Replicated anterior zeugopod (raz): a polydactylyous mouse mutant with lowered Shh signaling in the limb bud

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Summary
A unique limb phenotype is described in a radiation-induced mutant mouse resulting from an inversion of a proximal segment of chromosome 5. The limb phenotype in the homozygous mutant presents with two anterior skeletal elements in the zeugopod but no posterior bone, hence the name replicated anterior zeugopod, raz. The zeugopod phenotype is accompanied by symmetrical central polydactyly of hand and foot. The chromosomal inversion includes the Shh gene and the regulatory locus, located ~1 Mb away, within the Lmbr1 gene. In homozygous mutants, the expression of Shh mRNA and Shh protein is severely downregulated to about 20% of wild-type limb buds, but Shh expression appears normal throughout the remainder of the embryo. Correspondingly, Gli3 expression is upregulated and posteriorly expanded in the raz/raz limb bud. We propose that the double anterior zeugopod and symmetrical central polydactyly are due to an increased and uniform concentration of the Gli3 repressor form because of lowered Shh signaling.

Supplemental data available online

Key words: Sonic hedgehog, Gli3, Central polydactyly, Radial and tibial dimelia, Mouse

Introduction
The subject of anteroposterior (AP) patterning of the developing vertebrate limb bud has been intensively studied since the demonstration that grafting posterior limb mesenchyme into the anterior margin of a host chick wing bud induces a mirror-image duplication with the most posterior digit of the duplicated limb adjacent to the graft (Saunders and Gasseling, 1968). Thus, this duplicating capacity was named polarizing activity and the area possessing this capability, the zone of polarizing activity (ZPA) (Balcun’s et al., 1970). The induction of mirror-image duplicates from ZPA grafting is most evident when the graft is placed at the mid-anterior margin of the host chick stage 20 wing bud. If the ZPA graft is placed at other sites within the host wing bud, other patterns of limb duplications are consistently induced. These observations led to the hypothesis that the ZPA provides positional information to limb bud cells in a graded fashion, highest posteriorly adjacent to the ZPA (Tickle et al., 1975). A point that is not often recognized is that the ZPA is temporally specific, dependent on the age of the host wing bud at the time of grafting (Summerbell, 1974), so that proximal structures, even the humerus, can be duplicated if grafting is carried out in a suitably young host (stage 16). It is not clear whether there is also a dosage sensitivity of proximodistal (PD) skeletal elements to duplication whereby more or less ZPA signaling would be needed to induce duplication of the autopod versus the zeugopod. Most experimental studies record autopod duplication, but this is mainly attributable to the stage of wing development in the host at the time of grafting, usually stage 20/21.

The molecular basis of polarizing activity is sonic hedgehog, Shh (Riddle et al., 1993). In the limb bud, Shh expression is localized exclusively to the posterior mesenchyme coincident with the ZPA, and ectopic Shh or Shh can induce limb duplications indistinguishable from those induced by ZPA grafts (Lopez-Martinez et al., 1995; Riddle et al., 1993; Yang et al., 1997).

Recently, it has become evident that the function of Shh in AP limb patterning is based on its regulation of the processing of the Zn finger transcription factor Gli3 (Littington et al., 2002; te Welscher et al., 2002). Gli3 is present in the vertebrate limb bud in two forms, a full-length transcriptional activator and a processed transcriptional repressor (Wang et al., 2000). The processing of Gli3 to its repressor form is prevented by Shh signaling leading to graded repressor activity, high anteriorly in the limb bud where Shh is not expressed and lower posteriorly in the presence of Shh (Wang et al., 2000). Notably, Gli3/Shh double-null mice possess a limb phenotype
indistinguishable from the Gli3-null phenotype (Litingtung et al., 2002; te Welscher et al., 2002). There is a similar interaction between Gli3 and Shh in forebrain morphogenesis (Raiu, 2002).

Evidence in the literature supports the concept that increased Shh signaling leads to extra digits, polydactyly, whereas lowered Shh signaling leads to fewer digits, ectrodactyly or oligodactyly. Thus, the loss of Shh signaling by targeted knockout induces loss of digits 2-5 (Chiang et al., 2001; Kraus et al., 2001) and this phenotype is reproduced in the chick ozd mutant, a regulatory mutation that induces a loss of Shh expression in the limb (Ros et al., 2003). A less severe loss of Shh signaling as seen in a conditional limb knockout of Shh (Lewis et al., 2001), in the Wnt7a knockout (Parr and McMahon, 1995), in teratogen-exposed limbs (Bell et al., 1999) and cyclopamine-induced regenerating axolotl limbs (Roy and Gardiner, 2002) leads to the loss of only the most posterior digits, usually digit 5 or 4 and 5. However, ectopic Shh signaling in the anterior limb bud mesenchyme (Yang et al., 1997) leads to an excess number of digits. Polydactyly is observed in mutants with ectopic anterior mesenchyme Shh expression domains (Chan et al., 1995; Masuya et al., 1995; Masuya et al., 1997; Sharpe et al., 1999; Lettice et al., 2002), decreased Ptc1 signaling (Milenkovic et al., 1999), activation of the pathway by ectopic Ihh (Crick et al., 2003; Yang et al., 1998) and altered vesicular transport function in the Rab23 mutation (Eggenschwiler et al., 2001).

Thus, we were surprised to recover a mutant mouse with polydactyly, but with undetectable Shh expression in the limb bud using whole-mount in situ hybridization. Further study of the phenotype indicated a consistent alteration of zeugopod morphogenesis, leading to a symmetrical appearance of the two skeletal elements having anterior structural features. This led us to name the mutant replicant anterior zeugopod (raze).

We have examined various aspects of Shh signaling in the limb buds of raze embryos and have found that Shh transcription and translation are downregulated to about 20% of that in wild-type limb buds. Assays for Shh signaling activity, including polarizing activity and a luciferase reporter Shh-LIGHT2 cell assay, were both negative in raze limb buds. Further down the Shh signaling pathway, Ptc1 and Gli1 expression were undetectable in raze limb buds, whereas the Gli3 expression domain was upregulated and expanded to include posterior limb bud mesenchyme. We propose that this near uniform, increased expression of Gli3, presumably in the repressor form due to low Shh signaling, leads to the symmetrical zeugopod phenotype and central polydactyly. This presumption is based on the idea that graded levels of Gli3 repressor underlie AP limb patterning (Wang et al., 2000).

Chromosomal localization and linkage analysis

The initial chromosomal localization was detected by segregation analysis of 83 animals from the (C3H/HeJ-raze x C57BL/6)F1 x C57BL/6 backcross following our standard laboratory protocol (Favor et al., 1997). A second more extensive backcross of 553 animals from the (C3H/HeJ-raze x BALB/c)F1 x C3H/HeJ backcross was used for fine mapping, using the chromosome 5 markers D5Mit346, D5Mit1345, D5Mit149, D5Mit13, D5Mit148, D5Mit386, D5Mit348, D5Mit353, D5Mit79, D5Mit297 and D5Mit80. Linkage analysis of the segregation data was carried out with Map Manager classic (Manly, 1998), and the gene order was determined by minimizing the number of double recombinants.

Cytogenetic analysis for the occurrence of anaphase bridges followed the procedure as previously described (Roderick, 1971).

Genotyping

The embryos were genotyped by PCR analysis of embryonic DNA extracted from the yolk sac after proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. The tightly linked polymorphic microsatellite D5Mit148 was used for genotyping the embryos.

Skeletal staining

The skin and viscera of perinates were removed and the conceptus fixed in 95% ethanol. Combined Alcian Blue staining of cartilage and Alizarin Red staining of bone was done as previously described (Kuczuk and Scott, 1984).

Polarizing activity

E11.5 mouse ZPA or anterior limb tissue derived from all three genotypes was grafted into the anterior margin of stage 20/21 chick wings and the resulting digit pattern evaluated as previously described (Bell et al., 1999).

Shh activity assay

Pregnant wild-type/raze females were sacrificed on E11.5 of gestation. Implantation sites were removed, frozen in liquid nitrogen, and stored at –80°C until use in the assay.

For each sample of cell extract, implantation sites were thawed and the embryos dissected free of their surrounding membranes. Forelimbs and hindlimbs of each genotype were pooled separately in Eppendorf tubes. Excess dissection fluid was removed and replaced with 200 μl serum-free DMEM. Samples were sonicated and DNA measured using the Hoefer DyNA Quant 200 Fluorimeter. After DNA quantification, fetal bovine serum was added so that the final concentration of serum was 0.5%.

Shh-LIGHT2 cells were cultured in DMEM containing 10% fetal bovine serum in a 96-well plate (2.5x10^5 cells/well) for ~24 hours (confluency). The medium was removed and limb bud lysate containing 50 or 25 ng/ml of DNA in 100 μl of DMEM (0.5% FBS) was added to duplicate wells. A duplicate set of wells contained only 100 μl of DMEM (0.5% FBS) to serve as background. Cells were incubated for 24 hours. Using the Dual-Luciferase Reporter Assay System (Promega, Catalog number E1910), luciferase activity was measured and normalized to a Renilla control.

RT-PCR

Total RNA was extracted from freshly dissected embryonic mouse tissue using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany). DNasel-treated total RNA was used as a template for first-strand cDNA synthesis using random hexamer primers (Gibco Invitrogen, Karlsruhe, Germany). Molony murine leukemia virus (MMLV) reverse transcriptase was used for the extension, according to the manufacturer’s instruction (Gibco Invitrogen, Karlsruhe, Germany). Second-strand cDNA was synthesized during a single PCR cycle with a thermostable polymerase (Qiagen, Hilden, Germany). Each PCR cycle was 94°C

Materials and methods

Animals

The raze mutant, originally designated D470, was recovered in the offspring of male DBA/2 mice exposed to 3 + 3 Gy X-irradiation and mated with untreated specific locus test-stock females (Favor et al., 1987). The mutation was backcrossed for more than 20 generations to strain C3H/HeJ. All inbred strain C3H/HeJ, C57BL/6 and BALB/c mice were obtained from the breeding colonies of the GSF, Neuherberg, Germany.
for 30 seconds; 60°C for 30 seconds (Shh and Gapdh) or 64°C for 30 seconds (Gli3); 2°C for 1 minute and a final extension of 5 minutes at 72°C. We used 35 cycles for each assay. The sense and antisense primers used for each gene and the size of the PCR product were as follows: Shh, 5‘TCTGTGATGACCACTGCCC and 5‘GCCACCG-AGTCTCTGGCTTT (241 bp); Gapdh, 5‘GTGGCGAAAGTGAGG-ATTGTGACC and 5‘GATGATGACCCGTTTGCTCC (289 bp); and Gli3, 5‘CACACCCCCACTACAAACCCAT and 5‘GGTGTCGAACT-CTCTGGTGCA (901 bp) (Takabatake et al., 1997). For control, RT-PCR was performed with the same reaction mixture as for test samples, but without RNA template.

**Western analysis**

Western blot analysis was performed on the excised limb buds from 4 E11.5 raz/raz, wild-type/raz, and four wild-type/wild-type embryos by first lysing in RIPA buffer in the presence of protease inhibitor cocktail. Samples were homogenized by passing through a narrow gauge needle and water bath sonication. Samples were centrifuged at 2000 g for five minutes. Laemmli buffer (5%) was then added to the samples and boiled for 10 minutes. Samples were then run on SDS-PAGE gels and transferred to nitrocellulose. After probing the membranes for Shh with a rabbit polyclonal antibody (Santa Cruz, H160), the membranes were stripped and probed using a monoclonal antibody to tubulin (β-tubulin mouse monoclonal, Sigma, TUB 2.1). Using ImageQuant 5.1 software, the signals from Shh were adjusted based on the signals from the unaffected tubulin protein levels. The level of Shh protein was normalized to the wild-type limb buds.

**Reagents**

RIPA buffer: 150 mM NaCl, 50 mM NaF, 10 mM NaPO₄ (pH 7.4), 2 mM EDTA, 1% NP-40, 1% DOC, 0.1% SDS, plus PIC Protease Inhibitor Cocktail (PIC) [1 mM Pefebloc, 0.01 mM benzamidine-HCl, aprotinin (10 µg/ml), leupeptin (10 µg/ml) and pepstatin A (10 µg/ml)].

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed as described by Bell et al. (Bell et al., 1999) with minor modifications. Embryos were treated with different concentrations of proteinase K (10-20 µg/ml) for 3 E10.5 or 7 minutes (E13.5) at 25°C. The anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche Diagnostics GmbH) was centrifuged for 5 minutes at 4°C to avoid nonspecific staining and diluted 1:3500-1:5000.

**Results**

**Chromosomal localization**

The initial segregation analysis localized the raz mutation to chromosome 5 with the following gene order (number of crossovers/number of offspring genotyped between genes given in parentheses): D5Mit346 – (4/56) – raz – (6/82) – D5Mit391 – (20/82) – D5Mit7 – (11/82) – D5Mit138. Results from the extensive backcross are given in Fig. 1. No crossovers were observed within the 16 cM region between the markers D5Mit348 and D5Mit354 suggesting that the raz mutation is associated with an inversion in the proximal part of chromosome 5. The presence of an inversion was confirmed by an increased level of anaphase bridges [22/174 in wild-type/raz versus historical control (31/884)] (Roderick et al., 1971)]. The inversion breakpoints are located between the microsatellites D5Mit346 and D5Mit348 proximally and between D5Mit354 and D5Mit353 distally. Thus, the inversion includes the Shh structural gene and those regulatory regions of Shh expression that lie within the Lmbr1 gene (Lettice et al., 2002).

**Phenotype**

**Non-limb phenotype**

Newborn homozygous raz/raz neonates die shortly after birth. They exhibit eye malformations that have been previously described (Favor et al., 1987), and have heart and brain malformations.

**Limb phenotype**

**Heterozygotes**

All eight heterozygotes subjected to skeletal analysis exhibited preaxial polydactyly, generally on all four limbs. Some forelimbs (7/16) had only a piece of freestanding cartilage anterior to digit 1, usually adjacent to the proximal phalanx.
Eight forelimbs had anterior duplication of digit 1 beginning at the metacarpal. (Fig. 2B, arrow).

All hindlimbs exhibited an extra preaxial digit (Fig. 3E) and 1/16 had two extra digits (Fig. 3K). The constitutive digit 1 often had three phalanges (Fig. 3E) rather than two (6/16) and in four of these the extra digit was also triphalangeal (data not shown).

Homozygotes

Abnormality of limb development began at the most proximal level of the forelimb, the scapula. Often, a large hole was present below the scapular spine; rarely, there was a small slit-like opening above the spine and/or a notch (Fig. 2C, arrow) along the vertebral border of the scapula (Fig. 2C). This scapular phenotype, especially the large infraspinatal foramen, has been noted in mouse mutants with lowered Hh signaling including extra toes (Gli3) and postaxial hemimelia (Wnt7a), or raised Hh signaling in doublefoot (Ihh) (Johnson, 1967; Lyon et al., 1996; Searle, 1964). The stylopod of forelimbs and hindlimbs appeared normal except for some slight irregularities at the distal end of the
femur where it articulates with the tibia (data not shown). Particularly apparent was the loss of the lateral (posterior) fabella (compare Fig. 3A,C), perhaps the first indication of the loss of posterior structures in the limb.

The signature feature of the raz/raz phenotype is found in the zeugopod, in both the forelimb and hindlimb of mutant fetuses, where the posterior skeletal element is transformed into a replica of the anterior skeletal element. We were led to this conclusion by the absence of an olecranon process (elbow) in the forelimb (compare Fig. 2D with 2F) and the equalization of bone size in the hindlimb (compare Fig. 3G with 3I). Further anatomical characteristics supporting this conclusion are provided in Data S1 at http://dev.biologists.org/supplemental/.

The concept of an anteriorized limb was carried distally into the proximal autopod. Wild-type fetuses have two skeletal elements in the proximal carpus and tarsus (Fig. 2G, Fig. 3D). raz/raz fetuses usually possess a single bone in the proximal carpus and tarsus that is structurally characteristic of the anterior skeletal element (Fig. 2I, Fig. 3F). However, there are physical signs of posterior patterning in the carpus and tarsus. In the tarsus there is frequently a small variable sized skeletal structure posteriorly that we consider an attempt to construct a calcaneus (Fig. 3L). Similarly, in the tarsus there was a variable presence of a recognizable falciform anteriorly and a pisiform posteriorly (data not shown). Delineation of the distal carpus was impossible in the forelimb because of fusions. Fusions were not as severe in the hindlimb, therefore the cuboid (d4) was usually recognizable (Fig. 3L), again indicating some posterior patterning activity in the raz/raz autopod. Furthermore, the structural relationship between the distal tarsals and the metatarsals indicates that the peripheral digits 1 and 5 are missing in raz/raz mutants (compare Fig. 3J with 3L).

Another feature of the raz/raz phenotype is the disorganization of the autopod across the dorsoventral (DV) axis. About half (9/20) of raz/raz forelimbs had some indication of digit rudiments in different DV planes; usually a single central digit was involved, but in rare cases most of the digits within a limb were duplicated (Fig. 4).

**Hedgehog status**

**Expression**

Shh expression in the posterior limb bud mesenchyme of the mouse embryo begins late on day 9 in the forelimb and early on day 10 in the hindlimb (Echelard et al., 1993). This typical expression pattern was seen in wild-type/wild-type and wild-
type/raz embryos, although the level of expression appeared downregulated in the heterozygote limb buds (compare Fig. 5D with 5E, and 5G with 5H). No expression of Shh was visible in the raz/raz limb bud (Fig. 5F,I), although expression throughout the remainder of the homozygous mutant embryo appeared normal (compare Fig. 5A with 5C). As the raz/raz limb phenotype does not mimic the Shh-null limb phenotype (Chiang et al., 2001; Kraus et al., 2001), we examined Shh expression by RT-PCR. From each genotype, RNA was isolated from entire limb buds and dissected anterior and posterior halves of E11.5 limb buds. RT-PCR analysis revealed a definitive, low level expression of Shh in raz/raz forelimbs and hindlimbs (Fig. 6A, lanes 2, 3 and 10) that was posteriorly restricted (data not shown). Band quantification by image analysis indicated that the level of Shh mRNA in mutant limb buds is about 10-20% of that in wild-type/wild-type embryos (Fig. 6B). Limb buds from wild-type/raz embryos had 40-80% of the Shh mRNA level of that in wild-type/wild-type limb buds (Fig. 6A,B, lanes 6 and 7), substantiating the in situ hybridization results (Fig. 5E,H). Shh mRNA levels in non-limb embryo samples were essentially the same in all three genotypes (Fig. 6A,B), indicating that Shh expression is affected by the raz mutation mainly in the developing limb bud.

Western blot analysis of E11.5 limb buds indicated the low level of Shh transcription was translated into Shh protein in the raz/raz mutant limb. As with mRNA, the amount of Shh was about 20% of that in wild-type/wild-type limb buds (Fig. 6C,D). The level of Shh in E11.5 wild-type/raz limb buds was about 60% of wild type (Fig. 6C,D).

Shh signaling activity of raz/raz limb buds

Polarizing activity

The ability of posterior limb bud mesenchyme to induce a limb duplication when grafted to the anterior margin of a chick wing bud is designated as polarizing activity (Balcuns et al., 1970). Results of grafting posterior limb mesenchyme from E11.5 wild-type/wild-type, wild-type/raz and raz/raz limb buds to the anterior margin of stage 20/21 chick wing buds are summarized in Fig. 7. The wild-type forelimb posterior mesenchyme induced a polarizing score of 74, consistent with our previous studies of mouse ZPA tissue (Bell et al., 1999). raz/raz embryos had no polarizing activity in their ZPA (Fig. 7). As expected, polarizing activity of the ZPA from wild-type/raz E11.5 forelimbs was about 50% of that from wild-type/wild-type embryos (Fig. 7). We also searched for polarizing activity in the anterior forelimb mesenchyme of wild-type/raz and raz/raz embryos as other mouse mutants with preaxial polydactyly have been shown to have ectopic anterior ZPA activity (Chan et al., 1995; Masuya et al., 1997; Masuya et al., 1995). There was no polarizing activity in the anterior mesenchyme of E11.5 forelimbs from wild-type/raz or raz/raz embryos (data not shown).

Polarizing activity in the hindlimbs of raz/raz embryos was also totally lacking (Fig. 7). Surprisingly, the polarizing activity from the ZPA of wild-type/raz hindlimbs was not reduced (Fig. 7), when compared with wild-type/wild-type limbs. This finding correlates well with Shh expression as analyzed by PCR, but not with Shh as measured by western blot. This difference in Shh and Shh status of wild-type/raz forelimb and hindlimb buds may reflect their differing developmental stages at the time of the collection.

Shh-LIGHT2 cell assay

A more specific test of Shh signaling activity is provided by the Shh-LIGHT2 cell assay. 3T3 cells stably transfected with a Gli/luc reporter and constitutively expressing a Renilla luciferase yield a quantitative response to Shh signaling (Taipale et al., 2000) and have been useful in monitoring Shh activity in the chick wing bud (Zeng et al., 2001). Lysates of E11.5 embryo limb buds, fore or hind, revealed that raz/raz embryos had no activity in this assay (Fig. 7). Hindlimb buds from wild-type/raz embryos had 45% of the...
activity of wild-type hindlimbs, whereas forelimb buds had 36% of wild-type activity (Fig. 7).

Ptch expression
Ptch acts as a receptor for Shh, and is upregulated at sites of Shh expression (Ingham and McMahon, 2001). Thus, the expression of Ptch is a sensitive indicator of Shh signaling. The expression of Ptch in the posterior limb mesenchyme is not detectable in raz/raz mutants (compare Fig. 5M with 5O and 5P with 5R), although non-limb expression of Ptch was not noticeably affected (compare Fig. 5J with 5L).

Gli expression
Downstream of Ptch, Shh signaling affects the expression of Gli family genes. Gli1 expression is positively regulated by Shh signaling in the posterior limb mesenchyme (Marigo et al., 1996; Büscher and Rüther, 1998) seen here in wild-type/wild-type E11.5 forelimb bud (Fig. 8A). The expression of Gli1 in raz/raz forelimbs on E11.5 is undetectable by whole-mount in situ hybridization (Fig. 8C) in keeping with the very reduced Shh signaling activity in the homozygous mutant limb bud.

Gli3 expression, by contrast, is negatively regulated by Shh signaling (Marigo et al., 1996). During early limb bud morphogenesis, Gli3 is expressed throughout the mouse limb bud (Bücher and Rüther, 1998). As limb morphogenesis progresses and Shh signaling begins, Gli3 expression is progressively repressed beginning posteriorly and advancing anteriorly (Marigo et al., 1996) (Fig. 8D,G). This process is somewhat attenuated in wild-type/raz limb buds (Fig. 8E,H). The expression pattern in raz/raz forelimbs on E10.5 reveals little or no regression from the posterior border (Fig. 8F), as would be expected if Shh signaling is greatly reduced and remains uniform throughout the AP axis on E11.5 (Fig. 8I).

The importance of the ratio of Gli3 to Shh expression for limb morphogenesis has recently been documented (Litingtung et al., 2002). These two studies clearly show that the major function of Shh signaling in the limb is to modify the activity of Gli3 signaling. Thus, we chose to examine Gli3 expression more closely, using PCR analyses of limb bud AP halves to examine the effect of the raz mutation on expression levels of this important limb gene. Values were normalized to the wild-type/wild-type body, and in keeping with the limb specific effect of the raz mutation, Gli3 expression in the body was the same regardless of genotype (Fig. 8J,K). This was certainly not the case in the limb bud where the raz mutation led to a greatly enhanced level of Gli3 expression, especially in the raz/raz limb buds. This enhanced expression level was generally seen in both the anterior and posterior halves of the limb, but even in wild-type/wild-type embryos we did not measure any huge difference in Gli3 expression between anterior and posterior halves. In keeping with the observation that Shh signaling

Fig. 7. Polarizing activity and Shh activity in the limb. Polarizing activity (red) and Shh signaling activity (blue) are plotted with the mean from wild-type/wild-type samples given a value of 100. The number above each column represents the number of assays (n) conducted.

Fig. 8. Gli1 and Gli3 expression. (A–C) Whole-mount in situ hybridization to reveal Gli1 expression in the left forelimb of E11.5 wild-type/wild-type, wild-type/raz and raz/raz embryos. Anterior is upwards. Note the posterior expression of Gli1 in wild-type/wild-type limbs (A), and absence of expression in raz/raz limbs (C). (D–I) Gli3 expression in forelimbs (D–F) or left forelimb (G–I) of wild-type/wild-type, wild-type/raz and raz/raz embryos. Scale bar: 0.25 mm. (J) PCR analysis for Gli3 mRNA of E11.5 forelimbs (FL), hindlimbs (HL) and remainder of embryo (body). Limb buds were split longitudinally to provide anterior (Ant.) and posterior (Post.) samples. (K) Image quantitation of PCR analyses with wild-type/wild-type body given a value of 100 and other tissues normalized to that value. Results are mean±s.d.
is a negative regulator, Gli3 expression is generally increased in \( raz/raz \) limb buds (Marigo et al., 1996) although the increased Gli3 expression in the posterior half of heterozygote and homozygote mutant hindlimb samples was not as great as expected.

Molecular markers of the A/P axis

We also examined other AP molecular markers of limb morphogenesis including \( Hand2 \) (previously \( dHAND \)) Pax9 and members of the Hoxd cluster. The \( raz/raz \) mutation leads to a severe downregulation of \( Hand2 \) expression so only a very small domain of expression remains at the posterior border (see Fig. S2 at http://dev.biologists.org/supplemental/). By contrast, the expression of Pax9 and Hoxd family members are observed across the AP axis of the limb instead of being anteriorly or posteriorly restricted (see Figs S1, S2 at http://dev.biologists.org/supplemental/). Combined, these findings indicate that the \( raz/raz \) limb bud acquires anterior characteristics early on in morphogenesis leading to the final replicated anterior zeugopod phenotype.

Discussion

The \( raz \) mutation induces a unique limb phenotype, a replicated anterior zeugopod, a loss of peripheral digits and an excess of interior digits; i.e. central polydactyly. This phenotype is created in a limb bud that by all accounts is severely deficient in Shh transcription, translation and signaling activity. This is contrary to the usual scenario where lowered Shh signaling leads to limb deficiencies and increased Shh signaling leads to limb excess deformities (Lewis et al., 1999).

We have shown that the \( raz \) mutation is associated with an inversion in the proximal region of chromosome 5 where a number of ‘limb’ genes are within or near this inversion including \( Shh, Msx1, Hc, Hm \) and \( Luxate \). Using whole-mount in situ hybridization we were unable to detect Shh expression in limb buds of \( raz/raz \) embryos, yet expression elsewhere in the embryo appeared normal. Cis regulatory elements that specifically affect limb Shh expression have recently been identified (Hill et al., 2003; Lettice et al., 2002; Sharpe et al., 1999), and these elements are embedded within the \( Lmbr1 \) gene on mouse chromosome 5 (Clark et al., 2000). Several independent mutations within the \( Lmbr1 \) gene lead to different limb phenotypes. A transgene insertion within intron 5 of murine \( Lmbr1 \) leads to preaxial polydactyly and the mutation was designated \( Sasquatch \) (Lettice et al., 2002; Sharpe et al., 1999). Preaxial polydactyly was mapped to \( 7q36 \) in humans and disruption of intron 5 of the \( LMBR1 \) gene (Lettice et al., 2002). Presumably this mutation in the human infant leads to ectopic anterior limb expression of \( Shh \) as it does in the \( Ssq \) mutation (Sharpe et al., 1999). Another human disorder, \( acheiroptopia \) (Toledo et al., 1972; Fett-Conte and Richieri-Costa, 1990) is caused by a 4-6 kb deletion including exon 4 of the \( LMBR1 \) gene (Ivanikiev et al., 2001). The \( acheiroptopia \) limb phenotype resembles that of the \( Shh \) knockout mouse (Chiang et al., 2001; Kraus et al., 2001; Toledo and Saldanha, 1969; Toledo et al., 1972), leading to the speculation that a 4-6 kb deletion disrupts a regulatory element responsible for normal ZPA expression of \( Shh \) (Hill et al., 2003; Lettice et al., 2002). We expect that the \( raz \) mutant likewise represents the alteration of a regulatory site responsible for ZPA expression of \( Shh \), but whether this site is within the \( Lmbr1 \) gene or elsewhere on chromosome 5 cannot be determined at this time. Recently, it has been suspected that the chick mutation \( ozd \), which abolishes \( Shh \) limb expression and induces loss of posterior skeletal elements, also represents an alteration of a regulatory domain responsible for ZPA expression of \( Shh \) (Ros et al., 2003).

The downregulation of \( Shh \) in the limb bud caused by the \( raz \) mutation, in contrast to the complete loss of \( Shh \) in \( ozd \) or targeted \( Shh \) knockout, leads invariably to the presence of two symmetrical skeletal elements in the fore and hindlimb zeugopod. From structural characteristics these bones appear to possess an anterior character. We have been unable to find a phenotype exactly analogous to \( raz/raz \). Clinically there are two observations of duplicated radius (Mennen et al., 1997; Peterfly and Jona, 1942), and in both cases the zeugopod had an ulna as well. A few other examples are summarized by O’Rahilly (O’Rahilly, 1951) who concludes that it is difficult to find a case of radial dimelia with ulna deficiency. This conclusion can be extended to mutants in the chick and mouse. \( Duplicate \) (Landauer, 1956), \( diplopodia 4 \) (MacCabe et al., 1975) and \( Strong’s luxoid \) (Forsthoefel, 1962) all display duplication of the anterior zeugopod skeletal element, but a posterior bone, the ulna or fibula is always present. In contrast to the diminished level of \( Shh \) expression in \( raz/raz \) limb buds, molecular analysis of \( diplopodia 4 \) wing buds revealed normal levels of posteriorly restricted \( Shh \) expression although ectopic anterior expression domains of Hoxd genes, \( Bmp2 \) and \( Fgf4 \) are observed (Rodriguez et al., 1996). \( Strong’s luxoid \) (\( Alx4 \)) limb buds revealed normal posterior and ectopic anterior expression domains of \( Shh, Hoxd12 \) and \( Fgf4 \) (Chan et al., 1995; Qu et al., 1997). Presumably, the normal posterior expression of these genes leads to the formation of posterior skeletal structures in contrast to the \( raz/raz \) phenotype with an anteriorized zeugopod with lowered \( Shh \) and unrestricted \( 5’ \) Hoxd expression.

However, increased Shh signaling in the limb bud can lead to a duplicated posterior zeugopod in the absence of any anterior structure. This phenotype has been induced by aberrant expression of the posteriorly restricted genes, \( Hoxb8 \) and \( Hand2 \), throughout the limb bud mesoderm (Charite et al., 1994; Charite et al., 2000). In both cases, an ectopic domain of \( Shh \) expression was documented in the anterior limb bud mesenchyme accompanied by ectopic expression of \( Hoxd11 \). A striking example of duplicated posterior zeugopod is seen clinically in the Laurin-Sandrow syndrome (LSS) (Kantaputra, 2001). The LSS phenotype has been suggested to be related to preaxial polydactyly based on a familial appearance of LSS and tibial hemimelia-polylysandyctaly-trihiphalangeal thumb syndrome (THPTTS), in father and daughter (Kantaputra, 2001). This is significant because THPTTS has been mapped to \( 7q36 \) (Balci et al., 1999; Heus et al., 1999; Zguricas et al., 1999), a location syntenic with the regulatory region for \( Shh \) expression in the limb bud (Lettice et al., 2002). Thus, it seems plausible that children with LSS who have ulnar and/or fibular dimelia are another example of excess Shh signaling.

The aforementioned zeugopod phenotypes that result from increased Shh signaling are reproduced in the chick wing after deposition of retinoic acid (RA) to the anterior limb mesenchyme (Summerbell, 1983). When RA was deposited at stage 17, the lowest dosage, 0.25 mg, induced a duplicated
radius with the ulna still present. As the dose of RA was raised, the duplicated radius was not seen again, but was replaced by a duplicated ulna with no radius present. RA induces higher Shh signaling (Riddle et al., 1993; Helms et al., 1994; Helms et al., 1996), providing support for the concept that the quantitative level of Shh signaling in the early limb bud will influence the anteroposterior character of the zeugopodal skeletal elements. The concept of a quantitative influence of Shh signaling on AP patterning in the autopod has been clearly shown by Yang et al. (Yang et al., 1997), but Fig. 1B in that manuscript also reveals a quantitative effect of Shh protein on AP zeugopod morphogenesis.

A summary of the varied zeugopod phenotypes and their relationship to the intensity of Shh signaling suggests that a quantitative relationship exists (Table 1). Loss of the ulna is associated with the absence of Shh signaling. At the other end of the relationship is the induction of a replicated posterior zeugopod with no anterior structure because of supernormal Shh signaling. Between these extremes is normal limb development at 50% Shh signaling, while 20% Shh signaling is associated with duplicated radius/tibia and absence of ulna/fibula.

We speculate that the raz/raz mutation leads to this unique limb phenotype through downregulation of Shh expression specifically in the limb bud. We suggest that this level of Shh expression and translation, about 20% of wild type, results in low ubiquitous Shh signaling throughout the AP axis of the limb. The ubiquitous Shh signaling would be caused by the inability of low Shh activity to induce Ptc1 expression, which is one mechanism that normally constrains Shh activity to the posterior limb mesenchyme. The low level of Shh signaling is supported by lowered expression of Ptc1, Gli1 and 5′ Hoxd genes, by the posterior expression of anterior restricted genes such as Gli3 and Pax9, and the downregulation of posterior restricted genes such as Hand2.

Furthermore, we suggest that a low ubiquitous level of Shh activity allows a posteriorly expanded and thus uniform expression of Gli3. Presumably, these limb mesenchymal cells retain only a small fraction of Gli3 as an activator rather than conversion to the repressor form, as normal Shh signaling is required for processing in the vertebrate limb bud (Litingtung et al., 2002; Wang et al., 2000). Thus, we predict that there is a high ratio of Gli3 repressor/Gli3 activator throughout the limb bud, mimicking what is usually present in the central to anterior limb bud mesenchyme where Shh signaling is normally low.

We take as support for this concept the zeugopod phenotype of ShhKO; Gli3+/- fetuses (Litingtung et al., 2002; te Welscher et al., 2002). Here, one can predict that Gli3 will be predominantly in the repressor form as there is no Shh activity; and that there is less than the usual amount of Gli3 due to one null allele of Gli3. The zeugopod phenotype in these Shh null, Gli3 heterozygotes is the closest in structural similarity to the raz/raz phenotype that we have seen. In ShhKO; Gli3+/-, the complete absence of a duplicated ulna with no olecranon process, suggesting it is the anterior skeletal element, the radius. Within a short distance distally, the bone divides into two bones, which continue partially fused to the wrist. The hindlimb zeugopod phenotype in ShhKO; Gli3+/- mice is not strikingly symmetrical across the AP axis (Litingtung et al., 2002). Yet the fibula, the posterior bone, does not have a typical wild-type appearance. Rather, it has a proximal epiphysis resembling that of the tibia and proceeds in parallel with the tibia to the ankle rather than following the tortuous course seen in wild-type legs.

In support of this general concept, many of the altered expression patterns of ‘limb’ genes were very similar in ShhKO; Gli3+/- limbs to those seen in raz/raz limbs. These include 5′ Hoxd genes, Ptc1, Gli1, Gli3 and Hand2 (Litingtung et al., 2002; te Welscher et al., 2002).

The concept that this putative high uniform ratio of Gli3R/Gli3A in raz/raz limbs might also account for the autopod phenotype is challenged by the dogma that low Shh activity leads to limb reduction, not limb excess deformities. However, the clinical condition of Pallister-Hall syndrome leads to central polydactyly (Hall et al., 1980) caused by truncating mutations of Gli3 (Kang et al., 1997). In the mouse model for Pallister-Hall syndrome (Bose et al., 2002) homozygous mutants have two features of limb morphogenesis similar to raz/raz limbs, including central/insertional polydactyly and digits localized in different dorsal/ventral planes (Bose et al., 2002). Moreover, the biochemical activity of Gli3 truncation proteins constructed to epitomize PHS mutations act as transcriptional repressors (Shin et al., 1999) so that limb buds developing in such an organism would experience high levels of Gli3 repressor activity. Thus, we believe that altered Gli3 processing induces both the raz/raz zeugopod phenotype and also leads to the unique autopod phenotype of central polydactyly with near mirror-image symmetry. Support for the concept of a role for Gli3 repressors/activator distribution within the limb bud as a mediator of AP patterning can also be found in the studies of talpid chick mutants that lead to an autopod with 7-10 similar digits with no recognizable AP polarity (Hinchliffe and Ede, 1967; Abbott et al., 1959). In contrast to our predictions for the raz/raz limb bud, the talpid2 mutant has a very high level of Gli3 activator throughout the limb bud (Wang et al., 2000) correlated with digits that all have posterior character (Caruccio et al., 1999). Conversely, the talpid2 mutant would be predicted to have a uniform Gli3 repressor/activator ratio throughout the limb bud, as Gli3 expression is expanded into the posterior mesenchyme and Ptc1 expression is low and uniform throughout the limb (Lewis et al., 1999), thereby

### Table 1. Correlation between zeugopod phenotype and the intensity of Shh signaling

<table>
<thead>
<tr>
<th>Zeugopod phenotype</th>
<th>Intensity of Shh signaling*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent ulna, normal anterior</td>
<td>0%</td>
</tr>
<tr>
<td>Duplicated radius or tibia with normal ulna or fibula</td>
<td>~20%</td>
</tr>
<tr>
<td>Normal</td>
<td>~50%</td>
</tr>
<tr>
<td>Duplicated radius or tibia with normal ulna or fibula</td>
<td>100%</td>
</tr>
<tr>
<td>Aplastic/hypoplastic radius or tibia with normal ulna or fibula</td>
<td>100%</td>
</tr>
<tr>
<td>Duplicated ulna or fibula with absent radius or tibia</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Approximation, generally from Shh expression pattern in the limb bud.
permitting Shh to diffuse throughout the limb. The 5′ Hoxd expression in talpid3 limb buds suggests that the digits have a posterior character. Clearly, the interaction of Shh signaling leading to Gli3 processing is crucial for normal limb patterning across the AP axis (Wang et al., 2000; Litingtung et al., 2002) and slight skewing of this balance can lead to a variety of limb phenotypes.

The raz/raz mutation skews this balance to heavily favor Gli3 repressor. However, it must be borne in mind that the mutant phenotype is derived from a large chromosomal inversion potentially affecting the expression of many other genes, some of which are known to participate in limb morphogenesis. Altered function of some of these genes, e.g. Luxate and Msx1, may contribute to the unique limb phenotype in raz/raz mutants. In addition, they might contribute to aspects of the phenotype not easily explained by reduced Shh signaling such as the scapular defects and preaxial polydactyly in heterozygotes. Additional genetic dissection of the raz/raz phenotype, perhaps through breeding to other mutants in this chromosomal region, will be required to assign the true cause of this unique limb phenotype.

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References


Di-anterior zeugopod due to low Shh signal


