Genomes & Developmental Control

Interactions between HOXD and Gli3 genes control the limb apical ectodermal ridge via Fgf10

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Abstract

The development of the vertebrate limb is dependent upon two signaling centers, the apical ectodermal ridge (AER), which provides the underlying mesenchyme with essential growth factors, and the zone of polarizing activity (ZPA), the source of the Sonic hedgehog (SHH) product. Recent work involving gain and loss of function of Hox genes has emphasized their impact both on AER maintenance and Shh transcriptional activation. Here, we describe antagonistic interactions between posterior Hoxd genes and Gli3, suggesting that the latter product protects the AER from the deleterious effect of the formers, and we present evidence that Fgf10 is the mediator of HOX-dependent AER expansion. Furthermore, the striking similarity between some of the hereby observed Hox/Gli3-dependent morphogenetic defects and those displayed by fetuses with severely altered retinoic acid metabolism suggests a tight connection between these various pathways. The nature of these potential interactions is discussed in the context of proximal–distal growth and patterning.

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Introduction

Genetic studies in mice have shown that limb growth and patterning critically depends upon Hox genes belonging to paralogy groups 9 to 13 of both the HoxA and HoxD clusters. Despite structural homology and genomic neighborhood, individual representatives of the different groups have distinct roles in the formation of particular limb regions. For example, in the absence of both Hoxa13 and Hoxd13 function, autopods (hands and feet) mostly fail to develop (Fromental-Ramain et al., 1996b; Kondo et al., 1997). Similarly, severe truncations of the zeugopod (forearm, or lower arm) were seen when removing group 11 function (Davis et al., 1995) and, likewise, group 9 deficit mostly affected the stylopod (humerus) (Fromental-Ramain et al., 1996a; Wellik and Capecchi, 2003). These analyses uncovered anatomical defects generally corresponding in space and time to the expression domains of the genes concerned. In developing limbs, both the timing of expression and the position of the functional domains of the various Hox genes reflect their linear order along the chromosome (Kmita and Duboule, 2003). However, at the most proximal end of the stylopod Hox gene function seems to be somewhat dispensable (Kmita et al., 2005).

The importance and necessity for such a strict temporal-spatial distribution of gene expression domains along the proximo-distal limb axis has been illustrated by several approaches. Extensive rearrangements in the HoxD cluster induced limb anatomical defects due to the abnormal expression of Hox genes, rather than to their loss of function. When group 13 products were ectopically expressed in growing zeugopods, these segments were strongly affected, reminiscent of group 11 functional deficits. Related examples of forced expression of group 13 or 12 products in developing chick or mouse limbs resulted in similar patterning defects (Goff and Tabin, 1997; Williams et al., 2006). These observations gave support to the existence of functional interactions between Hox gene products, following the rule of
posterior prevalence (Duboule, 1991; Duboule and Morata, 1994), whereby a ‘posterior’ or ‘distal’ gene product (e.g. HOXD13) can abrogate the function of a more ‘anterior’ or ‘proximal’ gene (e.g. group 11), likely at the post-transcriptional level (Herault et al., 1997; Spitz et al., 2003; van der Hoeven et al., 1996). During limb development, posterior prevalence has been documented at rather late stages, i.e. at times and in domains corresponding to distal pieces of the appendages, and the functional relevance of excluding distal Hox gene products from the early limb bud, such as to prevent distal structures to form at proximal locations, has not been assessed.

At the molecular level, posterior prevalence may result from interactions between HOX proteins either with various HOX partners, or with other gene products, leading for instance to the modulation of their functional activities and concurrent impact upon the regulation of target genes (Williams et al., 2006; Zappavigna et al., 1994). Among the few confirmed protein partners of HOX products (Capellini et al., 2006; Chen et al., 2004), the zinc finger domain transcription factor Gli3 is of particular interest in this context. The Gli3 gene product is critical for proper limb development, mainly through its antagonistic genetic interaction with Shh, as the stimulation of Shh signaling prevents the default processing of Gli3 from an activator to a repressor form (Litingtung et al., 2002; te Welscher et al., 2002; Wang et al., 2000), thereby up-regulating Shh target genes. In addition to this involvement in Shh signaling, Gli3 and Hoxd genes were reported to interact during early limb development, in two different contexts. First, genetic evidence suggested that GLI3 acts as a negative regulator of several Hoxd genes, such as Hoxd13 and Hoxd12 during early limb budding (Buscher et al., 1997; Zuniga and Zeller, 1999). Secondly, GLI3 was shown to physically interact with the HOXD12 protein during distal limb patterning. In this latter case, the GLI3/HOXD12 interaction modified digit patterning, likely as a consequence of direct protein/protein contacts (Chen et al., 2004).

Mice carrying the Extra-toes (Xt) mutation lack the function of Gli3. These mice have a range of anomalies, among which a severe polydactyly of both fore-and hindlimbs, likely due to the de-repression of Hox genes, and concurrent ectopic expression of Shh, at the anterior margin of the developing limb (Buscher et al., 1997). In order to assess whether the wild-type pentadactyly was indeed due to a Gli3-dependent anterior repression of Hox genes, in other words whether the polydactyly observed in Xt mutant mice is dependent upon the gain of Hox gene function(s), we crossed Xt mice with mice carrying either a full, or a partial, deletion of the HoxD cluster (Zakany et al., 2001, 2004). Here, we show that removing all Hoxd gene function, in addition to Gli3 in the developing autopod, does not significantly reduce the number of digits when compared to mice mutant for Gli3 alone.

In striking contrast, however, the combination of the Gli3 mutant allele with a partial deletion of the HoxD cluster (deletion of Hoxd1 to Hoxd10 included) gave mice with heavily truncated limbs, a situation drastically different from the phenotype observed with the same deletion, but in the presence of Gli3 function. In this latter case, gain of function of the remaining ‘posterior’ Hoxd genes lead to an ectopic Shh domain anteriorly and consequent bilateral symmetry of an otherwise weakly truncated limb (Zakany et al., 2004). This observation indicates that widespread and early expression of Hoxd13 and Hoxd12 can severely impair stylopod development, but only when Gli3 function is either reduced or removed, suggesting that Gli3 function protects against the prevalent function of posterior genes over their more anterior neighbors. Such severe limb truncations involved defects in the apical ectodermal ridge (AER), likely due to a dramatic decrease of Fgf10 expression in limb bud mesenchyme. We discuss the potential roles of these various players in the growth and patterning of the limbs.

Materials and methods

Mouse stocks, crosses and genotyping of mid-gestation embryos and near-term fetuses

The mouse lines carrying the Hoxd cluster alleles used in this study were produced by loxP/Cre-mediated site-specific recombination. del(1–13) is an approximately 100-kb large deletion encompassing from the Hoxd1 to the Hoxd13 loci. In this deletion, the entire Hoxd function is lost (Zakany et al., 2001). Del(1–10) was generated by targeted meiotic recombination (Herault et al., 1998) using del(1–13) as one of the parental alleles to produce an approximately 70-kb large targeted deletion from Hoxd1 to Hoxd10 included (Zakany et al., 2004). The two deficiencies have the same breakpoint near Hoxd1. All Hoxd alleles were genotyped in a multiplex PCR reaction, using the 5′-CCACCGTGCTAAATAAACGCTG-3′ Hoxd11 forward primer, and the 5′-GGGTGCGCTCTTTTCTCTGTCTC-3′ Hoxd10 reverse primer for wild-type and the 5′-CTATTCAAGTGCGGGGGACGCATC-3′ Hoxd1 reverse primer for mutant allele. Gli3 Xt alleles were genotyped with the 5′-TACCCCAGGAGACTCAGATTAG-3′ forward and 5′AAACCGTGGTGTCAAGGAAG-3′ reverse primers, while the Gli3 wild-type allele with the 5′-GGGTGAAACAGCATCAAATGGAG-3′ forward and 5′-ATAGCCATGTGGTGGTTCCATG-3′ reverse primers.

Heterozygous males or females of either Hoxd deficiencies were crossed over Xt heterozygous males or females to obtain compound heterozygous Xt+/−; del(1–13)/+ and Xt+/−; Del(1–10)/+ males and females. Both compound mutants were obtained in near Mendelian proportions, and most individuals of both genotypes displayed characteristic digit defects in forelimbs: olygodactyly in the Xt/+; del(1–13)/+ and Xt+/−; Del(1–10)/+ parents on the 18th day post-fertilization (E18) in order to minimize losses of individuals with compound genotypes. Genomic DNA was extracted from tail biopsies or yolk sac (E10, see below) and genotyped by PCR reactions, using the specific primers indicated above.

RNA in situ hybridization

To evaluate early limb development in the various genotypic classes, F2 embryos were collected on the morning of the 10th day of development (E10) and processed for whole mount RNA in situ hybridization following standard procedures (see e.g. www.eumophlia.org/EMPreSS/servlet/EMPreSS Doc. Number: 13..003). Yolk sac samples were collected individually and genomic DNA was isolated for genotyping, whereas individually fixed embryos were stored at minus 20 °C in methanol. Once genotypes were established, representatives of the selected genotypes were grouped and processed together for any given probe. Forelimb buds of all specimens were photographed and the same representative of the selected genotypes were grouped and processed together for any given probe. Forelimb buds of all specimens were photographed and the same representatives of the selected genotypes were grouped and processed together for any given probe. Forelimb buds of all specimens were photographed and the same representatives of the selected genotypes were grouped and processed together for any given probe.
Skeletal preparations

Fetuses were collected by cesarean section on E18, photographed, tail biopsied, eviscerated and skinned for the Alizarin red and Alcian blue standard skeletal staining procedure (see e.g. www.eumorphia.org/EMPReSS/servlet/EMPReSS Doc. Number: 12_005). Forelimb skeletons were dissected off, equilibrated into 80% glycerol, flat-mounted and photographed at identical magnification.

Results

Protective role of Gli3 against posterior prevalence

We crossed Xt mice (Figs. 1A, D) with mice either lacking the full HoxD cluster (Figs. 1B, E) or carrying a deletion from Hoxd11 to Hoxd10 (Figs. 1C, F) in order to assess whether the polydactyly induced by the null mutation of Gli3 (Xt/Xt;+/+; Del(1–10)/+; Del(1–10)/Del(1–10)) is due to the de-repression, in time and space, of Hox gene function. Mice double homozygous for Xt and the del(1–13) allele (full deletion) were obtained and still displayed a severe polydactyly, as exemplified by forelimbs bearing seven to eight digital rods (Figs. 1H, K). This polydactyly was induced by the removal of Gli3 function, as forelimbs of animals homozygous for the del(1–13) allele alone were mostly pentadactylous (Fig. 1H). In addition to the digit phenotype, forelimbs of double mutant animals were significantly and globally smaller than controls, the reduction including distal as well as proximal limb segments (Fig. 1K). This was unexpected as the absence of the entire Hoxd cluster (del(1–13)/del(1–13))...
produced only a minor reduction of the proximal limb (Fig. 1H). Furthermore, Xt/Xt mice also show such a reduction in the length of their hindlimbs (Barna et al., 2005; Chen et al., 2004). A reduction in the length of the limbs was also scored in the second stock used in these crosses. We had previously reported that mice homozygous for the Del(1–10) allele displayed a double posterior digit pattern (Zakany et al., 2004), as a consequence of the establishment, at the anterior margin, of a second zone of Shh expression. This ectopic domain was triggered by the widespread expression of the remaining Hoxd11, Hoxd12 and Hoxd13 genes, likely under the transcriptional control of regulatory sequences located in 3′ of the cluster, which upon deletion would influence the expression of these genes (Tarchini and Duboule, 2006; Zakany et al., 2004). This gain of function phenotype showed some variability and a fair proportion of these limbs displayed both a reduced number of digits (oligodactyly) and a more or less severe limb shortening (Fig. 1I). Limb shortening was particularly evident in the stylopod (the humerus) and was substantially less pronounced in del(1–13) than in Del(1–10) mutant animals (Figs. 1H, I), supporting a gain of function effect as the causative factor. Altogether, these observations indicated that the abnormally early and proximal expression of Hoxd11, Hoxd12 and Hoxd13, in Del(1–10) mice, impacted upon the growth potential of the limbs, in addition to the loss of function effect following the deletion of several Hoxd genes.

Surprisingly, animals carrying the partial deletion of the HoxD cluster Del(1–10), associated with the absence of Gli3 function, almost completely lacked their forelimbs (Fig. 1L). This drastic phenotype was not scored with full deletions of the HoxD cluster (Fig. 1K), which suggested that a gain of function of the remaining Hoxd genes was likely involved. In such mice, the stylopod was completely lost and a single and truncated cartilage model, in the worst case, was observed at the position of the zeugopod. The autopods were just as severely reduced, displaying a single digit in the continuation of the zeugopod cartilage (Fig. 1L).

Because Xt homozygous mice, with or without a HoxD cluster, do not display such defects, we concluded that neither Gli3 nor the HoxD cluster are strictly necessary for proximal limb development, even though their combined absence generated somewhat smaller limbs, suggesting a genetic interaction between Gli3 and HoxD genes. In those mice where the absence of Hoxd1 to Hoxd10 was associated with ectopic expression of Hoxd11, Hoxd12 and Hoxd13 in the early limb bud (Zakany et al., 2004), subsequent growth was dramatically dependent on the presence of the Gli3 product. This genetic analysis thus identified GLI3 as a factor protecting the developing limb from the deleterious effect of an early gain of function of ‘posterior’ Hoxd genes.

To document this interpretation, we looked at the level of both Gli3 and Hoxd13 transcripts in E9 limb buds of Del(1–10)/Del(1–10) mice. Indeed a number of Del(1–10)/+ animals display hindlimb polydactyly (Zakany et al., 2004) and the majority of Xt/+;Del(1–10)/+ mice (Fig. 1C) show anterior polydactyly, reminiscent of fetal Xt/Xt forelimbs, suggesting that Gli3

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**Fig. 2.** Expression patterns of Hoxd13 (A and D) and Gli3 (B, C, E and F) in wild-type (A–C) and Del(1–10) homozygous (D–F) mid-gestation (E9) mouse embryos. All these specimen are wild-type at the Gli3 locus. (A) Hoxd13 expression is not yet detectable at this stage in wild-type, while the entire limb bud shows strong ectopic and premature Hoxd13 transcript accumulation in Del(1–10) homozygous (D). Expression pattern of Gli3 does not differ appreciably between wild-type and Del(1–10) homozygous (compare panels B to E and C to F).
expression may be modified in these stocks. The comparison between Figs. 2A and D demonstrates the ectopic and premature expression of Hoxd13 in Del(1–10)/Del(1–10), with a pattern including the entire incipient forelimb bud. At the same stage, Gli3 transcript accumulation is comparable in these two genotypes (compare Figs. 2B, C and E, F), suggesting that the effect of ectopic posterior Hoxd gene expression is not mediated by a transcriptional suppression of Gli3. Furthermore, the co-expression of Gli3 and posterior Hoxd genes in the limb bud mesenchyme make potential direct molecular interaction between these gene products possible.

Quantitative interactions

The Gli3-dependent suppression of Hoxd13 and Hoxd12 gain of function clearly depended upon the doses of both Gli3 and the remaining Hoxd genes, as shown by the phenotypic distribution detailed in Fig. 3. Forelimbs either lacking a single dose of Gli3 (Xt/+;+/-), or having a weak Hoxd gain of function in the presence of a full complement of Gli3 (+/+;Del(1–10)/+) generally displayed a wild-type phenotype (Fig. 3; group II). Forelimbs lacking two doses of Gli3 were polydactylous, but their humeri were of normal size (Fig. 3; I).

Reduction in the size of the stylopod started when trans-heterozygous animals were considered, i.e. those with only one dose of Gli3 and one dose of gained Hoxd11, Hoxd12 and Hoxd13 (Fig. 3; III, IV). This latter reduction was close to that routinely seen in the homozygous Del(1–10) mutants, in the presence of two doses of Gli3 (Fig. 3; IV).

Animals with three mutant alleles displayed slightly but significantly different phenotypic outcomes. In the presence of two doses of ectopic posterior Hoxd alleles combined with the loss of one dose of Gli3, the humerus was either severely reduced (7 out of 10; Fig. 3; V, VI) or virtually absent. The situation was similar when only one dose of ectopic posterior Hoxd alleles was combined with a complete absence of Gli3. This constitution also increased the severity of the phenotype (6 out of 10). Finally, animals lacking both doses of Gli3 and harboring two doses of ectopic posterior Hoxd alleles had no trace of humerus, even though the remnants of both a zeugopod and an autopod were recognizable (Fig. 3; VI–VIII).

Effect upon the AER

The extent of skeletal truncations suggested that mutant limb development was compromised from a very early stage.
Accordingly, we collected F2 embryos at E10 from Xi/+;Del(1–10)/+ compound heterozygous parents. Genotyping and anatomical examination revealed that all possible genotypes were represented in near Mendelian proportions and that all mutant embryos showed the clear presence of limb buds. Therefore, initial limb budding occurs in compound mutants, indicating that size reductions mostly developed later, during and after E10, a stage corresponding to the establishment of the apical ectodermal ridge (AER).

Several genetic activities are necessary for the formation, maintenance and function of the AER, such as the mesenchymal factors Fgf10 and Shh, or ectodermal factors like Fgf8. These genes are normally expressed at the time when ectopic Hoxd11, Hoxd12 and Hoxd13 expression is scored in the Del(1–10) allele, which also induces an ectopic Shh domain. We examined the expression of Hoxd13, Fgf10, Shh and Fgf8 in the various genotypic classes described in Fig. 3. Representative forelimb buds of four of these genotypic classes are documented in Fig. 4. Examples of the corresponding skeletal patterns at E18 are included for direct comparison.

In wild-type limb buds, Hoxd13 and Hoxd12 expression is first restricted to the most posterior part of the budding limb (early phase in Tarchini and Duboule, 2006). Soon after, expression of these genes de novo appears in the future digit domain, at the postero-distal part of the outgrowing bud, under a different transcriptional control (Tarchini and Duboule, 2006). Consequently, E10 limb buds show both this emergent domain (Fig. 4A; arrow), as well as more proximal weakly expressing cells, remnant of the early posterior domain seen at E9 (Fig. 4A; arrowhead). An important effect of this early and posterior expression domain is to trigger Shh expression, which will thus be confined to posterior cells (Fig. 4C; Tarchini et al., 2006). At

Fig. 4. Expression analyses of Xi, Del(1–10) and compound mutant forelimb buds. Whole mount in situ hybridization using Hoxd13, Fgf10, Shh and Fgf8 probes were carried out on wild-type, +/+;Del(1–10)/Del(1–10), Xi/Xi;+/, Xi/Xi;Del(1–10)/Del(1–10) and Xi/+;Del(1–10)/Del(1–10) E10 embryos. Genotypes are indicated on the left, probes on the top. Panels in the right are representative skeletal E18 preparations to indicate the fate of those buds shown on the left panels. In +/+;Del(1–10)/Del(1–10) embryos, the expression patterns of Hoxd13 (compare panels A and F), Fgf10 (compare panels B and G) and Shh (compare panels C and H) were extended into more anterior regions, whereas Fgf10 level was reduced and Fgf8 seemed relatively normal (compare panels D and I). In Xi/Xi;+/+Hoxd13 (K), Fgf10 (L) and Shh (M), transcript profiles were extended into more anterior regions as well, whereas Fgf10 level was somewhat reduced (compare panels B and L) and Fgf8 seemed relatively normal, if not slightly increased (N). In Xi/Xi;Del(1–10)/Del(1–10) embryos, expressions of Hoxd13 (P), Fgf10 (Q) and Shh (R) were scored only in anterior regions. Interestingly both Hoxd13, Fgf10 and Shh transcripts were hardly detectable in their normal domains and Fgf8 was confined to a very tiny cluster of cells overlying the Hoxd13, Fgf10 and Shh-positive domain (compare panels Q, R and S). Overall, limb buds from +/+;Del(1–10)/Del(1–10) and Xi/Xi;Del(1–10)/Del(1–10) genotypes appeared smaller than wild-type, which was consistent with the subsequently observed reduction in the size of these limbs. As compared to Xi/Xi; Del(1–10)/Del(1–10) (P and Q) in Xi/+;Del(1–10)/Del(1–10) embryos the limb bud size (U and V) and Fgf10 signal intensity (V) were significantly rescued by the presence of one wild-type copy of Gli3 in presence of a well detectable ectopic Hoxd13 signal throughout the anterior limb bud (U).
E10, 

Fgf10 is expressed with a medial-posterior specificity, in a rather large domain. Positive cells nevertheless are excluded from the most distal parts of the bud (where Hoxd13 appears stronger), as well as from most anterior cells (Fig. 4B). Throughout limb budding and early outgrowth, Fgf8 expression extends along the anterior to posterior rim of the limb bud (Fig. 4D), indicating the presence of a well-established AER (Lewandoski et al., 2000).

Mutant limb buds showed clear deviations from these expression patterns. In Del(1–10) homozygous, general accumulation of Hoxd13 transcripts in E9 buds (Zakany et al., 2004) leads to a visible anterior ectopic domain, mirroring wild-type posterior expressing cells (Fig. 4F; arrowheads). The late domain appeared in a more distal position, pre-figuring the bilateral symmetry of the limb. Following the de-localization of Hoxd13 and Hoxd12 expression domains, Shh appeared along the entire distal rim of the bud (Fig. 4H), to be subsequently split into two opposing domains, leading to the double-posterior morphology (Zakany et al., 2004). Expression of Fgf10 in these mutant buds was clearly down-regulated, and scored both anteriorly and posteriorly, whereas mostly non-detected in the distal part where the late Hoxd13 domain was visible (Fig. 4, compare F and G). Here again, expression matched the bilateral symmetry subsequently observed. Consistently, Fgf8 expression did not substantially change, albeit the overall length of the AER was slightly reduced, consistent with the general reduction in the size of the limb bud (Fig. 4I).

Interestingly, Xtx/Xt homozygous limb buds showed modifications of these expression patterns not drastically different from those observed in Del(1–10) deleted animals. Hoxd13 early expression extended into the most anterior part of the bud (Zuniga and Zeller, 1999), and a remnant of this pattern was still detected at E10 (Fig. 4K, arrowhead), along with a rather distal domain for the late Hoxd13 pattern. Shh expression was also somewhat extended along the anterior-distal margin (Fig. 4M), although much less extensively than Hoxd13, especially as anterior proximal, and also most part of the distal limb domains remained devoid of Shh transcripts until later, when an ectopic Shh domain was generally scored anteriorly (Buscher et al., 1997). Fgf10 was slightly down-regulated but again extended into anterior distal and proximal domains (Fig. 4L). Fgf8 expression was essentially identical to wild-type, indicating the presence of a near normal AER, corresponding to the seemingly normal aspect of these early limb buds, despite their subsequent polydactyly (Fig. 4N).

Xtx/Xt;Del(1–10)/Del(1–10) compound animals displayed drastically different expression patterns. Hoxd13 transcripts accumulated in the anterior limb bud, suggesting a complete shift in the anterior-posterior polarity of the bud. While this was observed for the late Hoxd13 domain, traces of the early expression suggested a similar inversion of polarity (Fig. 4P; arrow). Accordingly, Shh-positive cells were found at an anterior-distal position, corresponding to cells expressing Hoxd13 (Fig. 4R). However, signal intensity was just above detection and only few cells were scored positive. Shh expression was not detected in its usual posterior domain. Fgf10 expression was also severely reduced in quantity, and mostly found in anterior mesenchymal cells, illustrating once again an inversion in the AP polarity (Fig. 4Q; see below). Finally, the pool of Fgf8-positive cells was also dramatically reduced. Only a small cluster of positive cells was detected in the anterior limb bud, precisely above the ectopic domains for all three Hoxd13, Fgf10 and Shh (Fig. 4S). This virtually non-existing AER coincided with an important reduction in the size of the entire limb bud when compared to all other genotypes.

In Xtx/+;Del(1–10)/Del(1–10) compound mutants, ectopic expression of Hoxd13 in anterior regions was clearly detected as well, and a shift of the late distal domain towards the anterior margin was also evident, giving an overall pattern that was intermediate between Xtx/Xt;Del(1–10)/Del(1–10) and +/-;Del(1–10)/Del(1–10). Accordingly, the intensity of the Fgf10 signal increased, and the size of the limb bud was also consistently bigger than that of the double homozygous. From this data set, we concluded that expressions of both Fgf10 in the mesenchyme and Fgf8 in the newly forming AER were severely altered in the presence of prematurely expressed Hoxd13 and Hoxd12, provided the quantity of GLI3 was either reduced, or completely absent. Because both Gl3 and Hox genes are expressed in mesenchyme, we favored an hypothesis whereby these latter gene products would act upon Fgf10 transcript accumulation. In double mutants, Fgf10 was massively affected, which prevented formation of a full-grown AER leading to the observed truncations.

**Fgf10 in early mutant limb buds**

We looked at the expression of Fgf10 in earlier mutant limb buds, i.e. at a stage where the AER was being established, hence gene expression was unlikely to depend upon AER derived signals (Fig. 5). Two major aspects were immediately scored: firstly, the presence of two copies of the Del(1–10) mutant allele drastically reduced the quantity of Fgf10 transcripts, regardless of the presence or absence of Gl3 function (Figs. 5B, C). Secondly, the absence of Gl3 function (Xtx/Xt) induced a spectacular inversion of AP polarity, independently of the presence or absence of the Del(1–10) allele (Figs. 5C, D; see also Fig. 4). Concerning the former aspect, it is likely that the down-regulation of Fgf10 depended upon the presence of gained posterior Hox genes, as two copies of the Del(1–10) allele were required to achieve substantial extinction of Fgf10, in the absence of Gl3 function.

The inversion of Fgf10 polarized expression was mostly dependent upon the absence of Gl3 function, as it started to occur even with a normal set of Hox genes (Fig. 4). In this case, a ‘rotation’ of the Fgf10 pattern was scored, along with the distalization of the Shh expressing domain (Fig. 4L, compare Fig. 4M). While Del(1–10) homozygous limb buds had a bilateral expression of Fgf10, following that of posterior Hox genes, further removing Gl3 function gave the limb a clear, yet not sustainable, inverted polarity (Figs. 4 and 5). Up-regulation of Fgf10 in the anterior bud may result from the observed gain of posterior Hoxd11 gene expression there upon loss of Gl3 function (Zuniga and Zeller, 1999). Likewise, Del(1–10) mutant limb buds may induce Fgf10 expression anteriorly. The
disappearance of Fgf10 expression from the posterior margin of the limb in Del(1-10)/Xt compound mutants may also reflect the down-regulation of posterior Hoxd genes in these cells, yet how the Xt mutation stimulates this remains elusive.

Discussion

Genetic analyzes have highlighted the role of the HoxA and HoxD clusters in tetrapod limb development (Davis et al., 1995; Kmita et al., 2005). Recently, their capacity to regulate the amount and position of Shh transcripts, hence to control both proximal to distal growth and the anterior to posterior polarity, was proposed (Tarchini et al., 2006). In this view, the late and posteriorly restricted expression of groups 10 to 13 Hox genes is mandatory for further development of the Shh-dependent, most distal part of the limb. However, combined HoxA/HoxD clusters deficient limbs showed more extensive truncations than those reported for Shh mutant mice (Chiang et al., 2001; Kmita et al., 2005), suggesting that Hox gene products are required early on, independent of their effect upon Shh transcription, likely to control the formation or maintenance of the AER. In this work, we provide evidence that the integrity of the AER depends on the interplay between posterior Hoxd genes and Gli3, probably mediated through the control of Fgf10 expression in early limb bud mesenchyme.

Antagonistic role of 5′ HOXD genes and GLI3 in controlling FGF10

When Gli3 was either half, or fully abrogated, in the presence of prematurely expressed Hoxd13 and Hoxd12, extreme limb truncations occurred. Interestingly, the most affected limbs, in this phenotypic series resembled those observed either after surgical removal of the AER in chick limb buds (Saunders, 1948) or after inactivation of Fgf signaling in mice. Genetic analyses of limb development in mice identified Fgf10 as a major early mesenchymal competence factor (Sekine et al., 1999) and further studies on Fgf receptors have associated this early step with the establishment of the AER (Li et al., 2005; Revest et al., 2001; Xu et al., 1998), which will be subsequently the source of Fgf4 and Fgf8. Inactivation of Fgf8 impacted upon the formation of the stylopod (Lewandoski et al., 2000) and additional inactivation of Fgf4 prevented the development of all three limb segments. Interestingly, in Fgf8,Fgf4 compound mutants, Fgf10 expression was severely reduced, whereas Shh transcription was abrogated (Boulet et al., 2004; Sun et al., 2002), pointing to feedback mechanisms in this complex process.

In our experiments, expression of Fgf10 in the mesenchyme and of Fgf8 in the forming AER were dramatically reduced, indicating a defect in the Fgf10 to Fgf8 arm of the positive circuit maintaining AER function. This important decrease in the amount of Fgf10 transcripts resulted from prematurely expressed Hoxd13 and Hoxd12 genes. Yet this effect was not observed, or at least not to this degree, in the presence of the Gli3 gene product. Therefore, Gli3 products were able to mitigate, or protect from, the effects of Hoxd gain of function upon Fgf10 activity, in a dose-dependent manner. In Del1-10 homozygous mice, initial Fgf10 expression was indeed readily detectable, whereas only trace amounts of Fgf10 could still be seen after additional removal of Gli3 function. In such double mutants, patches of AER were occasionally scored with only dispersed Fgf8-positive cells, suggesting that, receiving weak Fgf10 signal, epidermal cells started responding by activating Fgf8 transcription, but the pool of responding cells was likely too small and failed to assemble a ridge. In the development of the final phenotype, these most severe constitutions are tantamount to a genetic AER ablation. From previous experiments, in particular those involving simultaneous inactivation of Fgf8 and Fgf4, it is expected that massive apoptosis is involved in bringing about the eventual truncations (Sun et al., 2002). Besides fibroblast growth factors, other signals are involved in AER establishment (Capellini et al., 2006; Hill et al., 2007).
...functions of GLI3 and posterior HOX products directly bind to the promoter of Gli3, providing a posterior input that is required for AER formation. However, whenever the dose of Gli3 was reduced, a full anteriorization of the patterns was observed, as a result of the reduction in (or absence of) Gli3-mediated repression. The full anterior pattern of posterior Hoxd genes, as illustrated by Hoxd13 (Fig. 4) was expectedly able to elicit Shh transcription in an anterior spot (Tarchini et al., 2006), underlying the few Fgf8-positive AER cells. A fully inverted limb was nonetheless impossible to produce since the protective effect of Gli3 against posterior prevalence had been removed, leading to the concurrent growth arrest (see above).

Hox genes; a link between RA and Fgf signaling in limb buds?

The drastic defects observed in Del(1–10);Xt compound mutants limbs are remarkably similar to those described in animals with altered levels of retinoic acid, suggesting that related developmental pathways were affected in both cases. In the absence of endogenous RA synthesis, i.e. in animals lacking the function of Raldh2, the modifications in the expression of Fgf10, Shh and Fgf8 were related to those reported in this paper. In particular, Shh was lost in the normal posterior limb bud of Raldh2 mutants, whereas appearing delocalized in the distal region (Mic et al., 2004; Niederreither et al., 2002). Furthermore, as a consequence of RA depletion, posterior Hoxd genes became prematurely and ectopically expressed in early limb buds (Niederreither et al., 2002). These analogies suggest that an important role for RA signaling is to prevent posterior Hox genes to be transcribed in the early bud. In the absence of RA, Hoxd12 and Hoxd13 transcription was activated...
prematurely and altered Fgf10 gene expression, thus precluding bud growth.

On the other hand, in embryos mutated for the Cyp261b gene, i.e. with an increased level of RA, Hoxd12 and Hoxd13 transcription in limb buds was delayed (Yashiro et al., 2004) leading to severe defects in the stylopod, zeugopod and autopod. Interestingly, the eventual phenotypes of Raldh2 and Cyp261b mutant limbs are quite alike, involving massive alterations of all limb regions, including the humerus. These phenotypes nevertheless arise through distinct mechanisms, as witnessed by the expression pattern of Fgf8 in the AER: while reduced in Raldh2 mutant, it was increased in Cyp261b mutants, in good correlation with either increased, or decreased Hoxd12 and Hoxd13 expression, respectively. The most severe genetic constitutions we report in this work are reminiscent to early vitamin A deficiencies. We tested the expression of Meis1, a gene that is under the control of RA signaling (Fig. S1) and observed normal expression patterns of this gene in our five key mutant combinations. We take this as an evidence that RA observed normal expression patterns of this gene in our five key mutant combinations. We take this as an evidence that RA signal was initially received from the flank and that Fgf-dependent suppression of Meis1 transcript accumulation in proximal bud was effective (Mercader et al., 2000).

The function of RA in the activation of Hox gene transcription has been abundantly documented, both on particular Hox genes, via their RAREs (Serpente et al., 2005) and at the level of entire clusters. In this latter case, RA was able to trigger collinear Hox genes activation in cultured EC cells (Simeone et al., 1990). It is thus possible that RA plays a role in the sequential activation of Hox genes during limb bud development by favoring expression of anterior Hox genes first, while delaying expression of the posterior members. This would allow for the AER to be established and functional, via Fgf10 regulation, before the massive expression of posterior genes in the autopods would abrogate it. In this task of maintaining posterior Hox genes silent or harmless, RA would be helped by Gli3, first by its repressive effect on posterior Hox gene transcription in anterior cells (Zuniga and Zeller, 1999) then by the protective effect against potential posterior HOX products, which we describe in this work.

However, this explanatory framework fails to account for the fact that no major proximal defect occurs in Gli3 minus animals, i.e. in the presence of detectable ectopic posterior Hox genes products in the early anterior limb bud (Zuniga and Zeller, 1999). We think that this may result from a dose effect, the amount and sustainability of these ectopic products being much below those observed in the Del(1–10) gain of function (Zakany et al., 2004). Also, in the Gli3 mutant limb buds, ectopic posterior products must ‘compete’ against (or abrogate) the full complement of anterior Hox genes, whereas the complete set of anterior Hox genes is removed in the Del(1–10) mutants, in addition to a strong gain of expression. Such a dosage effect is well supported by the trans-heterozygous phenotypes described above. The fact that limbs develop well in the absence of Gli3 function, despite the strong expression of posterior Hox genes in the distal autopod (late phase in Tarchini et al., 2006), suggests that the dramatic phenotypes observed in this work derive from perturbations occurring at a very early stage of limb bud development, a stage critical for the formation of the AER, in agreement with the described kinetics of the posterior Hoxd gene gain of function in Del(1–10) animals (Zakany et al., 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.517.

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