Anterior-posterior differences in HoxD chromatin topology in limb development

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SUMMARY
A late phase of HoxD activation is crucial for the patterning and growth of distal structures across the anterior-posterior (A-P) limb axis of mammals. Polycomb complexes and chromatin compaction have been shown to regulate Hox loci along the main body axis in embryonic development, but the extent to which they have a role in limb-specific HoxD expression, an evolutionary adaptation defined by the activity of distal enhancer elements that drive expression of 5’ Hoxd genes, has yet to be fully elucidated. We reveal two levels of chromatin topology that differentiate distal limb A-P HoxD activity. Using both immortalised cell lines derived from posterior and anterior regions of distal limb bud mesenchyme, and analysis in E10.5 dissected limb buds themselves, we show that there is a loss of polycomb-catalysed H3K27me3 histone modification and a chromatin decompaction over HoxD in the distal posterior limb compared with anterior. Moreover, we show that the global control region (GCR) long-range enhancer spatially colocalises with the 5’ HoxD genomic region specifically in the distal posterior limb. This is consistent with the formation of a chromatin loop between 5’ HoxD and the GCR regulatory module at the time and place of distal limb bud development when the GCR participates in initiating Hoxd gene quantitative collinearity and Hoxd13 expression. This is the first example of A-P differences in chromatin compaction and chromatin looping in the development of the mammalian secondary body axis (limb).

KEY WORDS: Autopod, Enhancer, Polycomb, Mouse

INTRODUCTION
Regulated Hox gene expression is important in anterior-posterior (A-P) patterning of the primary embryonic axis, with Hox genes being first activated at gastrulation (Wyngaarden et al., 2011; Deschamps and van Nes, 2005). The HoxD cluster has also been co-opted more recently in evolution into regulating the growth and patterning of the limb and digits.

Polycomb repressive complexes (PRC1 and PRC2) are required to maintain Hox genes in a silent compact chromatin state in embryonic stem (ES) cells (Boyer et al., 2006; Lee et al., 2006; Endoh et al., 2008; Stock et al., 2007; Eskeland et al., 2010). Whereas roles of polycomb at Hox loci are well established in early embryonic development and in differentiation along the primary embryonic axis (Vonken et al., 2003; Faust et al., 1998; Chambeyron et al., 2005; Soshnikova and Duboule, 2009), whether polycomb-mediated chromatin changes are involved in Hox regulation in the secondary body axis is unclear.

Two phases of HoxD expression are important in limb development and patterning (Tarchini and Duboule, 2006; Zakany and Duboule, 2007). The first phase results in expression of 3’ Hoxd genes (Hoxd1-9) earlier than the 5’ genes. This restricts 5’ HoxD expression to the posterior side of the distal limb bud and is required for limb outgrowth, proximal limb development, limb A-P polarity and the posterior expression of sonic hedgehog (Shh). This 3’-5’ temporal and spatial collinearity is reminiscent of regulation in the main embryonic axis, which is accompanied by progressive loss of histone H3 lysine 27 tri-methylation (H3K27me3) catalysed by PRC2 (Soshnikova and Duboule, 2009). However, it might also require as yet undefined regulatory elements 3’ of HoxD (Spitz et al., 2005).

A later phase [embryonic day (E) 10.5] of Hoxd expression in the distal limb is required for digit morphogenesis (Spitz et al., 2003). This is characterised by ‘quantitative collinearity’ in which expression of the most 5’ gene, Hoxd13, is initially strongest in the posterior distal mesenchyme, with progressively less strong expression of Hoxd12 to Hoxd10. The Hoxd13 expression domain then spreads anteriorly, so that by E12.5 it is the only Hoxd gene for which expression is robust enough to be detectable on the most anterior side (Montavon et al., 2008). This is driven by enhancer elements including a ~40 kb global control region (GCR) located 180 kb 5’ (centromeric) of Hoxd13 beyond Evx2 and Lnp, and the Prox enhancer located between Evx2 and Lnp (Fig. 4A) (Spitz et al., 2003; Tschopp and Duboule, 2011). It is not clear whether late phase HoxD regulation involves polycomb-mediated repression.

Here, we use immortalised mesenchymal cells derived from either the anterior (A) or the posterior (P) distal limb of E10.5 embryos, which show high levels of Hoxd13 expression in the posterior-derived cells, to show that the ancestral role of polycomb in regulating the HoxD cluster appears to be maintained during distal limb development. There is a loss of H3K27me3 and a decompaction of higher order chromatin structure over HoxD in the distal posterior, compared with the anterior, limb cells. This is confirmed by analysis in dissected limb buds. Furthermore, we show a spatial colocalisation of the GCR and 5’ HoxD that is restricted to the distal posterior limb, consistent with the notion of physical association between this enhancer and its target genes. This is the first demonstration of differential chromatin compaction and enhancer-gene colocalisation across the A-P axis of the developing limb.

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MATERIALS AND METHODS

Immortal mouse cell lines
The posterior third and the anterior two thirds of distal forelimb buds were dissected from E10.5 mouse embryos from an Immortal mouse (H2k-tsA58 × CD1) cross (Jat et al., 1991). After washing in PBS, limb buds were treated using trypsin (0.2 g/l)/Versene for 15-20 minutes, and dispersed gently. Cells were plated into 6-well plates in DMEM, 10% foetal calf serum, 20 ng/ml γ-interferon (Peprotech) and grown at 33°C, the permissive temperature for the temperature-sensitive T antigen. A1/P1 and A2/P2 cell lines pairs were derived from separate litters.

RNA expression
RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer’s protocol [for limb buds, dissected anterior and posterior tissue was dissociated into single cells in Trizol using a syringe with a 25G (0.5 mm) needle (BD Microlance)], followed by phenol:chloroform extraction and digestion with 2U DNasel (Ambion) for 30 minutes at 37°C. cDNA was made using a First Strand Synthesis Kit (Roche) and amplified by PCR or real-time qRT-PCR using specific primers (supplementary material Table S1).

For expression arrays, 400 ng of RNA from A2/P2 cells were amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion). Amplified, biotinylated cRNAs (1.5 μg) were labelled and hybridised to Illumina MouseRef6 Gene Expression beadchip arrays (Gene Expression Omnibus (GEO) accession platform number GPL6887, expression data GSE38370). Data were analysed in R using limma (Smyth et al., 2005) and beadarray (Dunning et al., 2007) bioconductor packages. Probes with detection P-values less than 0.01 were excluded from further analysis. Signals were quantile normalised to remove technical variation, and differential expression was assessed using limma’s lmFit, eBayes and topTable function. P-values were corrected for multiple testing (Benjamini and Hochberg, 1995).

The 5944 genes with significantly differential A:P expression (adjusted P<0.05) were ranked by P-value and searched for enriched gene ontology (GO) terms using the Gene Ontology enrichment analysis and visualisation tool (GOrilla) (Eden et al., 2009). To avoid false positives P<10^-7 was set as the cut off for terms to be considered, based on the Bonferroni correction.

Acid extraction of histones and western blot analysis
Nuclei were isolated from 3-6×10^6 cells and histones acid-extracted and analysed as described (Eskeland et al., 2010).

Chromatin immunoprecipitation
Native chromatin immunoprecipitation (nChIP) from cell lines was performed as previously described (Eskeland et al., 2010), except that 40-46 Boehringer units of MNase ( Worthington) was used to digest 1-6×10^8 cells.

For tissue, distal anterior and posterior forelimb buds were dissected from 50-55 E10.5 embryos. Owing to the lower cell numbers (~10^7) 8-9 Boehringer units of MNase (Worthington) was used to digest 1-6 MNase (Worthington). Released chromatin (10-30 μg) was incubated with 3-5 μg prebound (to Protein A or G magnetic beads, Invitrogen) H3K27me3 antibody (Millipore) in the presence of 25 μg BSA for 3 hours at 4°C.

For Ring1B ChIP, 0.5-3×10^7 anterior and posterior limb tissue cells dissected from the distal forelimb buds of 70-75 E10.5 embryos were fixed with 1% formaldehyde (25°C, 10 minutes) and stopped with 0.125 M glycine. Sonication was as described (Eskeland et al., 2010). Released chromatin (30-50 μg) was incubated with 1 μg of prebound (Protein A magnetic beads, Invitrogen) Ring1B antibody (MBL, D139-3) or mouse IgG (Santa Cruz, sc-2025).

ChIP analysis
Relative quantification of ChIP by qPCR was as previously described (Eskeland et al., 2010) using primers described in supplementary material Table S2.
can be derived from the transgenic ‘immortomouse’, which expresses temperature-sensitive SV40 T antigen (Jat et al., 1991). Such cell lines appear to retain many biological properties of the cells from which they were derived, including gene expression patterns and response to signalling pathways (Kohn et al., 2010). We derived two sets of cell lines from dissected E10.5 forelimb buds to represent the most posterior third of the distal limb (cell lines P1 and P2) or the anterior two-thirds (A1 and A2) (Fig. 1A).

The embryonic limb bud consists of two main cell types: mesenchyme and the surface ectoderm, which at the most distal margin forms the apical ectodermal ridge (AER). The morphology of the cell lines indicated that they were likely to be mesenchymal in origin and RT-PCR analysis confirmed this (Fig. 1B). Fgf10 is expressed in limb bud mesenchyme and signals to the Fgfr2b receptor expressed in the AER to induce expression of Fgfb there. By contrast, Fgfr2c is expressed in both the mesoderm and ectoderm of the developing limb bud (Lizarraza et al., 1999; Duboc and Logan, 2011). The detection of mRNAs from Fgf10 and Fgfr2c, but not Fgfb or Fgfr2b in immortomouse-derived limb bud cell lines thus indicates their derivation from the limb mesenchyme (Fig. 1B). The origin of these cells from the distal, rather than proximal, margin of the limb is supported by the expression of Etv4 (Mao et al., 2009). Analysis by expression microarrays provided further evidence for this. Genes with proximally restricted expression domains, such as Tbx15, Cart1 (Akit – Mouse Genome Informatics), Emx2, Pax1 (chondrogenesis), Pax3 (myogenesis) (Kuijper et al., 2005), Vcan, Ebf2 (neurogenesis) (Krawchuk and Kania, 2008) and Shox (chondrogenesis, myogenesis, neurogenesis) (Vickerman et al., 2011), could not be detected (data not shown). We conclude that the four cell lines retain at least some of the specific development functions expected of their limb origin (Robert and Lallemand, 2006; Hill, 2007).

Expression microarrays provided further insight into the differences between the anterior- and posterior-derived cell lines (A2 and P2) (GEO accession number GSE38370). For example, levels of Etv4 and Etv5 mRNAs (known to be expressed in the distal limb mesenchyme and important for the posterior restriction of Shh expression) (Mao et al., 2009) were higher in the anterior cells than in posterior cells, whereas expression of other ETS factors that define the spatial boundary of Shh were higher in the posterior cells (Ets1) or similar (Gabpa) throughout the distal limb bud (Lettice et al., 2012) (Fig. 1C). Genes involved in mesenchymal-epithelial signalling and distal limb patterning were upregulated in anterior (Bmp4, Spry4) and posterior (Twist1) cells or expressed evenly across the A-P axis (Fgfb10). Among the genes with the highest ratio of expression in the posterior cells were those involved in retinoid signalling, and in chondrogenesis, osteogenesis or myogenesis (Fig. 1C).

Gene ontology analysis (GOriilla) indicated that the most significantly different A/P expression levels were for genes enriched for GO Biological Process terms (P<10⁻⁹) such as A-P axis and pattern specification, anatomical structure morphogenesis and embryonic skeletal system morphogenesis – categories that reflect the cell lines’ origins from mesenchymal tissue of the A-P axis of a developing appendage (supplementary material Fig. S1).

**Cell lines reflect differential 5’ Hoxd gene expression in distal posterior limb bud**

Spatial expression domains of Hoxd genes in E10.5 limb buds are well characterised (Spitz et al., 2003; Spitz et al., 2005; Tarchini and Duboule, 2006; Zakany and Duboule, 2007). We used RT-PCR and qRT-PCR on both sets of cell lines and on tissue dissected from E10.5 anterior and posterior forelimb buds to determine how relevant the limb cell lines are for analysis of HoxD spatial regulation. Hoxd1 mRNA was not detected in any of the cell lines and only very faintly in anterior limb tissue (Fig. 2A). Hoxd5 expression was detected at generally low levels (Fig. 2A) that are similar between the anterior and posterior limb tissue and the corresponding cell lines, except for even lower levels in P2 cells (Fig. 2B). There was no significant A/P difference in Hoxd3 expression in either cell line pairs or limb tissue (Fig. 2C). Conversely, at the 5’ end of HoxD, Hoxd13 expression levels were 17-fold higher in posterior limb and limb-derived cell lines than in anterior equivalents (Fig. 2C). A2/P2 cell lines showed expression levels similar to those in anterior and posterior tissue, but levels in A1/P1 were both proportionally lower (Fig. 2B). We observed slightly higher Hoxd12 expression in the posterior limb tissue than in the anterior, and higher Hoxd11 in P1 compared with A1 cells, but their expression levels of were both too low for reliable quantification. Hoxd10 expression showed a high A/P difference in both cell line pairs; the apparent large difference (Fig. 2C) measured in the A1/P1 cell lines is due to the very low expression levels in A1. Hoxd8 expression showed an A/P differential (threefold higher in P2 than in A2) in the second cell line pair only due to its increased expression in P2 cells, expression

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**Fig. 1. Characterisation of cell lines from distal posterior and anterior mouse forelimb.** (A) Schematic of E10.5 forelimb bud showing the dissection into anterior (A) and posterior (P) distal regions. (B) RT-PCR to detect the expression of mesenchymal (Fgf10, Fgf2cR, Etv4) and epithelial (Fgfb, Fgf2cR) markers in immortomouse cell lines derived from the anterior (A) or posterior (P). The two cell line pairs derive from embryos from different litters. Primer sequences are indicated in supplementary material Table S1. –, negative control lacking reverse transcriptase. (C) Log2 A/P expression from microarray analysis of A2/P2 cells for selected genes categorised according to their function.
levels in A2, A1 and P1 being similar to limb tissue (Fig. 2B,C). No A/P difference in Hoxd8 expression was detected in the E10.5 limb tissue. This suggests that both cell line pairs capture cells that have activated 5′ Hoxd genes specifically in the posterior compartment at the start of the second wave of HoxD activation in the distal limb and that this activation has a greater extension 3′ in the 2P cell line (to Hoxd8) than it does in 1P cells (Hoxd10). Gene activation extends more 3′ in both posterior-derived cell lines than in the limb tissue. This could be due to the outgrowth of cells that have more extensive gene activation than the average in the posterior limb or, more likely, that the regulatory mechanisms driving progressive 5′-3′ HoxD activation continue operating for some time after tissue dissection and during cell immortalisation.

Beyond Hoxd13, Evx2 expression was not be detected in cell lines (data not shown) suggesting that the cells might originate from outside of the small Evx2 expression domain at the extreme distal margin of the E10.5 forelimb bud (Tarchini and Duboule, 2006). Shh expression was also not detected, suggesting that it is not required for maintenance of the second wave of HoxD activation, at least in cell lines, as has been previously proposed (Harfe et al., 2004).

Although both immortomouse-derived distal limb cell line pairs show an A/P difference in 5′ HoxD activity, the A2/P2 pair show a more extensive domain of activation (Hoxd13 to Hoxd8) and so were chosen for further study.

**Loss of H3K27me3 over the HoxD cluster in posterior cells**

The PRC2 polycomb complex is fundamental to regulation of Hox gene clusters during ES cell differentiation and early embryogenesis and is responsible for blanketing HoxD with H3K27me3 (Boyer et al., 2006; Lee et al., 2006). Polarised (3′-5′) loss of H3K27me3 accompanies 3′ Hoxd gene activation during ES cell differentiation (Eskeland et al., 2010) and in the tail bud of the primary axis during early embryogenesis (Soshnikova and Duboule, 2009). PRC2 function in the limb is required for cell survival and for proximodistal elongation of the limb (Wyngaarden et al., 2011), but whether differential polycomb-mediated chromatin changes are involved in regulating A-P Hoxd gene expression during the late phase of distal limb patterning is unclear.

Immunoblotting shows similar H3K27me3 levels globally in A2 and P2 cells (Fig. 3A). Using native ChIP (nChIP) combined with custom high-density tiling arrays covering multiple regions of the mouse genome, including Hox loci (Eskeland et al., 2010), we determined the H3K27me3 profile of the A2/P2 distal forelimb-derived cell lines (Fig. 3B) (GEO accession number GSE38526).

H3K27me3 is pervasive over HoxD in anterior cells, with both the 5′ and 3′ (Hoxd1-d4) genes densely covered (Fig. 3B). The dip in H3K27me3 between Hoxd4 and Hoxd8, is similar to that seen in ES cells (Eskeland et al., 2010). Whereas H3K27me3 still blankets Hoxd1-d3 in posterior (P2) cells, it is largely absent over the 5′ genes (Fig. 3B,C, upper two tracks). Beyond Hoxd13, H3K27me3 covers Evx2 in both cell lines. In contrast to ES cells, in which both HoxB and HoxD loci are both blanketed (Eskeland et al., 2010), H3K27me3 is largely absent from HoxB in A2 and P2 cell lines, with only Hoxb13 enriched, emphasising the particular regulation of HoxD across the A-P limb axis. As controls, the Pax6 polycomb target was blanketed with H3K27me3 in both anterior and posterior, whereas H3K27me3 was absent from the widely expressed Brd3.

Of those arrayed probes significantly enriched in H3K27me3 in anterior cells (log2 H3K27me3/input≥1) (Fig. 3E, upper pie chart) only 8% are from HoxD. Yet 93% of probes with an at least twofold (log2≥1) A/P difference in H3K27me3 enrichment are from this locus and 88% are from 5′ HoxD (Fig. 3E, lower pie chart). By contrast, only 2% of probes with at least twofold A/P H3K27me3 difference are from HoxA. Therefore, extensive A/P differences in H3K27me3 are specific to HoxD in these cell lines. Quantitative PCR (qPCR) confirmed the lower H3K27me3 levels in P2 versus A2.
Fig. 3. H3K27me3 and Ring1B distribution in E10.5 limb cell lines and mouse forelimb tissue. (A) Western blot of H3K27me3 in A2 and P2 cells. Levels of H3 are shown for comparison. (B) Mean log2 H3K27me3/input at HoxD, HoxB, Pax6 and Brd3 loci in A2 and P2 cell lines (top two rows, n=2 biological replicates) and for anterior and posterior forelimb tissue (bottom two rows, n=4 – 2 biological and 2 technical – replicates). (C) As in B, but with an expanded view of the HoxD cluster. (D) Mean log2 Ring1B/input at the HoxD region for anterior and posterior forelimb tissue. n=2 biological replicates. (E) Pie charts showing the genomic distribution of different probes categories enriched for: (top) H3K27me3 in A2 cells (log2 H3K27me3/input≥1) versus (bottom) the proportion with an A2/P2 difference of log2≥1. (F) Mean (± s.e.m.) log2 H3K27me3/input at HoxD and neighbouring genes and promoters in distal forelimb anterior and posterior tissue. Average log2 values were calculated from each individual probe value that covered the genomic locations. The statistical significance of A:P differences in H3K27me3 enrichment over each gene and promoter were examined by two-sample t-test (*P<0.01, **P<0.0001).
cells at Hoxd10 (Fig. 4A), with a less dramatic decrease at Hoxd1. There was no significant A-P difference in H3K27me3 at Olig2 (positive control) or Actb (β-actin, negative control).

The limb cell lines are apparently a relatively homogenous mesenchymal cell population. By contrast, more heterogeneous cell populations are inevitably present in dissected limb tissue, where AER- and differentiating mesenchyme-derived cells expressing different cohorts of Hox genes might mask differences in H3K27me3 levels between the anterior and posterior mesenchyme. Nevertheless, we analysed H3K27me3 in E10.5 anterior and posterior dissected distal forelimbs (Fig. 3B,C, lower two tracks) by nChIP. An H3K27me3 block covered the 3’ end of HoxD (d1-d4) in both limb regions. Both anterior and posterior limb samples also show a second block of modification over the 5’ end of HoxD, from Hoxd9 through to Evx2, but this was at a significantly higher level in anterior compared with the posterior region (Hoxd3-Hoxd10; P=0.0001) (Fig. 3F; supplementary material Fig. S2A). Whereas in the anterior distal limb Hoxd27me3 remains high from Evx2 to beyond Hoxd12, in the posterior H3K27me3 declines from the Evx2-Hoxd13 intergenic region up to Hoxd11 (Fig. 3C,F). This A-P difference in H3K27me3 levels in limb tissue is specific to 5’ HoxD (supplementary material Fig. S2B), being significantly greater (P<0.0001) than that for all Hox loci combined. The A-P difference for the 3’ HoxD region is not significant (P=0.57). H3K27me3 was more pervasive over the HoxB locus in cells dissected from the anterior or posterior limb tissues than in the cell lines (Fig. 3B), probably reflecting the heterogeneity of the former cell populations, and, unlike for HoxD, there were no differences between H3K27me3 at HoxB in anterior versus posterior limb.

Lower H3K27me3 levels at the Hoxd10 promoter in the posterior versus anterior limb tissue cell populations was confirmed by qPCR, whereas levels at the Hoxd1 promoter were similar in both distal limb samples (Fig. 4B). These data are consistent with a role for polycomb-mediated repression in regulating A-P differences in Hoxd expression during the patterning of the distal limb.

**PRC1 levels are reduced over HoxD in distal posterior cells**

The PRC1 complex recognises and binds to H3K27me3-modified chromatin to bring about chromatin compaction and gene repression. The ChIP profile of the PRC1 component Ring1B (Rnf2 – Mouse Genome Informatics) correlates with that of H3K27me3 in E10.5 anterior and posterior dissected limb bud tissues at HoxD (Fig. 3C,D), HoxB and Pax6 (supplementary material Fig. S2C). A block of Ring1B extends from Evx2 to Hoxd9 in anterior distal limb, whereas in posterior tissue Ring1B coverage is more sparse over Hoxd13-11. The A/P difference in Ring1B levels was significant throughout 5’ HoxD (promoters, genes and intergenic; P<0.0001) (supplementary material Fig. S2D) and was greater than at all the Hox loci combined (P<0.0001) (supplementary material Fig. S2E).

**A-P differences in chromatin compaction at HoxD in the distal limb bud**

PRC1 brings about a chromatin compaction at target loci that is detectable by measuring the spatial separation of fluorescence in situ hybridisation (FISH) signals from closely apposed probe pairs (Eskeland et al., 2010) (Fig. 5A). Using these approaches, a decompaction of HoxD is seen as Hox genes are activated during ES cell differentiation and in the tail-bud of the embryo (Morey et al., 2007). We first used 2D FISH to assay chromatin compaction (d²) across HoxD in limb bud cell lines. Any difference in inter-probe distances due to variation in nuclear size between cell lines was also assessed by normalising d² to the nuclear radius (r²). However, in practice the same conclusions were reached when considering inter-probe separation without normalisation to nuclear size (supplementary material Table S4).

Chromatin across HoxD (Hoxd3-Hoxd13) was significantly less compact in both distal posterior cell lines compared with the anterior cell lines (P=0.0002 and P=0.03 for A1/P1 and A2/P2, respectively) (Fig. 5B; supplementary material Table S4). This was restricted to the region with differential H3K27me3, as there is no significant A-P difference in compaction of the GCR-Lnp region 5’ of HoxD where similarly low levels of H3K27me3 are seen between A and P cell lines (Fig. 3B). Nor was there differential compaction at a control region around Pax6 (Rcn-Rpl10) on the same chromosome (MMU2) as HoxD, and which contains genes with no known role in limb development and no differential expression between A and P cell lines.

We sought confirmation of differential HoxD compaction by 3D FISH in E10.5-11.0 embryo tissue sections cut through the anterior and posterior regions of the forelimb (Fig. 6A). Here, not only were we able to compare the distal anterior and posterior regions, but also more proximal limb regions and indeed the flank region adjacent to the forelimb bud tissue where Hox genes are not expressed. As observed in the cell lines, HoxD chromatin was significantly less compact (d²) at the distal posterior forelimb bud compared with the distal anterior (P=0.0008) but also compared with other limb and non-limb regions analysed (proximal posterior, P=0.02; proximal anterior, P=0.0002; flank, P=0.0002) (Fig. 6B; supplementary material Table S5). There was no significant difference in HoxD chromatin compaction between any of the other limb regions or even between these other limb regions and the flank tissue. Likewise, no significant differences in chromatin compaction could be identified between any of the tissue regions, including the distal forelimb bud, at the GCR-Lnp and the Rcn-Rpl10 control regions (Fig. 6B; supplementary material Table S5).

Analysis of the frequency of hybridisation signals separated by defined distance (d) intervals also demonstrates the less compact chromatin conformation of the HoxD locus compared with the adjacent GCR-Lnp region. The proportion of colocalised signals (<200 nm) was less for Hoxd3-Hoxd13 than both GCR-Lnp (with a similar genomic distance) and the Rcn-Rpl10 control region, which has a larger genomic distance separating the two probes (supplementary material Fig. S3). Conversely, the proportion of signal pairs >400 nm apart was greater for Hoxd3-Hoxd13 than for the other two regions. The subpopulation of HoxD probe pairs separated by >600 nm
(~25%) was significantly greater in the distal posterior forelimb compared with the rest of the limb regions and the adjacent flank (distal anterior, P=0.0001; proximal posterior, P=0.0001; proximal anterior, P=0.001; flank, P<0.0001) (supplementary material Fig. S3 and Table S6). We conclude that chromatin unfolding at HoxD accompanies the start of the second wave of Hoxd gene activation during limb development.

**Nuclear colocalisation of the GCR and 5’ HoxD in distal posterior cells**

Developmental stage E10.5 marks the start of the later phase of distal limb development, characterised by ‘quantitative collinearity’ in which strong expression of Hoxd13 is initiated in the distal margin of the posterior mesenchyme. This is driven by enhancer elements located 5’ of HoxD, the best characterised of which is the GCR ~180 kb centromeric of Hoxd13 (Fig. 3B, Fig. 5A) (Montavon et al., 2008).

One possible mechanism for long-range cis-regulation invokes the spatial colocalisation of the enhancer and target promoter (Williamson et al., 2011). Indeed, modelling of gene expression changes that occur as a consequence of 5’ HoxD deletions led to the suggestion that the first step in 5’ Hoxd gene activation in the distal posterior limb bud might be a long-range interaction (looping) between regulatory sequences such as GCR and the Hoxd13 region (Montavon et al., 2008). To test this model, we analysed the hybridisation signals for the GCR and Hoxd13 regions and determined the proportion that colocalised (d<200nm). Multiple areas within the E11.0 limb bud were analysed (Fig. 7A), including those where late phase gene activation initiates (distal posterior); where it is poised for later activation (distal anterior); where the early phase of 3’-5’ Hoxd gene activation, which does not depend on the GCR, has occurred (proximal limb); and then in the control flank mesoderm.

In contrast to the Hoxd3-d13 region, average GCR-Hoxd13 interprobe distances (d2) were significantly smaller in the posterior distal limb bud compared with distal anterior (P=0.04) and also compared with the flank (P<0.0001), the proximal anterior (P=0.02) and proximal posterior (P=0.002) (Fig. 7B; supplementary material Table S7). This was not seen for distances between GCR and a probe at the 3’ end of Lnp (Fig. 6B) indicating that there is not a simple compaction of the whole region 5’ of Hoxd13. Moreover, the proportion of alleles for which GCR and Hoxd13 probe signals were spatially colocalised (d<200nm) was far higher in posterior distal limb (>30%) than in all other areas.
tested (Fig. 7C; supplementary material Fig. S4A and Table S8). These data are consistent with the formation of a chromatin loop between \(Hoxd13\) and the GCR regulatory module at the time and place of distal limb bud development when the GCR participates in initiating HoxD collinearity and \(Hoxd13\) expression.

Recently, 4C analysis was used to identify sequences that could be captured together with \(Hoxd13\) by cross-linking in tissue from across the distal limb at E12.5, a later stage of limb development than that studied here (Montavon et al., 2011). This identified multiple new potential regulatory regions in the extensive gene desert centromeric of HoxD and located far beyond the GCR. It was suggested that these elements might simultaneously interact with each other and with 5’ HoxD in the distal limb to form a compact regulatory hub. One of these new elements, island III, is ~200 kb centromeric of the GCR. We measured the physical separation between the island III region and the GCR – a genomic distance equivalent to that separating GCR from \(Hoxd13\) (~200 kb) – and also between the island III region and \(Hoxd13\) (Fig. 5A) in E11.0 limb buds. We did not detect any significantly increased frequency of colocalisation between island III and \(Hoxd13\) or between island III and GCR in posterior distal limb, compared with other forelimb regions (Fig. 7C; supplementary material Fig. S4B,C and Table S8). However, average interprobe distances (\(d^2\)) between 5’ HoxD and island III are similar to those between 5’ HoxD-GCR and GCR-island III despite the former being double the genomic distance, and the compaction of the island III-Hoxd13
region is higher in distal limb than in the proximal region (Fig. 7; supplementary material Table S7). These data suggest that the entire ~400 kb genomic region from the 5’ end of HoxD into the centromeric gene desert is configured in a relatively compact, yet flexible, conformation.

DISCUSSION

Differential 5’ Hoxd gene expression across the limb A-P axis in mouse E10.5 embryos

We have shown that immortalised cell lines derived from the anterior versus posterior E10.5 distal limb sustain global expression patterns consistent with their mesenchymal origin from the limb during A-P axis specification (Fig. 1). Moreover, they maintain some of the differential Hoxd gene expression patterns of the temporal developmental stage from which they are derived (Fig. 2). Limb-specific secondary activation of HoxD initiates in the distal posterior forelimb bud at ~E10.5 in the mouse. We confirmed that 5’ Hoxd gene expression is significantly higher in distal posterior E10.5 cells relative to distal anterior (Fig. 2), commensurate with the spatial and temporal aspects of late phase collinearity (Spitz et al., 2003). This A-P difference is lost by E11.5-12.5 as the 5’ genes are activated in more distal anterior regions, but with Hoxd13 still being the most strongly expressed (supplementary material Fig. S5) (Montavon et al., 2008).

A model for late phase HoxD activation in the distal limb proposes a reciprocal activation pathway involving restriction of earlier 5’ Hoxd expression to the posterior margin of the limb bud that then ensures posterior-only Shh activation, which in turn effects the initiation of secondary 5’ Hoxd expression in the distal posterior limb bud (Harfe et al., 2004; Robert and Lallemand, 2006). We did not detect Shh expression in either (P1 or P2) posterior-derived cell lines, which nonetheless maintain upregulated 5’ Hoxd gene expression, especially of Hoxd13 (Fig. 2). However, late phase Hoxd gene expression still occurs in Shh−/− Gli3−/− mice, and is only reduced in Shh−/− mice (Litingtung et al., 2002). Shh expression prevents Gli3 repression of 5’ Hoxd genes, both by repressing Gli3 expression posteriorly and by antagonising Gli3 processing to Gli3R (repressor form). Gli3R has been suggested to be the repressor of 5’ Hoxd gene expression in anterior limb (Tarchini and Duboule, 2006). It is likely that posterior Shh expression in the embryonic limb, up to the point of cell immortalisation, was sufficient to activate late phase HoxD expression. The subsequent strong maintenance of Hoxd13 expression in the posterior limb-derived cell lines suggests that Shh is not required for maintaining HoxD activity. Lack of Shh expression in cell lines could be due to the loss of interaction between the mesenchyme and the overlying AER (Laufer et al., 1994; Ogura et al., 1996).

Posteriorly restricted Hoxd expression is accompanied by chromatin decompaction and reduced polycomb binding

We identified differential H3K27me3 over 5’ Hoxd genes between anterior- and posterior-derived limb cell lines. In anterior cells, there is extensive H3K27me3 over Hoxd13 towards Hoxd10 that is
absent from posterior cells (Fig. 3). That this contributes to the repression of 5’ Hoxd genes in the anterior distal limb is consistent with the ectopic anterior expression of Hoxd11 and Hoxd13 in E10.5 limb buds after ablation of the PRC2 component Ezh2 (Wyngaarden et al., 2011). As Hox genes have been transcriptionally active during the earlier phase of limb development, we cannot determine whether there is active H3K27me3 removal from 5’ HoxD in cells of the posterior distal E10.5 limb, or re-establishment of H3K27me3 in anterior distal cells. The presence of H3K27me3 over 3’ HoxD in both anterior and posterior cell lines and in limb tissue probably represents a re-imposition of this histone mark after the initial phase of Hoxd gene expression in early, proximal limb bud development (Fig. 3).

Late phase 5’ Hoxd gene expression in the posterior distal limb bud is also accompanied by reduced Ring1B binding (Fig. 3; supplementary material Fig. S2). Consistent with a requirement for PRC1 to maintain Hox clusters in a silent, compact chromatin state in ES cells (Eskeland et al., 2010), we found differential chromatin compaction at HoxD both between the anterior- and posterior-derived limb cell lines and in limb tissue (Figs 5, 6), with a greater degree of decompaction in cells from the posterior distal region than in cells measured in any other region of the limb bud at this stage of development (Fig. 6). Our data suggest that dynamic polycomb-mediated control of HoxD expression and higher-order chromatin structure, previously described as occurring along the main A-P body axis (Morey et al., 2007; Soshnikova and Duboule, 2009) is recapitulated during limb-specific Hoxd gene expression.

**Enhancer-promoter looping restricted to the posterior distal region of the limb bud**

We observed a closer average proximity, and higher absolute colocalization frequency, of the GCR to Hoxd13 (Montavon et al., 2008). Our analysis at single cell resolution is consistent with the formation of a chromatin loop between enhancers in ES cells (Chambeyron et al., 2011), although there was no analysis of anterior versus posterior regions of the limb in that study. By contrast, we did not find significantly increased colocalisation of 5’ HoxD and a more distal regulatory region (island III) (Fig. 7C; supplementary material Fig. S4C) previously identified by 4C analysis. However, that study was conducted at a later developmental stage (E12.5) and any colocalisation might therefore occur later than E11. Our analysis does indicate that the ~400 kb region extending out from the 5’ HoxD locus into the adjacent gene desert is in a generally compact chromatin conformation in all the limb regions tested (Fig. 7A,B; supplementary material Table S7). Thus, the entire regulatory domain may be configured to minimise the nuclear search space for enhancer-gene interactions to then occur at the correct time and place. Further analyses over the entire temporal stage of late phase HoxD activity (E10.5-12.5) and across the A-P distal axis should clarify the spatial and temporal specificity of the multiple regulatory elements. We could not assess the colocalisation of the Prox regulatory element (Fig. 5A) as it is too close to Hoxd13 for us to resolve by FISH.

This article demonstrates that complex higher-order chromatin dynamics is implicated in the regulation of Hoxd gene expression during distal limb development, with two different processes occurring: chromatin decompaction and looping. It also establishes immortomouse-derived cell lines as a tractable model system to investigate chromatin states in relation to spatiotemporally regulated gene expression in development. This is the first demonstration of differential chromatin compaction and enhancer-gene colocalisation across the A-P axis of the developing limb, as a previous study analysing the spatial colocalisation of Shh and its limb enhancer reported similar colocalisation in both posterior (Shh-expressing) and anterior (no Shh expression) limb regions (Amano et al., 2009).

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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