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Hoxd and Gli3 interactions modulate digit number in the amniote limb

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Abstract

During limb development, Sonic hedgehog (SHH) and HOX proteins are considered among the most important factors regulating digit number and identity. SHH signaling prevents the processing of GLI3 into a short form that functions as a strong transcriptional repressor. Gli3 mutant limbs are characterized by a severe polydactyly and associated ectopic anterior expression of 5′Hoxd genes. To genetically determine the involvement of 5′-located Hoxd genes in the polydactyly of Gli3 mutants, we have generated a compound mutant that simultaneously removes the three 5′-located Hoxd genes and Gli3. Remarkably, the limbs that form in the absence of all four of these genes show the most severe polydactyly so far reported in the mouse. The analysis of gene expression performed in compound mutants allows us to propose that the increase in the number of digits is mediated by the gain in function of Hoxd10 and Hoxd9. Our results also support the notion that an adequate balance between positive and negative effects of different Hoxd genes is required for pentadactyly.

Keywords: Gli3; Hoxd; Hoxa; Shh; Limb development; Extra-toes; Polydactyly; Posterior prevalence

Introduction

Pentadactyly is the ancestral amniote digit formula. All extant tetrapods descend from an ancestor with a pentadactyl limb, although many species have reduced the number of digits or even lost the limb (Cohn and Tickle, 1999; Cohn, 2001). Despite the multiple variations on the basic pentadactyly pattern, a limit of 5 in the number of digits appears to be constant.

The detailed way in which the number and identity of the digits is established and controlled during limb development is not completely understood and remains a subject of active investigation. In humans, alterations in the number of digits, preferentially polydactyly, are among the most frequent congenital malformations (Lamb et al., 1982; Graham and Ress, 1998).

It is known that the zone of polarizing activity (ZPA), through its production of Sonic hedgehog (SHH), controls distal limb patterning including the number and identity of the digits (Saunders and Gasseling, 1968; Tickle et al., 1975; Riddle et al., 1993). SHH signaling in vertebrates is mediated by the three Gli genes (Gli1, Gli2 and Gli3). Of these, Gli3 is the one that is essential for limb development as evidenced by the polydactyly phenotype of the Extra-toes (Xt) mutation, which represents the complete loss of function of Gli3 (Schimmang et al., 1992; Hui and Joyner, 1993; Maynard et al., 2002). In the absence of Shh, GLI3 is processed to a short form that acts as a strong transcriptional repressor (GLI3R) (Dai et al., 1999; Aza-Blanc et al., 2000; Wang et al., 2000). Therefore, as a consequence of the posterior secretion of SHH during normal limb development, a gradient of GLI3R is established along the anteroposterior axis of the bud with maximum levels at the anterior border (Wang et al., 2000; Litingtung et al., 2002; Bastida et al., 2004). The molecular study of Xt homozygous limbs and several other polydactyly mutations in mice revealed an ectopic spot of Shh expression at the anterior border, which was considered responsible for the polydactyly phenotype (Chan et al., 1995; Masuya et al., 1995; Buscher et al., 1997; Masuya et al., 1997). However, the subsequent study of Shh;Gli3 double mutant embryos, phenotypically indistinguishable from Gli3 mutants, demonstrated that the
polydactyly in the absence of Gli3 was independent of Shh (Litingtung et al., 2002; te Welscher et al., 2002b). Consequently, two types of polydactylous phenotypes can be distinguished with different underlying molecular mechanisms. Type 1 depends on the ectopic anterior activation of Shh, with intact Gli3 function, and preaxial mirror-image duplication of digits with clear identities. Type 2 polydactyly disrupts Gli3 function, is Shh independent and shows unidentifiable digits (Litingtung et al., 2002; te Welscher et al., 2002b).

It has also been clearly demonstrated that the morphogenesis of the vertebrate digits requires the function of genes of the HoxA and HoxD clusters (Davis et al., 1995; reviewed by Zakany and Duboule, 1999). During limb development Hoxa and Hoxd genes are activated sequentially in time and space, following their genomic topography, a phenomenon known as temporal and spatial collinearity (Lewis, 1978; Gaunt et al., 1988). Genes located more 3′ in the chromosome (also referred to as anterior or proximal) are expressed earlier and at more anterior locations than genes located more 5′ (also referred to as posterior or distal). The division between anterior and posterior Hox genes has not been clearly defined although a separation between Hox genes expressed throughout the limb bud and those excluded from anterior cells and capable of inducing Shh has been precisely mapped between Hoxd9 and Hoxd10 (Tarchini et al., 2006). Interestingly, the forced expression of a posterior gene earlier and/or more anteriorly than normal results in a posteriorization phenotype (Duboule, 1991; Duboule and Morata, 1994), an observation that led to the model of “posterior prevalence” meaning that the product of a posterior gene can inactivate the function of a more anterior one, likely at the posttranscriptional level (van der Hoeven et al., 1996; Herault et al., 1997; Williams et al., 2006).

The generation of an extensive series of mutations in the HoxA and HoxD clusters including inversions, deletions, duplications and compound mutations has shed light on the regulation and function of Hox genes during limb development (Kmita et al., 2002; Spitz et al., 2003; Kmita et al., 2005; Tarchini and Duboule, 2006). Hoxd genes are activated in two consecutive waves under different transcriptional control. The first phase of Hoxd gene expression, essential for the development of the limb up to the forearm, relies on two opposite regulations: one, the early limb control region (ELCR), located at the telomeric end of the complex, and the other located at the centromeric end of the complex (Zakany et al., 2004; Tarchini and Duboule, 2006). The ELCR acts as a timer activator relying on relative distance to the promoter while the centromeric enhancer acts by preventing expression in anterior cells (Tarchini and Duboule, 2006). The second phase of Hoxd activation, regulated by a global control region (GCR) located centromeric to the cluster, is essential for digit development (Spitz et al., 2001; Spitz et al., 2003).

The HoxA and the HoxD gene clusters are also required for the initiation of Shh expression in the ZPA (Kmita et al., 2005). Furthermore, the study of a series of Hoxa/Hoxd double mutant mice showed that correct Shh expression depends on quantitative and qualitative combinations of Hoxa and Hoxd genes of the paralogous groups 10 to 13 (Tarchini et al., 2006). Therefore, the notion that anterior-posterior patterning of the limb bud is a consequence of the intrinsic colinearity of Hox gene expression has emerged (Tarchini et al., 2006).

Interestingly, the 5′-located Hoxd genes are broadly and ectopically expressed in the anterior limb bud mesoderm of Xr mutants, indicating that GLI3R represses their transcription (Zuniga and Zeller, 1999; Litingtung et al., 2002; te Welscher et al., 2002b). Since overexpression of these genes cause preaxial polydactyly (Morgan et al., 1992; Goff and Tabin, 1997; Knezevic et al., 1997; Kmita et al., 2002), it has been proposed that the polydactyly in Xr mutants is mediated by the ectopic anterior expression of posterior Hoxd genes. Interestingly, it has been recently shown that GLI3 and HOXD12 interact genetically and physically, and that this interaction modulates GLI3R function (Chen et al., 2004). This finding indicates that HOXD proteins may function semiquantitatively to regulate digit pattern and identity through the interaction with GLI3 (Chen et al., 2004). The graded posteroanterior level of 5′ HOXD proteins during their second phase of expression in the developing limb can be envisaged as functioning to counteract the transcriptional function of GLI3R, therefore potentiating SHH function (Chen et al., 2004).

To directly determine the involvement of 5′ Hoxd genes in the polydactyly of Gli3 mutants, we have generated a compound mutant that simultaneously removes the three most 5′-located Hoxd genes and Gli3. SHH prevents the processing of GLI3 while HOXD12 and HOXD13 convert its function from a transcriptional repressor to a transcriptional activator (Wang et al., 2000; Chen et al., 2004). Therefore, removal of the 5′ Hoxd genes in the absence of Gli3 may also help to determine other functions of 5′ Hoxd genes not related with their interaction with GLI3.

Remarkably, the limbs deficient in all four of these genes are extremely polydactyly, arguing against most 5′-located Hoxd genes mediating the polydactyly characteristic of the Xr phenotype. Compound mutants differ from Gli3 mutants in the number, length and chondrogenic differentiation of the digits, indicating that posterior Hoxd genes have functions independent of GLI3. Based on the molecular study of the compound mutant, we propose that the polydactyly phenotype is mediated by the gain of function of HOXD10 and HOXD9. Our results also support the notion that an adequate balance between positive and negative effects of different Hoxd genes is required for pentadactyly.

Materials and Methods

Mice mutant genotyping

The Extra-toes (Gli3lox/loxJackson allele; Hui and Joyner, 1993), the HoxdDel(11–13) (Zakany and Duboule, 1996) and the Shh (Chiang et al., 1996) mutant lines were maintained in a mixed background. The HoxdDel(11–13) allele is the deletion of the Hoxd13 and Hoxd12 locus plus the insertion of the lacZ reporter gene within the Hoxd11 gene, thus representing the loss-of-function of Hoxd13, 12 and 11 (Zakany and Duboule, 1996).

The embryos were obtained by caesarean from pregnant mice and dissected in cold phosphate buffered saline (PBS). Genotyping was performed by PCR as described (Hui and Joyner, 1993; Chiang et al., 1996; Zakany and Duboule, 1996). Noon of the day the vaginal plug was observed was considered as
embryonic day 0.5 (E0.5). Besides the gestational age, the external characteristics of the limbs were also used to stage the embryos (Fernandez-Tern et al., 2006).

In situ hybridization

Digoxigenin-labeled antisense riboprobes were prepared, and whole mount in situ hybridization analysis performed according to standard procedures (Nieto et al., 1996). The probes used were Shh, Fgf8, Fgf8, Grem1, Jagl1, Hox4, Hoxa11, Hoxa13, Gli1 and Hand2 (kindly provided by P. Beachy, D. Duboule, G. Martin and A. Joyner). For each gene analyzed, the complete genotypic series of limbs were processed jointly in the same hybridization tube to minimize possible differences in the staining due to the procedure.

Skeletal analysis

Skeletal staining was performed according to standard protocols. Briefly, embryos were skinned, eviscerated and fixed in 95% ethanol. Cartilages were stained with Alcian blue and bones with Alizarin red. The skeletal analysis was performed on newborns except for fetters including the Shh mutant allele, which were collected at E16.5 to avoid the late fetal lethality of the Shh mutation.

Results

Analysis of limb phenotypes

It has been suggested that the polydactyly characteristic of Gli3\textsuperscript{XtJ} mutants depends on the anterior upregulation of 5′ Hoxd genes (Zuniga and Zeller, 1999; Littingtung et al., 2002; te Welscher et al., 2002b). To test this hypothesis directly, we have generated the compound mutation for Gli3 and the three most 5′-located Hoxd genes. We also reasoned that this combined mutation would allow the analysis of 5′Hoxd function in digit patterning, independent of GLI3.

For this genetic approach, we analyzed progeny from crosses between Gli3\textsuperscript{XtJ}·Hoxd\textsuperscript{Del(11–13)−} double heterozygous mice (Fig. 1). At birth all genotypes were recovered at the expected Mendelian ratios but homozygous Gli3\textsuperscript{XtJ} mutants died immediately after birth. The limb skeletons of newborn homozygous for the two mutations were analyzed and all of them showed an identical phenotype characterized by an extreme polydactyly (Figs. 1Q–T).

As described, Gli3\textsuperscript{XtJ} homozygous limbs exhibit a severe polysyndactyly with 7–9 digits in the forelimb and 6–7 in the hindlimb (Figs. 1I, J; Hui and Joyner, 1993; Mo et al., 1997), whereas heterozygous mutants show a variable degree of duplication of digit 1 (Figs. 1E, F). Gli3\textsuperscript{XtJ} digits have three phalanges but the chondrogenesis of the proximal phalanx is very defective (Chen et al., 2004; Hilton et al., 2005). The digits in the Gli3\textsuperscript{XtJ} limb lack distinct anteroposterior identities (Figs. 1I, J).

Homozygous Hoxd\textsuperscript{Del(11–13)} mice also show polysyndactyly but of a very different morphology from that of Gli3\textsuperscript{XtJ} mutants (Figs. 1K, L; Zakany and Duboule, 1996). The autopod is characterized by brachydyctaly and central/postaxial polydactyly of 6–7 digits mostly bidental and with disorganized cartilage pattern (Figs. 1K, L). In our background, we only observed 6 digits in forelimbs while hindlimbs were pentadactyly. A prominent excrescence in the first metatarsal, a trait of the Hoxd13 deficiency, is always present (arrowhead in Fig. 1L; Dolle et al., 1993). Heterozygous Hoxd\textsuperscript{Del(11–13)} mutants frequently show a slight reduction in the second phalanx of digits II and V (Figs. 1G, H).

Remarkably, mice double homozygous for Gli3\textsuperscript{XtJ} and Hoxd\textsuperscript{Del(11–13)} had a more severe polysyndactyly phenotype than in any individual mutation (Figs. 1Q–T). Compound mutants developed 10 to 11 short and syndactylous digits in the forelimb and 8 to 9 in the hindlimb (Figs. 1S, T). To our knowledge this is the most severe polydactyly reported so far in the mouse. All digits in the compound mutant were bidental and looked identical with undefined identity, except for the posterior slender extradigit typical of the Hoxd\textsuperscript{Del(11–13)} deficiency, which was also found in the compound mutant (Figs. 1Q, R and Fig. S1). Distinct anteroposterior identities were only retained at the level of the hindlimb proximal autopod but not in the digits proper, neither in the whole forelimb autopod (Figs. 1Q, R and Fig. S1). The terminal phalanx was always clearly identifiable and normally ossified (marked as P3 in Fig. S1) but which of the two proximal phalanges had been eliminated was difficult to discern (therefore marked as P2? in Fig. S1). The absence of one phalanx is the main cause of the characteristic brachydyctaly but a pronounced ventral bending of the whole autopod also contributed to making the digits appear even shorter than they were (the ventral bending is best noted in the sections in Fig. S1). Remarkably, while the hindlimb autopod showed a conspicuous primary ossification center in each metatarsal, forelimb metacarpals showed no sign of ossification at birth except for some occasional and small and randomly located foci (Fig. 1Q and Fig. S1).

Interestingly, the syndactyly phenotype typical of the Gli3\textsuperscript{XtJ} limbs was increased by the additional removal of the three posterior Hoxd genes. In Gli3\textsuperscript{XtJ} limbs the soft tissue syndactyly does not affect the distal (ungueal) phalanx. However, in the compound mutation the syndactyly also affected the distal phalanx (Fig. 1 and not shown). The extreme polysyndactyly, together with the marked ventral flexion of the digits, resulted in a cup-shape autopod, particularly in forelimbs (Fig. 1Q and Fig. S1), reminiscent of the cup-shaped hands described in human syndactyly type IV (Sato et al., 2007).

An increase in the potential to form digits was already observed when one copy of the three posterior Hoxd genes was deleted in the absence of Gli3 (Gli3\textsuperscript{XtJ}·Hoxd\textsuperscript{Del(11–13)}−), suggesting a quantitative dose-dependent effect. This was more clearly observed in the hindlimb that showed a range of 6–9 digits, but with most specimens showing 7 or 8 digits, a number intermediate between the 6 and 7 typical of the Gli3\textsuperscript{XtJ} mutant and the 8–9 typical of the compound homozygous mutant (Figs. 1M, N). Equally interesting was the observation that the additional inactivation of one Gli3 allele in the absence of Hoxd11-13 (Gli3\textsuperscript{XtJ}·Hoxd\textsuperscript{Del(11–13)}−) resulted in the variable duplication of digit 1, typical of Gli3\textsuperscript{XtJ} heterozygous, added to the phenotype of Hoxd\textsuperscript{Del(11–13)} homozygous (Figs. 1O, P).

Thus, the development of Gli3\textsuperscript{XtJ}·Hoxd\textsuperscript{Del(11–13)}− resulted in digit abnormalities ranging from brachydyctyly and syndactyly to severe polydactyly. The presence of Hoxd13 deficiency is a common aspect of all the mutants, leading to a more severe phenotype, which emphasizes the importance of Hoxd13 in the control of digitization.
combined mutation also proves that the polydactyly of the Gli3 mutant limb does not require the three most 5′-located Hoxd genes and suggests that posterior Hoxd genes may have some negative effect in determining digit number since its additional removal, in the absence of Gli3, leads to an increase in the number of digits.

\[ \text{SHH signaling is irrelevant for the phenotype of the compound } \text{Gli3}^{XtJ/XtJ};\text{Hoxd}^{Del(11-13)/+} \]

It is known that Shh expression is ectopically activated in the anterior mesoderm of Gli3\(^{XtJ/XtJ}\) limbs (Masuya et al., 1995; Buscher et al., 1997; Masuya et al., 1997). This ectopic activation of Shh may depend on the ectopic anterior expression of posterior Hoxd genes as overexpression of HOXD proteins in mice and chicks results in anterior activation of Shh (Morgan et al., 1992; Charité et al., 1994; Goff and Tabin, 1997; Knezevic et al., 1997; Caronia et al., 2003; Chen et al., 2004; Zakany et al., 2004). Thus, we asked whether Shh ectopic activation would also occur when the three posterior Hoxd genes are deleted from the Gli3\(^{XtJ/XtJ}\) background. The analysis of Shh expression in several compound mutants between E10.5 and E13.5 failed to detect any anterior ectopic domain, while the posterior normal domain was spatially and temporally similar to normal (Figs. 2A, B, see also Fig. 3D and data not shown). The forelimb in Fig. 2A does not show the posterior domain of Shh expression because it corresponds to the developmental time at which Shh expression terminates.

Fig. 1. Skeletal phenotypes of single and compound mutants of Gli3\(^{XtJ}\) and Hoxd\(^{Del(11-13)}\). Genotypes are indicated at the top. For each genotype the autopod skeleton of the forelimb (left) and the hindlimb (right) are shown. The complete fore and hindlimb skeleton is also shown for the control (C, D) and compound mutant (S, T). All specimens are shown in dorsal views and with distal to the right and anterior to the top. Note that the number of digits increases as the dose of Hoxd11, Hoxd12 and Hoxd13 is reduced in the absence of Gli3. The polydactyly is maximal when Gli3 and the three most 5′-located Hoxd genes are simultaneously removed. The arrow in panels K and Q points to the posterior slender digit typical of Hoxd\(^{Del(11-13)}\) homozygous. The arrowhead in panel L indicates the protuberant first metatarsal typical of the Hoxd13 deficiency.
However, since the ectopic activation of Shh at the anterior border of Gli3XtJ mutants is usually small, transitory and sometimes difficult to detect, we decided to analyze the expression of Gli1, a well-established target of SHH (Ahn and Joyner, 2004). Interestingly, Gli1 transcripts were clearly detected at the anterior border of E12 double mutant limbs (Figs. 2C, D). The ectopic anterior expression was clearly observed in the hindlimb (arrowhead in Fig. 2D) but not in the forelimb, a difference that can be attributed to the normal difference in developmental stage between fore and hindlimbs. These results allow us to conclude that Shh is indeed activated at the anterior border of the compound mutant limb, disregarding the failure in detecting Shh transcripts. Therefore, in the context of absence of Gli3, the three most 5′-located Hoxd genes are dispensable for Shh anterior ectopic activation. It should be noted here that Hoxd10, capable of triggering Shh expression (Tarchini et al., 2006), is upregulated at the anterior border of the compound mutant limb (see below).

The double Shh;Gli3 mutant limb showed that SHH signaling was irrelevant for the Gli3XtJ polydactyly, disregarding its anterior ectopic activation (Litingtung et al., 2002; te Welscher et al., 2002b). However, SHH signaling is operative in Gli3XtJ mutant limbs as it induces Gli1 and Ptc1 (Litingtung et al., 2002; te Welscher et al., 2002b), possibly through GLI2. In the neural tube and other systems, GLI2 has been shown to play activator functions necessary for SHH signaling (Sasaki et al., 1999; Mill et al., 2003). Also, the reduction in bone length observed in Gli2−/− mutant limbs is increased by the additional removal of one copy of Gli3 (Mo et al., 1997). Therefore, to completely rule out the involvement of SHH signaling in the polydactyly of the compound mutant, we performed the triple mutation in which Gli3, the three most 5′-located Hoxd genes and Shh were simultaneously removed.

Embryos from crosses between triple heterozygotes (Gli3XtJ; HoxdDel(11–13)y; Shh−/−) were collected at E16.5 due to the late fetal lethality of the Shh mutant allele. Although endochondral ossification could not be completely evaluated because of the relatively early developmental age of the embryos, we found that the additional removal of Shh did not modify the polydactyly of double compound homozygous embryos (Figs. 2E, F). This result confirmed that Shh was irrelevant for the phenotype of the compound Gli3XtJ; HoxdDel(11–13)y; Shh−/− mutant.

**Gene expression analysis in mutant limbs**

Compound mutant limbs developed relatively normally during early stages, the phenotype not being distinguishable by morphologic changes up to E11. Between E11 and E12 the double mutant limb developed a dramatic expansion of the autopod (please note the shape of the compound mutant limb in Figs. 3 and 4). Besides the posterior Hoxd genes, other factors known to be downstream of GLI3 are Fgf8, Fgf4, Grem1, Hand2 and Jag1. All of them are upregulated in the anterior mesoderm of

![Fig. 2. Shh expression is irrelevant for the polydactyly phenotype of compound mutants. Panels A and B are E12 compound mutant forelimb (A) and hindlimb (B) hybridized for Shh showing no detectable expression at the anterior border. Note that the normal Shh expression in the posterior mesoderm has already terminated in the forelimb. Panels C and D are E12 compound mutant forelimb (C) and hindlimb (D) hybridized for Gli1 showing ectopic expression at the anterior border of the hindlimb (arrowhead). Panels E and F are skeletal preparations of E16.5 Gli3XtJ; HoxdDel(11–13)y; Shh−/− triple fore (E) and hindlimb (F) mutants.](image-url)
Gli3XtJ limbs, and therefore possibly implicated in the polydactylosus phenotype (Zuniga and Zeller, 1999; Litingtung et al., 2002; te Welscher et al., 2002b; McGlinn et al., 2005). In situ hybridization for Fgf8, which is normally expressed along the entire anteroposterior extension of the apical ectodermal ridge (AER; Martin, 1998), revealed a marked anterior extension of the AER in the compound mutant (Fig. 3D), similar to Gli3XtJ limbs (Fig. 3B) while Fgf8 expression in HoxdDel(11–13) homozygous limbs was comparable to normal (Figs. 3A, C). The limbs in Figs. 3A–D were hybridized conjointly for Fgf8 and Shh; note that the expression domain of Shh in the compound mutant looked similar to normal as previously mentioned (compare Figs. 3A and D).

Expression of Fgf4 is normally restricted to the posterior AER, parallel to the mesodermal expression of Grem1 (Martin, 1998; Zuniga et al., 1999; Fig. 3E). As reported, the expression of these two genes was anteriorly expanded in Gli3XtJ mutant limbs (Litingtung et al., 2002; te Welscher et al., 2002b; Fig. 3F). Compound mutants also showed a clear anterior expansion of the domain of expression of Fgf4 and Grem1, but curiously the level of expression was weaker than in Gli3XtJ limbs (Fig. 3H). Fgf4 expression appeared patched and irregular all along the extension of the AER, whereas Grem1 domain of expression appeared less well defined than in control and Gli3XtJ limbs. Expression of both Fgf4 and Grem1 was within normal limits in HoxdDel(11–13)/Del(11–13) limbs (Fig. 3G). The difference in pattern of expression between the limbs in Figs. 3G and E was merely due to a slightly different developmental stage between both limbs.

Hand2 (formerly dHand) is a transcription factor involved in the establishment of the anteroposterior patterning of the limb bud prior to Shh through a reciprocal antagonism with Gli3 (Charite et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002a). Accordingly, Hand2 is, in fact, upregulated in Gli3XtJ anterior mesoderm (Fig. 3J; te Welscher et al., 2002a, 2002b; Litingtung et al., 2002). In the compound mutant Hand2
expression also became upregulated in the anterior mesoderm of E11.5 limb buds, but in a pattern more distally restricted than in Gli3XtJ homozygous (Fig. 3L). At later stages, Hand2 upregulation occurred in the whole autopod of compound mutants, in a pattern similar to Gli3XtJ/XtJ (not shown). Expression of Hand2 in HoxdDel(11–13)/Del(11–13) limbs was similar to control limbs (Figs. 3K and I).

Using microarray technology, the Notch ligand Jag1 was identified as highly repressed by GLI3 (McGlinn et al., 2005). Since Jag1 is also an excellent marker for the distal mesoderm, the site of the phenotype in the double mutant, we decided to analyze its expression. As reported, Jag1 was highly upregulated in the anterior mesoderm of Gli3XtJ/XtJ limb buds by E11.5, in accordance with it being repressed by GLI3R (Fig. 3N; McGlinn et al., 2005; Panman et al., 2006). In the compound mutant limb, Jag1 was also anteriorly upregulated (Fig. 3P) while the pattern of expression in triple Hoxd deletants was comparable to normal (Figs. 3O, M).

In short, our results are compatible with Fgf8, Hand2 and Jag1 having a role in the polydactyly of Gli3XtJ and Gli3XtJ;HoxdDel(11–13)/Del(11–13) mutants and indicate that their anterior ectopic expansion, besides not requiring SHH signaling (Litingtung et al., 2002; te Welscher et al., 2002b), does not require the input of the three most 5′Hoxd genes either. However, the similarity in the patterns of expression of Fgf8, Hand2 and Jag1 between Gli3XtJ and Gli3XtJ;HoxdDel(11–13)/Del(11–13) limbs does not provide an explanation for the increase in the polydactyly potential exhibited by the compound mutant. The fact that the expression of Grem1 and Fgf4, although spatially expanded, is quite faint in the compound mutant argues against these two genes playing a major role in the polydactyly since a substantial decrease in

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**Fig. 4.** Expression of Hoxd9, Hoxd10 and Hoxa13 in the different genotypes. All the panels show dorsal pictures of forelimbs hybridized with the specific probe indicated on the left. The developmental stage of the limbs is also indicated on the left. Genotypes are marked at the top of each column. Note the strong upregulation of Hoxd10 expression in Gli3 and compound mutants. The arrowheads in panels A and C delimit the anteroposterior extension of the Hoxd9 autopodial domain. The arrowhead in panels I–L points to the anterior limit of Hoxa13 expression in the distal mesoderm.
their level of expression associates with an increase in the polydactylous potential of the limb.

Effects of the compound mutation on the expression of other Hox genes

One possible explanation for the exacerbation of the polydactyly in compound mutants is that the deletion of the three most 5′ Hoxd genes causes misexpression of other Hox genes necessary for proper autopod formation. Since the pattern of expression of a particular Hoxd gene changes following its location in the cluster and relative distance to the enhancers (Kmita et al., 2002; Tarchini and Duboule, 2006), we first analyzed the expression of Hoxd10 and Hoxd9, the genes nearest to the deletion point. We concentrated on the second phase of expression since it is the one required for the morphogenesis of the digits (Sordino et al., 1995; Tarchini and Duboule, 2006). At E11.5, Hoxd9 expression mainly occurs in a proximal forearm domain that corresponds to the early phase of expression but in addition also shows a faint autopodial domain corresponding to the late activation phase. The autopodial domain appeared centrally located in the distal wild-type and homozygous HoxdDel(11-13) autopods (delimited by arrowheads in Figs. 4A and C). However, Gli3XtJ and Gli3XtJ;HoxdDel(11-13) homozygous showed a marked expansion of Hoxd9 domain of expression all along the anteroposterior distal mesoderm (Figs. 4B and D). At this stage, the early phase of Hoxd9 expression, as reflected by the forearm domain, appeared much stronger in HoxdDel(11-13) and compound mutants, probably reflecting a stronger expression at earlier stages.

Normal Hoxd10 expression is posteriorly restricted and, at E11, occurs in two bands, corresponding to the zeugopod and autopod (early and late phases) domains of expression, separated by a transversal strip devoid of transcripts (Fig. 4E). This pattern is conserved in single HoxdDel(11-13)Del(11-13) mutants as previously reported (Fig. 4G; Zakany and Duboule, 1996). Remarkably, expression of Hoxd10 in both Gli3XtJ and double homozygous limbs lacked its characteristic posterior restriction as well as the separation between the autopodal and zeugopodal domains, except for a small area or lesser expression at the very posterior margin of the bud (Figs. 4F and H). Therefore, the compound mutant limb develops with a strong and uniform expression of Hoxd10 across the whole anteroposterior axis of the bud (Fig. 4H). It is worth noting here that the upregulation of Hoxd10 provides an explanation for the ectopic activation of Shh at the anterior border of the compound mutant (Figs. 2A, B), as it is known that Hoxd10 can trigger Shh expression (Tarchini et al., 2006).

Since several Hoxa and Hoxd genes have been shown to function cooperatively in a redundant and quantitative manner (Davis et al., 1995; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Zakany et al., 1997), we also analyzed the expression of Hoxa11 and Hoxa13 in the double mutant. In E10.5 compound mutant limbs the expression of Hoxa13 appeared clearly expanded in the anterior mesoderm (Fig. 4L), similarly to Gli3XtJ limbs (Fig. 4J; te Welscher et al., 2002b). The more distal restriction observed in compound (Fig. 4L) versus Gli3XtJ (Fig. 4J) mutant limbs at E10.5 is explained by the slight developmental delay of the compound mutant relative to the Gli3XtJ limb and was not observed at later stages. At E11.5 compound mutants were characterized by an elevated and homogeneous expression of Hoxa13 throughout the distal mesoderm, similarly to Gli3XtJ (Figs. 4N, P). HoxaDel(11-13) homozygous showed a pattern and level of expression similar to wild type (Fig. 4O, compare with Fig. 4M). Finally, the analysis of expression of Hoxa11 in E11.5 double mutant limb buds showed restriction to the junction between the zeugopod and autopod in a normal pattern (not shown).

Altogether, the results of our gene expression analysis revealed that the compound mutant limb developed with uniform expression of Hoxd9, Hoxd10 and Hoxa13 across the distal limb mesoderm in a pattern similar to Gli3 mutant limbs. However, the expression of Hoxd10 and Hoxd9, although similarly upregulated in these two genotypes, is predicted to have a higher functional impact in compound mutants because of the absence of the negative effect of posterior Hox genes (posterior prevalence).

Discussion

Deletion of Hoxd11-13, in the absence of Gli3, increases digit number

In this work, we have removed the three most 5′-located Hoxd genes simultaneously with Gli3 in order to further analyze the role these genes play during digit development. It is currently accepted that the polydactyly of Gli3XtJ mutants depends on the ectopic anterior expression of 5′-located Hoxd genes (Zuniga and Zeller, 1999; Litingtung et al., 2002; te Welscher et al., 2002b). Therefore, we reasoned that the number of digits would decrease if the most 5′Hoxd genes were deleted from the Gli3XtJ background. Unexpectedly, Gli3XtJ; Hoxa11, Hoxa13XtJ; HoxdDel(11-13)Del(11-13) mutant limbs, although relatively normal at proximal level, were severely polydactylous; the number of digits being larger than in the single Gli3 mutant. Indeed, to our knowledge, these compound mutant limbs bear the largest number of digits so far reported in the mouse, 10–11 in the forelimb and 8–9 in the hindlimb. This phenotype shows that the loss-of-function of Hoxd11 to 13, in the absence of Gli3, increases the polydactyly potential of the limb and reveals a negative effect of posterior Hoxd genes in determining digit number.

The polydactyly in compound mutants can be classified as type 2 polydactyly because of symmetric unidentifiable digits and disruption of Gli3 function (Litingtung et al., 2002; te Welscher et al., 2002b). As occurs in Gli3 mutants, ectopic Shh expression, reported by Gli1 expression, is observed at the anterior border of compound mutant limbs demonstrating that it does not require HOXD11-13 inputs, although it likely relies on the upregulation of Hoxd10, which is also capable of triggering Shh expression (Tarchini and Duboule, 2006). To thoroughly rule out a possible involvement of Shh in the polydactyly, we performed the triple Gli3XtJ; HoxaDel(11-13)Del(11-13)XtJ Shh−/−
mutant that showed identical limb phenotype as the double mutant proving that the phenotype was independent of SHH signaling. Also, the fact that the Gli3$^{XtJ/XtJ}$, Hoxd10$^{Del(11-13)/Del(11-13)}$ mutant limbs are not identical to Gli3$^{Xe/Xe}$ limbs, as both types of limbs differ in the number, morphology and chondrogenic differentiation of the digits, indicates that 5′ HOXD proteins do, indeed, have functions independent of GLI3, either acting as monomers or in association with multiple partners (Svingen and Tonissen, 2006).

The phenotype of the compound mutants indicates that Hoxd11-13 do not contribute to the polydactylous phenotype in Gli3$^{Xe/Xe}$ limb. This phenotype may appear difficult to reconcile with multiple experiments demonstrating that overexpression of Hoxd12 and other posterior Hox genes results in anterior polydactyly (Morgan et al., 1992; Goff and Tabin, 1997; Knezevic et al., 1997; Chen et al., 2004; Zakany et al., 2004). However, the physical interaction between HOXD and GLI3R proteins, which reverses the transcriptional activity of the latter from a repressor into an activator, provides an explanation for the preaxial polydactyly caused by 5′ Hoxd overexpression, in the presence of Gli3 (Chen et al., 2004). Indeed, overexpression of Hoxd12 requires Gli3 in order to cause anterior polydactyly (Chen et al., 2004).

**Chondrogenic defects in the mutants**

Targeted disruption in mice as well as overexpression experiments have shown that Hox genes are required for the appropriate condensation, proliferation and differentiation of skeletal elements (Morgan et al., 1992; Davis and Capecchi, 1996; Zakany and Duboule, 1996; Goff and Tabin, 1997; Yueh et al., 1998). In particular, loss of function of posterior Hox genes associates with a noticeable delay in the ossification pattern of the autopod bones revealing a strong deregulation of bone formation (Davis and Capecchi, 1996; Zakany and Duboule, 1996). Expression of Hoxd genes occurs in chondrocytes but is normally switched off as differentiation progresses to the prehypertrophic state (Zakany and Duboule, 1996; Suzuki and Kuroiwa, 2002). Interestingly, in the Hoxd10$^{Del(11-13)}$ mutant, expression of Hoxd11 lacZ, the marker gene present at the 5′ end of the Hoxd10$^{Del(11-13)}$ allele (Zakany and Duboule, 1996) persists in chondrocytes concomitantly with the delay in differentiation. Also, overexpression of Hoxc8 and Hoxd4 in the chondrogenic lineage results in a marked chondrogenesis delay (Yueh et al., 1998). These observations raise the possibility that the chondrogenic phenotype in the absence of posterior Hox genes may be mediated by abnormal persistent expression of remaining Hoxd genes instead of a more direct requirement of 5′ HOXD products in chondrogenesis. In this regard, it would be interesting to analyze whether expression of Hoxd9 and Hoxd10 abnormally persists in the growing skeleton of the compound mutant.

Besides the delayed chondrogenic differentiation resulting from the removal of posterior HOXD proteins, the defects in ossification in the double mutant should also combine modifications in IHH signaling due to the absence of GLI3 (Hilton et al., 2005; Koziel et al., 2005). It is worth noting the puzzling difference between the ossification of metacarpals and metatarsals in the newborn compound mutants. While ossification is completely absent in metacarpals, it is prominent in metatarsals. The reason for this variation remains unknown but differences in gene expression between the fore and hindlimb may account for this difference. In particular, several Hox genes show differential patterns of expression between fore and hindlimbs (Nelson et al., 1996). The chondrogenic differentiation of the three genotypes used in this work is an issue that deserves further investigation.

**Functional upregulation of Hoxd10 and Hoxd9 is responsible for the exacerbation of the polydactyly in compound mutants**

The examination of the pattern of expression of Fgf8, Fgf4, Grem1, Hand2 and Jag1, all of them previously associated with the Gli3 mutant phenotype (Zuniga and Zeller, 1999; Litingtung et al., 2002; te Welscher et al., 2002b; McGlinn et al., 2005), failed to detect changes that could account for the increase in digit number observed in the compound mutant relative to the Gli3 mutant. Indeed, the expression of Fgf4 and Grem1 was weaker in the double mutant suggesting that the modifications in pattern of expression of these genes may not be involved in the generation of the phenotype but rather associated with the broadening of the mutant limb. We also examined the expression of other Hox genes that could play a role in the formation of the autopod. The elimination of a group of Hoxd genes should impact on the expression of remaining Hoxd genes because of their reallocation in the chromosome (Tarchini and Duboule, 2006). However, the deletion we have used here contains a LacZ reporter transgene positioned at the place of Hoxd11 and hence it is the transcription of this reporter gene that results upregulated rather than that of the next gene (Hoxd10) in the chromosome (Zakany and Duboule, 1996). Accordingly, we observed that the late transcriptional profile of Hoxd9 and 10 was not significantly modified in Hoxd10$^{Del(11-13)}$ homozygous, confirming previous studies for Hoxd10 and Evx2 (Zakany and Duboule, 1996). GLI3 is a firm candidate to interact with the centromeric enhancer in the early phase of Hoxd expression and with the digital enhancer embedded in the Global Control Region implicated in the second phase of Hoxd expression (Spitz et al., 2003; Tarchini and Duboule, 2006). The fact that the spatial upregulation of the late Hoxd10 and Hoxd9 expression domains was similar in compound and Gli3$^{Xe/Xe}$ mutants, but absent in Hoxd11-13 deletants, indicates that it was mainly induced by the removal of GLI3.

Considering the functional suppression exerted by posterior HOXD products over more anterior products (Duboule and Morata, 1994; van der Hoeven et al., 1996; Herault et al., 1997; Williams et al., 2006), we propose that the expansion of the distal mesoderm and subsequent generation of an elevated number of short and identical digits typical of the Gli3$^{Xe/Xe}$, Hoxd10$^{Del(11-13)/Del(11-13)}$ compound mutation is mediated by the gain in function of HOXD10 and HOXD9. This gain in function relies on the derepression due to the absence of GLI3R and on...
the concomitant absence of the prevalent effect of posterior HOXD products. Even if the transcriptional profile of Hoxd10 and Hoxd9 is similar in Gli3 and compound mutants, the absence of posterior Hox genes in the later generates an important functional difference that would explain the different grade of polydactyly between both genotypes. The fact that, in the absence of Gli3, removal of the whole HoxD cluster does not modify the polydactyly (Zakany et al., 2007) while removal of only Hoxd11 to 13 increases the polydactyly (our work), strongly indicates that the gain in digit number is mediated by the remaining anterior Hox genes. The results of the genetic analysis performed by Zakany et al. (2007) along with our results strongly support the notion that “anterior” Hox genes have a positive effect on digit number, while “posterior” Hox genes restrict digit number and that a correct balance between anterior and posterior HOX products and GLI3 is required for pentadactyly.

In the context of our proposal, it is also important to consider why the upregulation of Hoxd10, which occurs in the absence of Hoxd11-13 but in the presence of Gli3, does not induce polydactyly (Kmita et al., 2002). Our interpretation is that the presence of GLI3, known to interact with HOX products (Chen et al., 2004), may interfere with HOXD10 function through a presently unknown molecular mechanism. Interestingly, the observation that a partial deletion of the HoxD cluster (Hoxd1 to Hoxd10 included), which carries a gain in function of Hoxd12 and 13 results in symmetric and frequently oligodactylous limbs (Zakany et al., 2004) but severely impairs limb development when combined with loss of Gli3 (Zakany et al., 2007), led Zakany et al. to suggest that GLI3 may exert a protective effect against the deleterious effects of these HOX proteins. Finally, the fact that Hoxd11 can trigger a spectacular polydactyly upon deletion of Hoxd12 and 13 but in the presence of Gli3 (Kmita et al., 2002) suggests specific GLI3/HOXD11 interactions. The capacity of Hoxd11 to trigger supernumerary digits indicates that the negative effect on digit number is indeed exerted by Hoxd13 and Hoxd12, but not Hoxd11.

Hoxa13 was also found to be upregulated in Gli3 and compound mutants. Previous published results indicate that the upregulation of Hoxa13 may mediate the polydactyly. For example, misexpression experiments in chick limb buds showed that Hoxa13 was involved in the adhesiveness of mesodermal cells by controlling homophilic cell interactions (Yokouchi et al., 1995). Hoxa13 was also found to be responsible for controlling cartilage growth and differentiation towards short bones characteristic of the autopod. The analysis of Hoxa13 mutant limbs further supported this interpretation and identified EphA7 as the molecule involved in this function (Stadler et al., 2001). Furthermore, it has recently been shown that Hoxa13 acts upstream of Sox9, since misexpression of Sox9 is able to rescue the hypodactyly phenotype characteristic of the Hoxa13 deficiency (Akiyama et al., 2007). Therefore, the upregulation in Hoxa13 observed in Gli3 and compound mutants may well contribute to the generation of an excessive number of chondrogenic rays. It is worth mentioning here that, on the basis of the phenotypes obtained after different combinations of loss-of-function alleles of HoxD and HoxA clusters, a predominant role for Hoxa13 in the generation of polydactylyous short-digited limbs was postulated (Zakany et al., 1997). The findings reported here are in complete agreement with this hypothetical role for Hoxa13.

Positive and negative effects of different Hoxd genes on determining digit number

In summary, the phenotype of the compound Gli3<sup>XtJ/XtJ</sup>; Hoxd<sup>Del(11–13)/Del(11–13)</sup> mutant indicates that the posterior Hoxd genes (probably only Hoxd13 and 12) play a negative role on digit number, considering that the polydactyly is clearly more severe than in Gli3<sup>XtJ/XtJ</sup> mutants. In accordance with the “posterior prevalence” model, we propose that the negative effect is exerted through the functional repression of HOXD10 and HOXD9, which have a positive effect on digit number. Therefore, anterior and posterior HOXD products need to be adequately balanced for the pentadactyl formula. The effects of modifications in this balance are more easily appreciated in the absence of Gli3, since GLI3, by directly binding HOXD proteins (Chen et al., 2004), may prevent or modify their function and interactions with other partners.

It has been speculated that the successive recruitment of the HoxA and HoxD clusters in developing appendages may have, at least partially, mediated the evolutionary transition from polydactyl to pentadactyl in ancestral tetrapods (Zakany et al., 1997). It is currently accepted that this recruitment may have carried the implementation of Shh activation (Tarchini et al., 2006). Our work suggests that the recruitment of the HoxD cluster had to include a correct balance between anterior and posterior products as well as with GLI3.

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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.2007.07.023.

References


