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 precedes 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 Velcher et al., 2002). Recently, it was shown that Hox9 genes are required to initiate Hand2 expression in the forelimb (Xu and Hand2, 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., 2005; 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
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 (Akasaka 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 methyltransferase 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 (Caretta et al., 2004; Erhardt et al., 2003; Etchegaray et al., 2006; Plath et al., 2003; Shet et al., 2008; Su et al., 2003; Su et al., 2005). Given the highly structurally conserved function and structure of polycomb genes and the role of E(z) and of Ezh2 in regulating cell identity, we hypothesised that Ezh2 would regulate cell fate determination in the mouse limb bud. Our data suggest that the histone methyl transferase 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 proximal-distal 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 (Aldrich) and bone with 75 mg/ml Alizarin Red (Aldrich) in 1% KOH. Skeletons were cleared with alternating washes of either 20% glycerol, 10% KOH or 20% 95% ethanol. They were stained with 0.3 mg/ml Alcian Blue (Aldrich) and 0.3760 mg/ml Alizarin Red (Mm00433973_m1) and B2 Microglobulin (Mm00437762_m1), 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 Qagen 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 YY1 consensus binding site (Milhky et al., 1998, Shuvartava and Calame, 1994) (ABI). Percent enrichment was determined by (CHip Hox gene/INPUT Hox gene). Forward and reverse primers were as follows, respectively: Hoxa11 (TCGTGGACAGAGCACGTAGTAGTTCTTCTTCTCCATGGCGTCAC); Hoxa13 (GCGCAAAAGCTCCTCCCTAC; CCGGGATGATTACATTTTCCTGT; CTTGAGGAGGTTCTAG); Hoxd11 (GCGAGCTGTGATAGATAGAAGGA, TTATCAGACTCCTGTTGTAATTAGTATC; TTCTTCTCATGAGGCGTAC); Hoxd13 (CTAATCTGGTGGAGCGG, CCGCGCAGACCCACC, CCGCGCAGCCGAT). Limb bud cell transpositions

Mouse embryos were harvested and manipulated in DMEM with 10% foetal calf serum. Limb bud tissue transpositions were performed as previously described (Wyngaarden and Hopyan, 2008). Embryos were then cultured in DMEM with 75% rat serum in overnight roller culture, fixed and in situ hybridisation was performed. Limb bud grafts were localised using the fluorescent Dil label under a dissecting stereomicroscope and scored for expression of the marker gene in a ‘yes or no’ binary fashion.

TUNEL

TUNEL staining was performed as previously described (Wyngaarden and Hopyan, 2008).

RESULTS

Ezh2 regulates anteroposterior pattern 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 longer and faded at E12.5 (Fig. 1A). Conditional mutants (Ezh2fl/fl; T::Cre) were generated by crossing homzygous floxed Ezh2fl/+; T::Cre) females with heterozygote floxed (Ezh2fl/+; T::Cre) males. The promoter element (Clements et al., 1996) drives expression of Cre recombinase in mesodermal lineages from E7.5 onward (Perantoni et al., 2005). 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 catalyses the methylation of H3K27 (Kuzmichev et al., 2002). Consistent with this fact, we found that in Ezh2<sup>fl/fl</sup>;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 Ezh2<sup>fl/fl</sup>;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 (5/5 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 a possible direct de-repression of Ptc1 and Gli1 in the absence of the H3K27me3 mark. As expected, expression of the anterior marker Alx4 (Pannun et al., 2003; Qi et al., 1997), which is negatively regulated by hedgehog signalling (Takahashi et al., 1998), was diminished in the Ezh2<sup>fl/fl</sup>;T::Cre mutants. The expression of Tbx5, in part a regulator of anterior skeletal pattern (Koshiba-Tekeuchi et al., 2006), was also diminished (Fig. 1D). Together, these data reflect the downstream consequences of an altered programme 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 (99.8%, s.d.=0.4) and Ezh2<sup>fl/fl</sup>;T::Cre mutants (98.2%, s.d.=0.7, P=0.02) on immunostained sections (Fig. 1E). Given the robustness of Hox expression in the E12.5 embryo limbs, this suggests that Hox expression is not linked to trimethylation of H3K27. Consistent with this fact, we found that in Ezh2<sup>fl/fl</sup>;T::Cre conditional mutant limb buds at E10.5, immunostaining against trimethylated H3K27, but not other histone modifications, in conditional mutants at E10.5. (C) At E10.5, Ezh2<sup>fl/fl</sup>;T::Cre mutant littermates exhibited ectopic anterior (upward) expression of Hoxa13, Hoxd11 and Hoxd13 (blue arrowheads). (D) Expression of markers of anteroposterior identity indicates that the limb bud is posteriorised in Ezh2<sup>fl/fl</sup>;T::Cre mutants. The prepattern genes Hand2 and Gli3 are regulated by Ezh2, as are downstream regulators. Despite the lack of ectopic anterior Shh, indicators of hedgehog signalling are ectopically activated in anterior mesenchyme. Expression of the anterior markers Alx4 and Tbx5 is diminished.

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Fig. 1. Posteriorisation prepattern in Ezh2<sup>fl/fl</sup>;T::Cre conditional mutants. (A) In situ hybridisation revealed that Ezh2 is expressed in a broad and distally biased domain in mouse limb buds. AS, antisense; FL, forelimb; WT, wild type. (B) Fewer cells stained specifically against trimethylated H3K27, but not other histone modifications, in conditional mutants at E10.5. (C) At E10.5, Ezh2<sup>fl/fl</sup>;T::Cre mutant littermates exhibited ectopic anterior (upward) expression of Hoxa13, Hoxd11 and Hoxd13 (blue arrowheads). (D) Expression of markers of anteroposterior identity indicates that the limb bud is posteriorised in Ezh2<sup>fl/fl</sup>;T::Cre mutants. The prepattern genes Hand2 and Gli3 are regulated by Ezh2, as are downstream regulators. Despite the lack of ectopic anterior Shh, indicators of hedgehog signalling are ectopically activated in anterior mesenchyme. Expression of the anterior markers Alx4 and Tbx5 is diminished.
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 (Prx1 – Mouse Genome Informatics) enhancer to drive expression of Cre.

Prxl is expressed in the forelimb forming region of the lateral plate mesoderm from the 14 somite stage onward, in the earliest forelimb bud mesenchyme from E9.5 onward, and later in the hindlimb bud (Hasson et al., 2007; Logan et al., 2002).

At E13.5, Alcian Blue cartilage staining revealed that Ezh2fl/fl;Prx1::Cre mutant limb segments were clearly shorter than those of control littermates (Fig. 3A). These findings correlated with those from later stages as these mutants survived well into adulthood. Skeletal preparations and faxitron X-ray from the neonatal period through to adulthood clearly demonstrated that the mutants exhibited shortened proximodistal limb segments. The zeugopod (forearm) segment was most severely shortened, whereas 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. = 1.2, mutant nuclei positive vs 99.6%, s.d. = 0.5, for wild type, P = 0.04). 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. = 1.5, mutant nuclei positive vs 99.0%, s.d. = 2.0, for wild type, P = 0.02). Consistent with this finding, the expression domain of all 5’ Hoxa 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 (Devis et al., 1995; Fromental-Ramain et al., 1996a, 1996b; Wellik and Capecchi, 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 (Fig. 2C). We identified somewhat greater reduction of those segments (Fig. 2D). Further similarity to the situation in Ezh2fl/fl;T::Cre mutants at E12.5 (Fig. 2B) was seen in the premature downregulation of 5’ Hox gene expression, especially that of Hoxd cluster genes (Fig. 4A). Further similarity to Ezh2fl/fl;T::Cre mutants at E12.5 was seen in the premature downregulation of 5’ Hox gene expression by subjecting dissected limb buds to real-time RT-PCR (Fig. 4C). This assay...
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

<table>
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<th></th>
<th>Ezh2Δ+</th>
<th>Ezh2Δ−;Prx1CRE</th>
<th>Ezh2Δ+</th>
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<td><strong>P0 forelimb</strong></td>
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<td>Scapula</td>
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<td>Stylopod</td>
<td>4.8</td>
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<tr>
<td>Zeugopod</td>
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<td>12.0</td>
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<tr>
<td>Autopod</td>
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<tr>
<td>Total</td>
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<td>12.3</td>
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<td><strong>P0 hindlimb</strong></td>
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<td>Stylopod</td>
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<td><strong>P3W forelimb</strong></td>
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For Ezh2Δ+ or Ezh2Δ−;Prx1CRE P0 forelimb and hindlimb, n=3.
For Ezh2Δ+ or Ezh2Δ−;Prx1CRE P3W forelimb and hindlimb, n=2.

Fig. 3. Lack of early Hox gene expression changes in Ezh2Δ−;Prx1::Cre 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 Ezh2Δ−;Prx1::Cre mutants compared with wild-type (WT) littermates. However, anteroposterior pattern was preserved relative to Ezh2Δ−;T::Cre mutants (Fig. 2D). (B) In Ezh2Δ−;Prx1::Cre 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 Ezh2Δ−;T::Cre mutants (Fig. 1B), and is likely to be due to the later onset of deletion by Prx1::Cre. (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 (Wyngaarden 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 the Hoxa13 expression domain of mutant or wild-type littermate donor embryos and labelled with DiI. 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.
Ezh2 regulates AP and PD limb axes

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), Ezh2fl/fl;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 (Misowander 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 (Gildea 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; Wygaarden 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 transdetermination (an indicator of fate plasticity) in E(spl) 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 Ezh2<sup> floxed</sup>, Prxl<sup>-/-</sup> 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 changes over time.

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 proximodalix axis is specified.

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References


Ezh2 regulates AP and PD limb axes


