarin Red (Alldrich) in 1% KOH. Skeletos were cleared with 20% glycerol and stored in 50% glycerol, 30% EOH.

Immunofluorescent staining

Limb crossections were fixed for 5 minutes in 1% paraformaldehyde (PFA) and washed in PBS three times. Sections were permeabilised with 0.05% Triton X-100 in PBS for 15 minutes, blocked for 30 minutes in 5% sheep serum in PBS and incubated with primary antibodies in a 1/3000 dilution in 1% sheep serum in PBS overnight at 4°C. After washing, sections were incubated with secondary antibodies for 1 hour, washed and mounted in Vectashield mounting medium (Vector Laboratories, Burlington, ON, Canada) with DAPI. Primary antibodies were from Upstate.

Quantitative RT-PCR

Total RNA was extracted from the dissected limbs of E12.5 mouse embryos using the RNeasy Kit (Ambion) following the manufacturer’s protocol. RNA was converted to cDNA using Oligo(dT) primers of the Superscript II First Strand Synthesis Kit (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR was performed using a standard format with 50 cycles of amplification on a 7900HT fast Real-Time PCR system (Applied Biosystems). TaqMan Assay On-Demand systems (ABI) were used to amplify the gene targets *Hoxa10* (Mm00433973_ml), *Hoxa11* (Mm00433960_ml), *Hoxa13* (Mm00433967_ml), *Hoxd10* (Mm00442839_ml), *Hoxd11* (Mm02602515_ml), *Hoxd13* (Mm00433973_ml) and *B2 Microglobulin* (Mm00437762_ml), a housekeeping gene used to normalise expression levels.

Chromatin immunoprecipitation (Chip)

Dissected E11.5 *Ezh2*, *Prx1*:Cre limbs were mechanically dissociated after a 20 minute 37°C incubation in 0.5% trypsin and 0.1% collagenase in DMEM. Cells were rinsed once in PBS then the CHIP assay was performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) according to the manufacturer’s protocol. DNA was recovered using the Qiagen PCR purification kit according to the manufacturer’s protocol. Antibodies against H3K27me3 and H3K9me3 (Abcam, 0.2 µg) were used for immunoprecipitation. Hox gene TaqMan primers were designed to amplify the presumptive regulatory sequences immediately upstream of a given Hox gene coding region that contained clusters of the CCAT core of the Y1 consensus binding site (Mihaly et al., 1998, Shrivastava and Calame, 1994) (ABI). Percent enrichment was determined by (Chip Hox gene/input Hox gene). Forward and reverse primers were as follows, respectively: *Hoxa11* (CTCGTGGACCAAGGCATTGATCTTAG, AACAGATGCTACCTGGTGTTCT, AAAGGAGGATGACATCTTTCTG, CTTGGGAGGGTCTAG); *Hoxa10* (CAGGCGAAAGCTCCACATGCT, CCCGCGACGCGCCAT). *Ezh2* regulates anteroposterior prepattern in the early limb bud

Owing to its role as the catalytic subunit of PRC2, we expected Ezh2 to repress Hox gene expression in the limb bud. To test this possibility, we generated conditional mutants. We found that the Ezh2 transcript in the mouse limb bud was expressed in a broad mesenchymal domain by in situ hybridisation. The Ezh2 signal became progressively diminished in the proximal region of the limb bud between E9.5 and E12.5. Distal expression was retained and faded at E12.5 (Fig. 1A). Conditional mutants (*Ezh2<sup>fl/fl</sup>*, *T*:Cre) were generated by crossing homozygous floxed Ezh2 (*Ezh2<sup>fl/fl</sup>* females with heterozygote floxed Ezh2, hemizygous *T*:Cre transgenic (*Ezh2<sup>fl/fl</sup>**, *T*:Cre) males. The T promoter element (Clemente et al., 1996) drives expression of Cre recombinase in mesoderm lineages from E7.5 onward (Perantoni et al., 2003). Cre is subsequently active in the lateral plate mesoderm posterior to the heart including the early limb bud. Complete deletion of floxed sequences by Cre in limb bud mesoderm is achieved by E10.0 (Verheyden et al., 2005). Cre recombinase deletes the essential SET domain from the floxed Ezh2 allele (Su et al., 2003).
Ezh2 catalyzes the methylation of H3K27 (Kuzmichev et al., 2002). Consistent with this fact, we found that in Ezh2fl/fl;T::Cre conditional mutant limb buds at E10.5, immunostaining against trimethylated H3K27, but not H3K36 or H3K9, was markedly diminished (Fig. 1B, 4.9%, s.d.=0.4, for wild type, P<0.001). In keeping with the repressive nature of this histone mark (Kimuzis et al., 2004), we observed ectopic anterior expansion of the expression domain of multiple Hox genes (Hoxd11, Hoxa13 and Hoxd13) by in situ hybridisation in the mutants (Fig. 1C, n=3 embryos per gene). These data are consistent with a model in which histone modifications precede and regulate the expression of numerous targets, including Hox genes (Soshnikova and Duboule, 2009). In the early limb bud, therefore, Ezh2 restricts the expression domain of some 5’ Hox genes as anticipated for a polycomb group gene.

Hox genes in the limb bud are linked closely to other important regulators of pattern formation. In particular, Hox gene expression is upregulated by Hand2 and downregulated by Gli3 (Chante et al., 2000; Galli et al., 2010; Sheth et al., 2007; Zakany et al., 2007). Consistent with these data, we found in Ezh2fl/fl;T::Cre conditional mutants that the early posterior marker Hand2 was ectopically expressed anteriorly (Fig. 1D, 4/4 embryos). Conversely, the expression of the anterior marker Gli3 was markedly diminished (Fig. 1D, 4/4 embryos). Given the decrease in the repressive histone methylation mark H3K27me3 that we found in the mutant limbs, this diminution of Gli3 expression was most likely to be secondary to repression from excessive Hand2. These findings indicate that anteroposterior axis prepattern was posteriorised in the mutants.

Other downstream regulators of anteroposterior pattern were affected. Whereas the expression of Shh, despite being downstream to Hand2, was not affected (6/15 embryos), the expression of Ptc1 and Gli1, both of which are indicators of hedgehog signalling, were ectopically expressed anteriorly (Fig. 1D). This finding is consistent with previous observations resulting from the overexpression of Hand2 in the chick embryo. It suggests that either Shh induction needs only to be transient or at levels below the detection limit of in situ hybridisation to induce target genes, or that Hand2 is an introductory factor that can independently initiate hedgehog signalling by inducing the expression of Ptc1 and Gli1 (Fernandez-Teran et al., 2000). Alternative explanations for this finding include the influence of Gli3 loss, as well as an indirect direct de-repression of Ptc1 and Gli1 in the absence of the H3K27m3 mark. As expected, expression of the anterior marker Alx4 (Panman et al., 2005; Qi et al., 1997), which is negatively regulated by hedgehog signalling (Takahashi et al., 1998), was diminished in the Ezh2fl/fl;T::Cre mutants. The expression of Tbx5, in part a regulator of anterior skeletal pattern (Koshita-Tekeuchi et al., 2006), was also diminished (Fig. 1D). Together, these data reflect the downstream consequences of an altered program of anteroposterior axis specification.

Ezh2 contributes to proximodistal elongation

Interestingly, Hox expression was disrupted differently at a later stage. In E12.5 embryo limbs, H3K27 trimethylation was no longer different between wild type (99.6%, s.d.=0.4) and Ezh2fl/fl;T::Cre mutants (98.2%, s.d.=0.7, P=0.02) on immunostained sections (Fig. 1E).
Consistent with this finding, the expression domain of 5' Hox genes was not expanded in Ezh2fl/fl;T::Cre mutants as it was at E10.5. Rather, Hox gene expression was prematurely downregulated in the mutants at E12.5 (Fig. 2B). In particular, a normally strong band of expression in the region of the prospective zeugopod was markedly diminished in the mutants. These genes represent the best available markers for the proximodistal segments prior to differentiation (Galloway et al., 2009), and are essential for the elaboration of those segments (Davis et al., 1995; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Wellik and Capocchi, 2003). Our results indicate that Ezh2, either directly or indirectly, maintains the late phase of 5' Hox gene expression in the limb bud and, therefore, might regulate proximodistal segment elaboration.

Limb bud morphology was clearly altered in Ezh2fl/fl;T::Cre mutants at E12.5 (Fig. 2B). To investigate further the consequences of loss of Ezh2 function on limb morphology, skeletal pattern analysis was performed at E13.5 because few embryos survived past this stage. Alcian Blue staining revealed shortening of all three primary proximodistal limb segments (stylopod, zeugopod and autopod) as well as anteroposterior patterning anomalies involving the autopod. Conditional mutant forelimb autopods contained four digits of disproportionate length, whereas hindlimb autopods primarily resulted in shortened segment lengths, affecting the autopod (hand) was relatively preserved (Table 1). Although digit one was shortened, the first metacarpal was present (Fig. 3A). This disproportionate shortening resembles human mesomelia (Jones, 2006) as well as the phenotype of Hoxa11;Hoxd11 double mutants to some degree (Davis et al., 1995). Therefore, the skeletal phenotype derived from the deletion of Ezh2 using Prx1::Cre primarily resulted in shortened segment lengths, affecting the anteroposterior axis to a far lesser extent than that obtained using T::Cre.

As anticipated, owing to the later deletion event in Ezh2fl/fl;Prx1::Cre conditional mutants, immunostaining at E10.5 revealed only a minor reduction of H3K27m3 (Fig. 3B; 94.4%, s.d. = 1.5, mutant nuclei positive vs 99.6%, s.d. = 0.5 for wild type, P = 0.03). Consistent with this finding, the early expression domain of all 5' Hoxa and Hoxd genes that are expressed in the limb bud was normal at E10.5 (Fig. 3C). By E12.5, H3K27m3 immunostaining remained only mildly affected (97.6%, s.d. = 1.2, mutant nuclei positive vs 99.6%, s.d. = 0.5, for wild type, P = 0.04), similar to the situation in Ezh2fl/fl;T::Cre mutants at the same stage (Fig. 4A). Further similarity to Ezh2fl/fl;T::Cre mutants at E12.5 was seen in the premature downregulation of 5' Hox gene expression, especially that of Hoxd cluster genes (Fig. 4B). We verified the diminished expression of Hox genes by subjecting dissected limb buds to real-time RT-PCR (Fig. 4C). This assay

The late role of Ezh2 primarily affects limb segment length rather than anteroposterior pattern formation

In order to isolate the late effect of Ezh2 from its early role, we generated conditional mutants in which the deletion event occurs later than with the T::Cre transgene. We chose the Prxl (Prxl – Mouse Genome Informatics) enhancer to drive expression of Cre.
revealed mild diminution of Hox gene expression in the whole limb bud, whereas in situ hybridisation highlighted a more dramatic loss of expression at the transverse zeugopod stripe of expression. Although it is conceivable that the zeugopod phenotype is partly the result of diminished zeugopod-specific Hox gene expression, increased cell death is likely to contribute substantially to overall proximodistal shortening in the mutants.

To investigate further whether histone modifications are associated with the role of Ezh2 in maintaining the late phase of Hox gene expression in the limb, we performed chromatin immunoprecipitation (ChIP). Limb buds were dissected from wild-type (WT) and conditional mutant E11.5 embryos. Histone methylation state-specific antibodies were used for immunoprecipitation, and real-time PCR was used to quantify immunoprecipitated DNA. Primers were designed to amplify the presumptive regulatory sequences immediately upstream of a given Hox gene coding region that contained clusters of the CCAT core of the YY1 consensus binding site (Mihaly et al., 1998; Shrivastava and Calame, 1994). We found a greater association of the repressive H3K9m3 mark with 5′ Hox genes, in particular with

Table 1. Proximodistal segmental measurements (mm) revealed predominantly mesomelic shortening of mutant limbs

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<th>Ezh2fl/+</th>
<th>Ezh2fl/+;Prx1Cre</th>
<th>Ezh2fl/fl;Prx1Cre</th>
<th>Ezh2fl/fl;Prx1Cre</th>
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<td>P0 forelimb</td>
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<td>Scapula</td>
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<td>Stylopod</td>
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<td>Zeugopod</td>
<td>5.0</td>
<td>2.8</td>
<td>12.0</td>
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<tr>
<td>Autopod</td>
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<td>Total</td>
<td>17.0</td>
<td>12.3</td>
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<td>P0 hindlimb</td>
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<td>Stylopod</td>
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<td>Zeugopod</td>
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<td>Total</td>
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<td>P3W hindlimb</td>
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For Ezh2fl/+ or Ezh2fl/+;Prx1Cre P0 forelimb and hindlimb, n=3.
For Ezh2fl/+ or Ezh2fl/+;Prx1Cre P3W forelimb and hindlimb, n=2.

Fig. 3. Lack of early Hox gene expression changes in Ezh2fl/fl;Prx1Cre conditional mutants at E10.5. (A) Alcian Blue staining of cartilage at E13.5 (top row), skeletal preparations in neonates (middle row) and faxitron X-ray of 4-week-old mice (bottom row) revealed mesomelic disproportionate shortening of proximodistal segments in Ezh2fl/fl;Prx1Cre mutants compared with wild-type (WT) littermates. However, anteroposterior pattern was preserved relative to Ezh2fl/fl;Prx1Cre mutants compared with wild-type (WT) littermates. However, anteroposterior pattern was preserved relative to Ezh2fl/fl;Prx1Cre mutants compared with wild-type (WT) littermates. (B) In Ezh2fl/+;Prx1Cre mutants there is only a subtle diminution of trimethylated H3K27 antibody uptake compared with wild-type littermates. This contrasts with the marked difference observed in Ezh2fl/fl;Prx1Cre mutants (Fig. 1B), and is likely to be due to the later onset of deletion by Prx1Cre. (C) Also, in contrast to T:Cre conditional mutants and consistent with the findings shown in B, in situ hybridisation against Hoxa and Hoxd transcripts revealed no differences in expression compared with wild-type littermates at E10.5.
Hoxd11, in mutant limbs (Fig. 4D). Changes in the association of 5′ Hox genes with other methylation marks, including H3K9m1, H3K27m2 and H3K27m3, were not identified (Fig. 4D; data not shown). Our finding regarding H3K9m3 correlates with the premature downregulation of Hox gene expression in the mutants.

**Ezh2 is a pro-determination factor**

We hypothesised that, by prolonging the duration of limb bud cell exposure to Hox gene expression, Ezh2 would act to consolidate positional identity. To test directly whether Ezh2 regulates a switch from plasticity to determination, we performed cell transposition experiments (Wyngarden and Hopyan, 2008). We chose to experimentally stress the expression of Hoxa13 because it was least affected in the mutants. Clumps of ~50 cells were harvested from within the Hoxa13 expression domain of mutant or wild-type littermate donor embryos and labelled with Dil. The cells were then grafted well proximal to the Hoxa13 domain in the opposite limb of the same embryo (Fig. 5A). TUNEL analysis of grafted, sectioned wild-type and mutant limb buds confirmed that, even at E12.5, excessive cell death did not occur within the graft relative to the surrounding tissue (Fig. 5B). At E10.5, Ezh2fl/fl; Prx1::Cre mutant cells lost expression of Hoxa13 following overnight culture. 

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**Fig. 4. Greater association of trimethylated H3K9 with Hox genes in Ezh2fl/fl; Prx1::Cre conditional mutants.** (A) Marginally diminished trimethylated H3K27 immunostaining in conditional mutants. (B) 5′ Hox gene expression was downregulated prematurely in mutants (arrowheads). (C) Quantitative RT-PCR confirmed diminished expression of 5′ Hox genes from dissected E12.5 conditional mutant (n=5 embryos) compared with wild-type limb buds (n=5 embryos; error bars represent s.e.m.). (D) ChIP analysis by real-time PCR revealed that there was greater association of the repressive mark H3K9m3 with Hox11 paralogues in mutant limb buds. Error bars represent standard deviation of three separate experiments performed with different litters. Real-time PCR was performed in triplicate.
Ezh2 regulates AP and PD limb axes

in 4/4 (100%) cases, whereas wild-type cells lost expression of Hoxa13 in 7/9 (78%) cases (Fig. 5C, D). This finding indicated that both the wild-type and mutant limb bud cells remained plastic at an early stage. When the experiment was performed at E12.5, Ezh2 mutant cells lost expression of Hoxa13 in 7/10 (70%) cases, whereas wild-type cells lost expression of Hoxa13 in 5/14 (36%) cases (Fig. 5C, D). Therefore, Hoxa13 expression was more plastic in mutant cells, and Ezh2 is a pro-determination factor during late limb bud stages.

DISCUSSION

The role of epigenetic regulators, including that of Ezh2, in development is being defined progressively (Chen et al., 2009; Juan et al., 2009; Pereira et al., 2010). This study expands the known developmental functions of Ezh2 to the limb bud, where the fundamental nature of this regulator with regard to axis formation is apparent. Our data suggest that Ezh2 is among the earliest known regulators of anteroposterior axial prepattern (Xu and Wellik, 2011). In particular, Ezh2 is essential for Gh3 expression and, therefore, for anterior identity. Unlike Gh3 mutants that exhibit polydactyly (Hui and Joyner, 1993), Ezh2+/−; T::Cre mice displayed a reduction in digit number, which is most likely to be related to increased cell death. In contrast to our T::Cre conditional Ezh2 mutants, anteroposterior pattern was less affected in Prx1::Cre mutants whereas segment lengths were affected in both. The anteroposterior and proximodistal axes are linked at the time that the Shh-Fgf positive feedback loop is established (Niswander et al., 1994), and our data provide no evidence that those axes are regulated together, at least by Ezh2, at an earlier stage. Our findings are consistent with a model in which the specification of limb anteroposterior pattern occurs early, and subsequent events that progressively determine positional identity contribute to the expansion and refinement of proximodistal limb segments.

Ezh2 has apparently distinct roles at different stages of limb development. In Drosophila and mouse, Polycomb group proteins usually, but not always, repress gene expression. A strong temperature-sensitive mutant in Drosophila revealed that E(z) both represses and maintains the expression of different Hox genes (LaJeunesse and Shearn, 1996). Several Polycomb genes, including E(z), can enhance Trithorax phenotypes (Glidea et al., 2000). In mammals, the polycomb genes Mel18 and Bmi1 maintain Hox gene expression (Akasaka et al., 2001), and EZH2 activates or maintains E2F target gene expression (Bracken et al., 2003). It has previously been suggested that genes with dual repressive and maintenance ability should be termed ‘Enhancer of Trithorax and Polycomb’. The early Polycomb-like transcriptional repression role of Ezh2 could be advantageous in the limb bud by preventing ectopic Hox gene expression at a time when cell positions are dynamically changing and cells cross between Hox gene boundaries (Vargesson et al., 1997). The subsequent role of Ezh2 in maintaining Hox gene expression would be useful to consolidate positional identities in the maturing limb bud.

It is not clear how some polycomb genes apparently maintain gene expression in certain contexts. Our data suggest that Ezh2 suppresses the association of trimethylated H3K9 with some 5’ Hox genes in the mature limb bud. Potential explanations for this finding include the possibility that, in the absence of Ezh2 activity, other H3K9 methyltransferases are potentiated, or that the composition of PRC2 varies in a context-specific fashion (Kuzmichev et al., 2005). Other dual regulators might also function...
by altering histone methylation of specific targets in unexpected and indirect ways. These findings highlight the complexity of developing systems, and underscore the importance of defining epigenetic functions in vivo.

The basis of plastic gene expression in certain developmental contexts (Trainor and Krumlauf, 2000; Wyngaarden and Hopman, 2008) is not well understood. Our transposition experiments (Fig. 5A-D) suggest that histone methylation regulates this process, and are consistent with the greater frequency of leg-to-wing transdegeneration (an indicator of fate plasticity) in E(z) mutant Drosophila imaginal disc cells (Lee et al., 2005). The ChIP data (Fig. 4D) show a trend toward increased H3K9 trimethylation of Hoxa13 in the Ezh2fl/fl;Prx1::Cre mutants. Although this increase is not enough to prematurely downregulate the expression of Hoxa13 within its usual domain (Fig. 4B), it might be enough to prevent maintenance of expression in the presence of new positional cues outside of its usual expression domain.

Modification of histone methylation might underlie an efficient mechanism of altering cell fate plasticity during development. We speculate that a context-specific balance between plasticity and commitment is essential to permit an appropriate duration of commitment to permit an appropriate duration of positional cues outside of its usual expression domain.

Epigenetic regulators other than Ezh2 are likely to influence axis formation. We anticipate that other limb conditional mutants will reveal exciting insights, including further details regarding the mechanisms by which the proximodistal axis is specified.

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Ezh2 regulates AP and PD limb axes


