**Gli3 (Xt) and formin (ld) participate in the positioning of the polarising region and control of posterior limb-bud identity**

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**SUMMARY**

During initiation of limb-bud outgrowth in vertebrate embryos, the polarising region (limb-bud organizer) is established upon activation of the Sonic Hedgehog (SHH) signaling molecule at the posterior limb-bud margin. Another hallmark of establishing anteroposterior limb-bud identities is the colinear activation of HoxD genes located at the 5’ end of the cluster (5’HoxD genes). The unique and shared functions of Gli3 and formin in these determinative events were genetically analyzed using single and double homozygous Extra-toes (Xt; disrupting Gli3) and limb deformity (ld; disrupting formin) mouse embryos. Analysis of the limb skeletal phenotypes reveals genetic interaction of the two genes. In addition to loss of digit identity and varying degrees of polydactyly, proximal skeletal elements are severely shortened in Xt;ld double homozygous limbs. The underlying molecular defects affect both establishment of the polarising region and posterior limb-bud identity. In particular, the synergism between Gli3- and formin-mediated mesenchyme-AER interactions positions the SHH signaling center at the posterior limb-bud margin. The present study shows that establishment and positioning of the polarising region is regulated both by restriction of Shh through Gli3 and its positive feedback regulation through formin. Concurrently, Gli3 functions independently of formin during initial posterior nesting of 5’HoxD domains, whereas their subsequent distal restriction and anterior expansion depends on genetic interaction of Gli3 and formin.

Key words: Mouse mutant, HoxA, HoxD, Limb bud, Patterning, Posterior limb identity, Shh activation

**INTRODUCTION**

Growth and patterning of vertebrate limb buds is controlled by epithelial-mesenchymal interactions of two main signaling centers. Apical ectodermal ridge (AER) signals promote outgrowth and maintain the posterior mesenchymal cells of the polarising region in an active state. The polarising region functions as a mesenchymal organizer expressing the Sonic Hedgehog (SHH) signaling molecule (reviewed by Cohn and Tickle, 1996; Johnson and Tabin, 1997). SHH signaling controls growth and patterning of distal limb structures including the autopod (digits; Riddle et al., 1993; Chiang et al., 1996). However, establishment of polarizing activity in the presumptive limb field long precedes Shh activation (Hornbruch and Wolpert, 1991). Experimental evidence indicates that combinations of Hox genes expressed by the flank mesenchyme position the presumptive limb field (Cohn et al., 1997) and that retinoid signaling induces polarizing activity (Lu et al., 1997; Stratford et al., 1997). In particular, Hoxb8 marks polarizing activity in the forelimb field and seems to participate in induction of SHH signaling during onset of limb-bud outgrowth (Charité et al., 1994). Furthermore, a positively acting AER factor has also been implicated in Shh activation (Grieshammer et al., 1996; Helms et al., 1996; Ros et al., 1996), but the mechanisms restricting Shh activation to the posterior limb-bud margin remained unknown.

Proliferation and commitment of limb-bud mesenchymal progenitors seem largely determined by differential expression of Hox genes located at the 5’ ends of the HoxA and HoxD clusters. For example, genetic analysis revealed both unique and redundant functions for paralogous 5’HoxA and 5’HoxD genes during limb-bud morphogenesis, which correlate well with distinct and overlapping distributions in limb buds (reviewed by Duboule, 1995; Johnson and Tabin, 1997). During the limb field to limb-bud transition, colinear activation of 5’HoxD genes results in their posteriorly restricted, nested early expression domains. During subsequent limb-bud outgrowth, the 5’HoxD domains expand anterior within the distal mesenchyme (late domains; reviewed by Duboule, 1992). These late 5’HoxD domains control formation of the autopod as indicated by genetic and evolutionary studies (Dollé et al., 1993; Sordino et al., 1995).

Analysis of classical mouse mutations has proven helpful in understanding the genetic hierarchies that control determinative steps during vertebrate limb pattern formation (reviewed by Niswander, 1997). In particular, alleles of the
haploinsufficient mouse Extra-toes (Xi) mutations (Johnson, 1967) disrupt the Gli3 gene (Hui and Joyner, 1993; Schimmang et al., 1992) and mutations in its human homologue cause several distinct genetic disorders. All known mutations in both human and mouse Gli3 genes affect limb morphogenesis among other developmental processes (reviewed by Biesecker, 1997). The polydactyly of Xr heterozygous (Xr/Xt) limbs was interpreted as being caused by induction of an ectopic anterior SHH cascade (polarizing activity) which in turn leads to anterior ectopic 5'HoxD expression during hand plate formation (Masuya et al., 1995; Buescher et al., 1997). Activation of Gli3 in the limb field slightly precedes initiation of limb-bud outgrowth and persists in all limb mesenchymal cells with exception of Shh-expressing cells (Marigo et al., 1996; Masuya et al., 1997). Their mutually exclusive distribution and the repression of Gli3 by retrovirus-mediated ectopic Shh in chicken limb buds led to the proposal that Gli3 and SHH repress one another (Masuya et al., 1995; Marigo et al., 1996).

In contrast, several recessive and most likely hypomorphic alleles of the mouse limb deformity (ld) mutation disrupt the formin gene (Maas et al., 1990; Woychik et al., 1990) and alter the patterning of distal limb skeletal elements, resulting in fusion and loss of digits (syndactyly). The positive SHH/FGF-4 feedback loop, controlling morphogenesis of distal limb structures (Lauffer et al., 1994; Niswander et al., 1994) is disrupted in ld homozygous (ld/ld) limb buds, which results in reduced polarising activity (Chan et al., 1995; Haramis et al., 1993; Kuhlman and Niswander, 1997). This disruption is due to a primary mesenchymal defect (Kuhlman and Niswander, 1997) preventing complete differentiation of the AER (Zeller et al., 1989) and the induction of Fgf-4 in the posterior AER. Taken together these studies establish that mesenchymal formin functions primarily in establishment of the signalling interactions between the polarizing region (SHH) and the AER (FGF-4). Most likely as a consequence of disrupting these mesenchyme-AER interactions, the transition from early to late 5'HoxD domains is delayed in ld/ld limb buds (Haramis et al., 1995). Consistent with a genetic function in establishing the SHH/FGF-4 feedback loop, formin is expressed by posterior and distal limb-bud mesenchymal cells (Trumpp et al., 1992; Chan et al., 1995). High levels of formin transcripts are also expressed by the AER during early limb-bud outgrowth, but transgene-mediated formin re-expression in the ld mutant AER fails to rescue the SHH/FGF-4 feedback loop and limb-bud patterning (A. G. Haramis, D. James, K. Brennan and R. Zeller, unpublished data). Furthermore, targeted disruption of the predominant formin isoform in the AER does not disrupt limb morphogenesis (Wynshaw-Boris et al., 1997). These studies agree with those of Kuhlman and Niswander (1997) and strengthen the conclusion that only mesenchymal formin is essential to establish the SHH/FGF-4 feedback loop.

Possible interactions and/or unique functions of Gli3 and formin during early limb-bud patterning were genetically studied by comparative analysis of both single and Xt/ld (Xt/Xt;ld/ld) double homozygous embryos. Our studies show that Gli3 functions initially independent of formin to posteriorly restrict 5’HoxD genes during onset of limb-bud outgrowth. In contrast, concurrent positioning and establishment of the Shh-expressing polarising region at the posterior limb-bud margin is controlled by a synergistic genetic interaction of Gli3 and formin. Transacting Gli3 and formin also regulate aspects of the transition from early to late 5’HoxD domains and of the differential distribution of 5’HoxD from 5’HoxA paralogues.

MATERIALS AND METHODS

Genetic crosses and genotyping of embryos

Previous analysis of the ld limb-bud phenotype established that different ld alleles disrupt the SHH/FGF-4 signaling feedback loop in the same way (Haramis et al., 1995; Chan et al., 1995). Therefore, the ldln2 allele was used because the genotypes can be easily determined by PCR (see below). All strains were maintained in a mixed C57Bl6/JL L1 background. Two alleles of the Xt mutation were used: in the Xt allele, the 3’ part of the Gli3 gene is deleted (Hui and Joyner, 1993), whereas the 5’ part of the Gli3 open reading frame is deleted in the Xt allele (Schimmang et al., 1992). Both Xt alleles display identical polydactylosus limb phenotypes in homozygous embryos (Hui and Joyner, 1993). Therefore, most analysis was performed by intercrossing ldln2 mutant mice with the Xt strain, as offspring can easily be PCR genotyped using genomic DNA isolated from embryonic heads (Haramis et al., 1995; Buescher et al., 1997). However, all results were confirmed by also analyzing ldln2/XtH intercrosses. No molecular differences were detected between limb buds of the following genotypes: wild-type (+/+; +/+, Xt/+; ld/+; Xt;+; +/+, +/+; ld/+; Xt;+; ld/+ and +/+; ld/+. Therefore, these genotypes were considered phenotypically wild type in agreement with previous studies detecting ectopic Shh only in Xt/Xt limb buds (Buescher et al., 1997). Similarly, no differences were detected between Xt/Xt;+/+ and Xt/Xt;ld/+ or ld/ld;+ and ld/ld; Xt/+ limb buds.

Age-matching of embryos

ld/+;Xt/+ females were crossed with either ld/+;Xt/+ or ld/ld; Xt/+ males to obtain single and double mutant litter mates. The day of vaginal plug detection is defined as embryonic day zero and embryos were age-matched by somite numbers (variation: ±2 somites; see Haramis et al., 1995).

Skeletal preparations

Due to perinatal lethality of both Xt/Xt (Johnson, 1967) and Xt/ld double homozygous embryos, the pattern of limb skeletal elements was analyzed during embryonic day E15, the oldest developmental stages at which these genotypes were recovered at expected ratios. Embryos were stained for cartilage and bones using standard Alcian blue and Alizarin red staining.

Whole-mount in situ hybridization

Whole-mount in situ hybridization (Haramis et al., 1995) was performed using the following probes: Shh (Echelard et al., 1993), Hoxa11 and Hoxa13 (Small and Potter, 1993), Hoxd9 to Hoxd13 (Dollé et al., 1989), Hoxb8 (Charié et al., 1994), Ptc (Platt et al., 1997), Gli3 (Buescher et al., 1997) and Gli (Hui et al., 1994). Formin transcripts were detected using a riboprobe complementary to their 3’ part (1.7 kb SstI-EcoRI probe, Woychik et al., 1990). For comparative studies, embryos were always hybridized in the same experiment to avoid variation. Reproducibility of all results was established by analyzing several embryos in independent experiments.

RESULTS

Xt/ld double homozygous limbs display a loss of digit identity and truncations of proximal skeletal elements

Possible direct dependence of formin on Gli3 (and vice versa) was examined by studying the distribution of formin transcripts
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in Xt/Xt and Gli3 transcripts in ld/ld limb buds. Fig. 1 shows that no significant alterations of formin transcripts are detected by comparing wild type (Fig. 1a) to Xt/Xt mutant limb buds (Fig. 1b). Despite the fact that Shh expression is not upregulated in ld/ld limb buds (Chan et al., 1995; Haramis et al., 1995; Kuhlman and Niswander, 1997), Gli3 remains excluded from posterior limb-bud mesenchyme in both wild-type (Fig. 1c; see also Masuya et al., 1997) and ld/ld limb buds (Fig. 1d). During the developmental stages relevant to this study (prior to embryonic day 11, see below), neither formin nor Gli3 expression is directly affected in either Xt/Xt or ld/ld limb buds, respectively.

Possible synergistic genetic interactions of formin and Gli3 were uncovered by analyzing the limb phenotypes of Xt;ld double homozygous embryos (Fig. 2). Wild-type mouse limbs of embryonic day 15 have five distinct digits (Fig. 2a), whereas ld/ld limbs are syndactylosus (Fig. 2b; see also Woychik et al., 1985). In contrast, Xt/Xt limbs display varying degrees of preaxial polydactyly and associated loss of digit identities (Fig. 2d,e; see also Johnson, 1967). Only rudimentary digits form in Xt;ld double homozygous limbs (Fig. 2g,h). The autopods of such limbs often display partial polydactyly and syndactyly (Fig. 2g). Their rudimentary digits lack phalanges, which indicates that coordinated outgrowth and patterning of distal limb skeletal elements are affected, resulting in autopods with apparently little (Fig. 2g) or no discernible anteroposterior polarity (Fig. 2h). Most strikingly, the stylopods are always severely reduced (humerus, white arrowhead, Fig. 2i; femur, not shown) and an enlarged foramen is present in the scapula of all Xt;ld double homozygous limbs (black arrowhead in Fig. 2i). In addition, ossification of all skeletal elements is always severely delayed as only a single ossification center has formed by this developmental stage (compare arrows, Fig. 2i to c,f).

Gli3 controls initial posterior restriction of the 5’HoxD domains in limb buds independent of formin

Sequential activation of 5’HoxD genes results in their distinct, posteriorly nested early expression domains in wild-type limb
buds (Fig. 3a,e; reviewed by Duboule, 1992). These early 5’HoxD domains are correctly established in ld/ld limb buds (Fig. 3b,f; see also Haramis et al., 1995). However, in limb buds of both Xt/Xt (Fig. 3c,g) and Xt;ld double homozygous embryos (Fig. 3d,h) posterior restriction and characteristic nesting of the early Hoxd11 and Hoxd13 domains are completely lost. No significant differences are observed between Xt/Xt and Xt;ld double homozygous limb buds (compare Fig. 3c,g to 3d,h; Hoxd12, data not shown), establishing that Gli3-mediated repression controls posterior nesting of 5’HoxD genes independent of formin. Furthermore, no striking changes of 5’HoxD distributions are observed in the posterior trunk of Gli3-deficient embryos, indicating that colinear activation of 5’HoxD genes is not affected (data not shown).

Gli3- and formin-mediated interactions regulate aspects of the transition from early to late 5’HoxD domains, but not of the spatial distribution of 5’HoxA paralogues

During subsequent limb-bud patterning, the 5’HoxD domains expand anteriorly and restrict distally in wild-type limb buds (transition to the ‘late domains’) as shown for Hoxd11 in Fig. 4a. As previously reported, this anterior expansion of 5’HoxD domains is delayed in ld/ld limb buds (Fig. 4b; for details see Haramis et al., 1995). Despite of the complete loss in initial posterior nesting (Fig. 3), distal restriction still occurs in the medial mesenchyme of Xt/Xt limb buds (arrow, Fig. 4c), giving the late 5’HoxD domains their U-shaped appearance (compare Fig. 4c to Fig. 3e for Hoxd11 and data not shown). In contrast, aberrant expression of the three 5’most HoxD genes (Hoxd11 to Hoxd13) persists in the proximal limb-bud mesenchyme of Xt;ld double mutant embryos (Hoxd11; arrow, Fig. 4d; Hoxd12 and Hoxd13, data not shown). Furthermore, the spatial distributions of 5’HoxD genes in Xt/Xt limb buds (Figs 3c,g, 4c) are reminiscent of the ones of their HoxA paralogues, which are expressed without anteroposterior, but with proximodistal restriction in wild-type limb buds (see e.g. Nelson et al., 1996). In particular, comparison of the Hoxd11 domain in Xt/Xt limb buds (Fig. 4c) to the Hoxa11 domain in wild-type limb buds (Fig. 4e) reveals the apparent similarity of their distributions during the transition from early to late domains. Furthermore, no changes are detected in the characteristic proximodistal restriction of 5’HoxA genes as the distributions of Hoxa11 and Hoxa13 transcripts are very similar between wild-type (Fig. 4e,g), Xt/Xt (Fig. 4f and data not shown) and Xt;ld double mutant limb buds (Fig. 4h and data not shown) during the same developmental period.

Gli3 and formin are part of the genetic mechanism that positions and upregulates the Shh expression domain at the posterior limb-bud margin

Deregulated 5’HoxD expression (Figs 3, 4) and disrupted autopod morphogenesis (Fig. 2) might reflect underlying defects in anteroposterior polarisation of the limb field and/or establishment of the polarising region. To detect possible alterations in initial limb field polarisation, the Hoxb8 distribution was studied in all relevant genotypes during onset of forelimb-bud outgrowth (Fig. 5a-d). No significant differences were observed by comparing wild-type (Fig. 5a) to either single (Fig. 5b,c) or Xt;ld double homozygous forelimb buds (Fig. 5d). Hoxb8 expression within the posterior compartment always extends to medial, but never anterior mesenchyme (arrowheads, Fig. 5a-d). During limb-bud emergence, Shh expression is activated by the mesenchymal cells of the polarising region, which are initially located at the posterior limb-bud margin (Riddle et al., 1993). In Xt/Xt limb buds, Shh expression is correctly initiated and upregulated to normal levels at the posterior limb-bud margin (Fig. 5e and data not shown) and weak ectopic anterior Shh expression is only apparent much later (around embryonic day 11 in forelimb buds, data not shown; for details see Masuya et al., 1995; Buescher et al., 1997). Activation and initial positioning of Shh is also normal in ld/ld limb buds (Fig. 5f), but subsequent upregulation and distal propagation of the Shh domain are disrupted (for details see Introduction). In contrast, Shh transcripts are barely or not at all detectable in most Xt;ld double homozygous limb buds analyzed. In fact, the limb buds shown in Fig. 5g,h are representative of the highest Shh levels detected by embryonic day 10.25 (32 to 34 somites). Most strikingly, Shh transcripts are not detected at the posterior limb-bud margin, but at variable subapical positions within the posterior half of the limb-bud mesenchyme (compare Fig. 5g to 5e,f) and are often displaced to the apex (Figs 5h, 6d; 5 of 10 embryos). However, Shh expression was never detected in the anterior half of the limb-bud mesenchyme. In addition, Fgf-4 is not expressed and Fgf-8 levels are reduced in Xt;ld double mutant AERs (data

![Fig. 3. Establishment of the posteriorly nested 5’HoxD expression domains during initiation of limb-bud outgrowth. (a-h) Forelimb buds of embryonic day 9.75 (28 somites) were hybridized with riboprobes to detect Hoxd11 (a-d) and Hoxd13 transcripts (e-h, contralateral limb buds). Asterisks mark the anterior margins of limb buds. (a,b) Hoxd11 is posteriorly restricted in wild-type (a) and ld/ld (b) limb buds. (c,d) Posterior restriction of Hoxd11 is lost in Xt/Xt (c) and Xt;ld double homozygous (d) limb buds. (e,f) Hoxd13 expression in wild-type (e) and ld/ld (f) limb buds. Note that the Hoxd13 domain is smaller than the Hoxd11 domain in the contralateral limb bud (revealing posterior nesting). (g,h) Posterior restriction of Hoxd13 is lost in Xt/Xt (g) and in Xt;ld double homozygous limb buds (h).]
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not shown) as previously described for ld/ld mutant AERs (Chan et al., 1995; Haramis et al., 1995; Kuhlman and Niswander, 1997).

Transcriptional activation of two mesenchymal targets of SHH signaling, Ptc and Gli (Marigo et al., 1996; Platt et al., 1997) was analyzed to determine whether low and/or displaced Shh expression results in transduction of a signal indicative of polarizing activity. These studies showed that transcription of Ptc is never significantly upregulated in Xt;ld double homozygous limb buds in contrast to single mutant and wild-type embryos (data not shown). However, Shh expression either more posterior (Fig. 6c) or apical (Fig. 6d) induces Gli transcription (Fig. 6g,h) as shown by comparative analysis of contralateral Xt;ld double homozygous limb buds. As in wild-type (Platt et al., 1997) and single mutant limb buds (Fig. 6e,f), Gli transcription is induced in mesenchymal cells responding to the SHH signal (compare e.g. Fig. 6a to e and Fig. 6d to h). However, Gli transcript levels are always lower in Xt;ld double mutant limb buds in comparison to their wild-type (data not shown) and single mutant counterparts (compare Fig. 6g,h to e,f).
DISCUSSION

Gli3 (Xt) and formin (ld) interact genetically during limb pattern formation

Our studies establish that Gli3 and formin interact during limb morphogenesis. Firstly, both proximal (humerus and scapula) and distal (digits) limb skeletal elements are more severely affected in Xt:ld double than single homozygous limb buds. Secondly, molecular analysis shows that both positioning of the Shh-expressing polarising region and spatial regulation 5’HoxD domains are disrupted in Xt:ld double mutant limb buds. As Gli3 and formin are co-expressed in the posterior limb-bud mesenchyme (Fig. 7b; for details see Trumpp et al., 1992; Chan et al., 1995; Masuya et al., 1997), these two proteins with postulated nuclear functions (Chan and Leder, 1996; Marine et al., 1997) might directly interact. However, biochemical studies using cultured embryonic fibroblasts co-expressing epitope-tagged Gli3 and formin proteins failed to provide any evidence for direct molecular interactions (data not shown). Therefore, the molecular and phenotypic alterations observed in Xt:ld double mutant limb buds are most likely caused by disruption of two distinct, but genetically interacting cascades. Presently, it is not known at what level these cascades are disrupted by the Xt mutation, but formin participates in these genetic interactions most likely through its primary function in establishing the SHH/FGF-4 feedback loop (as indicated by the green loop in Fig. 7; for details see Introduction).

Fig. 7 summarizes the Gli3- and formin-mediated interactions in correlation with their respective distributions during early limb-bud outgrowth. During initiation of limb-bud outgrowth, mesenchymal cells activate Gli3 expression (Fig. 7a; Masuya et al., 1997) and Gli3, but not formin, is essential to posteriorly restrict the early 5’HoxD domains (Fig. 7a, upper right). In contrast to Gli3, formin is initially predominantly expressed by the AER (Fig. 7a; Trumpp et al., 1992; Chan et al., 1995), but expression in the posterior limb-bud mesenchyme is upregulated during early limb-bud outgrowth (Fig. 7b; for details see Trumpp et al., 1992). Firstly, correct positioning and establishment of the Shh-expressing polarising region at the posterior limb-bud margin depends on a genetic synergism involving both Gli3 and formin (Fig. 7b, upper left). Secondly, Gli3- and formin-mediated interactions regulate aspects of the coordinated spatial restriction during the transition from early to late 5’HoxD domains (Fig. 7b, upper right). The ectopic proximal 5’HoxD expression in Xt:ld double mutant limb buds provides a likely molecular explanation for the observed defects in proximal skeletal elements as ectopic 5’HoxD expression is known to induce truncations (Goff and Tabin, 1997; Hérault et al., 1997; Peichel et al., 1997). Finally, the rudimentary autopods of Xt:ld double homozygous limbs are probably a consequence of the combined effects of low levels of aberrant SHH signaling and deregulated 5’HoxD expression, as these gene products are essential for morphogenesis of distal limb structures (see Introduction and below).

Formin and Gli3 are part of the genetic mechanism that establishes the Shh-expressing polarising region at the posterior limb-bud margin

Low levels of polarizing activity are already established in the posterior half of the limb field (Hornbruch and Wolpert, 1991) and is defined by the Hoxb8 expression domain in the forelimb field (Fig. 7a; upper left, green area; Charité et al., 1994; Lu et al., 1997; Stratford et al., 1997). In particular, Hoxb8 seems to be one of possibly several mesenchymal factors that establish competence to induce Shh expression in the posterior limb-bud mesenchyme (Fig. 7b; upper left, green area) as ectopic anterior Hoxb8 expression results in establishment of an ectopic anterior SHH domain (Charité et al., 1994). Interestingly, Hoxb8 expression is normal in Xt:ld double homozygous limb buds and the observed low levels of Shh transcripts are always confined to the posterior half of the limb-bud mesenchyme. These results suggest that limb field polarisation and competence to induce Shh expression are not affected in Xt:ld double mutant limb buds. In addition, the involvement of a positively acting AER signal has been postulated to restrict Shh activation to the subapical mesenchyme (Fig. 7b; upper left, yellow area) (Charité et al., 1994; Helms et al., 1996) and FGF-8 signaling has been implicated in Shh activation (Grieshammer et al., 1996; Ros et al., 1996). Indeed, the low levels of aberrant Shh expression detected in Xt:ld double homozygous limb buds are always restricted to mesenchymal cells in close proximity to the AER. Most relevant to the present study, polarising activity is restricted to a group of mesenchymal cells located at the posterior limb-bud margin during initiation of limb-bud outgrowth. These cells

![Fig. 6. Transcriptional activation of Gli in limb-bud mesenchymal cells responding to SHH signaling. Comparative analysis of Shh (a-d) and Gli (e-h) distributions in contralateral hindlimb buds of embryos at embryonic day 11 (about 40 somites). Arrowheads indicate either the Shh or Gli expression domains and asterisks mark posterior limb-bud margins. (a) Xt/Xt. Shh expression is comparable to wild-type hindlimb buds at this developmental stage (data not shown). (b) ld/ld, Shh expression remains proximal at low levels. Note that the ld phenotype is already apparent in hindlimb buds at this developmental stage. (c) Xt:ld double homozygous hindlimb bud with posterior Shh expression. (d) Xt:ld double homozygous hindlimb bud with apical Shh expression. (e-h) Gli expression in contralateral hindlimb buds of the same embryos as shown in a-d. Note that Gli expression is highest in cells close to the Shh domain. (e) Xt/Xt hindlimb bud. (f) ld/ld hindlimb bud. (g) Xt:ld double homozygous hindlimb bud with posterior Gli expression. (h) Xt:ld double homozygous hindlimb bud with apical Gli expression.

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begin to express the SHH signaling molecule and define the polarising region (Fig. 7b; upper left, purple area; Hornbruch and Wolpert, 1991; Riddle et al., 1993). Our studies establish that Gli3- and formin-mediated positive feedback regulation of Shh expression are both required to stably position the polarising region at the posterior limb-bud margin. Subsequent maintenance and distal propagation of SHH signaling depends on mesenchymal formin, as the SHH/FGF-4 feedback loop is never established in ld/ld limb buds (Fig. 7b; panel ‘Limb Patterning,’ Chan et al., 1995; Haramis et al., 1995; Kuhlman and Niswander, 1997). In addition, signals specifying dorsoventral limb fates (reviewed by Zeller and Duboule, 1997) also participate in establishment and maintenance of SHH signaling, which indicates its coordinated spatial regulation along all three limb-bud axes. For example, mice deficient for the dorsalizing Wnt-7a signaling molecule also display reduced Shh levels and associated patterning defects of distal limb skeletal elements (Parr and McMahon, 1995).

**Initial posterior restriction of 5'HoxD domains in limb buds depends on transacting Gli3, whereas subsequent regulation requires both Gli3 and formin**

Considering previous studies (Masuya et al., 1995; Buescher et al., 1997), one unexpected finding of the present study is that Gli3 functions first to posteriorly restrict 5'HoxD genes (Hoxd11 to Hoxd13). These results show that their characteristic posterior nesting is not only dependent on successive activation (reviewed by Duboule, 1992), but on Gli3-mediated anterior repression during initiation of limb-bud outgrowth (Fig. 7a, panel ‘5'HoxD’). Gli3 belongs to a family of zinc finger proteins and analysis of Xenopus laevis Gli3 indicates that it can act as a transcriptional repressor (Marine et al., 1997). Therefore, Gli3 could mediate repression by direct binding to cis-acting negative regulatory HoxD elements. Indeed, identification of several such negative
regulatory elements within or close to the HoxD gene cluster has revealed the importance of transcriptional repression in shaping of their expression domains (van der Hoeven et al., 1996; Hérault et al., 1997; Peichel et al., 1997; Kondo et al., 1998).

Interestingly, deregulation of 5’HoxD expression in Xt/Xt limb buds (by embryonic day 9.5; this study) long precedes detection of weak anterior ectopic SHH signalling and activation of its downstream targets Ptc and Gli (around embryonic day 11; Masuya et al., 1995; Buescher et al., 1997 and data not shown). Therefore, it is rather unlikely that deregulated anterior 5’HoxD expression in Xt/Xt limb buds is a consequence of ectopic SHH signaling as previously proposed (Masuya et al., 1995; Buescher et al., 1997). On the contrary, the ectopic Shh induction in Xt/Xt limb buds might rather be a consequence of deregulated 5’HoxD expression as transgene-mediated ectopic Hoxd12 expression in wild-type limb buds also results in establishment of an anterior SHH signaling domain and digit polydactyly (Knezevic et al., 1997). The analysis of several other polydactylous mouse mutations has begun to uncover an ‘anterior negative regulatory’ cascade (reviewed by Johnson and Tabin, 1997), which might regulate aspects of 5’HoxD distributions and/or polarizing activity in concert with/or independent of Gli3. For example, inactivation of the Alx-4 gene by gene targeting (Qu et al., 1997) or lst mutations in the mouse (Qu et al., 1998) also results in preaxial polydactyly and anterior SHH signaling in older limb buds. The de novo induction of a well-separated, anterior Hoxd13 domain together with normal Gli3 expression in Alx-4 deficient limb buds (and vice-versa) suggests that Alx-4 acts either downstream or independent of Gli3. However, genetic evidence also indicates that wild-type formin function is required for the semidominant, polydactylous lst limb phenotype (Vogt and Leder, 1996).

The results of this study indicate that Gli3- and formin-mediated signalling interactions regulate aspects of the dynamic spatial distributions of 5’HoxD genes, which result in the temporally and spatially coordinated establishment of their late expression domains during progression of limb-bud development. Genetic and evolutionary evidence indicates that the late 5’HoxD domains are essential for formation and patterning of the vertebrate autopod (see e.g. Dollé et al., 1993; Sordinio et al., 1995). The deregulated expression of 5’HoxD genes in both single and XtId double homozygous limb buds provides a likely direct molecular explanation for the observed digit patterning defects (see also Haramis et al., 1995; Masuya et al., 1995; Buescher et al., 1997). Furthermore, the expression of paralogous 5’HoxA genes is apparently normal in both single and XtId double homozygous limb buds, indicating that Gli3- and formin-mediated genetic interactions regulate specifically the limb-bud domains of 5’HoxD genes. Therefore, these observations might have evolutionary relevance as differential expression of paralogous Hox genes has been proposed as important during evolution of paired appendages (reviewed by Shubin et al., 1997; Coates and Cohn, 1998).

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REFERENCES


