Short Communication

Genetic interaction of \textit{Gli3} and \textit{Alx4} during limb development

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ABSTRACT The \textit{Gli3} and \textit{Alx4} transcriptional regulators are expressed in the anterior limb bud mesenchyme and their disruption in mice results in preaxial polydactyly. While the polydactyly phenotype of \textit{Alx4} deficient limb buds depends on SHH, the one of \textit{Gli3} deficient limb buds is completely independent of SHH signalling, suggesting that these genes act in parallel pathways. Analysis of limb buds lacking both \textit{Gli3} and \textit{Alx4} now shows that these two genes interact during limb skeletal morphogenesis. In addition to the defects in single mutants, the stylopod is severely malformed and the anterior element of the zeugopod is lost in double mutant limbs. However, limb bud patterning in \textit{Gli3}\textsuperscript{-/-}; \textit{Alx4}\textsuperscript{-/-} double mutant embryos is not affected more than in single mutants as the expression domains of key regulators remain the same. Most interestingly, the loss of the severe preaxial polydactyly characteristic of \textit{Gli3}\textsuperscript{-/-} limbs in double mutant embryos establishes that this type of polydactyly requires \textit{Alx4} function.

KEY WORDS: \textit{Hox} gene, limb development, preaxial polydactyly, radius, SHH, tibia

The semi-dominant mouse mutations \textit{Extra-toes} (\textit{Xt}) and \textit{Strong's Luxoid} (\textit{Lst}), are loss-of-function mutations disrupting the zinc-finger encoding transcription factor \textit{Gli3} (Schimmang \textit{et al.}, 1992; Hui and Joyner, 1993) and the \textit{aristaless} related homeobox gene \textit{Alx4} (Qu \textit{et al.}, 1998; Takahashi \textit{et al.}, 1998), respectively. In the homozygous state, both mouse mutations cause pleiotropic and lethal congenital malformations with distinct preaxial limb polydactylies. In both mutants, an anterior ectopic \textit{Sonic Hedgehog} (SHH) signalling centre is established in addition to the one in the posterior limb bud mesenchyme (Masuya \textit{et al.}, 1995; Buscher \textit{et al.}, 1997; Qu \textit{et al.}, 1997). SHH is a morphogen produced by the polarizing region (or ZPA) in the posterior limb bud and is essential for controlling antero-posterior patterning of the limb skeleton (zeugopod and autopod; reviewed by Zeller, 2004). Only one anterior zeugopodal element and digit develop in limbs of embryos lacking SHH function (Chiang \textit{et al.}, 2001; Kraus \textit{et al.}, 2001). Interestingly, the polydactylyous phenotypes caused by the mutations in \textit{Gli3} and \textit{Alx4} differ with respect to their dependence on SHH signalling. The polydactyly of \textit{Gli3} deficient limbs is SHH independent as limbs lacking both \textit{Gli3} and \textit{Shh} display a polydactyly identical to \textit{Gli3} single mutants. In contrast, the one caused by disrupting \textit{Alx4} is SHH dependent as only one digit forms in limbs lacking both \textit{Alx4} and \textit{Shh} (for details see te Welscher \textit{et al.}, 2002b). Indeed, \textit{Gli3} functions first in a mutually antagonistic interaction with \textit{dHand}, which seems to pre-pattern the limb bud mesenchyme already prior to \textit{Shh} activation (te Welscher \textit{et al.}, 2002a). In contrast, \textit{Alx4} does not seem to function during this early determinative phase upstream of SHH mediated limb bud patterning. Additional striking differences between the two mutant limb phenotypes (Qu \textit{et al.}, 1997; Zuniga \textit{et al.}, 1999; te Welscher \textit{et al.}, 2002b) are a likely consequence of their differential functions: anterior expansion of the normally posteriorly restricted expression domains of \textit{Gremlin} and 5\textsuperscript{5}'\textit{Hoxd} genes is apparent from the earliest limb bud stages onwards in \textit{Gli3} deficient limb buds. In contrast, small anterior ectopic expression domains appear only much later in \textit{Alx4} deficient limb buds. Furthermore, \textit{Alx4} expression is reduced in \textit{Gli3} deficient limb buds, indicating that \textit{Gli3} acts genetically upstream of \textit{Alx4} (te Welscher \textit{et al.}, 2002a). However, the remaining, more proximally restricted \textit{Alx4} expression in \textit{Gli3}\textsuperscript{-/-} mutant limb buds also indicates that \textit{Alx4} may function at least in part independent of \textit{Gli3}. Taken together, these and other studies establish that \textit{Gli3}, but not

Abbreviations used in this paper: \textit{Xt}, extra toes.

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Alx4, acts initially up-stream of SHH to polarise the nascent limb bud mesenchyme (Masuya et al., 1995; Buscher et al., 1997; te Welscher et al., 2002a). Subsequently, SHH mediated inhibition of Gli3 protein processing produces a Gli3 repressor gradient that has been proposed as essential to antero-posterior limb bud patterning (Wang et al., 2000). During progression of limb bud development, Alx4 becomes critical to suppress anterior ectopic Shh expression (Qu et al., 1997). To uncover possible genetic interactions of Gli3 and Alx4, we have generated Gli3 and Alx4 compound mutant embryos for analysis. Up to gestational day 14.5 (E14.5), all genotypes are recovered from litters at expected ratios (Fig. 1 and data not shown), but by E16.5 no double homozygous embryos could be collected due to embryonic lethality (data not shown). The skeletons of four embryos lacking both Gli3 and Alx4 at E14.5 were analysed and all double homozygous limbs display skeletal defects that are distinct from the ones of all other genotypes (Fig. 1, 2 and data not shown; for summary see Table 1). In addition, severe craniofacial clefting of the nose region affects these embryos by E14.5 (data not shown).

**Skeletal Phenotypes of Forelimbs**

The scapula and stylopod of Gli3-/-; Alx4-/- double homozygous forelimbs are malformed (Fig. 1D; arrowhead points to malformed humerus) and the latter lacks a well-formed deltoid tuberosity. In contrast, the humerus of Gli3 (Fig. 1B) and Alx4 (Fig. 1C) single mutant embryos appears normal, while additional inactivation of either one Gli3 or Alx4 allele causes slight malformation of the humerus (arrowheads Fig. 1E, F). These results are indicative of a dose-dependent requirement of these two genes during humerus morphogenesis. In contrast to all other genetic combinations (Fig. 1A–C, 1E, F and data not shown), the zeugopod of Gli3-/-; Alx4-/- double homozygous forelimbs

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**TABLE 1**

<table>
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<tr>
<th>Skeletal Phenotypes</th>
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<th>Alx4-/-</th>
<th>Gli3-/-; Alx4-/-</th>
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<td>wild-type</td>
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<tr>
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<td>slightly abnormal</td>
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<tr>
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<td>6</td>
<td>~ 7</td>
<td>6</td>
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**Fig. 1.** Skeletal preparations of limbs of single and compound mutant embryos lacking Gli3 and/or Alx4 at gestational day E14.5. (A–F) Forelimbs, (G–L) hindlimbs, cartilage appears blue as stained by alcian blue. As ossification is just being initiated, none or only few ossification centres become apparent as darker areas (alizarin red). (A) Wild-type forelimb. (B) Gli3-/- homozygous forelimb. Note the preaxial polydactyly and loss of digit identity. (C) Alx4-/- homozygous forelimb. Asterisk indicates a duplicated preaxial digit. (D) Gli3-/-; Alx4-/- double homozygous forelimb. Arrowhead points to the malformed humerus. Arrow points to the anterior zeugopodal region lacking the radius. Note the duplication of the distal phalange of the most anterior digit. (E) Gli3-/-; Alx4+/- mutant forelimb, arrowhead indicates the malformed humerus. The preaxial polydactyly of the autopod is similar to the one of a forelimb lacking Gli3 alone. (F) Gli3+/-; Alx4-/- mutant forelimb. Arrowhead indicates the duplicated preaxial digit. Arrow points to the humerus lacking the deltoid tuberosity. Note that the phenotype of the autopod is identical to the one of a forelimb lacking Alx4 alone. (G) Wild-type hindlimb. (H) Gli3-/- homozygous hindlimb. Arrow points to the rudimentary tibia. (I) Alx4-/- homozygous hindlimb. Arrow points to the deformed tibia. Asterisk indicates the duplicated preaxial digit. (J) Gli3-/-; Alx4-/- double homozygous hindlimb. Arrow points to the region lacking the tibia. Arrowhead indicates the malformed femur. (K) Gli3-/-; Alx4+/- mutant hindlimb. The femur (arrowhead) and tibia (arrow) are affected. (L) Gli3+/-; Alx4-/- mutant hindlimb. Arrow points to the malformed tibia. Asterisk points to the duplicated preaxial digit. All panels are oriented with anterior to the top and distal to the right. a, autopod; dt, deltoid tuberosity; hu, humerus; ra, radius; sc, scapula; ul, ulna; fe, femur; fi, fibula; pg, pelvic girdle; ti, tibia.
lacks the radius (arrow, Fig. 1D), while the ulna forms correctly. Finally, the autopod of double homozygous limbs is distinct from all other mutant combinations (Fig. 1D and Fig. 2A-F, for details see below).

**Skeletal Phenotypes of Hindlimbs**

Similar to the forelimbs, the pelvic girdle and femur of Gli3-/-; Alx4-/- double homozygous hindlimbs are severely malformed (arrowhead, Fig. 1J). The femur of Gli3-/-; Alx4-/- compound mutant hindlimbs (arrowhead Fig. 1K) is affected similar to Gli3-/- limbs (arrowhead in Fig. 1H), while it is normal in embryos lacking Alx4 (Fig. 1I, L). In contrast to forelimbs, the tibia is missing in both Gli3-/-; Alx4-/- and Gli3-/-; Alx4-/- limbs (arrows in Figs. 1J, K) and its medial part is deleted in Gli3-/- single (arrow, Fig. 1H) and Gli3+/-; Alx4-/- compound mutant hindlimbs (arrow, Fig. 1L). Furthermore, the tibia is also deformed in Alx4-/- single mutant embryos (Fig. 1I), which shows that both genes are required and apparently fulfill only partially overlapping functions during tibia morphogenesis. Development of the hindlimb autopod (Fig. 1G-L) is affected similarly to forelimbs.

**Phenotypes of the Autopod**

An additional preaxial digit forms in Alx4-/- limbs (Fig. 1C, 2C) in comparison to wild-type limbs (Fig. 1A, 2A), irrespective of the presence of either both (Fig. 1C, 2C; see also Qu et al. 1997) or only one functional Gli3 allele (Fig. 1F, 2F). In contrast, the autopods of Gli3 deficient limbs display severe preaxial polydactylies with up to eight digits (Fig. 1B, 2B). In agreement with the conclusions of recent studies (Ahn and Joyner, 2004; Harfe et al., 2004), the posterior 3 digits retain distinct identities, while the five anterior-most digits lack defined identity (as indicated by question marks in Fig. 2B). Rather unexpectedly, complete inactivation of Alx4 and Gli3 (Fig. 1D and Fig. 2D) reduces digit numbers from eight (Fig. 2B, E) to five or six (Fig. 1D, 2D, 2J). The posterior three digits retain at least partial posterior identities (similar to Gli3-/-; compare Fig. 2D to 2B), while the anterior two digits display no defined identities. Most importantly, these results establish that the severe preaxial polydactylous limb phenotype of Gli3-/- deficient embryos (Fig. 1B, 2B) depends on Alx4 function. While digits 2 to 5 are patterned under the influence of SHH-mediated graded inhibition of Gli3 repressor formation, the most anterior digit 1 and the anterior zeugopod (radius and tibia) are specified by a so far unknown other mechanism (reviewed by Zeller, 2004). The results of our study now suggest that interaction of the Alx4 transcription factor with the Gli3 (repressor) protein may be involved in specifying the anterior-most limb skeletal elements (digit 1, radius and tibia). In support of this proposal, the characteristic duplicated preaxial digit observed in forelimbs lacking Alx4 but not Gli3 (Fig. 2C) in comparison to wild-type limbs (Fig. 2A, 2B), irrespective of the presence of either both (Fig. 2C, 2D; see also Qu et al. 1997) or only one functional Gli3 allele (Fig. 2F, 2G). 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1C, F and Fig. 2C, F) is also absent in most double mutant forelimbs (Fig. 1D, 2D). However, the distal most phalange of the most anterior digit is often duplicated (Fig. 1D, 2D). In contrast to inactivation of both Alx4 alleles (arrows, Fig. 2C, F), the loss of Gli3 functions causes interdigital webbing (arrowheads, Fig. 2B, E) by blocking interdigital apoptosis (Macias et al., 1999). This interdigital webbing is not altered by concurrent removal of Alx4 in double homozygous limbs (Fig. 2D), which points to a non-redundant and essential function of Gli3 in interdigital apoptosis.

Phenotypes of the Zeugopod
The most severe additional limb skeletal defect observed in embryos lacking both Gli3 and Alx4 in comparison to single homozygous mutants is the loss of the anterior zeugopodal element (radius and tibia, Fig. 1). Analysis of advanced limb bud patterning stages (E12.5, Fig. 2G-L) shows that the cartilage model giving rise to the radius does not form in double homozygous limbs (Fig. 2J). Unexpected from the analysis of older stages (Fig. 1E, 2E), the radius primordia is also less prominent in Gli3-/-; Alx4+/− compound mutant limb buds at this stage (arrow, Fig. 2K) than in wild-type (Fig. 2G) and the other genotypes (Fig. 2H, I, L and data not shown). These results indicate that formation of the radius is delayed in limbs lacking Gli3 and one copy of Alx4 (Fig. 2K), but catches up during its subsequent development (Fig. 1E, 2E) in contrast to the resulting aplasia in double homozygous forelimbs (Fig. 1D, 2D, 2J).

Normal Expression of Molecular Markers of Limb Bud Patterning
As in particular formation of anterior skeletal elements is disrupted in double homozygous limbs, the expression of relevant key regulators was analysed. Shh remains expressed normally in the posterior mesenchyme of single and double homozygous limb buds (Fig. 3A-D). No precocious anterior ectopic Shh expression is observed in double homozygous limb buds (Fig. 3D) as is the case later in single mutant limb buds (Masuya et al., 1995; Buscher et al., 1997; Qu et al., 1997). Expression of Hoxa11 in wild-type and mutant forelimb buds (E11.5). Note that the Hoxa11 expression domain in Gli3-/-; Alx4-/- limb buds (H) is identical to the one in Gli3-/- limb buds (F). Hoxd11 expression in wild-type and mutant forelimb buds (E11.75). Again the Hoxd11 expression domain in Gli3-/-; Alx4-/- limb buds (L) and Gli3-/- limb buds (U) are similar. Hoxd13 expression in the distal limb mesenchyme of wild-type and mutant limb buds (E11.75). The Hoxd13 expression in Gli3-/-; Alx4-/- limb buds (P) again resembles the one of Gli3-/- limb buds (N). Arrowheads in (I-P) indicate the anterior boundaries of the distal (autopod) expression domains. Asterisk in (K) indicates the ectopic anterior domain of Hoxd11 expression (Qu et al., 1997). Sox9 expression in wt and mutant limb buds (E11). Sox9 marks pre-cartilaginous condensations of mesenchymal cells. No significant differences are apparent. Arrowheads point to the approximate position of the condensations giving rise to the primordia of the radius. S’: prospective stylopod; Z’: prospective zeugopod; A’: prospective autopod. All limb buds are oriented with anterior to the top and distal to the right.
et al., 1997; Qu et al., 1997). Genetic analysis has established that the paralogous Hoxa11 and Hoxd11 genes interact to specify the ulna and radius (Davis et al., 1995) and proximal miss-expression of Hoxd13 inhibits zeugopod morphogenesis (Goff and Tabin, 1997). Therefore, these three Hox genes are good candidates to detect alterations affecting zeugopod patterning. However, expression and distribution of neither Hoxa11 nor Hoxd11 transcripts (Fig. 3H, L) is significantly altered in Gli3-/−; Alx4-/− double homozygous forelimb buds in comparison to Gli3 deficient counterparts (Fig. 3F, 3J). Furthermore, no significant changes in Hoxd13 expression are observed in Gli3-/−; Alx4-/− double homozygous limb buds (Fig. 3P) in comparison to single mutant and wild-type limb buds (Fig. 3M-O). Therefore, the absence of the radius is not paralleled by consistent changes in the expression patterns of these three Hox genes during stages of limb bud patterning. In particular, the anterior limits of the Hoxd11 and Hoxd13 expression domains in the distal limb bud mesenchyme of Gli3-/−; Alx4-/− embryos (arrowheads, Fig. 3L, P) are identical to the ones in Gli3-/− limb buds (arrowheads, Fig. 3N) and differ significantly from the ones in Alx4-/− and wild-type limb buds (arrowheads, Fig. 3K, K, M, O). These results indicate that Gli3, but not Alx4, is required to regulate the anterior limits of the Hoxd11 and Hoxd13 expression domains. The establishment of these expression domains is regulated by Gli3 already during onset of limb bud development (Zuniga and Zeller, 1999). Finally, the Sox9 transcription factor is expressed by the condensing mesenchymal cells that prefigure the limb cartilage elements (Wright et al., 1995). Analysis of Sox9 expression reveals that the mesenchymal condensations are induced apparently normal in Gli3-/−; Alx4-/− double homozygous limb buds (Fig. 3T; compare to Fig. 3Q-S). Taken together, the results shown in Figure 3 indicate that the patterning events directing initiation of mesenchymal condensations and cartilage models, in particular the anterior limb bud, occur to the same extent in Gli3-/−; Alx4-/− and Gli3-/− limb buds.

The present study reveals overlapping functions of Gli3 and Alx4 in formation of the radius, while truncations (but not complete loss) of the tibia have also been previously reported for Alx4-/− and Gli3-/− single mutant limbs (Johnson, 1967; Qu et al., 1998). In forelimb buds lacking both Fgf4 and Fgf8, the skeletal progenitors forming the zeugopodal condensations are reduced, which most likely directly causes loss of the radius (Sun et al., 2002). In contrast, the apparently normal Sox9 distribution in Gli3-/−; Alx4-/− double mutant limb buds indicates that the skeletal progenitor population is not reduced and induction of the mesenchymal condensations occurs apparently normally. Defects affecting the zeugopod are also observed in a variety of other mouse mutations, in particular those affecting 5′Hoxd and 5′Hoxa genes (Zakany and Duboule, 1999). For example, the mouse mutation Unlaless (Ul) affects cis -regulation of 5′Hoxd genes (Spitz et al., 2003). Ectopic proximal Hoxd13 together with reduced Hoxd11 expression causes severe reduction of both zeugopod skeletal elements in limbs of Ul mutants embryos (Herault et al., 1997; Peichel et al., 1997). Moreover, Chen et al. (2004) have established that the Gli3 repressor interacts directly with Hoxd proteins to promote digit specification. Therefore, it is possible to assume that the specific genetic interaction of Gli3 with Alx4 impacts on limb bud morphogenesis at the level of Hox protein regulation and function rather than their expression.

Experimental Procedures

Gli3+/−; Alx4+/− double heterozygous mice were inter-crossed to obtain Gli3+/−; Alx4+/− double mutant embryos. Embryos and mice were genotyped as described by te Welscher et al. (2002b). Day of vaginal plug detection was defined as embryonic day 0.5. Embryos of gestational days 10.5-11.75 were dissected in PBS, fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization using digoxigenin-labelled antisense riboprobes as described by Haramis et al. (1995). Embryos were age-matched by determining their somite number (variation ±2 somites). To visualize cartilage, embryos of gestational day E12.5 were fixed 5% TCA and subsequently stained with alcian green to visualize the cartilage. Embryos were cleared in methyl salicylate. Embryos of gestational days older than E14.0 were stained for cartilage and bone using standard alcian blue and alizarin red staining (Zeller et al., 1989). However, either no or only small ossification centres (red) were detected by E14.5 (Fig. 2, 3).

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References


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