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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 Welscher 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., 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 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 Capecchi, 2003), zeugopod (Davis et al., 1995; Wellik and Capecchi, 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 an increased frequency of the phenomenon of leg-to-wing transdetermination (Klebes et al., 2005; Lee et al., 2005), an indication of cellular plasticity. There is evidence that epigenetic modifiers regulate pattern formation in the vertebrate limb as well. 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 (Yakushiji 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.

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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* (Caretti et al., 2004; Erhardt et al., 2003; Etchegaray et al., 2006; Plath et al., 2003; Sher et al., 2008; Su et al., 2003; Su et al., 2005). Given the highly conserved structure and function 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 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 (Aldrich) and cleared with alternating washes of either 20% 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 (Aldrich) and bone with 75 μg/ml Alizarin Red (Aldrich) in 1% KOH. Skeletons were cleared with 20% glycerol and stored in 50% glycerol, 50% EtOH.

Immunofluorescent staining

Limb cryosections 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 RNAqueous 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_m1), *Hoxa11* (Mm00439360_m1), *Hoxa13* (Mm00433967_m1), *Hoxd10* (Mm00442839_m1), *Hoxd11* (Mm02602515_mH), *Hoxd13* (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 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 YY1 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* (TCGTGGACCAGGACCATGTAG, AACAGATCGTCACTCGGTGTTCT, AAAGGAGAGCTGCCC); *Hoxa13* (CAGGCGAAAGCTCCCACAT, CCAGGGATGGATACATCTTTCTG, CTTGGGAGGGTCTAGC); *Hoxd11* (GCGAGCGCTGATATAGATATAAGGA, TGATTGATTCTGGTGGTAATTATGTCA, TTTCTCTCCATGGCGTCAC); *Hoxd13* (CTAACCTGTTGGAGGGCGG, CCCGCGCAGGACACC, CGCAGCGCGCCAT).

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 DiI 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 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 longer and faded at E12.5 (Fig. 1A). Conditional mutants (*Ezh2$^{fl/fl}$;T::Cre*) were generated by crossing homozygous floxed *Ezh2* (*Ezh2$^{fl/fl}$*) females with heterozygote floxed *Ezh2*, hemizygous *T::Cre* transgenic (*Ezh2$^{fl/+}$;T::Cre*) males. The *T* promoter element (Clements et al., 1996) drives expression of *Cre recombinase* in mesoderm 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).

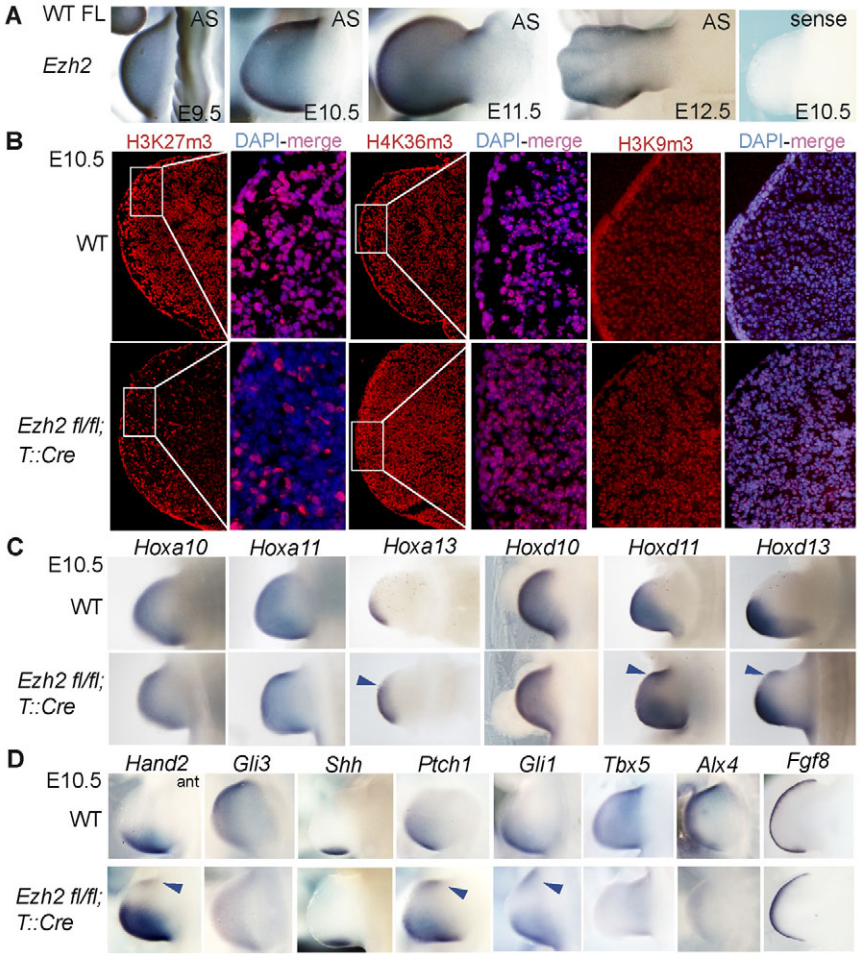

Fig. 1. Posteriorised prepattern in *Ezh2$^{fl/fl}$;T::Cre* conditional mutants. (**A**) In situ hybridisation revealed that *Ezh2* is expressed in a broad and distally biased domain in mouse limb buds. AS, antisense; FL, forelimb; WT, wild type. (**B**) Fewer cells stained specifically against trimethylated H3K27, but not other histone modifications, in conditional mutants at E10.5. (**C**) At E10.5, *Ezh2$^{fl/fl}$;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$^{fl/fl}$;T::Cre* mutants. The prepatterning 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.

Ezh2 catalyses the methylation of H3K27 (Kuzmichev et al., 2002). Consistent with this fact, we found that in *Ezh2$^{fl/fl}$;T::Cre* conditional mutant limb buds at E10.5, immunostaining against trimethylated H3K27, but not H3K36 nor H3K9, was markedly diminished (Fig. 1B; 4.9%, s.d.=2.6, mutant nuclei positive vs 99.6%, s.d.=0.4, for wild type, $P<0.001$). In keeping with the repressive nature of this histone mark (Kirmizis 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* (Charite et al., 2000; Galli et al., 2010; Sheth et al., 2007; Zakany et al., 2007). Consistent with these data, we found in *Ezh2$^{fl/fl}$;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 (5/5 embryos), the expression of *Ptch1* 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 *Ptch1* 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 *Ptch1* and *Gli1* in the absence of the H3K27m3 mark. As expected, expression of the anterior marker *Alx4* (Panman et al., 2005; Qu et al., 1997), which is negatively regulated by hedgehog signalling (Takahashi et al., 1998), was diminished in the *Ezh2$^{fl/fl}$;T::Cre* mutants. The expression of *Tbx5*, in part a regulator of anterior skeletal pattern (Koshiba-Takeuchi 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$^{fl/fl}$;T::Cre* mutants (98.2%, s.d.=0.7; $P=0.02$) on immunostained sections (Fig.

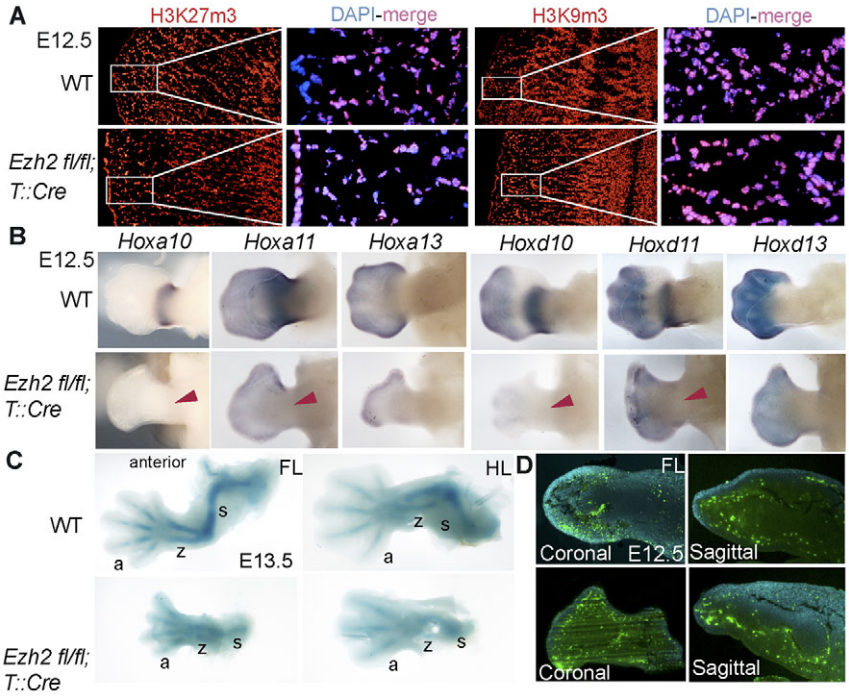

Fig. 2. Hox gene expression is prematurely downregulated in the maturing *Ezh2$^{fl/fl}$;T::Cre* limb bud. (**A**) In contrast to earlier limb buds, at E12.5 immunostaining against trimethylated H3K27 was marginally diminished, whereas trimethylated H3K9 remained comparable between wild type and conditional mutant mouse limb buds. (**B**) By E12.5, the expression of multiple Hox genes was prematurely downregulated in mutant forelimbs. A prominent band of expression in the zeugopod region was particularly diminished (red arrowheads). Similar findings were observed in mutant hindlimbs (data not shown). (**C**) *T::Cre* conditional mutants exhibited shortened limb segments and anteroposterior patterning anomalies involving the autopod. a, autopod; s, stylopod; z, zeugopod. (**D**) Increased cell death as assessed by TUNEL assay at a preceding stage. FL, forelimb; HL, hindlimb; WT, wild type.

2A). Consistent with this finding, the expression domain of 5′ Hox genes was not expanded in *Ezh2$^{fl/fl}$;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 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 *Ezh2$^{fl/fl}$;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 contained three digits (Fig. 2C). We identified somewhat greater cell death by TUNEL staining in the mutant limbs (Fig. 2D). Therefore, *Ezh2* is essential for proximodistal elongation in addition to anteroposterior pattern formation, at least in part by promoting cell survival.

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 *Prx1* (*Prrx1* – Mouse Genome Informatics) enhancer to drive expression of *Cre*. *Prx1* 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 *Ezh2$^{fl/fl}$;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 *Ezh2$^{fl/fl}$;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 99.0%, s.d.=0.5 for wild type, P=0.02). 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 *Ezh2$^{fl/fl}$;T::Cre* mutants at the same stage (Fig. 4A). Further similarity to *Ezh2$^{fl/fl}$;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

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

	Ezh2$^{fl/+}$	*Ezh2*$^{fl/fl}$*;Prx1Cre*	*Ezh2*$^{fl/+}$	*Ezh2*$^{fl/fl}$*;Prx1Cre*
	P0 forelimb		**P3W forelimb**	
Scapula	3.8	2.8	7.5	5.0
Stylopod	4.8	4.0	9.5	6.5
Zeugopod	5.0	2.8	12.0	5.5
Autopod	3.3	2.7	8.5	6.3
Total	17.0	12.3	37.5	23.3
	P0 hindlimb		**P3W hindlimb**	
Stylopod	4.5	4.0	11.5	8.5
Zeugopod	4.8	4.0	12.8	9.0
Autopod	5.2	5.2	17.3	14.8
Total	14.5	13.2	41.5	32.3

For *Ezh2*$^{fl/+}$ or *Ezh2*$^{fl/fl}$*;Prx1Cre* P0 forelimb and hindlimb, *n*=3.
For *Ezh2*$^{fl/+}$ or *Ezh2*$^{fl/fl}$*;Prx1Cre* P3W forelimb and hindlimb, *n*=2.

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

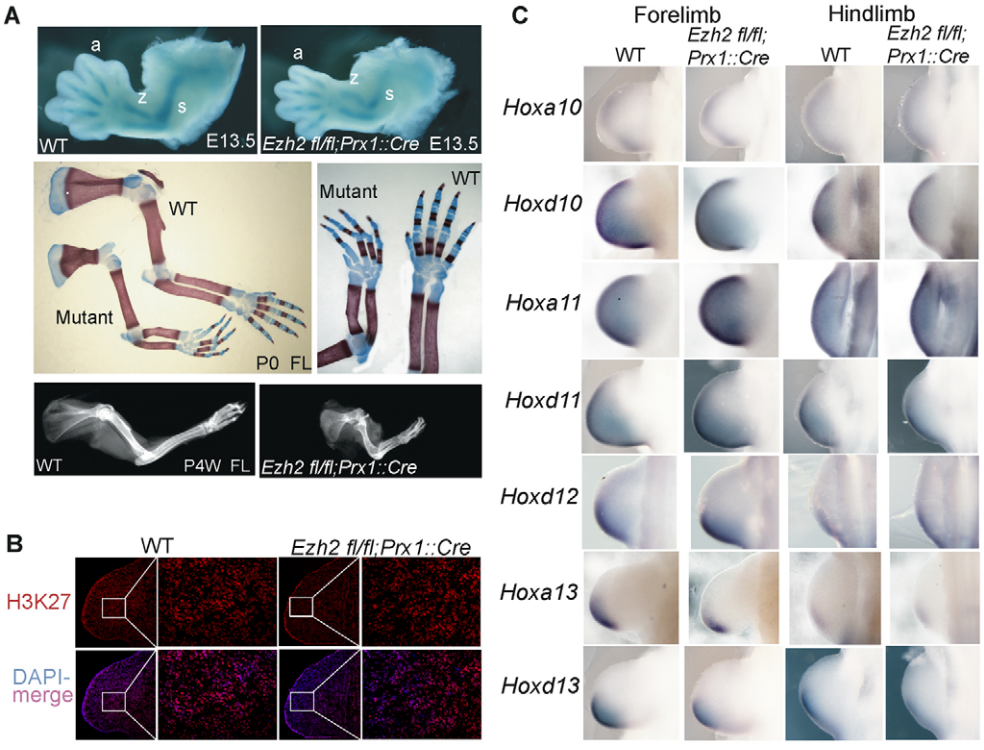

Fig. 3. Lack of early Hox gene expression changes in *Ezh2*$^{fl/fl}$*;Prx1::Cre* conditional mutants at E10.5. (**A**) Alcian Blue staining of cartilage at E13.5 (top row), skeletal preparations in neonates (middle row) and faxitron X-ray of 4-week-old mice (bottom row) revealed mesomelic disproportionate shortening of proximodistal segments in *Ezh2*$^{fl/fl}$*;Prx1::Cre* mutants compared with wild-type (WT) littermates. However, anteroposterior pattern was preserved relative to *Ezh2*$^{fl/fl}$*;T::Cre* mutants (Fig. 2D). (**B**) In *Ezh2*$^{fl/fl}$*;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 *Ezh2*$^{fl/fl}$*;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.

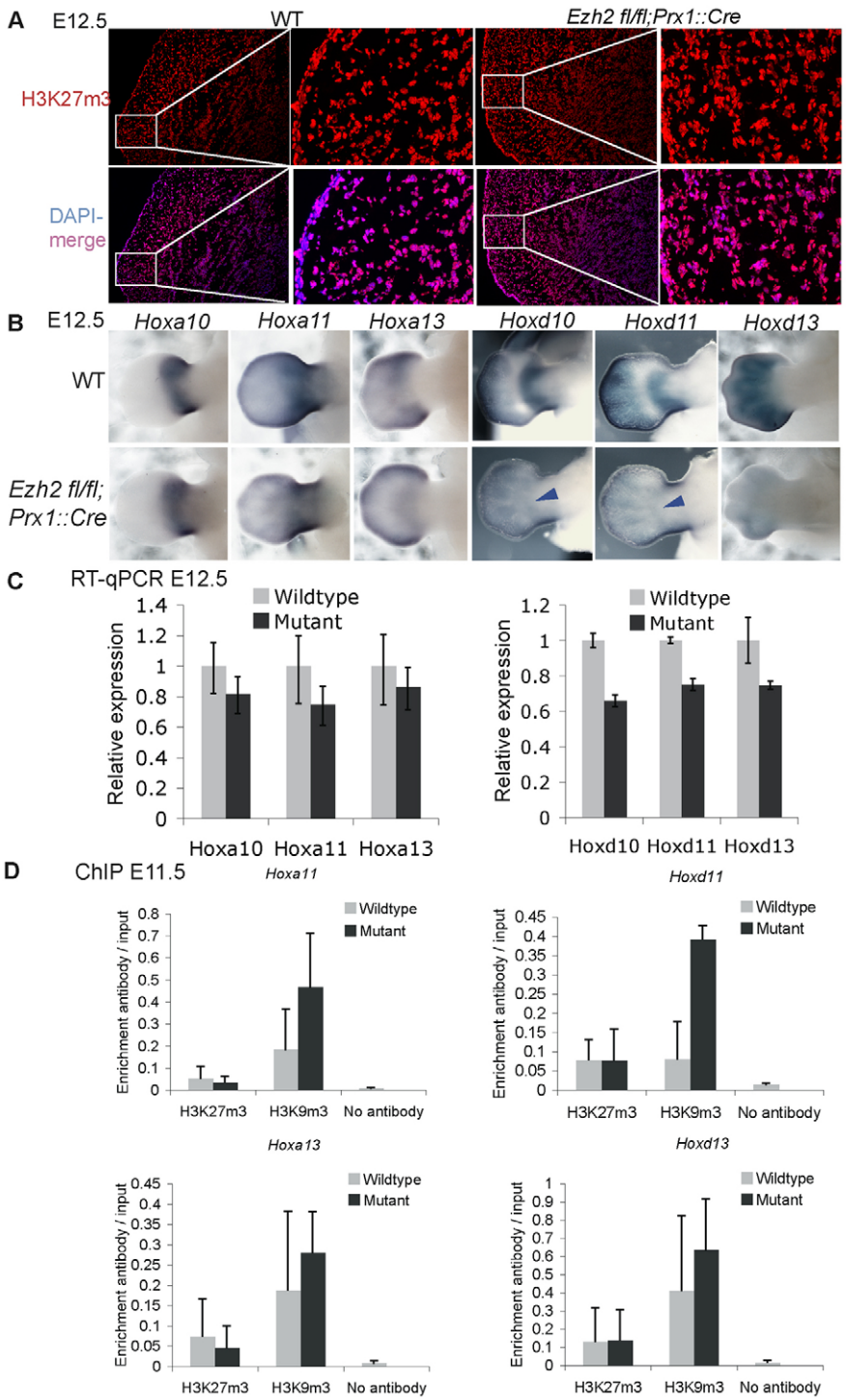

Fig. 4. Greater association of trimethylated H3K9 with Hox genes in *Ezh2$^{fl/fl}$;Prx1::Cre* conditional mutants. (**A**) Marginally diminished trimethylated H3K27 immunostaining in conditional mutants. (**B**) 5′ Hox gene expression was downregulated prematurely in mutants (arrowheads). (**C**) Quantitative 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.

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 within 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, *Ezh2$^{fl/fl}$;Prx1::Cre* mutant cells lost expression of *Hoxa13* following overnight culture

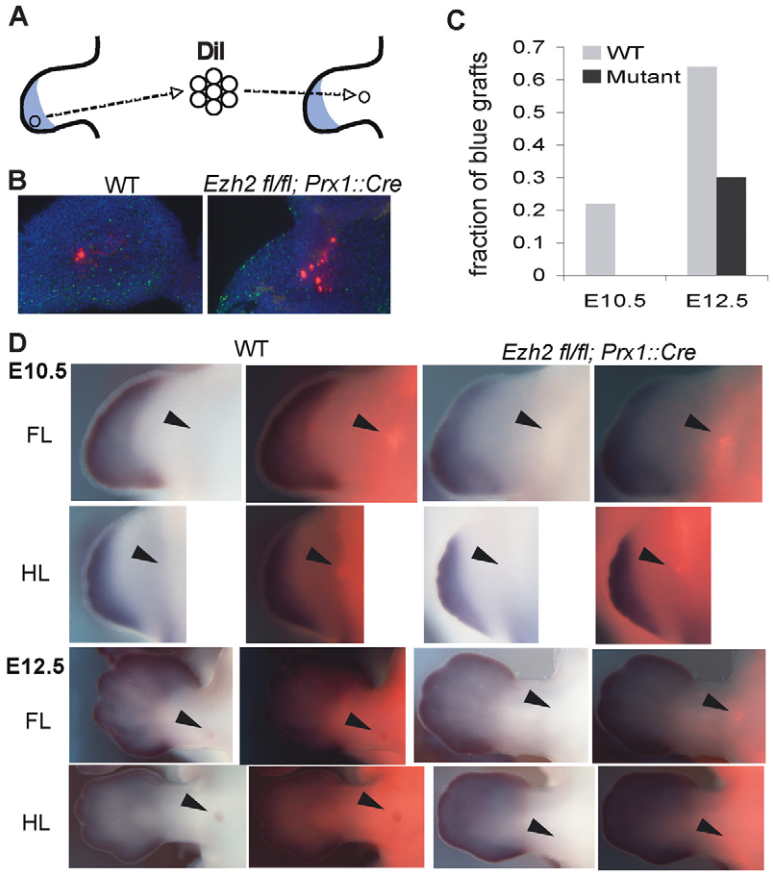

Fig. 5. Ezh2 positively regulates a switch between plasticity and determination. (**A**) Scheme of transposition of small clumps of cells from within a distal *Hoxa13*-expressing domain (blue shading) of a donor embryo to a proximal non-expressing domain of a recipient embryo. (**B**) Cell death as assessed by TUNEL assay was not different in either the wild type (WT) or mutant graft relative to the surrounding mesenchyme after culture. (**D**) Cell transpositions were carried out as shown in A and embryos were cultured overnight in rat serum media using aerated roller culture (Wyngaarden and Hopyan, 2008). *Hoxa13* expression is represented by blue, and graft location is marked by DiI as seen in the accompanying merged fluorescent/bright field views (arrowheads). Both WT and *Ezh2*$^{fl/fl}$*;Prx1::Cre* mutant distal cells lost expression of *Hoxa13* when transposed to a proximal location at E10.5. By E12.5, the expression of *Hoxa13* was unchanged by transposition in WT cells, but not in mutant cells in which expression was lost (see text for quantitative data). (**C**) The fraction of grafts retaining expression of *Hoxa13*, a measure of determination, in their new proximal environment.

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 *Gli3* expression and, therefore, for anterior identity. Unlike *Gli3* mutants that exhibit polydactyly (Hui and Joyner, 1993), *Ezh2*$^{fl/fl}$*;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 (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 (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; Wyngaarden and Hopyan, 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(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 *Ezh2$^{fl/fl}$;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 position-specific gene expression in growing tissue in which the location of and distance from instructional cues, potentially retinoic acid and Fgf (Cooper et al., 2011; Rosello-Diez et al., 2011), 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 proximodistal axis is specified.

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Competing interests statement

The authors declare no competing financial interests.

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