Chapter 2

Genetic interaction between Gli3 and Alx4 during limb and craniofacial development

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Summary

Anterior-posterior patterning of distal limb skeletal elements is controlled by the polarizing region (ZPA). The function of the ZPA during limb development is mediated by the signalling molecule Sonic Hedgehog (SHH). Mutual genetic antagonism between Gli3 and dHand prepatters the limb bud prior to SHH signalling resulting in establishment of the ZPA in posterior limb bud mesenchyme. Subsequently, the Shh expression domain is kept posteriorly restricted by Gli3 and Alx4, which are both expressed in anterior limb bud mesenchyme. Disruption of either Gli3 or Alx4 results in establishment of an ectopic Shh domain and preaxial polydactyly. However the type of polydactyly observed in Gli3 deficient limb differs from that of Alx4 mutant limbs, suggesting that these genes might act in parallel pathways during limb development. Indeed, analysis of Gli3 \(^{-/-}\); Alx4 \(^{-/-}\) double homozygous mutant limbs reveals that Gli3 and Alx4 interact synergistically during patterning of all three groups of skeletal elements. The stylopod is severely malformed and the anterior skeletal element of the zeugopod is consistently lost in double mutant limbs. Furthermore digit numbers and identities are affected. However, no alterations in molecular markers of early limb patterning are observed in Gli3 \(^{-/-}\); Alx4 \(^{-/-}\) double mutant embryos, indicating that the two genes interact only during later developmental stages. In addition, Gli3 \(^{-/-}\); Alx4 \(^{-/-}\) double mutant embryos exhibit severe craniofacial defects.

Introduction

Tetrapod limb skeletal elements show a high variation in sizes, shapes and numbers between different species, although three skeletal segments are evident in all tetrapod limbs: proximally the stylopod, medially the zeugopod and distally the autopod. Limb skeletal elements are formed by endochondral ossifications, whereby mesenchymal cells condense and subsequently differentiate into chondrocytes to form a cartilaginous skeleton. These cartilage templates are ultimately replaced by bone during ossification (reviewed by Kronenberg, 2003). The patterning of limb skeletal elements is controlled by two signalling centres that integrate growth and anterior-posterior patterning of the limb (reviewed by Johnson and Tabin, 1997). The zone of polarizing activity (ZPA) functions as limb bud mesenchymal organizer by secreting the signalling molecule Sonic hedgehog (SHH; Riddle et al., 1993). SHH signals to the apical ectodermal ridge (AER), which in turn promotes limb bud outgrowth. Fibroblast growth factor (FGF) signalling by the AER regulates Shh expression (Niswander et al., 1993; Laufer et al., 1994) and results in establishment of a feedback loop between these signalling centres. SHH signalling regulates patterning of distal limb skeletal elements and ectopic SHH signalling causes digit duplications, while the lack of SHH signalling abolishes development of distal limb structures (Riddle et al., 1993; Chiang et al., 2001; Kraus et al., 2001).
Several independent mutations in mouse embryos disrupt the posterior restriction of Shh, which results in establishment of an anterior ectopic polarizing region and polydactyous limbs (Masuya et al., 1995; Buscher et al., 1997; Qu et al., 1997). Well characterized are the semidominant mouse mutant strains Extra-toes (Xt) and Strong's luxoid (Lst), which both show preaxial polydactyous limbs. The zinc-finger encoding transcription factor Gli3 is disrupted in the Xt mouse strain (Schimmang et al., 1992; Hui and Joyner, 1993), while the aristaless-related homeobox gene Alx4 is mutated in the Lst mouse strain (Qu et al., 1998; Takahashi et al., 1998). However, the type of polydactyly differs between these two mouse mutants. For example, the polydactylous limb phenotype of Gli3 deficient limbs is independent of SHH signalling, while polydactyly caused by disruption of Alx4 is dependent on SHH signalling (te Welscher et al., 2002b). In addition, Gli3 genetically interacts with dHand to prepattern the limb bud prior to SHH activation, while Alx4 does not interact with dHand during these early stages (te Welscher et al., 2002a). The difference between Gli3 and Alx4 mutant limbs is also reflected by the differences in gene expression. For example, the Hoxd13 domain is expanded anteriorly in Gli3 deficient limbs from early stages onwards, while only a small anterior spot of 5'HoxD expression appears late in Alx4 deficient limb buds (Qu et al., 1997; Zuniga et al., 1999; te Welscher et al., 2002b). This indicates that Gli3 and Alx4 act in different pathways during limb bud development. As Alx4 expression is reduced in Gli3 deficient limbs, Gli3 acts genetically most likely upstream of Alx4 (te Welscher et al., 2002a). However the residual, more proximally restricted Alx4 expression in Gli3 -/- mutant limbs is in agreement with the proposal that Gli3 and Alx4 function in different pathways. To uncover possible redundant roles and synergistic interactions of Gli3 and Alx4 during limb bud patterning, we have generated Gli3 -/-; Alx4 -/- double mutant embryos. Analysis of their mutant limbs reveals that Gli3 and Alx4 interact to pattern all three types of limb skeletal elements. Furthermore Gli3 -/-; Alx4 -/- double mutant embryos display severe craniofacial defects.

**Results**

**Gli3 and Alx4 interact during patterning of limb skeletal elements**

To reveal possible redundant roles for Gli3 and Alx4 during limb bud development, we have crossed Gli3 +/-; Alx4 +/- double heterozygous mice to generate Gli3 +/-; Alx4 +/- double homozygous embryos. At E16.5, no Gli3 +/-; Alx4 +/- double homozygous embryos were recovered from litters due to embryonic lethality. However at E14.5, all genotypes were recovered at expected ratios. In total we have analyzed the skeletons of four Gli3 +/-; Alx4 +/- double homozygous embryos. All limbs analyzed are of the same genetic background. Skeletal stains of E14.5 Gli3 +/-; Alx4 +/- mutant forelimbs and hindlimbs reveals defects in stylopod, zeugopod and autopod skeletal elements with complete penetrance that are distinct from defects observed with any other genetic combination (Figs. 1-3). Gli3 +/-; Alx4 +/- double mutant
Fig. 1. Skeletal stains of single and compound Gli3/Alx4 mutant limbs. All skeletal stains shown are of forelimbs of E14.5 embryos. Skeletal analysis shows that genetic interaction between Gli3 and Alx4 is required for patterning of the scapula, stylopod, radius and autopod. (a) Wild-type forelimbs. (b) Gli3 +/- heterozygous forelimbs. Arrow points to small ectopic cartilage condensation. (c) Alx4 +/- heterozygous forelimbs, which is phenotypically wild-type. (d) Gli3 +/-; Alx4 +/- double heterozygous mutant forelimbs, which is similar to Gli3 +/- heterozygous mutant forelimbs. Arrow points to small ectopic cartilage condensation. (e) Gli3 +/- homozygous forelimbs. Note the polydactylyous forelimb phenotype and the loss of digit identity. (f) Alx4 +/- homozygous forelimbs. Arrow points to a duplicated preaxial digit 2. (g) Gli3 +/-; Alx4 +/- double homozygous forelimbs. Arrow points to the region lacking the radius. Arrowhead points to the humerus, which is severely affected and lacks the deltoid tuberosity. Note the loss of digit identities. (h) The humerus of Gli3 +/-; Alx4 +/- mutant forelimbs is mildly affected (arrowhead). The autopodal phenotype is similar to Gli3 +/- mutant forelimbs. (i) The humerus of Gli3 +/-; Alx4 +/- mutant forelimbs lacks the deltoid tuberosity (arrowhead). Arrow points to a duplicated preaxial digit 2. Note that the autopodal phenotype is identical to Alx4 +/- mutant limbs. 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.

forelimbs develop a slightly malformed shoulder girdle. However, the humerus of Gli3 +/-; Alx4 +/- double mutant forelimbs is severely affected and lacks the deltoid tuberosity (dt; arrowhead, Fig.1g). In contrast Gli3 +/- (Fig. 1e) and Alx4 +/- (Fig. 1f) single mutant limbs develop a normal humerus. However, additional removal of one copy of either Alx4 or Gli3 in single homozygous mutant limbs results in a slightly enhanced phenotype (arrowheads Fig. 1h and i), suggesting a Gli3 and Alx4 dose-dependent requirement for patterning of the humerus. Most strikingly, Gli3 +/-; Alx4 +/- double mutant forelimbs lack the radius (arrow, Fig. 1g), which is normal in all other genetic combinations analyzed. Furthermore, the phenotype of the Gli3 +/-; Alx4 +/- double homozygous autopod is distinct from the autopodal phenotype observed in any other genetic combination (Fig. 1 and 3). The autopods of Gli3 +/- mutant
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limbs are polydactylous and form six to eight digits without distinct identities (Fig. 1e and Fig. 3b). Autopods of Gli3−/−; Alx4−/− mutant limbs display a similar phenotype (Fig. 1h and Fig. 3e). In contrast, six digits with distinct identities develop in Alx4−/− mutant polydactylous limbs (Fig. 1f and Fig. 3c). Again, the Alx4−/− mutant autopod phenotype is not changed by additional removal of one copy of Gli3 (Fig. 1i and Fig. 3f). However, removal of both Gli3 and Alx4 results in a complete loss of digit identity in forelimbs (Fig. 1g and Fig. 3d) as seen in Gli3−/− single mutant limbs (Fig. 1e). In addition, digit numbers of Gli3−/−; Alx4−/− double homozygous forelimbs varies between five and six. We never observed more than six digits in double mutant limbs, which is in contrast to the up to eight digits that form in Gli3−/− single mutant limbs (compare Fig. 1g with Fig. 1e). The distinct forelimb autopod phenotype of Gli3−/−; Alx4−/− double homozygous embryos suggest that Gli3 and Alx4 interact to pattern the autopod of the forelimb (Fig. 1 and 3). No significant differences are observed between Gli3+/-; Alx4+/- double heterozygous (Fig. 1 and Fig. 2d) and Gli3−/− single heterozygous mutant limbs (Fig. 1b and Fig.2b).

Fig. 2. Skeletal phenotypes of single and compound Gli3/Alx4 mutant hindlimbs of E14.5. Skeletal analysis reveals that Gli3 and Alx4 genetically interact during patterning of the pelvic girdle, femur, tibia and autopod. (a) Wild-type hindlimbs. (b) Gli3−/− heterozygous hindlimbs.
Arrow points to a split digit 1. (c) Alx4 +/- heterozygous hindlimbs. Arrow points to a duplicated preaxial digit 2. (d) Gli3 +/-; Alx4 +/- double heterozygous hindlimbs, which is similar to Gli3 +/- heterozygous hindlimbs. Arrow points to the split digit 1. (e) Gli3 +/- homozygous hindlimbs. Arrow points to the rudimentary tibia. Note the polydactylylimb phenotype and the loss of digit identities. (f) Alx4 +/- homozygous hindlimbs. Arrow points to a duplicated preaxial digit 2. (g) Gli3 +/-; Alx4 +/- double homozygous mutant hindlimbs. Arrow points to the region lacking the tibia. Arrowhead indicates the malformed femur. In addition the pelvic girdle is affected. 4 to 5 digits form without distinct identities. (h) The femur of a Gli3 +/-; Alx4 +/- mutant hindlimb is mildly affected (arrowhead), while the tibia is truncated (arrow). 5 digits form, which do not show distinct identities. (i) Gli3 +/-; Alx4 +/- mutant hindlimbs. Arrow points to the region lacking the tibia, which is truncated. Arrowhead points to a duplicated preaxial digit 2. Panels are oriented with anterior to the top and distal to the right. a: autopod; fe: femur; fi: fibula; pg: pelvic girdle; ti: tibia.

Similar to the forelimbs, Gli3 and Alx4 have redundant functions during hindlimb development (Fig. 2). The pelvic girdle and femur of Gli3 +/-; Alx4 +/- mutant hindlimbs are severely malformed (arrowhead, Fig. 2g). The femur of Gli3 +/-; Alx4 +/- compound mutant limbs (arrowhead Fig. 2h) is slightly affected, while Gli3 +/- single homozygous limbs (Fig. 2e) develop a normal femur, suggesting a dose dependent requirement of Gli3 and Alx4 during femur development. In contrast to the forelimb, the zeugopod of Gli3 +/- single and Gli3 +/-; Alx4 +/- and Gli3 +/-; Alx4 +/- compound mutant hindlimbs are affected (arrow in Fig. 2e, h, i). In these mutant limbs only a rudimentary tibia is formed. The tibia is totally missing in Gli3 +/-; Alx4 +/- double mutant hindlimbs (Fig. 2g), suggesting a dose dependent requirement for Gli3 and Alx4 during tibia formation. Finally, patterning of the hindlimb autopod is also affected in Gli3 +/-; Alx4 +/- mutant limbs as only four to five digits without distinct identities form (Fig. 2g). In contrast to the forelimb, the Gli3 +/-; Alx4 +/- double homozygous hindlimb autopod phenotype resembles that of Gli3 +/- single and Gli3 +/-; Alx4 +/- compound mutant hindlimbs, which also develop five digits lacking distinct identities (compare Fig. 2g with 2e and 2h). In contrast digits of Alx4 +/- single (Fig. 2f) and Gli3 +/-; Alx4 +/- (Fig. 2i) compound mutant hindlimbs retain distinct identities. Analysis of skeletons cleared with KOH for only a short period reveals that removal of the interdigital mesenchyme by apoptosis (Macias et al., 1999) is delayed in Gli3 deficient limbs (Fig. 3). At E14.5 mesenchymal cells of the interdigital regions have undergone apoptosis in wild-type, Alx4 +/- single and Gli3 +/-; Alx4 +/- compound mutant limbs, thereby eliminating the interdigital mesenchyme (arrows in Fig. 3a, c and f). In contrast, webbing is apparent in mutant limbs lacking both copies of Gli3 gene (arrowheads Fig. 3b, d, and e).
Fig. 3. Skeletal stains of E14.5 embryos that have been cleared by KOH for only a short period to visualize the remaining interdigital mesenchyme. In wild-type (a), Alx4 " single (c) and Gli3 "; Alx4 " (f) mutant forelimbs the interdigital mesenchyme is regressing (arrows). (b, d, e) Resorption of the interdigital mesenchyme is delayed in Gli3 deficient forelimbs (arrowheads). Digits are numbered according to their identities. Question marks indicate digits with unclear identities (b, d, e). (b, d, e) Digit identities are lost in Gli3 deficient forelimbs. (c, f) Digits of Alx4 " single and Gli3 "; Alx4 " mutant forelimbs have distinct identities. All limbs shown are forelimbs. Panels are oriented with anterior to the top and distal to the right.

With respect to losing the radius, it is well possible that the cartilage element giving rise to the radius initially forms in Gli3 "; Alx4 " mutant limbs, but that it is not maintained. However, analysis of cartilage elements of E12.5 forelimbs by Alcian green staining reveals that in absence of both Gli3 and Alx4, the cartilage model giving rise to the radius is also absent at this stage (arrow, Fig. 4d). Interestingly, also in Gli3 "; Alx4 " compound mutant limb buds, the radius cartilage model is only weakly apparent (arrow, Fig. 4e), which indicates that the formation of the radius cartilage model is delayed in Gli3 "; Alx4 " mutant limbs.

Fig. 4. Cartilage elements of single and Gli3/Alx4 compound mutant forelimbs of E12.5 embryos. The cartilage was visualized by Alcian green staining. (a, b, c, f) The cartilage element giving rise to the
radius is detectable in (a) wild-type, (b) Gli3^+/-, (c) Gli3^+/-, Alx4^+/-, and (f) Gli3^+/-, Alx4^+/- mutant embryos. (d, e) In contrast the radius cartilage element is lacking in Gli3^+/-, Alx4^-/- double homozygous limbs (arrow in d) and only weakly apparent in Gli3^+/-, Alx4^+/- mutant limbs (arrow in e). Limbs are oriented with anterior to the top and distal to the right. hu: humerus; ra: radius; ul: ulna.

Skeletal abnormalities in Gli3^-/-; Alx4^-/- double homozygous mutant limb are not caused by alterations of early limb bud patterning

SHH signalling is essential for anterior-posterior patterning of the zeugopod and autopod (Chiang et al 2001; Kraus et al., 2001). A single zeugopodal element forms in Shh deficient limb buds similar to Gli3^-/-; Alx4^-/- double mutant limbs. Therefore, we analyzed Shh expression in Gli3^-/-; Alx4^-/- mutant limb buds. However, Shh remains expressed in Gli3/Alx4 single and compound mutant limb buds of E10.75 (Fig. 5a-d and data not shown), indicating that the defects in patterning of distal skeletal elements in Gli3^-/-; Alx4^-/- mutant limb are not due to altered SHH signalling at this stage.

5'Hoxa and 5'Hoxd genes regulate patterning of limb skeletal elements (Zakany et al., 1997). Therefore we analyzed the expression of Hoxa11, Hoxd11 and Hoxd13 in wild-type and Gli3/Alx4 single and compound mutant limbs (Fig. 5e-p). The paralogous Hoxa11 and Hoxd11 genes interact to specify the ulna and radius (Davis et al., 1995). The absence of radius in Gli3^-/-; Alx4^-/- double mutant limb buds suggest that Hoxa11 or Hoxd11 expression might be altered. However, normal levels of Hoxa11 (Fig. 5h) and Hoxd11 (Fig. 5i) transcripts are detected in Gli3^-/-; Alx4^-/- double mutant limbs in comparison to wild-type limbs (Fig. 5e and i). At 11.5 Hoxd13 expression is restricted to the distal limb mesenchyme in wild-type embryos (Fig. 5m), consistent with the role of Hoxd13 in autopod patterning (Dolle et al., 1993). Misexpression of Hoxd13 inhibits the formation of the zeugopod (Goff and Tabin, 1997). In Gli3^-/-; Alx4^-/- double mutant limbs of E11.5 Hoxd13 expression domain is confined to the autopodal region and no ectopic proximal expression is observed (Fig. 5p), indicating that the absence of the radius is not caused by altered Hoxd13 expression. The expression domains of Hoxa11, Hoxd11 and Hoxd13 (Fig. 5h, I, p) in Gli3^-/-; Alx4^-/- double mutant limbs are identical to Gli3^-/- single mutant limb buds (Fig. 5f, j, n) and different from Alx4^-/- single mutant limb buds (Fig. 5g, k, and o), indicating that Gli3 acts upstream of Alx4 in regulating Hox gene expression.
Fig. 5. Molecular analysis of genes involved in limb bud patterning in (a, e, i, m, q) wild-type (wt), (b, f, j, n, r) Gli3 -/-, (c, g, k, o, s) Alx4 -/- single homozygous and (d, h, l, p, t) Gli3 -/-; Alx4 -/- double homozygous mutant limb buds. (a-d) Shh expression in wild-type and mutant limb buds (E10.75). There are no significant differences in Shh expression between wt and mutant limb buds. (e-h) Expression of Hoxa11 in wild-type and mutant limb buds (E11). Note that the Hoxa11 expression domain in Gli3 -/-; Alx4 -/- mutant limb is identical to the one in Gli3 -/- mutant limb buds. (i-l) Hoxd11 expression in wt and mutant limb buds (E11.5). Again the Hoxd11 expression domain in Gli3 -/-; Alx4 -/- double and Gli3 -/- single mutant limb buds are similar. (m-p) Hoxd13 expression is detected in distal limb mesenchyme of wt and mutant limb buds (E11.5). The Hoxd13 expression in Gli3 -/-; Alx4 -/- double mutant limbs resembles the one of Gli3 -/- single mutant limb buds. (q-t) Sox9 expression in wt and mutant limb buds (E11). Sox9 is a marker of precartilagenous condensations. Comparison of wt and mutant embryos shows that no significant differences are apparent. S': prospective stylopod; Z': prospective zeugopod; A': prospective autopod. All limb buds are oriented with anterior to the top and distal to the right.
Sox9 is expressed in cells of mesenchymal condensations that prefigure the limb skeletal elements (Wright et al., 1995). Analysis of Sox9 expression reveals that the formation of mesenchymal condensations takes place in Gli3−/−; Alx4−/− double mutant limbs of E11 (Fig. 5t), in a pattern similar to wild-type, Gli3−/− and Alx4−/− single mutant limb buds (Fig. 5q-s). These results indicate that the initial steps in cartilage formation are not affected in Gli3−/−; Alx4−/− double mutant limbs.

As no striking changes are observed in expression of genes involved in limb bud patterning, this analysis cannot explain the Gli3−/−; Alx4−/− double mutant limb skeletal phenotype. This suggests that the alterations specific for the Gli3−/−; Alx4−/− limb phenotype are not a consequence of alterations in early limb bud patterning, but might rather result from defects in cartilage formation and/or differentiation.

![Fig. 6. Analysis of the craniofacial skeleton reveals defects Gli3; Alx4 compound mutant embryos. Pictures show a dorsal view of the skulls of E14.5 embryos. Arrows point to the nasal bones in (a) wild-type, (b) Gli3−/− single, (c) Alx4−/− single, (d) Gli3−/−; Alx4−/− double, (e) Gli3−/−; Alx4−/− and (f) Gli3−/−; Alx4−/− mutant embryos. (d) The nose region of Gli3−/−; Alx4−/− double mutant embryos is clefted (arrow). (f) Gli3−/−; Alx4−/− compound mutant embryos also show a cleft nasal tip (arrow) and a clefted nasal septum (arrowhead). n: nasal bone.](image)

**Gli3 and Alx4 interact synergistically during craniofacial development**

In addition to the limb patterning defects, we detected craniofacial abnormalities in embryos deficient for both Gli3 and Alx4 (Fig. 6). Analysis of skeletal stains of E14.5 Gli3/Alx4 single and double mutant skulls reveals synergistic genetic interactions between Gli3 and Alx4 during craniofacial development. The craniofacial phenotype observed in Gli3−/−; Alx4−/− double mutant embryos includes severe clefting of the nose region (arrow, Fig. 6d), which is completely penetrant. The two lateral halves of the nasal cartilage are spaced wide apart. Gli3−/−; Alx4−/− mutant skulls also display a split nasal tip and a cleft nasal septum (arrow, Fig. 6f), while no facial clefting is observed in all other genotypes.
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Discussion

Skeletal analysis of Glis3/Alx4 single and compound mutant limbs has uncovered additional roles for Glis3 and Alx4 in patterning of limb skeletal elements. The studies reveal a dose dependent requirement of Glis3 and Alx4 for normal stylopod patterning. Malformation of the stylopod has not been observed in Glis3 and Alx4 single homozygous mutant limbs, likely because of functional compensation. In addition, the zeugopod of Glis3 +/-; Alx4 +/- double mutant limbs lacks the cartilage elements giving rise to the radius in forelimbs and the tibia in hindlimbs. The specific loss of the anterior zeugopodal cartilage element in Glis3 +/-; Alx4 +/- double mutant limbs points to complementary functions of Glis3 and Alx4 in formation of the radius. However, truncation of the tibia in Alx4 +/- and Glis3 +/- single homozygous limbs has been reported before (Johnson, 1967; Qu et al., 1998), suggesting that both Glis3 and Alx4 function specifically during formation of the tibia. As disruption of either Glis3 or Alx4 results in polydactyly in limbs, we expected a possible enhancement of the polydactyly phenotype in Glis3 +/-; Alx4 +/- double mutant limbs. However, only five to six digits form in forelimbs and -in some cases-only four digits in hindlimbs of Glis3 +/-; Alx4 +/- double mutant mice. One possible explanation for the mild or lack of polydactyly in Glis3 +/-; Alx4 +/- double mutant limbs is the possible redundant or antagonistic functions of Glis3 and Alx4 during chondrogenesis in the anterior limb. Furthermore, digit identities are lost in limbs lacking Glis3. In contrast, Alx4 +/- single and Alx4 +/-; Glis3 +/- compound mutant limbs have digits with distinct identities, suggesting a specific role for Glis3 in specification of digit identities (see also te Welscher et al., 2002b). Furthermore, Glis3 is expressed by the interdigital mesenchyme (Hui and Joyner, 1993), whose regression is delayed in Glis3 deficient and compound mutant limb buds. These results indicate that GLI3 normally promotes apoptosis of the interdigital mesenchyme. In agreement, apoptosis is reduced in limb buds of Glis3 deficient embryos (Aoto et al., 2002). Severe nasal clefting is observed in Glis3 +/-; Alx4 +/- double mutant embryos. Both Glis3 and Alx4 are expressed in mesenchyme of the frontonasal processes (Hui et al., 1994; Qu et al., 1997), where they might complement one another as nasal clefting is not observed in the skulls of Glis3 +/- and Alx4 +/- single homozygous embryos. Mild nasal clefting occurs already in Glis3 +/-; Alx4 +/- compound mutant skulls, suggesting a dose dependent requirement for Glis3 in the context of an Alx4 deficiency during craniofacial development. Previously, it has been reported that Alx4 is functionally redundant with Cart1 and Alx3 during patterning of the frontonasal regions (Qu et al., 1999; Beverdam et al., 2001). Double homozygous Alx3 +/-; Alx4 +/- and Alx4 +/-; Cart1 +/- embryos also display severe nasal clefting. The cleft nose phenotype of Alx3 +/-; Alx4 +/- double homozygous embryos has been attributed to increased cell death affecting the presumptive nasal processes (Beverdam et al., 2001). In addition, the authors observed abnormal lateral outgrowth of nasal processes from early stages onwards, which may also underlie the defects in Glis3 +/-; Alx4 +/- double mutant embryos.

Specific loss of radius and tibia has also been observed in limbs of other mouse mutant strains. For example, the zeugopod of forelimbs lacking both Fgf4 and Fgf8 consists of a single ulna cartilage (Sun et al., 2002). The radius also fails to form in the hyperplastic forelimb buds of retinoic acid-
rescued $Raldh2^{-/-}$ mutant embryos (Niederreither et al., 2002). Furthermore, hindlimbs of mice lacking the transcription factors $Pitx1$ and $Pitx2$ lack the tibia (Marcil et al., 2003). Reduction in limb bud size may explain the loss of limb skeletal elements in $Fgf4;Fgf8$ and $Pitx1^{+/+};Pitx2^{-/-}$ double mutant limbs (Sun et al., 2002; Marcil et al., 2003). However, the sizes of $Gli3^{-/-};Alx4^{-/-}$ double mutant limb buds is comparable to wild-type limb buds. Therefore, reduction of cell numbers cannot account for the absence of the radius in $Gli3^{-/-};Alx4^{-/-}$ double mutant limbs. The mouse mutation $Ulnaless$ ($Ul$) affects limb development resulting in a severe reduction of both zeugopod skeletal elements. The $Ul$ mutant phenotype can be attributed to deregulated 5’$Hoxd$ gene expression (Spitz et al., 2003), as proximal ectopic $Hoxd13$ together with reduced $Hoxd11$ expression is observed (Herault et al., 1997; Peichel et al., 1997). However, no specific alterations of 5’$Hoxd$ expression domains are observed in $Gli3^{-/-};Alx4^{-/-}$ double mutant limb bud in comparison to $Gli3^{-/-}$ and $Alx4^{-/-}$ single mutant limb buds. Therefore, the molecular alterations causing truncation of the stylopod and loss of the anterior zeugopodal element in $Gli3^{-/-};Alx4^{-/-}$ double mutant limbs remain unknown.

In forelimbs lacking both $Fgf4$ and $Fgf8$, the numbers of skeletal progenitor cells that form the zeugopodal elements are reduced, likely resulting in loss of the radius (Sun et al., 2002). However, analysis of the $Sox9$ distribution in $Gli3^{-/-};Alx4^{-/-}$ double mutant limb buds indicates that the skeletal progenitor cells are normal. The apparently normal $Sox9$ expression in $Gli3^{-/-};Alx4^{-/-}$ double mutant limb buds suggests that formation of the chondrogenic mesenchymal condensations may be normal in these mutant limbs. In conclusion, the absence of the radius cartilage element in $Gli3^{-/-};Alx4^{-/-}$ mutant embryos is most likely not caused by an early patterning defect, but rather caused by defects in the precursor cells, which differentiate into chondrocytes to form the radius cartilage model.

**Materials and Methods**

**Mouse strains and embryos**

To obtain $Gli3^{-/-};Alx4^{-/-}$ double homozygous mutant embryos $Gli3^{+/-};Alx4^{+/-}$ double heterozygous mice were intercrossed. Embryos and mice were genotyped as described by te Welscher et al. (2002b). No double homozygous mutant embryos were recovered after embryonic day E15. Day of vaginal plug detected was considered as embryonic day 0.5.

**Whole-mount in situ hybridization**

Embryos dissected in PBS were fixed in 4% paraformaldehyde (PFA) and processed as described by (Haramis et al., 1995). Whole-mount in situ hybridization using digoxigenin-labeled antisense riboprobes was performed as described by Haramis et al. (1995). Embryos were age-matched by determining their somite number (variation ± 2 somites).

**Cartilage and bone staining**

Embryos of E12.5 were fixed 5% TCA and subsequently stained with Alcian green to visualize the cartilage. Embryos were cleared with methyl salicylate.
Embryos of E14.5 were stained for cartilage and bone using standard Alcian blue and Alizarin red staining.

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