Development 130, 527-537

doi:10.1242/dev.00245

The chick *oligozeugodactyly* (*ozd*) mutant lacks sonic hedgehog function in the limb

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Accepted 31 October 2002

SUMMARY

We have analyzed a new limb mutant in the chicken that we name *oligozeugodactyly* (*ozd*). The limbs of this mutant have a longitudinal postaxial defect, lacking the posterior element in the zeugopod (ulna/fibula) and all digits except digit 1 in the leg. Classical recombination experiments show that the limb mesoderm is the defective tissue layer in *ozd* limb buds. Molecular analysis revealed that the *ozd* limbs develop in the absence of *Shh* expression, while all other organs express *Shh* and develop normally. Neither *Ptc1* nor *Gli1* are detectable in mutant limb buds. However, *Bmp2* and *dHAND* are expressed in the posterior wing and leg bud mesoderm, although at lower levels than in normal embryos. Activation of *Hoxd11-13* occurs normally in *ozd* limbs but progressively declines with time. Phase III of expression is more affected than phase II, and expression is more severely affected in the more 5′ genes. Interestingly, re-expression of *Hoxd13* occurs at late stages in the distal mesoderm of *ozd* leg buds, correlating with formation of digit 1. *Fgf8* and *Fgf4* expression are initiated normally in the mutant AER but their expression is progressively downregulated in the anterior AER. Recombinant Shh protein or ZPA grafts restore normal pattern to *ozd* limbs; however, retinoic acid fails to induce Shh in *ozd* limb mesoderm. We conclude that Shh function is required for limb development distal to the elbow/knee joints, similar to the *Shh*$^{-/-}$ mouse. Accordingly we classify the limb skeletal elements as Shh dependent or independent, with the ulna/fibula and digits other than digit 1 in the leg being Shh dependent. Finally we propose that the *ozd* mutation is most likely a defect in a regulatory element that controls limb-specific expression of *Shh*.

Key words: Chick mutant, ZPA, Shh, Shh pathway, Limb development, Pattern formation

INTRODUCTION

Patterning of the amniote limb is organized by three well-defined signaling centers (reviewed by Capdevila and Izpisua Belmonte, 2001; Schaller et al., 2001). The apical ectodermal ridge (AER) permits limb bud elongation and the determination of skeletal elements along the proximal-distal (PD) axis, through the action of fibroblast growth factor (FGF) family members (Martin, 1998; Moon et al., 2000; Sun et al., 2002). The non-ridge limb bud ectoderm controls dorsal-ventral (DV) polarity through the action of Wnt7a and engrailed1 respectively (reviewed by Chen and Johnson, 1999). The anterior-posterior (AP) limb axis is controlled by a small group of mesodermal cells along the posterior limb bud border called the zone of polarizing activity (ZPA) through the activity of Sonic hedgehog (Shh), which is synthesized by ZPA cells (reviewed by Pearse and Tabin, 1998).

Evidence from a variety of sources points to an interdependence of the limb bud signaling centers for continued synthesis of effector molecules and signaling function. The AER is necessary for the induction (Crossley et al., 1996; Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996) and maintenance of *Shh* expression (Riddle et al., 1993) by ZPA cells. AER induction and maintenance has long been known to be dependent on the limb bud mesoderm (e.g. Saunders, 1977). Recent data suggest the Bone morphogenetic protein (BMP) inhibitor gremlin (Gre) is downstream of Shh and required for AER maintenance (Capdevila et al., 1999; Merino et al., 1999; Zúñiga et al., 1999). Thus, the molecular framework of a possible AER-to-ZPA-to-AER feedback loop is emerging. The possibility that FGF10 is the effector growth factor of AER induction has been suggested (Ohuchi et al., 1997). The complexity of limb signaling center interaction is further demonstrated by the observation that *Wnt7a*$^{-/-}$ mice showed reduced *Shh* expression and posterior limb deficiencies (Parr and McMahon, 1995).

The mechanisms that precisely define the location and subsequent maintenance of the limb bud signaling centers are poorly understood. This is especially true of the ZPA (Tanaka et al., 2000). There is evidence of a role for retinoids in *Shh* induction and maintenance from studies using retinoid inhibitors and retinoid deficiency models (e.g. Lu et al., 1997;

Power et al., 1999; Stratford et al., 1997; Stratford et al., 1999), and from *Shh* induction after treatment with retinoids (reviewed by Tickle and Eichele, 1994). Misexpression of *Hoxb8* in anterior limb mesoderm results in ectopic *Shh* expression but only in the proximity of the AER (Charité et al., 1994). Interestingly, *Hoxb8* expression precedes retinoid-induced *Shh* expression in the anterior limb bud mesoderm (Lu et al., 1997; Stratford et al., 1999). Similarly, ectopic expression of the transcription factor dHAND results in ectopic *Shh* expression (Charité et al., 2000; Fernandez-Teran et al., 2000; McFadden et al., 2002) and *dHAND*$^{-/-}$ mice fail to express *Shh* in the limb (Charité et al., 2000). At present, it is not clear how these observations can be integrated to explain how the ZPA is spatially delineated or how *Shh* expression is maintained.

While it would appear that a cohort of cells in the emerging limb bud has the competence to express *Shh* when exposed to FGFs (Ros et al., 1996), analyses of mouse mutants with anterior polydactyly, and other studies, indicate the existence of negative regulators that restrict *Shh* expression to the posterior bud. The transcription factors Alx4 and Gli3 have domains of expression in the limb bud complementary to that of *Shh* and have been proposed to repress *Shh* expression in the anterior limb mesoderm (Büscher et al., 1997; Marigo et al., 1996b; Masuya et al., 1997; Masuya et al., 1995; Qu et al., 1997; Qu et al., 1998; Takahashi et al., 1998). A gradient of the repressor form of Gli3 has been described in the limbs of mice and chickens with the highest concentration in the anterior portion of the limb (Litingtung et al., 2002; Wang et al., 2000). It has also been proposed recently that Gli3 is an obligate component of ZPA function, required in responding cells for Shh mediated polarizing activity (Litingtung et al., 2002).

The mechanisms involved in skeletal patterning downstream of Shh are being actively investigated. *Bmp2* has been considered a candidate Shh effector gene because it is expressed in a domain that overlaps *Shh* expression and because it is induced in the anterior limb mesoderm by ectopic Shh expression (Duprez et al., 1996; Yang et al., 1997). Recently, it was proposed that Shh acts to specify digit formation, while concurrently setting up a gradient of Bmp2 that subsequently specifies digit identity in a dose-dependent manner (Drossopoulou et al., 2000). It has been demonstrated that BMP activity in the interdigital mesoderm at autopod stages is required for the interdigits to specify digit identity (Dahn and Fallon, 2000).

We have analyzed a new limb mutant in the chicken first described by Smyth et al. (Smyth et al., 2000) and previously named *Ametapodia 2*. These chickens develop limbs that lack ulna and fibula and all digits except digit 1 (d1) of the foot. Digit identity was proposed on the basis of genetic evidence. Here we rename this mutation as *oligozeugodactyly* (*ozd*) meaning reduced zeugopod and digits and report data consistent with the complete absence of *Shh* expression and activity specifically in the developing limb buds. We report that the stylopod is normal in *ozd* limbs and the zeugopod develops with only radius or tibia. While the wing lacks digits, the leg develops a clearly identifiable d1. Consistent with the absence of Shh signaling, neither *Ptc1* nor *Gli1* are detectable in mutant limb buds, and we observe that the expression of *Bmp2*, *dHAND* and the 5′ *Hoxd* genes in posterior wing and leg bud mesoderm is comparable to that observed in the limbs of *Shh*$^{-/-}$ mice. We conclude that Shh becomes necessary for limb skeletal patterning distal to the elbow and knee joints, similar to *Shh*$^{-/-}$ mice (Chiang et al., 2001; Kraus et al., 2001). The data presented are consistent with a developmental model proposing the PD axis is specified in the limb field, and that the radius/tibia and d1 are Shh independent, while the ulna/fibula and other digits are Shh dependent.

MATERIALS AND METHODS

Embryos

Oligozeugodactyly (*ozd*) mutant and normal chick embryos were obtained from a heterozygous mating flock maintained at the University of Wisconsin Poultry Science Department (Madison, WI). Normal chick embryos were also obtained from Granja Santa Isabel (Cordoba, Spain) and from a white leghorn flock supplied by the S&R egg farm (Whitewater, WI). Eggs were incubated, opened, and staged as described previously (Hamburger and Hamilton, 1951; Ros et al., 2000). Visualization of cartilage patterns was achieved by routine Victoria Blue or Alcian Green staining.

To analyze gene expression in *ozd* embryos by whole-mount in situ hybridization, we used two methods: the batch method and the hemisection method. By the batch method we analyzed groups of appropriately staged embryos from the *ozd* flock, of which approximately one quarter should be homozygous for the *ozd* mutation. For each gene expression analyzed we used a minimum of 16 embryos of the *ozd* flock, giving a probability of 0.99 that at least one of them is homozygous. The hemisection method was based on the fact that limb buds of *ozd* homozygous embryos do not express *Shh* at any stage. Embryos were hemisected along their midline and one half was hybridized for the gene of interest and the other half for *Shh*. Embryos in which *Shh* was not detected were confirmed *ozd* mutants. In order to analyze gene expression before the *ozd* mutant phenotype was discernible, we surgically removed the right wing buds from embryos in ovo and allowed the embryo to develop to show the phenotype (Carrington and Fallon, 1988). Similar results were obtained by all three methods.

Recombinant limb experiments

Right wing buds of stage (st.) 19-21 embryos from the *ozd* flock were removed in ovo, and embryos allowed to develop to confirm the phenotype. The isolated buds were incubated in 0.5% trypsin for 1 hour at 4°C to separate the ectoderm from the mesoderm. The isolated ectoderm was recombined with wild-type mesoderm. Using the same approach, isolated limb bud mesoderm from the *ozd* mutant flock was recombined with ectoderm from wild-type embryos. The recombinant limbs were allowed to heal for 1 hour and then grafted to the flank level somites of host embryos as described previously (Fernandez-Teran et al., 1999).

Grafts of ZPA and applications of Shh or RA

The ZPA was removed in ovo from st. 19-20 embryos of the *ozd* flock, and donor embryos were allowed to develop to confirm the phenotype. ZPA grafts were performed as described previously (Tickle, 1981). Heparin acrylic beads (Sigma, H5263) were soaked in recombinant mouse Shh (4 mg/ml). The beads were implanted into the posterior wing bud mesoderm of st. 20 embryos from the *ozd* flock.

For application of retinoic acid (RA; all-*trans*-retinoic acid, Sigma), beads (AG1X2, Bio-Rad) were soaked for 20 minutes at room temperature in 0.1 mg/ml, 0.6 mg/ml or 1 mg/ml RA suspended in DMSO and rinsed several times in saline before use. RA-soaked beads were implanted under the AER at the anterior or posterior border of the developing wing and leg buds (Tickle et al., 1985).

In situ hybridization in whole mounts and to tissue sections

Digoxigenin-labeled antisense riboprobes were prepared, and whole-mount in situ hybridization analysis performed according to standard

procedures (Nieto et al., 1996). S^{35}-labeled riboprobes were prepared and hybridized to tissue sections as described previously (Wilkinson and Nieto, 1993). The probes used were *Shh, Fgf4, Fgf8, Bmp2, Hoxd11, Hoxd12, Hoxd13, Gli1, Gli3, Ptc1, Hoxb8* and *dHAND* (kindly provided by C. Tabin, T. Jessel, J-C Izpisua-Belmonte, P. Beachy and D. Srivastava).

Cell death analysis

In situ detection of DNA fragmentation was performed using terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine-triphosphate (dUTP) nick end-labeling (TUNEL) with the In Situ Cell Death Detection Kit, Fluorescein (Boehringer-Mannheim).

RESULTS

The *oligozeugodactyly* mutation in the chicken

Recently, a new mutation was reported in the chicken, characterized by a limb phenotype resembling the *Ametapodia* mutation (Cole, 1967) and was named *Ametapodia-2* (Smyth et al., 2000). We have performed a detailed analysis of the *Ametapodia-2* limbs. Because *Ametapodia* refers to a dominant mutation resulting in reduced or absent metapodial bones (metacarpal and metatarsal bones) (Cole, 1967; Ede, 1969) we renamed the mutant *oligozeugodactyly* (*ozd*) indicating fewer than normal elements at zeugopod and autopod levels. This mutation is inherited as a simple Mendelian recessive trait, and the gross overall morphology of *ozd* embryos appeared normal except for the limb. *ozd* mutants hatch normally and are alert, but have impaired mobility; death occurs for unknown reasons within the first days of hatched life [personal observations and (Smyth et al., 2000)].

Anatomy of *ozd* limbs

Limb development in *ozd* embryos proceeds normally until st. 23/24 when the limb buds become abnormally narrow across the AP axis. The narrowing becomes more evident during subsequent stages of development; by st. 26 the mutant limb buds acquire a pointed and hooked shape; eventually, the mutant limbs adopt a spiked shape (Fig. 1).

Skeletal preparations at 10 days of incubation (Fig. 1A,B) showed *ozd* mutant wings composed of humerus, radius and a hypoplastic carpal element while the ulna, metacarpals and digits were absent (Fig. 1B). *ozd* mutant legs displayed femur, tibia, tibiale and first toe with a total absence of fibula, and digits 2, 3 and 4 (Fig. 1B). It is important to emphasize that the skeletal elements present in the mutant limb were of normal morphology and easily recognizable except for the rudimentary carpal. The single element present in the leg autopod showed the characteristic morphologies of the first metatarsal and proximal phalanx of d1, making the identification unequivocal (Fig. 1C). According to the current classification of limb mutations, *ozd* can be considered a longitudinal postaxial defect (Stoll et al., 1998).

Alcian Green staining of st. 25 and 27 mutant and wild-type limbs failed to detect evidence of cartilage condensations corresponding to the absent skeletal elements in the day-10 mutant limb, indicating the development of these elements was never initiated (Fig. 1D).

Unexpected patterns of apoptosis in *ozd* limb buds

To determine whether *ozd* limb bud narrowing resulted from

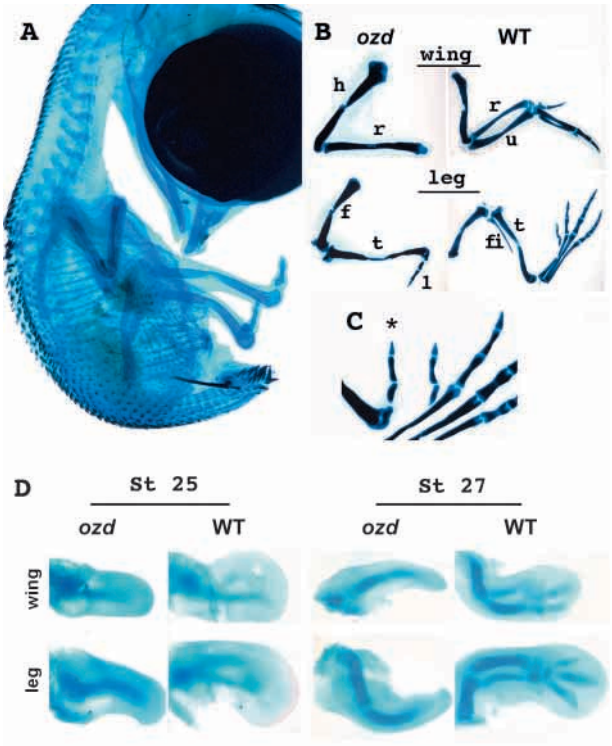

Fig. 1. Skeletal pattern and chondrogenesis in the *ozd* mutant limbs. (A) A homozygous *ozd* embryo at day 10 of development showing limb-specific skeletal deficiencies. (B) Skeletal preparations of *ozd* wing and leg compared with stage-matched wild-type limbs. (C) Higher magnification image comparing morphology of the *ozd* leg digit (asterisk) with wild-type leg d1. (D) Alcian Green preparations comparing chondrogenic condensations in st. 25 and 27 wild-type and *ozd* limbs. f, femur; fi, fibula; h, humerus; r, radius; t, tibia; u, ulna.

abnormal cell death we performed TUNEL analysis in wild-type and *ozd* limbs at st. 24, when the mutant phenotype became discernible (Fig. 2). Wild-type wing buds showed two areas of well-defined mesodermal apoptosis, one in the center of the wing bud, known as the opaque patch (OP), and another along the posterior border called the posterior necrotic zone (PNZ; Fig. 2A) (Fell and Canti, 1934; Hinchliffe, 1982; Hurlé et al., 1995; Saunders and Fallon, 1967). In contrast, comparably staged *ozd* wing buds showed extensive abnormal apoptosis in the anterior border mesoderm that extended into the distal mesoderm (Fig. 2B) as well as increased apoptosis in the OP (Fig. 2A,B). However, cell death was not detected in the posterior border mesoderm in mutant wing buds (arrow in Fig. 2B, compared with the control in Fig. 2A). TUNEL analysis of leg buds gave similar results. st. 24 wild-type leg buds show apoptosis in a fairly extensive anterior zone, called the anterior necrotic zone (ANZ) as well as in the OP and a small PNZ (Fig. 2C). The *ozd* leg buds showed massive apoptosis along the anterior and distal borders of the limb and increased central cell death (Fig. 2D). No evidence of cell death in the posterior mesoderm was found (arrow in Fig. 2D). During subsequent development of the mutant limb, the anterior-distal area of cell death persisted, but posterior apoptosis was not observed (not shown). The absence of posterior cell death was a surprising result since the shape of

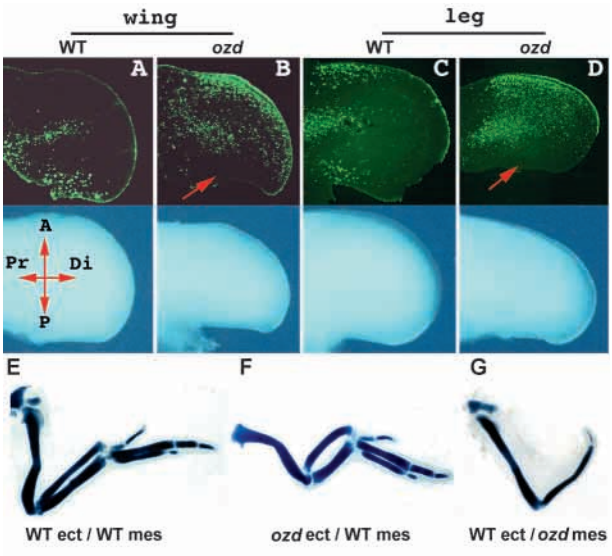

Fig. 2. Pattern of cell death and affected tissue layer in *ozd* limbs. (A-D) TUNEL analysis of sectioned, st. 24 wild-type and *ozd* limb buds reveals an abnormal pattern of cell death in *ozd*. Beneath each section is a picture of the limb bud prior to embedding, shown to better understand its shape. Arrows in B and D indicate the lack of cell death along the posterior border. (E) Normal skeletal pattern of a recombinant limb constructed with wild-type ectoderm and mesoderm. (F) Recombined *ozd* ectoderm and wild-type mesoderm result in a normal skeletal pattern. (G) Recombined *ozd* mesoderm and wild-type ectoderm produce wings with an *ozd* skeletal pattern. Axial orientations are indicated in A. A, anterior; Di, distal; P, posterior; Pr, proximal.

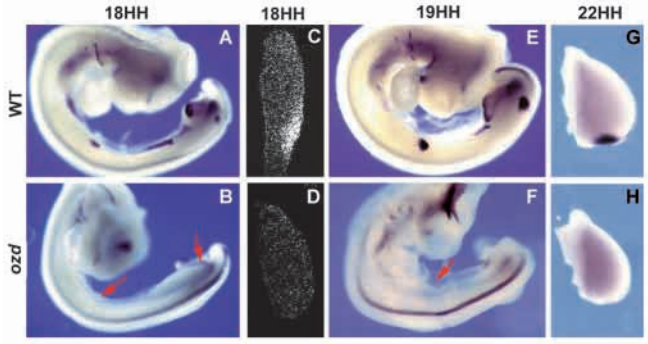

Fig. 3. *Shh* expression is undetectable in *ozd* limb buds. (A) Normal pattern of *Shh* expression in a wild-type st. 18 embryo. (B) Comparable stage *ozd* embryo lacks detectable *Shh* expression in the limbs (arrows). In situ hybridization to sectioned limb buds confirms this result, showing posterior *Shh* expression in a wild-type st. 18 wing bud (C) while failing to detect *Shh* expression in *ozd* wing buds (D). Normal *Shh* expression is shown for wild-type st. 19 (E) and 22 (G) embryos, while comparable *ozd* (F) and (H) embryos exhibit normal domains of *Shh* expression except in the limb buds (arrow).

the mutant buds gives the appearance of a less substantial posterior border that eventually formed a concavity. Our results indicate that the increased apoptosis in the mutant contributes to the progressive narrowing of the bud to a pointed shape over the course of development. However, the predominantly anterior pattern of apoptosis in the mutant cannot account for the loss of posterior structures characteristic of *ozd* wings and legs.

The mesoderm is the defective tissue layer in *ozd*

To investigate which tissue layer is affected by the *ozd* mutation we performed recombination experiments interchanging mesoderm and ectoderm between mutant and normal donors (Fernandez-Teran et al., 1999). Control experiments exchanging mesoderm and ectoderm from normal limb buds resulted in completely normal skeletal patterns (Fig. 2E). Recombinant limbs constructed with mutant ectoderm and wild-type mesoderm also developed into limbs with a normal skeletal pattern (Fig. 2F), indicating that the *ozd* ectoderm is capable of supporting normal development. However, mutant mesoderm recombined with normal ectoderm resulted in limbs exhibiting the mutant phenotype (Fig. 2G). These experiments demonstrate that the mesoderm is defective in the mutant while the ectoderm is capable of normal function.

Shh expression is undetectable in *ozd* limb buds

The lack of posterior elements in both the zeugopod and autopod of the *ozd* mutants indicated a defect along the AP axis, so we began our molecular analysis by looking at *Shh* expression.

Batch analysis of st. 19 and older embryos revealed that approximately one quarter (14/50) lacked normal posterior *Shh* expression (Fig. 3E-H). This correlated with the expected percentage of homozygous embryos, suggesting *ozd* mutants did not express detectable levels of *Shh* in the limb.

We detected *Shh* transcripts in st. 17/18 wild-type embryos (cf. Riddle et al., 1993) by whole-mount in situ hybridization (n=10; Fig. 3A). But, since there is some variability in the developmental time at which *Shh* expression is initiated in the limb bud (cf. Riddle et al., 1993), the batch method was not completely satisfactory for the study of these stages. In order to determine if mutant embryos expressed transient levels of detectable *Shh* prior to st. 19, we analyzed *Shh* expression in st. 17/18 wing buds of confirmed *ozd* embryos. For this specific experiment, we removed the right limb buds in ovo and allowed the embryo to develop to determine the phenotype. Confirmed *ozd* limbs were embedded, sectioned and hybridized with ^{35}S-labeled *Shh* riboprobe. We found that *Shh* expression was undetectable in all confirmed *ozd* buds (n=4, Fig. 3D) while control buds, acquired in the same way, expressed *Shh* (n=11, Fig. 3C). Thus, *ozd* embryos do not express detectable levels of *Shh* in the posterior limb bud at any stage. We stress at this point that the defect in *Shh* expression is specific for the limb bud, since expression at other embryonic sites, e.g. the floor plate of the neural tube, appeared normal and these structures had no morphological defects (Fig. 1A-B and Fig. 3).

We also analyzed the posterior *ozd* mesoderm for polarizing activity. ZPA grafts from confirmed *ozd* limbs gave no duplications (n=3, not shown) while ZPA tissue from non-*ozd* siblings gave the expected digital duplications (n=8, not shown); polarizing activity of 71.8%, calculated according to the method of Drossopoulou et al. (Drossopoulou et al., 2000).

The Shh pathway is not activated in *ozd* posterior limb bud mesoderm

To confirm that Shh was not expressed in *ozd* limbs, we

analyzed the expression of *Patched1* (*Ptc1*) and *Gli1*, genes directly regulated by Shh and considered to be highly sensitive indicators of Shh signaling (Ingham and McMahon, 2001).

During normal limb development *Ptc1*, the receptor for Shh, and *Gli1*, a target of Shh signaling, are expressed in domains overlapping the expression domain of *Shh* but extend more anteriorly (Fig. 4A). Using the batch method, we found that roughly 25% of embryos from the *ozd* flock did not express detectable levels of *Ptc1* in the wing bud (5/22; Fig. 4A). Utilizing the hemisection technique, expression of *Ptc1* was never detected in st. 18/19 *ozd* mutant limb buds (st. 18-19, 36-38 somites: n=5; Fig. 5B). Similar to *Ptc1*, we found that roughly 25% of hybridized embryos (3/18) did not express detectable *Gli1* in the limb (compare Fig. 4C with 4D). These data confirm that detectable Shh activity is not present in *ozd* limb buds.

Gli3 and *Shh* have mutually exclusive expression domains in the developing limb and are believed to repress one another's expression (Büscher et al., 1997; Marigo et al., 1996a; Masuya et al., 1997; Schweitzer et al., 2000). By the batch method, at st. 18/19, no differences in *Gli3* expression were detected among embryos of the *ozd* flock (Fig. 4E). This was confirmed in mutant limb buds (n=4) as early as late st. 18/19 (37-40 somites) by hemisection analysis. However, at st. 21, *ozd* embryos failed to down-regulate *Gli3* expression at the posterior border of the limb (25% of batch, Fig. 4F).

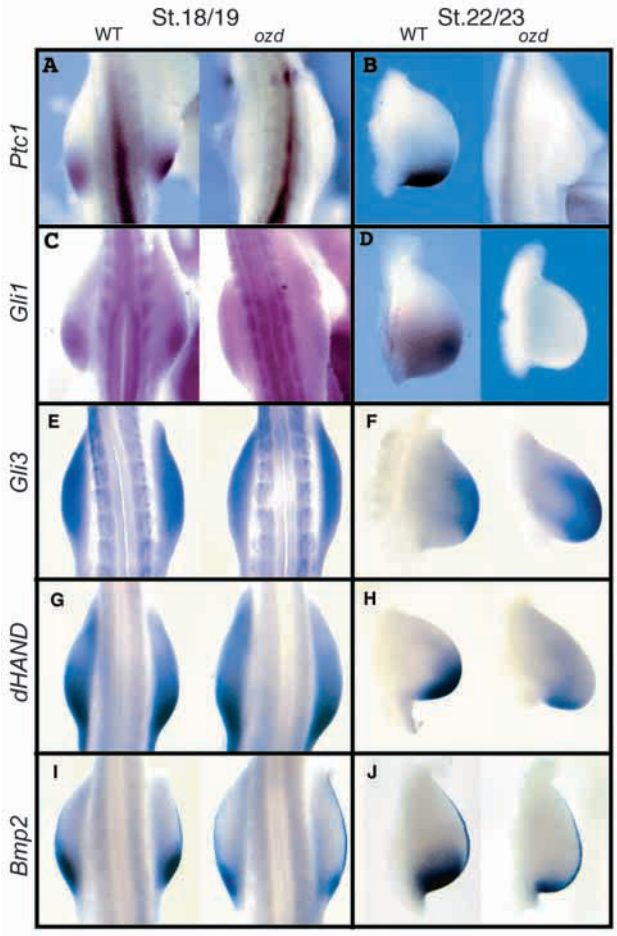

Fig. 4. Expression of putative *Shh* target genes in *ozd* limb buds. Both *Ptc1* (A,B) and *Gli1* (C,D) expression is undetectable in *ozd* limb buds at early (A,C) or later stages (B,D). (E-F) *Gli3* expression is normal in mutant limbs at st. 18/19 (E), but abnormally extends to the posterior border at later stages (F). (G,H) *dHAND* expression is normal in early stage *ozd* wing buds (G), but is posteriorly restricted at later stages. (I,J) *ozd* limb buds express *Bmp2*, but expression levels are reduced and the spatial domain posteriorly restricted relative to wild type. In every panel anterior is up.

We next compared expression patterns of the bHLH transcription factor dHAND which has been proposed to act upstream of *Shh* and establish a positive feedback loop with *Shh* later in development (Charité et al., 2000; Fernandez-Teran et al., 2000). Expression of *dHAND* in the *ozd* limbs started normally (batch method), but then was reduced to a weak domain of expression restricted to the posterior border of the limb, in a very similar pattern to that observed in the limbs of the $Shh^{-/-}$ mice (Fig. 4G,H) (Charité et al., 2000; Fernandez-Teran et al., 2000).

We also analyzed the expression patterns of other genes considered to be major downstream targets of Shh. *Bmp2*, previously thought to be a downstream target of Shh, was expressed in the mesoderm and AER of both mutant wing and leg buds as early as st. 18/19 (Fig. 4I,J). It was expressed in a reduced area and at a slightly lower level than normal as determined by both batch (n=2/4, st. 20-23) and hemisection methods (st. 19, 37-39 somites; n=3).

Expression of *Hoxd11-13* was also analyzed in *ozd* limbs. Using the batch method, it was determined that *Hoxd11-13* expression was initiated in a temporally and spatially normal pattern (not shown), but progressively declined with time (Fig. 5). *Hoxd11* pattern of expression was virtually normal in *ozd* wings up to st. 25, although its level of expression was slightly reduced compared to wild-type (Fig. 5A-B). During subsequent stages *Hoxd11* expression in the mutant wing was restricted to the posterior border (Fig. C,D). In the *ozd* leg bud *Hoxd11* expression was very reduced compared to wild type at st. 21/22 (Fig. 5A), becoming undetectable at st. 24/25 (Fig. 5B-D). *Hoxd12* expression was reduced in the *ozd* wing buds as early as st. 21/22 (Fig. 5E) and its expression continued restricted to the posterior border (Fig. 5F-H). Expression of *Hoxd12* was much more affected in the mutant leg where it became undetectable at st. 24 (Fig. 5E-H). In the mutant wing and leg, *Hoxd13* expression was very reduced and became undetectable by st. 23/24 (Fig. 5I-L). Interestingly, *Hoxd13* was re-expressed in the distal mutant leg mesoderm at st. 27 (Fig. 5K) and persisted in the distal leg mesoderm (Fig. 5L). Re-expression of *Hoxd11 or 12* was never observed.

Genes involved in PD and DV patterning were normally expressed in *ozd* limbs. For PD specification we analyzed the expression of *Meis1* and *2* and *Hoxa11* and *Hoxa13* genes. We found that expression of *Meis1* and *2* was not modified in *ozd* limbs (not shown). While the expression of *Hoxa11* was normal in *ozd* wing buds, the expression of *Hoxa13*, considered a marker for the autopod, was dramatically diminished to a thin low-level stripe of distal expression in the mutant wing mesoderm. In the mutant leg *Hoxa13* expression was similar to normal (not shown). For DV specification we analyzed the expression of *Wnt7a* and *Lmx1*; both showed a normal pattern of expression in *ozd* limbs (not shown).

Gene expression in the *ozd* AER

Although our molecular characterization and experimental

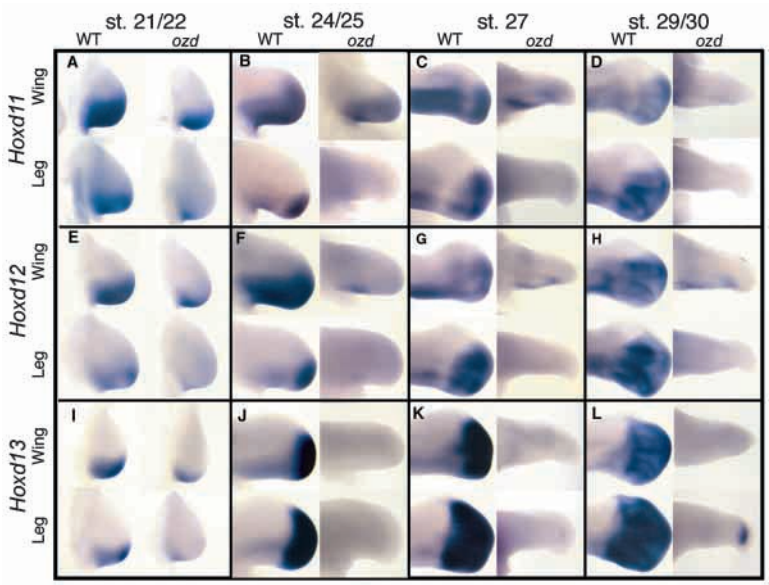

Fig. 5. Expression of *Hoxd11*, *Hoxd12* and *Hoxd13* in *ozd* limb buds. (A-D) *Hoxd11* expression in wild-type and *ozd* limbs. (A,B) Up to st. 25, *Hoxd11* shows a normal pattern of expression in the mutant wing, although its expression level is slightly reduced. (C,D) From st. 27, *Hoxd11* expression in the *ozd* wing is confined to the posterior border. (A-D) In *ozd* leg buds, *Hoxd11* expression is more affected, showing a reduced domain of expression by st. 21/22 (A), and becomes undetectable by st. 24/25 (B-D). (E-H) *Hoxd12* expression in wild-type and *ozd* limbs. (E) The pattern of *Hoxd12* expression is close to normal in st. 21/22 *ozd* wing buds, but expression levels are reduced. (F-H) From st. 24/25, *Hoxd12* becomes confined to the posterior border of the mutant wing. (E-H) *Hoxd12* expression in the *ozd* leg bud is only observed at early stages and at very reduced levels. (I-L) *Hoxd13* expression in wild-type and *ozd* limbs. (I) *Hoxd13* expression occurs at low levels and is posteriorly restricted in st. 21/22 *ozd* wing and leg buds, relative to wild type. (J-L) From st. 23/24 *Hoxd13* expression becomes undetectable both in wing and leg buds. (K-L) Expression is re-initiated at st. 27 in the distal mesoderm of *ozd* legs. In all the panels anterior is up.

study of the *ozd* mutant limb indicates that the defect is in the mesoderm, reciprocal interactions between the mesoderm and the AER are well documented (Deng et al., 1997; Ohuchi et al., 1997). Therefore, we analyzed the expression of *Fgf8* and *Fgf4* in the AER of *ozd* limbs. The mutant AER always expressed high levels of *Fgf8* throughout development of both the wing and leg (Fig. 6A-F). Coincident with the progressive narrowing of the mutant limb, the posterior extent of the AER was reduced, showing an abrupt end at the posterior border at the point of the posterior concavity in the mutant limb shape (Fig. 6B). *Fgf8* expression persisted in the mutant AER up to st. 27 in the wing and st. 28 in the leg. At later stages, *Fgf8* was dramatically reduced throughout the anterior AER (Fig. 6C). The anterior loss of *Fgf8* together with its reduced posterior extension resulted in a discrete point of *Fgf8* expression at the very tip of the mutant limbs at st. 28 (not shown). The expression of *Fgf4* appeared reduced except in the most posterior of the mutant AER (Fig. 6D), where a spot of elevated expression became apparent by st. 22/23 (Fig. 6E). *Fgf4* expression was not maintained in the mutant AER and declined with time, so that by st. 25 it was undetectable except for residual levels of expression in the posterior spot of high-level expression seen at st. 22/23 (Fig. 6F, compare with Fig. 6E).

Recently, it was proposed that *Fgf4* upregulation by Shh in the posterior AER is mediated by the BMP antagonist Gre and expression of *Gre* in the limb mesoderm is considered necessary for AER maintenance (Capdevila et al., 1999; Zúñiga et al., 1999). During development of the *ozd* limb buds *Gre* expression appeared reduced and restricted to the posterior border (Fig. 6G-I) as confirmed by the hemisection technique (st. 20/21, 40-44 somites; n=5). This pattern of *Gre* expression is similar to that reported in the *Shh* mutant mice (Zúñiga et al., 1999) and is consistent with the reduced *Fgf4* expression observed in *ozd* limb buds.

ZPA or SHH application rescues the *ozd* phenotype

Since *Shh* expression and signaling is undetectable in mutant limbs, we tried to rescue the mutant phenotype by grafting a normal ZPA or applying exogenous SHH-N to the posterior border of st. 20 mutant limb buds. ZPA fragments from st. 20 wild-type limb buds were grafted under the posterior AER of either the wing or leg of embryos from the mutant flock. For

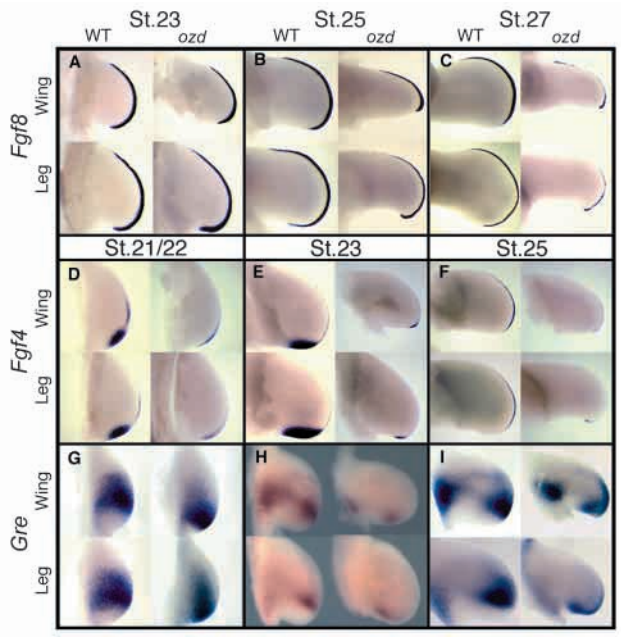

Fig. 6. Expression of *Fgf8*, *Fgf4* and *Gre* in *ozd* limb buds.(A,B) *Fgf8* is expressed at high level throughout the *ozd* AER. The AP extent of the AER is reduced in the narrowed *ozd* limb. (C) At st. 27, *Fgf8* appears down regulated in the anterior AER but expression persists at high levels in the posterior AER. (D,E) *Fgf4* expression in the mutant AER is reduced except at the most posterior edge, where a spot of elevated expression became apparent by st. 23. Note that the specimens in D and E have been analyzed for both *Fgf4* and *Shh* expression. (F) By st. 25, *Fgf4* expression has declined in the mutant AER except for the posterior spot of high-level expression. (G-I) *Gre* expression in *ozd* and wild-type limbs. *ozd* limb buds express *Gre* but, contrary to normal, its spatial domain of expression expands to the posterior border. In all the panels anterior is up.

wings, pieces of leg ZPA were used and for legs, pieces of wing ZPA were used. When the ZPA was grafted to an *ozd* limb, the mutant phenotype was restored to normal (*n*=2; Fig. 7B). In the specimen showed in Fig. 7B, the piece of ZPA of leg origin has also formed a digit characteristic of the leg (asterisk in Fig. 7B). The appearance of a digit of graft (leg) origin may occur if the grafted ZPA is large. ZPA grafts into the *ozd* leg buds gave equivalent results (not shown).

Next, heparin acrylic beads loaded with SHH-N protein (4 mg/ml) were applied to the posterior border, attempting to mimic a normal ZPA. In these cases, a total pattern restoration of the AP axis was observed at the zeugopod level with formation of a normal ulna, and improved development of carpals, although the limbs were truncated at wrist level (Fig. 7C). The sequential application of a second SHH-N loaded bead 24 hours after the first restored wing patterning at both zeugopod and autopod levels (Fig. 7D).

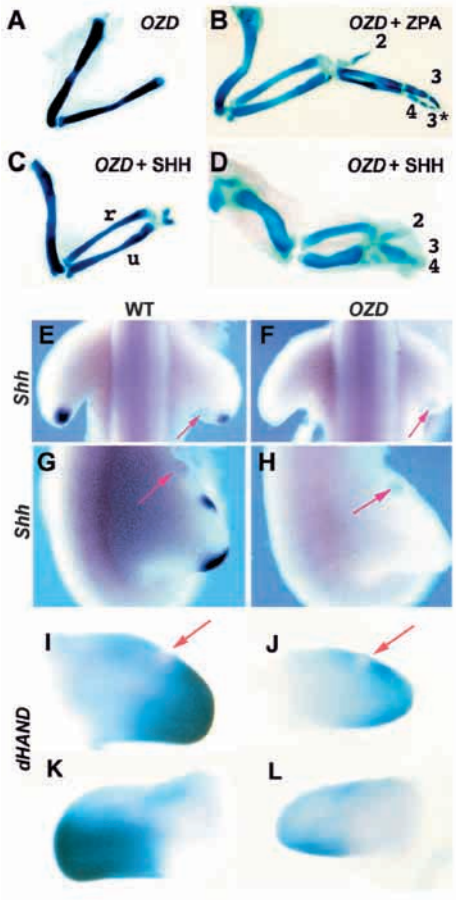

Fig. 7. *ozd* mesoderm is Shh-responsive, but cannot express *Shh*. The *ozd* mutant wing phenotype (A) was completely rescued by a ZPA graft (B). The ZPA graft was of leg origin and contributed a leg digit marked here as 3*. (C) Application of an Shh-N-soaked bead to posterior st. 20 *ozd* wing buds restores zeugopod development. (D) Two sequential applied SHH beads rescues both zeugopod and autopod formation in *ozd* wings. (E) Reduction of *Shh* expression 24 hours after application of an RA bead to the posterior border of a wild-type wing bud. (F) *ozd* posterior mesoderm does not express *Shh* 24 hours after RA application. (G) Induction of *Shh* at the anterior border of a wild-type leg bud 24 hours after implantation of a RA bead. (H) RA application at the anterior border of *ozd* leg buds does not induce ectopic *Shh* expression. (I-L) RA application induces ectopic anterior *dHAND* expression in both normal (I) and mutant (J) wing buds. *dHAND* expression in the unmanipulated contralateral wild-type (K) and *ozd* (L) limb buds. The position of the RA bead is indicated by the red arrow.

Retinoic acid is unable to induce *Shh* expression in the *ozd* mutant limb mesoderm

Retinoic acid (RA) induces *Shh* expression when applied to the anterior wing bud mesoderm (Helms et al., 1994; Riddle et al., 1993) and is implicated in the normal induction of the ZPA (Lu et al., 1997; Stratford et al., 1997). We applied RA to either the anterior or posterior mesoderm of st. 20/21 *ozd* limb buds to determine if *Shh* could be induced and the mutant phenotype rescued. We first applied beads soaked in RA (0.1 and 1 mg/ml) under the posterior AER. In wild-type limb buds (*n*=10), the level of normal *Shh* expression was reduced when analyzed 24 hours after the operation (Fig. 7E) and resulted in a range of skeletal alterations varying from a loss of digits (*n*=9) to the complete inhibition of outgrowth (*n*=1). These data are consistent with previous reports (Tickle et al., 1985). Application of an RA bead to the posterior mesoderm of *ozd* wings did not induce *Shh* expression after 24 hours (*n*=3; Fig. 7F) and resulted in the total absence of the right wing (*n*=2; not shown).

RA-soaked beads (0.1 and 1 mg/ml) applied to anterior *ozd* limb mesoderm did not induce *Shh* at 24 hours (*n*=2; compare Fig. 7H with Fig. 7G) or 48 hours (*n*=1; not shown) after the operation and the mutant phenotype was not modified (*n*=2). It has been shown in the wing that the induction of *Shh* by RA may be mediated by the early, transient activation of *Hoxb8* (Lu et al., 1997; Stratford et al., 1997), and that it is also preceded by the activation of *dHAND* expression (Fernandez-Teran et al., 2000). Thus, we analyzed at what point RA induction of *Shh* failed in the mutant. RA-soaked beads (1 mg/ml and 0.6 mg/ml) were placed in the anterior border of wing buds, and embryos were fixed after 5 hours to analyze *Hoxb8* expression, and after 12 or 20 hours to analyze *dHAND* expression. *Hoxb8* was normally expressed by *ozd* limb mesoderm in response to RA signaling (*n*=5; confirmed by hemisection technique; not shown). RA applications also induced *dHAND* expression in the anterior mutant limb mesoderm, similar to the normal limb (*n*=5; Fig. 7I-L). These observations indicate that the *ozd* mutation lies downstream of *Hoxb8* and *dHAND* activation by RA.

DISCUSSION

The anatomical, molecular and experimental analyses presented here indicate that *ozd* limbs develop in the absence of Shh signaling. Our data establish that the defect in the Shh signaling pathway lies upstream of *Shh* transcriptional activation, suggesting the *ozd* mutation affects a regulatory element that controls limb-specific expression of *Shh*. Our analysis further demonstrates that the limb buds develop with an AP identity independent of Shh function. The identification of a naturally occurring ‘targeted knockout’ of *Shh* in the developing limbs of a experimentally tractable model system offers a unique tool to address its role in amniote limb patterning.

AP molecular asymmetry in the absence of Shh function

Bmp2 and 5′ *Hoxd* genes are considered to be downstream effectors of Shh signaling since Shh application to the anterior border induces their ectopic expression (Laufer et al., 1994; Riddle et al., 1993; Yang et al., 1997). We report that in *ozd* limbs these genes are activated in a pattern similar to that in normal limbs. The 5′ *Hoxd* genes were also shown to be asymmetrically expressed in the *limbless* mutant limb bud in the absence of detectable *Shh* expression (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996) and in the *Shh*$^{-/-}$ mouse (Chiang et al., 2001; Kraus et al., 2001). Phase II of 5′ *Hoxd* genes expression, proposed to be Shh dependent (Nelson et al., 1996), starts normally in *ozd* but is not fully developed and expression declines with time. Phase III of expression, which corresponds to the autopod (Nelson et al., 1996), is dramatically affected. The more 5′ the *Hoxd* gene, the earlier and more severely its pattern of expression is affected. For example, in the st. 25 *ozd* wing bud, *Hoxd11* is expressed in a pattern similar to normal, while *Hoxd12* and *Hoxd13* expression is progressively diminished. This may indicate a progressive differential requirement for Shh among 5′ *Hoxd* genes. However, it is of interest that the distal tip of the *ozd* leg bud re-expresses *Hoxd13* at later stages correlating with the formation of d1 and, interestingly, precedes activation of *Indian hedgehog* (*Ihh*) in the digital cartilage (data not shown). This late *Hoxd13* expression was also reported to occur in the *Shh*$^{-/-}$ hindlimb (Chiang et al., 2001; Kraus et al., 2001). Also, *dHAND* is expressed in a reduced but posteriorly polarized domain of expression in *ozd* limb buds. Thus, activation and polarization of *Bmp2*, the 5′ *Hoxd* and *dHAND* expression in the posterior limb bud does not require Shh and reflects AP patterning asymmetries in the early limb bud that are independent of Shh. However, Shh inputs are required to stabilize and augment initial gene expressions so that the AP polarization of the limb bud is realized.

Shh-dependent and -independent limb skeletal elements

Because Shh signaling is absent in the limbs of *ozd* embryos, it is useful to compare the limb phenotype of *ozd* mutants and *Shh*$^{-/-}$ mice (Chiang et al., 2001; Kraus et al., 2001). Interestingly, both types of limbs show a very similar phenotype forming a complete PD axis with a normal stylopod. One digit, identified as d1, forms in the *Shh*$^{-/-}$ hindlimb (Chiang et al., 2001; Kraus et al., 2001; Lewis et al., 2001) and also d1 forms in the *ozd* leg. The main differences between *ozd* and *Shh*$^{-/-}$ limbs occur at the zeugopod. The skeletal elements in the zeugopod of the *Shh*$^{-/-}$ mice (one in forelimb and two in hindlimbs) are abnormal while the morphology of the single fore and hindlimb zeugopod element in *ozd* mutants are virtually normal. Despite the differences, both genotypes demonstrate the necessity for Shh distal to the elbow/knee region, since either loss of AP identity and/or posterior deficits are observed without it. Thus, it is possible to classify the skeletal elements of the limb according to their requirement for Shh signaling. The *ozd* mutation indicates that in the chick the humerus/femur, radius/tibia and d1 are Shh independent, while the ulna/fibula and rest of the digits require Shh inputs for normal development (Fig. 8). However, the Shh-independent potential of the limb varies between chick and mouse at the zeugopod level since the element that forms in chick is well shaped while it is unidentifiable in mouse.

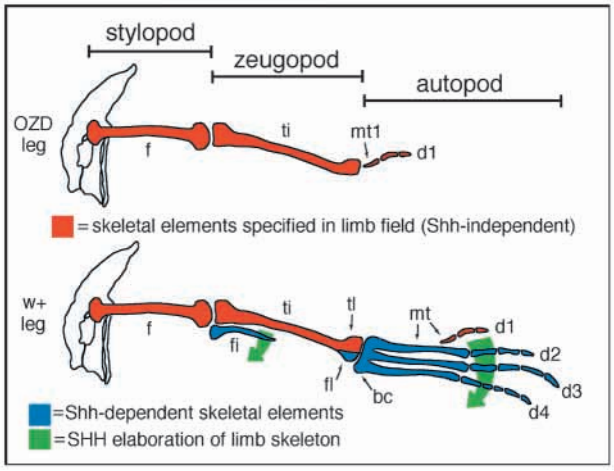

Fig. 8. SHH function in generating the amniote limb skeleton. *ozd* legs (top) develop with a single, identifiable, *Shh*-independent skeletal element at each PD level (red), forming a linear series of the anterior-most elements found in wild-type legs (bottom). Therefore, given a functional AER, the limb field contains all the information necessary to form a distally complete limb. In normal development, *Shh* signaling is differentially required at each PD level to elaborate the formation and patterning of additional limb skeletal elements (blue) along the AP axis. We propose that while the femur (f) is *Shh* independent, *Shh* acts in the zeugopod context of the prepatterned tibia (ti) to specify the fibula (fi). In the autopod, *Shh* acts in the context of the prepatterned d1 to progressively specify the posterior digits (d2, d3, and then d4). We note that *Shh* is also required to elaborate the posterior fibulare (fl) and basal commune (bc) elements of the tarsus, which respectively fuse to the distal tibia (ti) and proximal metatarsals (mt) by 8.5 days of development (data not shown). tl, tibiale.

Experimental removal of the posterior wing mesoderm in chick, including the whole ZPA leads to limbs with a phenotype very similar to *ozd* limbs (Pagan et al., 1996; Todt and Fallon, 1987). The operated wings form a normal radius with or without d2 and since the surgery is performed at st. 20, before the determination of the zeugopod (Summerbell, 1974), it can be concluded, on the basis of various approaches to this issue, that a completely normal radius can develop in the chick in the absence of Shh input.

Morphological differences between the wing and the leg reflect differences in the response to common molecular signals that pattern them. Moreover, wing buds and leg buds may respond differently to experimental manipulation (e.g. Todt and Fallon, 1987; Wada and Nohno, 2001). The formation of a properly patterned digit in the leg but not the wing indicates that Shh is required for the most anterior digit to form in the wing. The identity of the three avian wing digits remains controversial (Burke and Feduccia, 1997) (see also Kundrát et al., 2002; Larsson et al., 2002). However, if we assume the conventional nomenclature of d2, d3, d4, our hypothesis that d1 is Shh independent predicts no wing digits will develop in the absence of Shh function. Admittedly, the loss of d1 in the *Shh*$^{-/-}$ mouse forelimb is difficult to explain. It is possible that global loss of Shh function has more deleterious effects on limb development than limb-specific loss of Shh function alone. A

conditional null of *Shh* in the mouse limb will permit a direct comparison of the mouse with the *ozd* limb.

The role of Shh in mesoderm cell survival and proliferation

Removal of posterior mesoderm was shown to cause cell death similar to our findings for *ozd* and was attributed to the loss of ZPA function (Todt and Fallon, 1987). It is notable that grafting a bead loaded with Shh protein prevents normal anterior cell death in the chick wing (Sanz-Ezquerro and Tickle, 2001), suggesting a role for Shh in regulating cell death in the limb.

Abnormal cell death correlates with the progressive narrowing of *ozd* limbs. Interestingly, while the anterior mesoderm undergoes increased apoptosis, neither the PNZ nor abnormal cell death are detected in the posterior border. However, there is a significant change in the shape of the posterior border, most notably in the leg, where a concavity forms that contributes to the spike shape of the *ozd* phenotype. Determination of the mechanism of posterior limb bud shape change is made more challenging by the observation that there are no gross differences in BrdU incorporation in posterior cells as compared to wild type at the stages examined (st. 19, 23 and 25; not shown). It is possible that those cells that will later contribute to posterior structures failed to proliferate and were left behind, beginning slightly before the phenotype becomes obvious, around st. 23/24. A slight change in proliferation at st. 17 and 18, or even at the stages analysed with BrdU, but below a detectable level could still account for the loss of posterior structures. Clarification of this point will require further investigation. Also, it is worth mentioning that a mitogenic effect for Shh has been reported in several developing systems (Bellusci et al., 1997; Duprez et al., 1998; Jensen and Wallace, 1997) and that Hh signaling can induce proliferation during development by promoting expression of *cyclin D* and *cyclin E* (Duman-Scheel et al., 2002). Thus, in the absence of Shh, stimulus from the AER would not be sufficient to support enough mesoderm to permit the specification of the whole anterior-posterior axis.

The *ozd* mutation potentially affects a *Shh* regulatory element

Disruptions in AP limb pattern are among the most common human birth defects (Castilla et al., 1996; Castilla et al., 1998), and understanding the affected developmental mechanisms is of significant clinical importance. Interestingly, studies in human and mouse have mapped several mutations and transgene insertions causing limb-specific AP patterning defects to a syntenic locus near or within the *Limb region 1* (*Lmbr1*) gene, located less than 1 Mbp from the *Shh* coding region [(Clark et al., 2001; Lettice et al., 2002), and references therein]. Recent genetic analyses demonstrate the *Lmbr1* gene is incidental to the limb phenotypes; rather, evidence suggests these mutations affect long-range *cis* regulatory elements, embedded within the *Lmbr1* locus, that control *Shh* expression in the limb. While the majority of these mutations cause dominant pre-axial polydactyly, the small deletion responsible for the autosomal recessive human disorder *Acheiropodia* maps within the *Lmbr1* locus (Ianakiev et al., 2001), and causes longitudinal postaxial deficiencies closely resembling the limb phenotypes of *ozd* chicks and $Shh^{-/-}$ mice. Here we have shown that *ozd* limb mesoderm is incapable of expressing *Shh*, clearly indicating that the mutation affects a limb-specific regulatory element of *Shh* expression. Although the data presented here are compatible with the mutation affecting either a *cis*- or *trans*-acting element, we hypothesize that the *ozd* mutation disrupts a *cis*-acting regulatory element directing *Shh* expression in the limb, which lies within the *Lmbr1* locus such as in *Acheiropodia* individuals (Ianakiev et al., 2001); this hypothesis is currently being investigated.

This work was supported by NIH Grant No. 32551 to J. F. F.; R. D. D. was supported in part by a Cremer Fellowship from the University of Wisconsin Medical School. Work in M. A. R.'s laboratory is supported by grants DGICYT-PM98-0151 and FIS 01/1219 from the Spanish Ministry of Science. We thank Prof. John Opitz for helpful discussions on naming the mutant, the Florsheim family for their financial support of this research, Allen W. Clark for help with Fig. 8 and S&R Egg Farm (Whitewater, WI) for providing the White Leghorn flock. This paper is dedicated to the memory of Genie Fallon Hall.

REFERENCES

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G. E. and Hogan, B. M. (1997). Involvement of Sonic Hedgehog (shh) in mouse embryonic lung growth and morphogenesis. *Development* **124**, 53-63.

Burke, A. C. and Feduccia, A. (1997). Developmental Patterns and the Identification of Homologies in the Avian Hand. *Science* **278**, 666-668.

Büscher, D., Bosse, B., Heymer, J. and Rüther, U. (1997). Evidence for genetic control of *Sonic hedgehog* by *Gli*3 in mouse limb development. *Mech. Dev.* **62**, 175-182.

Capdevila, J. and Izpisua Belmonte, J. C. (2001). Patterning Mechanisms Controlling Vertebrate Limb Development. *Annu. Rev. Cell. Dev. Biol.* **17**, 87-132.

Capdevila, J., Tsukui, T., Rodríguez Esteban, C., Zappavigna, V. and Izpisúa Belmonte, J. C. (1999). Control of vertebrate limb outgrowth by the proximal factor *Meis2* and distal antagonism of BMPs by Gremlin. *Mol. Cell* **4**, 839-849.

Carrington, J. L. and Fallon, J. F. (1988). Initial limb budding is independent of apical ectodermal ridge activity: evidence from a limbless mutant. *Development* **104**, 361-367.

Castilla, E. E., Lugarinho da Fonesca, R., da Graca Dutra, M., Bermejo, E., Cuevas, L. and Marinez-Frias, M. L. (1996). Epidemiological analysis of rare polydactylies. *Am. J. Hum. Genet.* **65**, 295-303.

Castilla, E. E., Lugarinho, R., da Graca Dutra, M. and Salgado, L. J. (1998). Associated anomalies in individuals with polydactyly. *Am. J. Hum. Genet.* **80**, 459-65.

Charité, J., de Graaf, W., Shen, S. and Deschamps, J. (1994). Ectopic expression of *Hoxb-8* causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. *Cell* **78**, 589-601.

Charité, J., McFadden, D. G. and Olson, E. N. (2000). The bHLH transcription factor dHAND controls *Sonic hedgehog* expression and establishment of the zone of polarizing activity during limb development. *Development* **127**, 2461-2470.

Chen, H. and Johnson, R. L. (1999). Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. *Cell Tiss. Res.* **296**, 67-73.

Chiang, C., Litingtung, Y., Harris, M. P., Simandl, B. K., Li, Y., Beachy, P. A. and Fallon, J. F. (2001). Manifestation of the Limb Prepattern: Limb Development in the Absence of Sonic Hedgehog Function. *Dev. Biol.* **236**, 421-435.

Clark, R. M., Marker, P. C., Roessler, E., Dutra, A., Schimenti, J. C., Muenke, M. and Kingsley, D. M. (2001). Reciprocal Mouse and Human Limb Phenotypes Caused by Gain- and Loss-of-function Mutations Affecting lmbr1. *Genetics* **159**, 715-726.

Cole, R. K. (1967). Ametapodia, a dominant mutation in the fowl. *J. Hered.* **58**, 141-146.

Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-136.

Dahn, R. D. and Fallon, J. F. (2000). Interdigital regulation of digit identity

and homeotic transformation by modulated BMP signaling. *Science* **289**, 438-441.

Deng, C., Bedford, M., Li, C., Xu, X., Yang, X., Dunmore, J. and Leder, P. (1997). Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. *Dev. Biol.* **185**, 42-54.

Drossopoulou, G., Lewis, K. E., Sanz-Ezquerro, J. J., Nikbakht, N., McMahon, A. P., Hofmann, C. and Tickle, C. (2000). A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. *Development* **127**, 1337-1348.

Duman-Scheel, M., Weng, L., Xin, S. and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature* **417**, 299-303.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M. and Tickle, C. (1996). Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol.* **174**, 448-452.

Duprez, D., Fournier-Thibault, C. and le Douarin, N. (1998). Sonic hedgehog induces proliferation of committed skeletal muscle cells in the chick limb. *Development* **125**, 495-505.

Ede, D. A. (1969). Abnormal dvelopment in the cellular level in talpid and other mutants. In *The Fertility and Hatchability of the Hen's Egg* (ed. T. C. Carter and B. M. Freeman) pp. 71-83. Edinburgh: Oliver and Boyd.

Fell, H. B. and Canti, R. G. (1934). Experiments on the development in vitro of the avian knee-joint. *Proc. R. Soc.* **116**, 316-351.

Fernandez-Teran, M., Piedra, M. E., Kathiriya, I. S., Srivastava, D., Rodriguez-Rey, J. C. and Ros, M. A. (2000). Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway. *Development* **127**, 2133-2142.

Fernandez-Teran, M., Piedra, M. E., Ros, M. A. and Fallon, J. F. (1999). The recombinant limb as a model for the study of limb patterning, and its application to muscle development. *Cell Tiss. Res.* **296**, 121-129.

Grieshammer, U., Minowada, G., Pisenti, J. M., Abbott, U. K. and Martin, G. R. (1996). The chick *limbless* mutation causes abnormalities in limb bud dorsal-ventral patterning: implications for the mechanism of apical ridge formation. *Development* **122**, 3851-3861.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Helms, J., Thaller, C. and Eichele, G. (1994). Relationship between retinoic and *sonic hedgehog*, two polarizing signals in the chick wing bud. *Development* **120**, 3267-3274.

Hinchliffe, J. R. (1982). Cell death in vertebrate limb morphogenesis. In *Progress in Anatomy*, Vol. 2 (ed. Harrison and Navatnam), pp. 1-9. Cambridge: Cambridge University Press.

Hurlé, J. M., Ros, M. A., Garcia-Martínez, V., Macías, D. and Gañan, Y. (1995). Cell death in the embryonic developing limb. *Scan. Microsc.* **9**, 519-534.

Ianakiev, P., van Baren, M. J., Daly, M., Toledo, S., Cavalcanti, M. G., Neto, J. C., Silveria, E. L., Freire-Maia, A., Heutink, P., Kilpatrick, M. W. and Tsipouras, P. (2001). Acheiropodia is caused by a genomic deletion in C7orf2, the human orthologue of the Lmbr1 gene. *Am. J. Hum. Genet.* **68**, 38-45.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Jensen, A. M. and Wallace, V. A. (1997). Expression of *Sonic hedgehog* and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124**, 363-371.

Kraus, P., Fraidenraich, D. and Loomis, C. A. (2001). Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech. Dev.* **100**, 45-58.

Kundrát, M. V., Seichert, A. P., Russell, K, and Smetana, K., Jr (2002). Pentadactyl pattern of the avian wing autopodium and pyramid reduction hypothesis. *JEZ (Mol. Dev. Evol.)* **294**, 152-159.

Larsson, H. C. E. and Wagner, G. P. (2002). Pentadactyl ground state of the avian wing. *JEZ (Mol. Dev. Evol.)* **294**, 146-151.

Laufer, E., Nelson, C., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.

Lettice, L. A., Horikoshi, T., Heaney, S. J. H., van Baren, M. J., van der Linde, H. C., Breedveld, G. J., Joosse, M., Akarsu, N., Oostra, B. A., Endo, N. et al. (2002). Disruption of a long-range cis-acting regulator for *Shh* causes preaxial polydactyly. *Proc. Natl. Acad. Sci. USA* **99**, 7548-7553.

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599-612.

Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F. and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418**, 979-983.

Lu, H.-C., Revelli, J.-P., Goering, L., Thaller, C. and Eichele, G. (1997). Retinoid signaling is required for the establishment of a ZPA and for the expression of Hoxb-8, a mediator of ZPA formation. *Development* **124**, 1643-1651.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996a). Biochemical evidence that Patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996b). Sonic hedgehog differentially regulates expression of *GLI* and *GLI3i* during limb development. *Dev. Biol.* **180**, 273-283.

Martin, G. R. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12**, 1571-1586.

Masuya, H., Sagai, T., Moriwaki, K. and Shiroishi, T. (1997). Multigenic control of the localization of the zone of polarizing activity in limb morphogenesis in the mouse. *Dev. Biol.* **182**, 42-51.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K. and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* **9**, 1645-1653.

McFadden, D. G., McAnally, J., Richardson, J. A., Charite, J. and Olson, E. N. (2002). Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. *Development* **129**, 3077-3088.

Merino, R., Macias, D., Gañan, Y., Rodriguez-Leon, J., Economides, A. N., Rodriguez-Esteban, C., Izpisua-Belmonte, J. C. and Hurle, J. M. (1999). Control of digit formation by activin signalling. *Development* **126**, 2161-2170.

Moon, A. M., Boulet, A. M. and Capecchi, M. R. (2000). Normal limb development in conditional mutants of *Fgf4*. *Development* **127**, 989-996.

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., Gonzales, E., Tessarollo, L., Parada, L. F. and Tabin, C. (1996). Analysis of *Hox* gene expression in the chick limb bud. *Development* **122**, 1449-1466.

Nieto, M. A., Patel, K. and Wilkinson, D. G. (1996). In situ analysis of chick embryos in whole mount and tissue sections. In *Methods in Cell Biology*, Vol. 51 (ed. M. Bronner-Fraser), pp. 219-235. New York: Academic Press.

Noramly, S., Pisenti, J., Abbott, U. and Morgan, B. (1996). Gene expression in the limbless mutant: polarized gene expression in the absence of Shh and an AER. *Dev. Biol.* **179**, 339-346.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M. et al. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235-2244.

Pagan, S. M., Ros, M. A., Tabin, C. and Fallon, J. F. (1996). Surgical removal of limb bud *Sonic hedgehog* results in posterior skeletal defects. *Dev. Biol.* **179**, 35-40.

Parr, B. A. and McMahon, A. P. (1995). Dorsalizing signal *Wnt-7a* required for normal polarity of D-V and A-P axes of mouse limb. *Nature* **374**, 350-353.

Pearse, R. V. and Tabin, C. J. (1998). The molecular ZPA. *J. Exp. Zool.* **282**, 677-690.

Power, S. C., Lancman, J. and Smith, S. M. (1999). Retinoic acid is essential for Shh/Hoxd signaling during rat limb outgrowth but not for limb initiation. *Dev. Dynam.* **216**, 469-480.

Qu, S., Niswender, K. D., Ki, Q., van der Meer, R., Keeney, D., Magnuson, M. A. and Wisdom, R. (1997). Polydactyly and ectopic ZPA formation in *Alx-4* mutant mice. *Development* **124**, 3999-4008.

Qu, S., Tucker, S. C., Ehrlich, J. S., Levorse, J. M., Flaherty, L. A., Wisdom, R. and Vogt, T. F. (1998). Mutations in mouse *Aristaless-like4* cause *Strong's luxoid* polydactyly. *Development* **125**, 2711-2721.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Ros, M. A., Lopez-Martinez, A., Simandl, B. K., Rodriguez, C., Izpisua Belmonte, J. C., Dahn, R. and Fallon, J. F. (1996). The limb field mesoderm determines initial limb bud anteroposterior asymmetry and budding independent of sonic hedgehog or apical ectodermal gene expressions. *Development* **122**, 2319-2330.

Ros, M. A., Simandl, B. K., Clark, A. W. and Fallon, J. F. (2000). Methods

for manipulating the chick limb bud to study gene expressions, tissue interactions and patterning. In *Development Biology Protocols* (ed. R. S. Tuan and C. W. Lo), pp. 245-266. Totowa, NJ: Humana Press.

Sanz-Ezquerro, J. J. and Tickle, C. (2001). 'Fingering' the vertebrate limb. *Differentiation* **69**, 91-99.

Saunders, J. W., Jr (1977). The experimental analysis of chick limb bud development. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, J. R. Hinchliffe and M. Balls), pp. 1-24. Cambridge, UK: Cambridge University Press.

Saunders, J. W. and Fallon, J. F. (1967). Cell death in morphogenesis. In *Major Problems in Developmental Biology* (ed. M. Locke), pp. 289-313. New York and London: Academic Press.

Schaller, S. A., Li, S., Ngo-Muller, V., Han, M. J., Omi, M., Anderson, R. and Muneoka, K. (2001). Cell biology of limb patterning. *Int. Rev. Cytol.* **203**, 483-517.

Schweitzer, R., Vogan, K. J. and Tabin, C. (2000). Similar expression and regulation of *Gli2* and *Gli3* in the chick limb bud. *Mech. Dev.* **98**, 171-174.

Smyth, J. R., Sreekumar, G. P., Coyle, C. A. and Bitgood, J. J. (2000). A new recessive ametapodia mutation in the chicken (*Gallus domesticus)*. *J. Hered.* **91**, 340-342.

Stoll, C., Duboule, D., Holmes, L. B. and Spranger, J. (1998). Classification of limb defects. *Am. J. Med. Genet.* **77**, 439-441.

Stratford, T., Kostakopoulou, H. K. and Maden, M. (1997). *Hoxb-8* has a role in establishing early anterior-posterior polarity in chick forelimb but not hindlimb. *Development* **124**, 4225-4234.

Stratford, T., Logan, C., Zile, M. and Maden, M. (1999). Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* **81**, 115-125.

Summerbell, D. (1974). A quantitative analysis of the excision of the AER from the chick limb bud. *J. Embryol. Exp. Morphol.* **32**, 651-660.

Sun, X., Mariani, F. V. and Martin, G. R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* **418**, 501-508.

Takahashi, M., Tamura, K., Büscher, D., Masuya, H., Yonei-Tamura, S., Matsumoto, K., Naitoh-Matsuo, M., Takeuchi, J., Ogura, K., Shiroishi, T. et al. (1998). The role of *Alx-4* in the establishment of anteroposterior polarity during vertebrate limb development. *Development* **125**, 4417-4425.

Tanaka, M., Cohn, M. J., Ashby, P., Davey, M., Martin, P. and Tickle, C. (2000). Distribution of polarizing activity and potential for limb formation in mouse and chick embryos and possible relationships to polydactyly. *Development* **127**, 4011-4021.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.

Tickle, C. and Eichele, G. (1994). Vertebrate limb development. *Annu. Rev. Cell Biol.* **10**, 121-152.

Tickle, C., Lee, J. and Eichele, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109**, 82-95.

Todt, W. L. and Fallon, J. F. (1987). Posterior apical ectodermal ridge removal in the chick wing bud triggers a series of events resulting in defective anterior pattern formation. *Development* **101**, 501-515.

Wada, N. and Nohno, T. (2001). Differential response of Shh expression between chick forelimb and hindlimb buds by FGF-4. *Dev. Dyn.* **221**, 402-411.

Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by *in situ* hybridization to tissues sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Yang, Y., Drossopoulou, G., Chuang, P.-T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A. et al. (1997). Relationship between dose, distance and time in *Sonic Hedgehog*-mediated regulation of anteroposterior polarity in the chick limb. *Development* **124**, 4393-4404.

Zúñiga, A., Haramis, A.-P. G., McMahon, A. and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401**, 598-602.