Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development

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SUMMARY

The correct patterning of vertebrate skeletal elements is controlled by inductive interactions. Two vertebrate hedgehog proteins, Sonic hedgehog and Indian hedgehog, have been implicated in skeletal development. During somite differentiation and limb development, Sonic hedgehog functions as an inductive signal from the notochord, floor plate and zone of polarizing activity. Later in skeletogenesis, Indian hedgehog functions as a regulator of chondrogenesis during endochondral ossification. The vertebrate Gli zinc finger proteins are putative transcription factors that respond to Hedgehog signaling. In Drosophila, the Gli homolog cubitus interruptus is required for the activation of hedgehog targets and also functions as a repressor of hedgehog expression. We show here that Gli2 mutant mice exhibit severe skeletal abnormalities including cleft palate, tooth defects, absence of vertebral body and intervertebral discs, and shortened limbs and sternum. Interestingly, Gli2 and Gli3 (C.-c. Hui and A. L. Joyner (1993). Nature Genet. 3, 241-246) mutant mice exhibit different subsets of skeletal defects indicating that they implement specific functions in the development of the neural crest, somite and lateral plate mesoderm derivatives. Although Gli2 and Gli3 are not functionally equivalent, double mutant analysis indicates that, in addition to their specific roles, they also serve redundant functions during skeletal development. The role of Gli2 and Gli3 in Hedgehog signaling during skeletal development is discussed.

Key words: skeletal development, Sonic hedgehog, Indian hedgehog, Gli, mouse

INTRODUCTION

The vertebrate skeleton is derived from three embryologically distinct lineages through two types of bone development (Erlebacher et al., 1995). The neural crest gives rise to the branchial arch derivatives of the craniofacial skeleton, the sclerotome generates most of the axial skeleton and the lateral plate mesoderm forms the bones of the sternum and limbs. The flat bones of the skull as well as the new bones added to the outer surfaces of the long bones are formed by intramembranous ossification in which mesenchymal precursor cells differentiate directly into bone-forming osteoblasts. The rest of the skeleton is formed by endochondral ossification where mesenchymal cells condense and differentiate into chondrocytes forming a cartilaginous skeleton which is subsequently transformed into a calcified skeleton. Later in development, longitudinal bone growth also proceeds through endochondral ossification in the growth plates at the epiphyses.

The correct patterning of skeletal elements is controlled by inductive interactions. The apical ectodermal ridge (AER) and zone of polarizing activity (ZPA) signal the growth and pattern formation of the developing limbs (reviewed by Cohn and Tickle, 1996). Signals from the notochord and floor plate direct the formation of sclerotome from the somites (reviewed by Christ and Ordahl, 1995). Epithelial-derived signals are required for the differentiation of facial cartilage (reviewed by Langille and Hall, 1993). Embryological explant studies have recently identified some of these signals – the AER signals are probably fibroblast growth factors (FGFs) and the signals from
the ZPA, notochord and floor plate are likely the secreted factor Sonic hedgehog (SHH) (see Cohn and Tickle, 1996).

Relatively little is known about the nuclear factors that respond to these secreted factors. The paired box containing transcription factor Pax1 has been shown to be induced by SHH during sclerotome differentiation (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) and is a key nuclear factor in mediating the notochord and floor plate signals (Koseki et al., 1993; Dietrich et al., 1993). Mutations in Pax1 results in multiple abnormalities of the vertebral column including defects of the vertebral body, absent or reduced intervertebral discs, and absent or reduced ribs (Wallin et al., 1994; Dietrich and Gruss, 1995).

In the developing limbs, homeobox genes situated 5’ in the Hoxa and Hoxd clusters are activated in response to FGF and SHH (Niswander et al., 1994; Laufer et al., 1994). These genes are initially expressed in overlapping domains centered on distal and posterior-distal regions of the limb (Dolle et al., 1989; Haack and Gruss, 1993) and are likely downstream mediators of the AER and ZPA signals. Inactivation of some of these Hox genes in mice have revealed their roles in limb development (detailed by Davis and Capecchi, 1996 and Fromental-Ramain et al., 1996). Furthermore, the analysis of compound mutants revealed synergistic limb malformations not present in the single mutants, indicating both specific and redundant functions of these Hox genes in limb development. Other homeobox genes, such as Msx1, Msx2 and MHOX, have also been implicated as regulators of inductive events during skeletal development (see Satokata and Maas, 1994; Martin et al., 1995). Gene disruption in mice indicates both specific and redundant functions of these Hox genes in limb development. Other homeobox genes, such as Msx1, Msx2 and MHOX, have also been implicated as regulators of inductive events during skeletal development (see Satokata and Maas, 1994; Martin et al., 1995). Gene disruption in mice has indicated that Msx1 is required for normal development of the craniofacial skeleton (Satokata and Maas, 1994) and MHOX plays a regulatory role in the growth of both chondrogenic and osteogenic precursors (Martin et al., 1995).

Members of the Gli gene family are potential mediators of SHH signaling during skeletal development. In both humans and mice, three closely related Gli genes, Gli1, Gli2 and Gli3 (Ruppert et al., 1988; Hui et al., 1994), encode zinc finger containing proteins that show significant sequence similarity to the product of the Drosophila segment polarity gene cubitus interruptus (ci; Orenic et al., 1990). Genetic analysis indicates that ci functions as a downstream mediator of hedgehog (hh) signaling (Forbes et al., 1993; Johnson et al., 1995; Motzny and Holmgren, 1995; Dominguez et al., 1996) as well as a repressor of hh expression (Dominguez et al., 1996). Extrapolating from these Drosophila observations, the Gli genes might be expected to play a similar role in signaling by vertebrate hh homologs such as SHH.

At the onset of gastrulation, the three mouse Gli genes are broadly expressed in the ectoderm and mesoderm (Hui et al., 1994). They are later expressed in the developing somites and limb, where SHH is a key inductive signal. During skeletogenesis, Gli1 expression is found in the condensing mesenchyme (blastema) and later is restricted to the perichondrium (Walterhouse et al., 1993; Hui et al., 1994). Gli2 and Gli3 are co-expressed during mesoderm differentiation – their expression can first be found in the undifferentiated mesenchyme and later in the mesenchyme surrounding the blastema but becomes undetectable upon the completion of organogenesis (Schimmang et al., 1992; Hui and Joyner, 1993; Hui et al., 1994). All three Gli genes are also expressed in the neural crest derivatives of the craniofacial skeleton (Hui et al., 1994).

Molecular analyses of the human malformation disorder Greig cephalopolysyndactyly syndrome (GCPS) and the mouse limb mutant extra-toes (Xt) have revealed a role of Gli3 in skeletal development (Vortkamp et al., 1991, 1992; Schimmang et al., 1992; Hui and Joyner, 1993). Loss of one copy of the human Gli3 gene (Vortkamp et al., 1991) results in craniofacial abnormalities such as frontal bossing and a broad nasal root as well as limb defects including preaxial and postaxial polysyndactyly of the hands and preaxial polysyndactyly of the feet in GCPS patients (Gollop and Fontes, 1985). Heterozygous Xt mice show craniofacial and limb phenotypes similar to those seen in GCPS patients. We and others have shown that the original Xt allele contains a 5’ deletion in Gli3 (Schimmang et al., 1992; Vortkamp et al., 1992) and the Xt/J allele contains a 3’ non-overlapping deletion in Gli3 (Hui and Joyner, 1993). Both Xt homozygotes completely lack Gli3 expression indicating that the dominant phenotypes observed in heterozygous Xt mutant mice and GCPS patients are due to haploinsufficiency of Gli3 (GLI3). Hereafter, the Xt allele will be designated as Gli3Xt and the Xt/J allele as Gli3XtJ. Gli3Xt homozygotes show a high incidence of neural tube closure defects and severe forebrain malformation (Johnson, 1967; Franz, 1994). They die at birth with severe defects in their craniofacial, axial, and appendicular skeletons including an enlarged maxillary region, open skull vault, severe fore limb polyductally, tibial hemimelia, an unfused sternum, and fusion of neural arches (Johnson, 1967). However, only a subset of Gli3-expressing tissues are affected in Gli3 null mutant animals suggesting that Gli1 and/or Gli2 may partially compensate for loss of Gli3 activity. To study the role of Gli2 in skeletal development, we have generated a targeted deletion of the zinc finger domain by homologous recombination in embryonic stem (ES) cells. We report here that mutant mice deficient for Gli2 and Gli3 exhibit different subsets of skeletal defects. While double homozygous Gli2;Gli3 mutants die before the onset of skeletogenesis, mutations of Gli2 and Gli3 show synergistic effects in compound mutants indicating that Gli2 and Gli3 have specific as well as redundant functions in skeletal development.

MATERIALS AND METHODS

Generation of the Gli2rd mutation

The human Gli2 gene was mapped to chromosome 2q14.1-21 and the mouse Gli2 gene was localized to chromosome 1E2-4 by fluorescence in situ hybridization (data not shown). The targeting vector was constructed from two overlapping mouse genomic clones that contain the Gli2 zinc finger domain isolated from a 129/Sv genomic library. To construct the targeting vector, a 2.5 kb Xbal 5’ homology fragment was subcloned into the Xbal site of pPNT (Tybulewicz et al., 1991). Subsequently, a 5 kb BamHI-Xbal fragment of 3’ homology was cloned into the XhoI site of the above subclone. Both the PGKneo and PGKtk cassettes are in opposite transcriptional orientation compared to the endogenous Gli2 gene. The culture, selection and electroporation of R1 ES cells was carried out as described by Wurst and Joyner (1993). ES cell colonies that are resistant to 150 µg/ml of G418 and 2 µM ganciclovir were analyzed by Southern blotting for homologous recombination events. Genomic DNA was prepared directly from 96-well plates for restriction enzyme digestion as described by Hogan et al. (1994). Genomic DNA was digested with EcoRV and hybridized with a 0.7 kb XbaI-BamHI 5’ probe, or with BamHI and hybridized with a 1.0 kb XbaI-EcoRI 3’ probe. Chimeras were generated by ES-morula aggregation with targeted ES lines (Nagy et al., 1994). Chimeric males...
were bred to CD1 or 129/Sv females to establish F1 heterozygotes. Genotypes of Gli2\(^{zd}\) mutant embryos and animals were determined by Southern blot analysis of tail DNA or by PCR analysis of ear punch or yolk sac (Hogan et al., 1994). The following primers (sense Gli2 primer: 5\'AAACAAAGCTCCTGTGACACG3\' antisense Gli2 primer: 5\'CACCCTCAAGCATGTGTTTT3\'; pPNT primer: 5\'ATGCCCT-GCTTTTACTGAAG3\') were used to detect a 0.3 kb wild-type band and a 0.6 kb mutant band. Samples were amplified for 30 cycles (95°C 80 seconds; 58°C 60 seconds; 72°C 90 seconds).

**Gli3\(^{XtJ}\) mutant mice**

Gli3\(^{XtJ}\) mice in a C3H background were obtained as heterozygotes from the Jackson laboratory (Bar Harbor, Maine) and maintained in a (C3H×CD1) F2 background. The genotypes of heterozygous and homozygous Gli3\(^{XtJ}\) mice were determined by their characteristic limb phenotypes (Johnson, 1967; Hui and Joyner, 1993).

**Generation of Gli2\(^{zd}\), Gli3\(^{XtJ}\) double mutants**

Initial intercrosses between single Gli2\(^{zd}\) and Gli3\(^{XtJ}\) heterozygotes were performed to generate double heterozygotes. Double heterozygotes were mated and progenies were analyzed by PCR analysis of yolk sac DNA and phenotypic analysis of the limbs as described above for the single mutants.

**Histological and skeletal analyses**

Animals were mated overnight and females examined for a vaginal plug the following morning. Noon of the day of evidence for a vaginal plug was considered day 0.5 post coitum (p.c.). Specimens (embryos or skinned fetuses) were either fixed in Bouin’s solution or phosphate-buffered 4% paraformaldehyde. Following fixation, specimens were rinsed with H\(_2\)O to remove excess picric acid, or phosphate-buffered saline to remove paraformaldehyde, dehydrated through increasing concentrations of ethanol, equilibrated in xylene, impregnated with paraffin wax, embedded, and sectioned at 6 µm. Sections were stained with hematoxylin and eosin.

The cartilaginous skeleton of day 14.5 p.c. mutant embryos (Gli2\(^{zd}\)/Gli2\(^{zd}\) [n=13], Gli3\(^{XtJ}\)/Gli3\(^{XtJ}\) [4], Gli2\(^{zd}\)/Gli3\(^{XtJ}\)+/+ [7], Gli2\(^{zd}\)/Gli2\(^{zd}\)/Gli3\(^{XtJ}\)+/+ [3] and Gli2\(^{zd}\)/Gli3\(^{XtJ}\)/Gli3\(^{XtJ}\) [6]) was analyzed by alcian blue staining (Jegalian and De Robertis, 1992). Skeletal analyses of fetuses and newborn animals were performed by using alcian blue/alizarin red bone staining (Jegalian and De Robertis, 1992). The cartilaginous skeleton of day 14.5 p.c. mutant embryos was analyzed by skeletal staining. Genotypes of the double mutants were determined by using PCR analysis of yolk sac DNA and phenotypic analysis of the limbs as described above for the single mutants.

**In situ hybridization analysis**

In situ hybridization analysis was carried out as described (Hui and Joyner, 1993). The in situ probes used were Shh (Echelard et al., 1993) and the three Gli genes (Hui et al., 1994).

**RESULTS**

**Gli2\(^{zd}\) mutant mice**

To study the function of Gli2, we generated a targeted mutation in ES cells by homologous recombination. A positive/negative targeting vector (Fig. 1A) containing 2.5 kb of 5′ and 5.0 kb of 3′ genomic sequences was designed such that homologous recombination would delete a 2.4 kb region of Gli2 that contains the exons coding for zinc fingers 3-5 as well as 71 amino acid residues carboxyl to the zinc fingers, replacing this DNA with the PGKneo cassette from the pPNT vector (Tybulewicz et al., 1991). Such a deletion would result in an out-of-frame mutation to the sequences 3′ to the deletion. A truncated protein would be made that should not be able to bind DNA since zinc fingers 4 and 5 are known to be important for DNA binding (Pavletich and Pabo, 1993; Zarkower and Hodgkin, 1993). This allele is referred to as Gli2\(^{zd}\) (zinc finger deletion). The linearized targeting vector was electroporated into ES cells and clones were selected for resistance to G418 and ganciclovir (see Materials and Methods). 340 double resistant clones were analyzed by Southern blot using a 3′ external probe and 16 cell

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**Fig. 1.** Targeted disruption of the Gli2 gene. (A) The targeting vector contains 7.5 kb of genomic sequence of the Gli2 locus. The black boxes represent the 5′ zinc fingers. The 5′ and 3′ probes used for Southern blot analysis are indicated. Homologous recombination replaces zinc fingers 3-5 with the PGKneo gene, resulting in a change in size of the EcoRV fragment detected by the 5′ probe from 6 kb (WT) to 3.5 kb (Mt). Similarly, the BamHI fragment detected by the 3′ probe shifts from 10 kb (WT) to 13 kb (Mt). (B) Southern blot and PCR analyses. Southern blot analysis of DNA from control R1 (+/+), and three targeted (+/−) ES lines. The wild-type (WT) and mutant (Mt) DNA fragments detected by the 5′ and 3′ probes are as described in A. Also shown in PCR analysis of yolk sacs from wild type (+/+), heterogeneous (+/−) and homozygous (−/−) embryos. The wild-type (WT) band is 0.3 kb and the mutant (Mt) band is 0.6 kb.
lines including G3 and G10 yielded the 13 kb BamHI band expected from a homologous recombination event (Fig. 1B). The 3.5 kb EcoRV band expected from a homologous recombination event was detected by a 5′ internal probe (Fig. 1B). The G3 and G10 targeted ES cell lines were used to generate chimeras by ES cell-morula aggregation (Nagy et al., 1994). These chimeras transmitted the mutation to their progeny. G3 was backcrossed to CD1 for two to four generations while G10 was maintained in a 129/Sv background. Similar phenotypes were obtained with mouse lines generated from each of the two clones.

Heterozygous Gli2<sup>zfd</sup> mutant mice do not show any detectable abnormalities. Heterozygous mice were intercrossed to generate homozygous mutant animals. Analysis of more than 500 offspring from heterozygous intercrosses at weaning did not reveal any homozygous mutant animals. When newborn animals were analyzed from these crosses, many stillborn pups were found and most of them were genotyped as homozygous Gli2<sup>zfd</sup> mutants (see Table 1). Analysis of homozygotes at various developmental stages (days 9.5-19.5 p.c.) revealed that they were present in the expected Mendelian ratio of roughly 25%. The percentage of homozygotes became somewhat lower at day 18.5 p.c. and at birth, suggesting that some lethality occurred during later embryonic development. Consistent with this, we frequently observed resorbed embryos at late gestational stages.

### Craniofacial abnormalities in homozygous Gli2<sup>zfd</sup> mutants

Homozygous Gli2<sup>zfd</sup> mutants could be first recognized at day 10.5 p.c. by their small size and microcephaly (Fig. 2A,B). At day 14.5 p.c., their heads appeared to be flattened and they frequently showed subcutaneous edema in the back (Fig. 2C,D). Craniofacial abnormalities could be clearly seen in homozygous Gli2<sup>zfd</sup> mutants at late gestational periods. In the skull, a deficiency of the medial portion of the frontal and parietal bones is observed in most mutants (Fig. 2E,F). The mutants also showed delayed ossification of the skull (Fig. 3C-F). Truncation of the distal maxilla and mandible was partly due to an absence of upper and/or lower incisors (Fig. 2G,H). The alveolar processes, which surround the molars, appear normal although mutant mandibles are reduced in size (see Fig. 8G). 91% (30/33) of the homozygotes did not have an upper incisor and 33% (11/33) did not have a lower incisor. Tympanic ring bones are absent in some mutants indicating that inner ear development is affected by the Gli2<sup>zfd</sup> mutation (Fig. 3C-F).

Many newborn homozygotes have a severely cleft secondary palate (Fig. 3A,B). Analysis of 45 homozygotes by histological sectioning (n=12) or skeletal staining (n=33) enabled us to quantitate the cleft palate defect as 64% penetrant. The palatal phenotype ranged from a complete absence of the presphenoid bone as well as the maxillary and palatine shelves (Fig. 3C) to normal looking palatal shelves (Fig. 3F). Serial coronal sections along the palate at days 13.5 to 14.5 p.c. showed that there was a delay or failure in the elevation of the mutant palatal shelves. At day 13.5 p.c., wild-type shelves are in the process of fusion with a clearly visible midline seam (Fig. 3G) and, by day 14.5 p.c., are properly fused with continuous mesenchyme (Fig. 3K). In contrast, mutant shelves were either not elevated at all (Fig. 3H,L) or were only partially elevated (Fig. 3I,M). Some of the mutant shelves did elevate but showed a delay in fusion (Fig. 3J,N). The former two mutant classes are presumed to develop into the subset of progeny exhibiting cleft palate.

### Table 1. Results of Gli2<sup>zfd</sup> heterozygote matings

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<td>63 (52)</td>
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**Fig. 2. Craniofacial abnormalities in homozygous Gli2<sup>zfd</sup> mutants.**

Lateral views of day 10.5 p.c. wild-type (A) and homozygous Gli2<sup>zfd</sup> (B) embryos show that the mutant is smaller than the wild-type littermate and exhibits microcephaly (indicated by the arrow). Later at day 14.5 p.c. (C,D), homozygous Gli2<sup>zfd</sup> mutants (D) show edema at the back (arrow) and the head appears to be flattened. At birth (E-H), skeletal staining reveals that the skull is shortened in homozygous Gli2<sup>zfd</sup> mutants (F) and, in some homozygous Gli2<sup>zfd</sup> mutants (H), both upper and lower incisors are missing (indicated by the arrows).
Specific and redundant functions of Gli2 and Gli3

Gli3 \textsuperscript{xtJ} mutant mice

Johnson (1967) provides a detailed analysis of Gli3 \textsuperscript{xtJ} mutant mice, in which the 5' region of Gli3 has been deleted (Schimmang et al., 1992). In this study, we confirm similar skeletal abnormalities in Gli3 \textsuperscript{xtJ} mutant mice, which contain a 3' deletion in Gli3 (Hui and Joyner, 1993). Homozygous Gli3 \textsuperscript{xtJ} mutants exhibit severe craniofacial abnormalities similar to those reported for Gli3 \textsuperscript{xtJ} homozygotes (Johnson, 1967): an enlarged maxillary region, reduced external nasal processes and failure of skull vault formation. In addition, homozygous Gli3 \textsuperscript{xtJ} mutants also revealed an incomplete penetration of cleft palate and tooth defects (data not shown). Consistent with their roles in palatal and tooth development, Gli2 and Gli3 are highly expressed in the developing facial mesenchyme (see Fig. 5M,N and unpublished data).

Gli2 \textsuperscript{zfd} and Gli3 \textsuperscript{xtJ} mutant mice exhibit distinct limb and sternum phenotypes

The limbs and sternum are derived from the lateral plate mesoderm. Both structures are severely affected in Gli3 \textsuperscript{xtJ} mutants (Fig. 4C,F,I). During limb development, undifferentiated mesenchymal cells first aggregate to form condensations. These prechondrogenic condensations (blastema) then segment and bifurcate along the proximodistal axis to form the stylopods (humerus and femur), the zeugopods (ulna/fibula and radius/tibia), and finally the autopods (carpals, metacarpals and digits). In Gli3 \textsuperscript{xtJ} mutants, the fore limbs exhibit severe polydactyly (seven to eight digits) and the hind limbs also show polydactyly (six digits). Some Gli3 \textsuperscript{xtJ} mutants, however, have only five digits in the hind limbs (see Fig. 4F). Gli3 \textsuperscript{xtJ} mutants also show a severe truncation of the tibia (Fig. 4F) and a slight shortening and thickening of the humerus, ulna and radius (Fig. 4C). These observations indicate that the Gli3 \textsuperscript{xtJ} mutation severely affects the development of the autopods. While the Gli3 \textsuperscript{xtJ} mutation has very little effect on the development of stylopods and zeugopods of the fore limbs, it causes deletion of some anterior components in the hind limbs, resulting in tibial hemimelia as well as a mild preaxial polydactyly (Fig. 4F). The sternum of Gli3 \textsuperscript{xtJ} mutants is unfused (Fig. 4I).

In contrast, except for a delay in the ossification of the digits, Gli2 \textsuperscript{zfd} mutants show no obvious abnormalities of the autopods (Fig. 4B,E). 100% (12/12) of day 17.5 p.c. mutant skeletons and 53% (8/15) of day 18.5 p.c. mutant skeletons did not show any sign of ossification in their digits. The length of Gli2 \textsuperscript{zfd} mutant limbs was significantly reduced. When compared with wild type counterparts, the ossification of humerus, ulna and radius was reduced to 75%, 85% and 50%, respectively, and

Fig. 3. Cleft palate in homozygous Gli2\textsuperscript{zfd} mutant mice is due to abnormal palatal shelf elevation. Ventral view of the upper jaw of wild-type (A) and homozygous Gli2\textsuperscript{zfd} (B) neonates. The arrows point to the open nasal cavities. Ventral view of the base of skulls of a wild-type (C) and three homozygous Gli2\textsuperscript{zfd} (D-F) neonates. The maxillary (m) and palatine (p) shelves, as well as the presphenoid (ps) and basisphenoid (bs) bones are completely absent in the mutant shown in D but are variably affected in others (E,F). Arrows indicate the tympanic ring which is absent in some Gli2\textsuperscript{zfd} mutant mice (D,E). Elevation of palatal shelves is abnormal in homozygous Gli2\textsuperscript{zfd} mutant mice. Frontal sections through the heads of days E14.5 (G-J) and E15.5 (K-N) wild-type (G,K) and homozygous Gli2\textsuperscript{zfd} (H,J,L-N) embryos illustrate a complete failure (H,L), partial failure (I,M) or delay (J,N) in the elevation of the palatal shelves in the mutants.
the ossification of femur, fibula and tibia was reduced to 85%, 75% and 60%, respectively. Although the length of Gli2\textsuperscript{zd} mutant sternums was reduced, ossification of the sternabrae was normal (Fig. 4H). Analysis of cartilaginous skeletons at day 14.5 p.c. confirmed the same phenotypes in both mutants (data not shown).

The different effects of Gli2\textsuperscript{zd} and Gli3\textsuperscript{XtJ} mutations on limb development correlate with the differential expression of Gli2 and Gli3 during embryogenesis. As shown in Fig. 5, all three Gli genes showed a dynamic pattern of expression during limb development. At day 10.5 p.c., while Gli1 expression is restricted to the distal mesenchyme in the posterior region of the limb buds, Gli2 and Gli3 are broadly expressed in the undifferentiated mesenchyme (Fig. 5B-D). Interestingly, a region in the posterior mesenchyme, which corresponds to the zone of polarizing activity and expresses Shh (Fig. 5A), consistently shows little or no expression of the three Gli genes (indicated by the arrows in Fig. 5A-D). Later in development, Gli1 expression is found in the condensing mesenchyme and eventually becomes restricted to the perichondrium (Fig. 5F and unpublished data). Although Gli2 and Gli3 are both expressed in the mesenchyme surrounding the condensing mesenchyme (Fig. 5G,H,K,L), Gli3 is expressed at a higher level in the autopods (Fig. 5H,L). Similar expression patterns were observed in both fore limbs and hind limbs (Fig. 5J,L). It is thus possible that the more severe autopod defects observed in Gli3\textsuperscript{XtJ} mutants are due to the preferential expression of Gli3 and functional requirement for Gli3 in the distal part of the developing limbs.

**Vertebral defects in Gli2\textsuperscript{zd} and Gli3\textsuperscript{XtJ} mutant mice**

The vertebral column is derived from the sclerotome – medially located sclerotome cells contribute to intervertebral discs and vertebral bodies, lateral sclerotome cells form the pedicles and ribs, and the dorsal part of the sclerotome contributes to the neural arches (Verbout, 1985). The development of the four occipital somites is, however, different; they develop into the basioccipital (the occipital equivalent of a vertebral body) and exoccipital (the occipital equivalent of a neural arch) bones without resegmentation (Theiler, 1988).

In all Gli2\textsuperscript{zd} mutants, the development of the basisphenoid bone (a derivative of the cephalic mesoderm) and the basioccipital bone was affected (Fig. 6B). In the vertebral column, no or little ossification of vertebral bodies could be found (Fig. 6E). Examination of individual mutant vertebrae indicated that the ossification of neural arches was normal (data not shown; see Fig. 6H). Histological sections of Gli2\textsuperscript{zd} mutants revealed that the intervertebral discs were absent or reduced and a bend in the vertebral column frequently occurred in the thoracic–lumbar region (data not shown). In addition, 36% (12/33) of the mutants showed a bilateral pair of extra ribs and 6% (2/33) had an unilateral extra rib (not shown). In contrast to Gli2\textsuperscript{zd} mutants, the basisphenoid bone, basioccipital bone and vertebral bodies developed normally in Gli3\textsuperscript{XtJ} mutants (Fig. 6C,F).

In contrast to Gli2\textsuperscript{zd} mutants, all Gli3\textsuperscript{XtJ} mutant mice exhibited abnormal development of the neural arches; the C1 and C2 neural arches were fused and the neural arches of other cervical vertebrae were expanded (Fig. 6I). They also showed a bilateral pair of extra ribs (data not shown). Interestingly, though Gli2 and Gli3 are both expressed in the developing sclerotome (Hui et al., 1994), Gli2 is preferentially expressed in the medial sclerotome cells while Gli3 is preferentially expressed in the dorsal part of the sclerotome (Fig. 5O,P).

**Redundant roles of Gli2 and Gli3 in skeletal development**

To determine whether Gli2 and Gli3 play redundant roles in skeletal development, we analyzed the skeletons of double mutant mice at days 14.5 and 18.5 p.c. Double heterozygous mutant mice are viable and were mated to one another. No double homozygotes were found among the six litters killed at day 14.5 p.c. and the eight litters at day 18.5 p.c. (Table 2). Our preliminary results indicated that double homozygotes die before day 10.5 p.c. Skeletal analysis revealed no significant difference between Gli2\textsuperscript{zd}/+;Gli3\textsuperscript{XtJ}/+ and Gli3\textsuperscript{XtJ}/+ mutants. In contrast, Gli2\textsuperscript{zd}/Gli2\textsuperscript{zd}/Gli3\textsuperscript{XtJ}/+ mutants showed an exacerbated phenotype in various skeletal elements. Gli2\textsuperscript{zd}/+;Gli3\textsuperscript{XtJ}/+ mutants also exhibited some enhancement of the Gli3\textsuperscript{XtJ}/Gli3\textsuperscript{XtJ} mutant phenotype. These synergistic effects of Gli2\textsuperscript{zd} and Gli3\textsuperscript{XtJ} mutations on skeletal development clearly illustrate the redundant functions of Gli2 and Gli3.

Gli3\textsuperscript{XtJ}/+ mutants showed mild preaxial polydactyly in both the fore limbs and hind limbs (Fig. 7F,L). The stylopod and

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**Fig. 4.** Limb and sternal defects in homozygous Gli2\textsuperscript{zd} and Gli3\textsuperscript{XtJ} mutant mice. The fore limbs (A-C), hind limbs (D-F) and sternum (G-I) of day 18.5 p.c. wild-type (A,D,G), homozygous Gli2\textsuperscript{zd} (B,E,F) and homozygous Gli3\textsuperscript{XtJ} (C,F,I) animals are shown. In homozygous Gli2\textsuperscript{zd} animals, the fore limbs show a shortening of humerus (h), radius (r) and ulna (u) and a bowed-shaped radius (indicated by the arrow in B) and the hind limbs show a similar shortening of femur (fe), tibia (t) and fibula (fi) (E). The sternum has seven ribs (r1-r7) attached and the sternabrae (st) and xiphoid (x) are grossly normal (H). In homozygous Gli3\textsuperscript{XtJ} animals, the fore limbs show a slight shortening and thickening of humerus, radius and ulna, and severe polydactyly (C) and the hind limbs show a severe truncation of tibia (E). In the hind limbs, the polydactyly is less severe and the anterior part appears to be deleted. The sternum is completely split (I). In this specimen, only the clavicle (cl) and six ribs are attached to the sternum.
Specific and redundant functions of \textit{Gli2} and \textit{Gli3}

Table 2. Results of \textit{Gli2}\textsuperscript{dd}; \textit{Gli3}\textsuperscript{XtJ} heterozygote matings

<table>
<thead>
<tr>
<th>\textit{Gli2}\textsuperscript{dd}</th>
<th>\textit{Gli3}\textsuperscript{XtJ}</th>
<th>+/+</th>
<th>+/+</th>
<th>+/+</th>
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<th>+/+</th>
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<td>Expected</td>
<td>6.2</td>
<td>12.5</td>
<td>6.2</td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
<td>6.2</td>
<td>12.5</td>
<td>6.2</td>
<td>12.5</td>
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</tr>
<tr>
<td>E14.5*</td>
<td>7.3(3)</td>
<td>19.5(8)</td>
<td>9.8(4)</td>
<td>19.5(8)</td>
<td>17.1(7)</td>
<td>14.6(6)</td>
<td>4.9(2)</td>
<td>7.3(3)</td>
<td>0(0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E18.5†</td>
<td>14.4(13)</td>
<td>21.1(19)</td>
<td>7.8(7)</td>
<td>14.4(13)</td>
<td>15.6(14)</td>
<td>5.6(5)</td>
<td>6.7(6)</td>
<td>14.4(13)</td>
<td>0(0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Expected percentage assumes Mendelian inheritance of wild-type and mutant alleles. Calculated frequencies of various genotypes are listed with the actual number of animals indicated in brackets.

*6 litters; †8 litters.

zeugopod elements of \textit{Gli3}\textsuperscript{Xl+} mutants were normal (Fig. 7C,I). When compared with \textit{Gli2}\textsuperscript{dd} homozygotes, \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants showed a significant reduction of the stylopod and zeugopod elements in both fore limbs (Fig. 7A,B) and hind limbs (Fig. 7G,H). In particular, the length of radius and tibia appeared to be more reduced than those of ulna and fibula (Fig. 7B,H). These observations suggest that both \textit{Gli2} and \textit{Gli3} are required for the normal development of stylopods and zeugopods. Furthermore, \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants exhibited a more severe preaxial polydactyly phenotype (Fig. 7E,F,K,L). In the fore limbs of \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants, there was also a postaxial nubbin (Fig. 7E). The lack of a strong autopod phenotype in \textit{Gli2}\textsuperscript{dd} homozygotes may thus be due to functional redundancy of \textit{Gli2} and \textit{Gli3}.

Functional redundancy of \textit{Gli2} and \textit{Gli3} could also be observed in the development of other skeletal elements, such as the sternum (derived from the lateral plate mesoderm; Fig. 8B), the vertebral column (somite; Fig. 8E) and the mandible (neural crest; Fig. 8H). In contrast to their single mutant counterparts (Fig. 8A,C), \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants showed a strong sternum phenotype (Fig. 8B). The sternum was split rostrally and was not properly segmented. As for the limbs, the functional redundancy of \textit{Gli2} and \textit{Gli3} may explain the mild sternum phenotype observed in \textit{Gli2}\textsuperscript{dd} homozygotes. In the vertebral column, \textit{Gli2}\textsuperscript{dd} homozygotes showed defects of chondrogenesis ventrally (Fig. 8D) corresponding to the thoracic-lumbar position of the bend of the mutant vertebral column (see above). However, in \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants, chondrogenesis was found to be affected in multiple regions along the ventral part of the vertebral column (Fig. 8E). This clearly indicates that \textit{Gli3} also plays a role in the development of the ventral vertebral processes and that the lack of a phenotype in the vertebral bodies of \textit{Gli3}\textsuperscript{Xl} mutants is due to \textit{Gli2} compensating for the absence of \textit{Gli3}. The mandibles of \textit{Gli2}\textsuperscript{dd}/\textit{Gli2}\textsuperscript{dd}, \textit{Gli3}\textsuperscript{Xl+}/+ mutants were severely malformed (Fig. 8H). Though \textit{Gli2}\textsuperscript{dd} homozygotes had a smaller mandible, the development of the coronoid (c),
Fig. 6. Vertebral defects in homozygous Gli2$^{zd}$ and Gli3$^{XJ}$ mutant mice. Ventral views of the caudal part of the skull show that the basioccipital (bo) and basisphenoid (bs) bones are severely affected in homozygous Gli2$^{zd}$ animals (indicated by the red arrows; B) but are normal in wild-type (A) and homozygous Gli3$^{XJ}$ (C) animals. Ventral views of the vertebral column show that only very few ventral vertebral components fuse with each other. Both the sternum and limb are more malformed in Gli3 null mutants. Most Gli3$^{XJ}$ homozygotes show a completely split sternum. Gli3 inactivation causes preaxial polydactyly in both fore limb and hind limb in heterozygous conditions and severe fore limb polydactyly and anterior deletion of the hind limb zeugopod and autopod elements in homozygous conditions. These observations indicate that Gli2 and Gli3 play specific roles in skeletal development.

The differential expression of Gli2 and Gli3 in the developing limb and sclerotome (Fig. 5) can partly explain why their mutations result in different skeletal malformations. The ventral vertebral defects of Gli2$^{zd}$ homozygotes are reminiscent of those observed in Pax1 null mutant mice (Wallin et al., 1994; Dietrich et al., 1995). Pax1 and a closely related gene, Pax9, are co-expressed in the developing sclerotome in response to the notochord signals; however, Pax1 is mainly expressed in medial sclerotomal cells which eventually form the vertebral body and intervertebral discs and Pax9 in the more lateral regions which give rise to the neural arches (Goulding et al., 1994; Neubuser et al., 1995). Interestingly, Gli2 is also preferentially expressed in medial sclerotomal cells while Gli3 is preferentially expressed in the more lateral regions. In correlation with this differential expression, the Gli2$^{zd}$ mutation affects ventral vertebral components and the Gli3$^{XJ}$ mutation affects the dorsal components. Similarly, Gli3 is strongly expressed in the distal part of the developing limb and Gli3$^{XJ}$ mutation causes severe malformation of the autopods.

The fact that double Gli2$^{zd}$;Gli3$^{XJ}$ homozygotes die around day 10 p.c. before the onset of skeletogenesis strongly suggests that Gli2 and Gli3 play redundant roles in embryogenesis. It will be interesting to examine whether Gli1 also plays redundant roles since all three Gli genes are expressed in partially overlapping domains during early embryogenesis (Hui et al., 1994). Our skeletal analyses of double mutant mice have revealed the redundant roles of Gli2 and Gli3 in skeletal development. Synergistic malformations were observed in the limb, sternum, vertebral column and mandible of Gli2$^{zd}$;Gli3$^{XJ}$+ mutant mice (Figs 7, 8). Taken together, our

condylar (co), angular (a) and dental (d) processes was normal (Fig. 8G). In contrast, all these processes were hypoplastic in Gli2$^{zd}$/Gli2$^{zd}$;Gli3$^{XJ}$/+ mutants.

In addition to the fusion of the C1 and C2 neural arches observed in Gli3$^{XJ}$ homozygotes (Fig. 6I), Gli2$^{zd}$/Gli3$^{XJ}$/+;Gli3$^{XJ}$/+ mutants showed neural arch fusion of other cervical vertebrae (not shown). Some Gli2$^{zd}$/Gli3$^{XJ}$/+;Gli3$^{XJ}$/+ mutants also appeared to have more duplicated distal phalanges than others (not shown).

DISCUSSION

Specific and redundant roles of Gli2 and Gli3

skeletal development

We have shown here that Gli2 is required for normal development of the skeleton. As previously observed in Gli3 null mutant mice, the disruption of Gli2 affects some, but not all, of the bones in the craniofacial, axial and appendicular skeletons. In the craniofacial skeleton, Gli2$^{zd}$ mutation affects the development of the basisphenoid bone (a derivative from the cephalic mesoderm), the basioccipital bone (derived from somitic mesoderm), the presphenoid bone, the maxillary and palatine shelves and the incisors (neural crest derivatives). In the axial skeleton, the development of ventral vertebral structures is severely affected with vertebral bodies and intervertebral discs are mostly absent. In the limbs, the stylopod and zeugopod bones are significantly shortened. Gli2$^{zd}$ mutation also results in a shortening of the sternum.

Gli2$^{zd}$ and Gli3$^{XJ}$ mutants both exhibit an incomplete penetrance of cleft palate and tooth defects. While this can be due to partially redundant functions of Gli2 and Gli3 in craniofacial development, it is possible that the genetic background of the mutants (129, C3H and CD1) may contribute to the variation in penetrance and expressivity. Interestingly, other than cleft palate and tooth defects, Gli2$^{zd}$ and Gli3$^{XJ}$ mutations show complete penetrance and affect different subsets of skeletal elements. While the development of the basioccipital and basioccipital bones appears normal, the skull vault is severely affected in Gli3 null mutants. In the vertebral column, Gli3 inactivation affects the development of dorsal structures. The neural arches of all cervical vertebrae tend to fuse with each other. Both the sternum and limb are more malformed in Gli3 null mutants. Most Gli3$^{XJ}$ homozygotes show a completely split sternum. Gli3 inactivation causes preaxial polydactyly in both fore limb and hind limb in heterozygous conditions and severe fore limb polydactyly and anterior deletion of the hind limb zeugopod and autopod elements in homozygous conditions. These observations indicate that Gli2 and Gli3 play specific roles in skeletal development.

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results indicate that although Gli2 and Gli3 exert distinct functions during skeletal development, they also implement partially redundant functions in the development of skeletal elements from all three embryonic lineages (the neural crest, somite and lateral plate mesoderm).

**Possible role of Gli2 and Gli3 in hedgehog signaling**

In *Drosophila*, hh plays a central role in cell patterning during embryonic and post-embryonic development (Forbes et al., 1993). The Gli homolog, ci, is an important component of the hh pathway. It is required for the activation of hh target genes, such as wingless (wg), decapentaplegic (dpp) and patched (ptc) (Motzny and Holmgren, 1995; Johnson et al., 1995; Dominguez et al., 1996). The activity of ci is regulated post-transcriptionally. The hh signal increases the level of ci protein while the ptc activity decreases it (Johnson et al., 1995; Dominguez et al., 1996). Increased ci protein levels have been shown to induce dpp expression in a hh-independent manner (Dominguez et al., 1996). Interestingly, besides acting as a downstream mediator of hh signaling, ci also functions as a repressor of hh expression (Dominguez et al., 1996).

In addition to multiple hh homologs (Echelard et al., 1993) and the three Gli genes described here, several other conserved components of the hh pathway have been identified in vertebrates. The vertebrate ptc homolog (Goodrich et al., 1996; Margio et al., 1996) and two highly homologous dpp homologs, Bmp2 and Bmp4 (Bitgood and McMahon, 1995; see Hogan, 1996), are expressed in regions complementary to, or overlapping with, hh-expressing cells during embryogenesis. As predicted by the *Drosophila* observations, the expression of the ptc homolog, Bmp2, and Bmp4, can be induced by ectopic Shh expression (Goodrich et al., 1996; Margio et al., 1996; Laufer et al., 1994; Niswander et al., 1994; Roberts et al., 1995). Furthermore, the role of cAMP-dependent protein kinase as a negative regulator of the hh pathway is conserved in *Drosophila* and vertebrates (Fan et al., 1995; Hynes et al., 1995; Hammerschmidt et al., 1996). These observations strongly suggest that similar signaling mechanisms by hh
homologs may be employed in the induction and morphogenesis of tissues and organs in vertebrates and invertebrates.

Two vertebrate hh homologs, Shh and Indian hedgehog (Ihh), have been implicated in skeletal development. During somite development, Shh mediates the inductive effects of the notochord in the differentiation of the sclerotome (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). In the developing limb, Shh functions as the ZPA signal in the specification of limb pattern (Riddle et al., 1993; Chang et al., 1994). Expression of Shh in the developing tooth and tongue epithelium also suggests that it may function as the inductive signal involved in the epithelial-mesenchymal interactions during tooth and palatal development (Bitgood and McMahon, 1995; Iseki et al., 1995; Koyama et al., 1995; Vaahakari et al., 1996). During skeletogenesis, Ihh is expressed in the pre-hypertrophic regions of the cartilage (Bitgood and McMahon, 1995) and is a regulator of chondrogenesis (Lanske et al., 1996; Vortkamp et al., 1996). If the hhcl-Hh/Gli signaling pathway is evolutionarily conserved in vertebrates, inactivation of the Gli genes should result in (i) defective signaling by Shh and Ihh as well as (ii) causing ectopic expression of Shh and Ihh. The results reported here are consistent with both possibilities.

Both single and double mutants of Gli2 and Gli3 exhibit clear somite defects. In particular, the disruption of normal Gli2 function results in a vertebral phenotype similar to that of Pax1 null mutants. Since Pax1 is a mediator of Shh signaling in the developing sclerotome, our results suggest that Gli2 can function as a positive regulator of Pax1. Since Gli2 and Gli3 are redundant functions of the paralogous Gli2 and Gli3 genes in forelimb development, both haplo- and null mutants exhibit more severe vertebral defects than Gli2 homozygotes, Gli3 also functions in the reception of the notochord Shh signal during sclerotome differentiation, albeit to a lesser extent. Since ectopic expression of Shh and Ihh in the anterior limb bud induces digit duplication (Riddle et al., 1993; Chang et al., 1994; Vortkamp et al., 1996), the polydactyly phenotype of Gli3 homozygotes may be due to ectopic expression of Ihh. Masuya et al. (1995) reported ectopic expression of Shh in Gli3 homozygotes.

Our results thus illustrate both specific and redundant functions of Gli2 and Gli3 in skeletal development. Strikingly, the spectrum of skeletal abnormalities is similar to those observed in the nevoid basal cell carcinoma syndrome or Gorlin’s syndrome which has recently been shown to be caused by mutation of the human PTC gene (Johnson et al., 1994; Hahn et al., 1996). Patients with Gorlin’s syndrome have a variety of craniofacial abnormalities, such as cleft palate, cyst jaws and limb malformation. Taken together with our observations reported here, these results emphasize the role of the Hh-Gli-PTC pathway in skeletal development. The Gli2 and Gli3 mutant mice described here will facilitate the genetic analysis of these pathways in vertebrates.

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