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The zebrafish *fgf24* mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation

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SUMMARY

The development of vertebrate limb buds is triggered in the lateral plate mesoderm by a cascade of genes, including members of the Fgf and Wnt families, as well as the transcription factor *tbx5*. Fgf8, which is expressed in the intermediate mesoderm, is thought to initiate forelimb formation by activating *wnt2b*, which then induces the expression of *tbx5* in the adjacent lateral plate mesoderm. Tbx5, in turn, is required for the activation of *fgf10*, which relays the limb inducing signal to the overlying ectoderm. We show that the zebrafish *fgf24* gene, which belongs to the Fgf8/17/18 subfamily of Fgf ligands, acts downstream of *tbx5* to activate *fgf10* expression in the lateral plate mesoderm. We also show that *fgf24* activity is necessary for the migration of *tbx5*-expressing cells to the fin bud, and for the activation of *shh*, but not *hand2*, expression in the posterior fin bud.

Key words: Zebrafish, Limb development, *fgf24*, *fgf10*, *tbx5*, *wnt2b*, *ikarus*, Pectoral fin, Apical ectodermal ridge

INTRODUCTION

Vertebrate paired appendages, or limbs, develop from primordia called limb buds, that arise as localized outgrowths from the flank of the embryo. Two distinct populations of cells in the flank contribute to the limb bud. These are the mesenchymal cells of the lateral plate mesoderm (LPM), which will form the core of the bud, and the overlying ectodermal cells, which will form the epithelial jacket of the bud. Two important questions arising in the context of this development are: how is limb development initiated in these cells, and how is it coordinated spatially and temporally between the mesenchyme and the ectoderm (reviewed by Johnson and Tabin, 1997; Martin, 1998; Tickle and Munsterberg, 2001; Capdevila and Izpisua Belmonte, 2001). Experimental removal of the intermediate mesoderm (IM), which lies between the somites and the LPM, leads to a reduction of limb structures in the chicken (Geduspan and Solursh, 1992), suggesting that the IM could be the source of a signal triggering limb development. The chicken *FGF8* gene has been proposed to encode this signal, based on the observation that it is transiently expressed in the IM adjacent to the limbs, and that it, and similar Fgfs, can initiate the development of additional limbs from the chicken embryonic flank when ectopically expressed (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Fgf8 is thought to exert its effect by activating the expression of *WNT2B* (at the forelimb level) and *WNT8C* (at the hindlimb level) in the IM of the chicken embryo (Kawakami et al., 2001). As *fgf8* is expressed in the same tissue as its target genes, *wnt2b* and *wnt8c*, this is a local signaling event. Like Fgf8, Wnt2b and Wnt8c are capable of inducing ectopic limbs in the chicken, and are thus thought to mediate the limb inducing activity of Fgf8 (Kawakami et al., 2001).

Another gene involved in limb induction is *Fgf10*. *Fgf10* is expressed in the limb mesenchyme beginning at very early stages, and when ectopically expressed, can induce additional limbs in the chicken (Ohuchi et al., 1997). Furthermore, *Fgf10* mutant mice fail to form limbs (Min et al., 1998; Sekine et al., 1999). These mutant embryos do not show activation of markers expressed in the limb bud ectoderm, suggesting that *Fgf10* relays limb induction from the mesenchyme to the ectoderm. Fgf10 belongs in a subclass of the Fgf family with highest affinity for the Fgf receptor 2 isoform b, Fgfr2b (Ornitz et al., 1996), which is expressed in epithelial cells (Orr-Urteger et al., 1993) (reviewed by Xu et al., 1999). *Fgfr2b* mutant mice share many phenotypes with *Fgf10* mutants (DeMoerlooze et al., 2000; Ohuchi et al., 2000), further supporting a model in which mesenchymally expressed Fgf10 activates Fgfr2b in the overlying ectoderm (reviewed by Xu et al., 1999). Fgf2, Fgf4 and Fgf8, however, have highest affinity for Fgfr2c (Ornitz et al., 1996), which is mesenchymally expressed (Orr-Urteger et al., 1993). This scenario suggests that the limb-inducing activity of Fgf8 and similar Fgfs is mediated by Fgf10, which relays the inductive event to the ectoderm. Consistent with this proposal, Fgf10 is able to induce ectodermal limb markers in the chicken flank even in the absence of mesenchyme, while Fgf2 and Fgf4 are not (Yonei-Tamura et al., 1999). The induction of *Fgf4* and *Fgf8* in the ectoderm by Fgf10 is not

direct, and appears to be mediated in the chicken by *WNT3A*, which is activated in the ectoderm in response to Fgf10 (Kengaku et al., 1998; Kawakami et al., 2001).

Once the limb bud has formed, *Fgf4* and *Fgf8* are expressed in the apical ectodermal ridge (AER), a signaling center that directs outgrowth of the limb bud, and these Fgfs have been shown to mediate the activity of the AER in the chicken and the mouse (Laufer et al., 1994; Niswander et al., 1994; Sun et al., 2002). *Fgf10* continues to be expressed in the mesenchymal cells underneath the AER, and forms a feedback loop of mutual dependence with the ectodermally expressed *Fgfs* (reviewed by Xu et al., 1999). *Fgf10* thus also directs ectodermal expression of *Fgf4* and *Fgf8* during the outgrowth phase of the limb.

Tbx5 encodes a T-box transcription factor that is expressed in the forelimb mesenchyme at very early stages, and has been shown to participate in the specification of limb identity in the chicken (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Recent results have indicated that *Tbx5* is also involved in limb bud initiation. Targeted knockdown of *tbx5*, or mutagenesis of the *tbx5* locus, leads to zebrafish embryos that lack pectoral fin buds (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). These mutants fail to activate expression of ectodermal fin bud markers, which correlates with the absence of *fgf10* in the mesenchyme. Furthermore, targeted knock-down of *wnt2b* causes loss of *tbx5* expression in the zebrafish pectoral fin primordium, suggesting that Tbx5 acts downstream of Wnt2b to induce *fgf10* during limb initiation (Ng et al., 2002).

The zebrafish has recently become established as a model system to study the development of vertebrate paired appendages, and a number of zebrafish mutants affecting the development of paired fins have been isolated in a large-scale genetic screen (van Eeden et al., 1996). We report the molecular and phenotypic analysis of one of these mutants, named *ikarus* (*ika*; *znfn1a1* – Zebrafish Information Network). We show that *ika* encodes *fgf24*, which is a new member of the Fgf8/17/18 subfamily of Fgf ligands, and has highest sequence similarity to Fgf18. In the absence of *fgf24* activity, we observe activation of early mesenchymal fin bud markers, such as *tbx5*, but the absence of all genes expressed in the fin bud ectoderm. We show that *fgf24* acts downstream of *tbx5* to activate *fgf10* expression. These results identify an additional layer controlled by Fgf signaling in the genetic hierarchy initiating limb development.

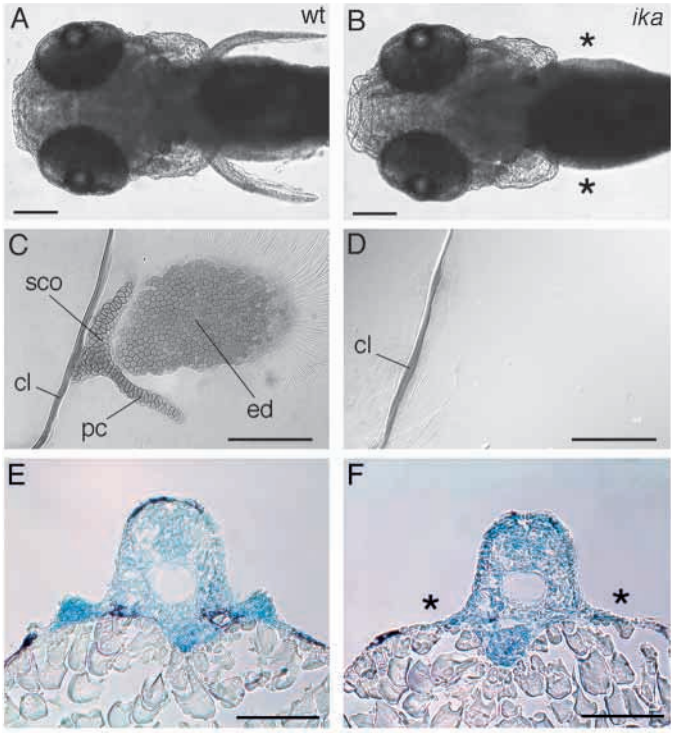

Fig. 1. The *ikarus* (*ika*) mutant phenotype. (A) Wild-type larva at 3 days of development. Note the prominent pectoral fins protruding from the flanks. (B) *ika* mutant larva at 3 days of development. Note the complete absence of pectoral fin structures (asterisks). (C) Alcian Blue staining of the endoskeleton of a wild-type larval pectoral fin. cl, cleithrum; sco, scapulocoracoid; pc, postcoracoid process; ed, endodermal disc. (D) Alcian Blue staining of the endoskeleton of an *ika* mutant larval pectoral fin. Note the complete absence of the fin endoskeleton, including the scapulocoracoid, or shoulder girdle. (E) Transverse section through a wild-type embryo at the level of the pectoral fin buds at 36 hpf. (F) Transverse section through an *ika* mutant embryo at the level of the pectoral fin buds at 36 hpf. Note the absence of pectoral fin buds (asterisks). Scale bars: 100 μm.

MATERIALS AND METHODS

Zebrafish lines

The *ika*^{tm127c} allele has been previously described (van Eeden et al., 1996). We isolated a new *ika* allele, *ika*^{hx118}, in a screen for ENU-induced mutations, which has been described in Habeck et al. (Habeck et al., 2002). As *ika*^{hx118} is the stronger of the two alleles, all experiments presented in this study have been performed with this allele.

Meiotic mapping and sequencing of *ika* alleles

We mapped the *ika* mutation to linkage group 14 by using a standard panel of SSLP markers (Knapik et al., 1998). For fine mapping single *ika* mutant embryos (938 in total) were tested against individual SSLP markers in the crucial interval. The cloning and physical mapping of *fgf24* are described elsewhere (B.W.D., D. W. Stock and C. B. Kimmel, unpublished). For sequencing of the *fgf24* gene, total RNA was extracted from wild-type or *ika* embryos at 36 hpf. RT-PCR was performed using the Superscript kit (Invitrogen) with the following primers: fgf24up (5′-TCCGGGGTTTTGTTTGTGAG-3′) and fgf24down (5′-TCTTTTCGGTAGCCATTGTTTATT-3′). PCR products from four independent PCR reactions on two different RNA samples were sequenced on both strands and analyzed using the MacVector software.

Morpholino injections

Morpholinos were purchased from GeneTools LLC. The following morpholinos were used: anti-*tbx5* oligonucleotide for the coding sequence, as described by Ahn et al. (Ahn et al., 2002). Anti-*wnt2b* oligonucleotide, as described by Ng et al. (Ng et al., 2002). For *fgf24*, we used an oligonucleotide targeted against the translation start site with the following sequence: 5′ GACGGCAGAACAGACATCTT-GGTCA-3′. As a control we used the standard control oligonucleotide available from GeneTools. All oligonucleotides were solubilized in 1×Danieau's solution and injected into one-cell stage zebrafish embryos at concentrations ranging from 5-10 ng/embryo.

Transplantation

Donor embryos were injected with 2.5% rhodamine-dextran, and cells transplanted into hosts at 30-70% epiboly. To target wild-type cells to

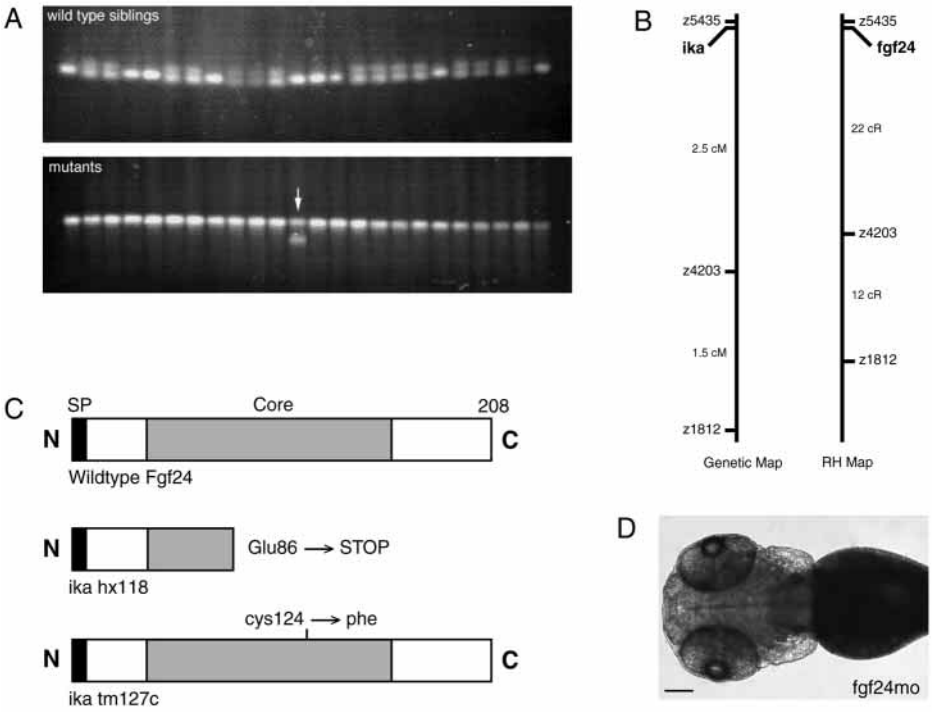

Fig. 2. *ika* encodes *fgf24*. (A) Mapping data for *ika* with the SSLP marker z5435. The upper gel shows the PCR products amplified from 24 siblings, while the lower gel shows the products amplified from 24 *ika* mutant embryos. The mutant embryos only show the (upper) Tuebingen band, except for a single embryo, which also shows the (lower) WIK band (arrow). This is the only recombination event we observed with this marker out of 938 embryos tested. (B) Schematic representation of the region of linkage group 14 to which *ika* and *fgf24* map, depicting the genetic map on the left, and the radiation hybrid (RH) map on the right. (C) Schematic representation of the alterations to the Fgf24 protein found by sequencing *fgf24* in the two *ika* alleles, ika^{hx118} and ika^{tm127c}. (D) The phenotype generated by injecting a morpholino targeted against the translation start site of *fgf24*. These embryos lack pectoral fins, but otherwise appear normal, thus phenocopying *ika* (compare with Fig. 1A,B). Scale bar: 100 μm.

the fin mesenchyme, transplantation was carried out as described previously (Ahn et al., 2002). To target the ectoderm, we transplanted cells to a region opposite the shield in early gastrula embryos.

Histochemical methods

Whole-mount in situ hybridization was performed as previously described (Kishimoto et al., 1997), using the following probes: *tbx5* (Begemann and Ingham, 2000); *msxc* (Akimenko et al., 1995); *dlx2* (Akimenko et al., 1994); *pea3* and *erm1* (Roehl and Nusslein-Volhard, 2001); *fgf8* (Reifers et al., 1998); *fgf10* (Ng et al., 2002); *shh* (Krauss et al., 1993); *bmp2* (Kishimoto et al., 1997); and *hand2* (Yelon et al., 2000). Alcian Blue staining was performed described previously (Grandel and Schulte-Merker, 1998). Histological sections were obtained by staining cryosections with Methylene Blue (Humphrey and Pittman, 1974).

RESULTS

Absence of pectoral fins in *ika* mutants

Of all the fin mutants from the Tuebingen I screen (van Eeden et al., 1996), *ika* shows the most severe reduction of pectoral fins. Larvae at 3 days post fertilization (dpf) have no detectable pectoral fins (Fig. 1A,B), and staining of the cartilage elements at this stage reveals that *ika* mutants lack all the skeletal elements of the pectoral fin, including the shoulder girdle (Fig. 1C,D). Examination of *ika* mutants at earlier stages reveals the absence of morphologically detectable pectoral fin buds at any time (Fig. 1E,F; data not shown). We also fail to detect any morphological signs of an AER in *ika* mutants (data not shown). Interestingly, even the strongest *ika* allele, ika^{hx118} (which is likely to be a null mutant, see below), is homozygous viable, giving rise to adults that lack pectoral fins, but that have normal pelvic fins, and have no other obvious defects (data not shown). We used the ika^{hx118} allele for all the experiments described in this study.

ika encodes *fgf24*

As the *ika* phenotype suggests it plays an important role during pectoral fin development, we were interested in identifying the molecular nature of *ika*. To this end, we mapped *ika* using SSLP markers (Knapik et al., 1998; Liao and Zon, 1999), and found it to be located on linkage group 14, between the markers z5435 and z4203 (Fig. 2A,B). We found z5435 to be very tightly linked to the *ika* locus (1 recombinant out of 938 embryos tested) (Fig. 2A). Comparison of this interval of the genetic map with the radiation hybrid map (Geisler et al., 1999) revealed that a cDNA encoding *fgf24* maps to a very similar location (Fig. 2B) (B.W.D., D. W. Stock and C. B. Kimmel, unpublished). Fgf24 is a new member of the Fgf8/17/18 subfamily of Fgf ligands and has highest sequence similarity to Fgf18 (B.W.D., D. W. Stock and C. B. Kimmel, unpublished).

In order to determine whether *ika* encodes *fgf24*, we sequenced the *fgf24* gene of the two *ika* alleles. In ika^{hx118}, we found the G at position 256 of the wild-type coding region changed to a T, causing the conversion of a glutamate codon to a stop codon at amino acid 86 (Fig. 2C and data not shown). In ika^{tm127c}, we found the G at position 271 of the wild-type coding region changed to a T, causing the conversion of a cysteine codon to a phenylalanine codon at position 124 (Fig. 2C; data not shown).

The stop codon in ika^{hx118} leaves a truncated protein lacking more than half of its C terminus (Fig. 2D), thus removing most of the core region, which has been shown to interact with the receptor in the case of other Fgf proteins (reviewed by Ornitz and Itoh, 2001). ika^{hx118} is thus likely to be a null allele, which is supported by the full phenotypic penetrance observed with this allele. The cysteine converted in ika^{tm127c} to phenylalanine is one of six amino acids that are conserved in the core region of all Fgf proteins (reviewed by Ornitz and Itoh, 2001),

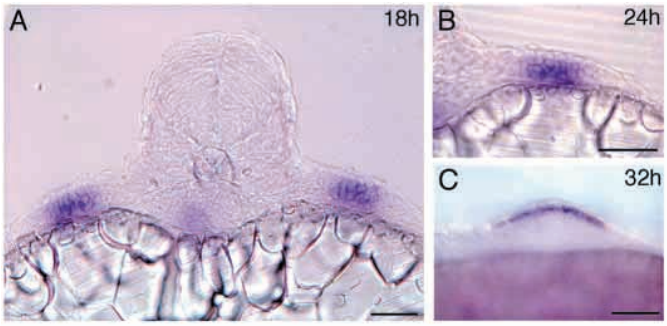

Fig. 3. The expression pattern of *fgf24* in the pectoral fin primordia. (A) Transverse section through a wild-type embryo at 18 hpf at the level of the pectoral fin primordia. Note the prominent expression in the mesenchyme. (B) Transverse section through the pectoral fin primordium of a wild-type embryo at 24 hpf. Note that *fgf24* is still strongly expressed in the mesenchyme, but not the ectoderm. (C) A wild-type pectoral fin bud at 32 hpf. *fgf24* has been downregulated in the mesenchyme, and is now expressed in the AER. Scale bars: 50 μm.

suggesting that it is crucial for normal function. Nevertheless, this allele appears to retain some activity, as it shows variable phenotypic penetrance and expressivity (van Eeden et al., 1996).

To further test the possibility that *ika* encodes *fgf24*, we designed an antisense morpholino oligonucleotide to block translation of *fgf24* (Nasevicius and Ekker, 2000). Injection of morpholinos targeted against *fgf24* generated larvae that specifically lack pectoral fins (n=72), but otherwise appear normal, thus phenocopying the *ika* mutation (Fig. 2D) (B.W.D., D. W. Stock and C. B. Kimmel, unpublished). We injected morpholinos within the range of 5-10 ng per embryo, and within this range, all injected embryos displayed complete absence of pectoral fins. The injection of a control morpholino had no effect (data not shown), and yielded individuals identical to the wild-type larva shown in Fig. 1A.

From these data, we conclude that *ika* encodes *fgf24* and hereafter refer to the mutant by its molecular name. The GenBank Accession number for *fgf24* is AY204859.

fgf24 is expressed in the mesenchyme of early pectoral fin buds

To better define the role played by *fgf24* during pectoral fin development, we localized the *fgf24* transcript using in situ hybridization. We first detect *fgf24* in the region of the pectoral fin primordia at 18 hpf (Fig. 3A). Apart from *tbx5*, which is first detected in this region at 17 hpf (Begemann and Ingham, 2000; Ruvinsky et al., 2000), *fgf24* is the earliest marker for the pectoral fin primordia. At this stage, the transcript is detectable only in the mesenchyme (Fig. 3A). Expression is still present in the fin bud mesenchyme at 24 hpf (Fig. 3B), but becomes downregulated in these cells between 28 and 30 hpf (Fig. 3C). At the same time, *fgf24* is activated in the overlying AER (Fig. 3C).

Failure to activate ectodermal marker gene expression in the pectoral fin buds of *fgf24* mutants

As both the *fgf24*$^{-/-}$ phenotype and the *fgf24* expression pattern suggest that it plays a role during early stages of pectoral fin development, we examined the expression of several genes expressed at these stages in *fgf24* mutants.

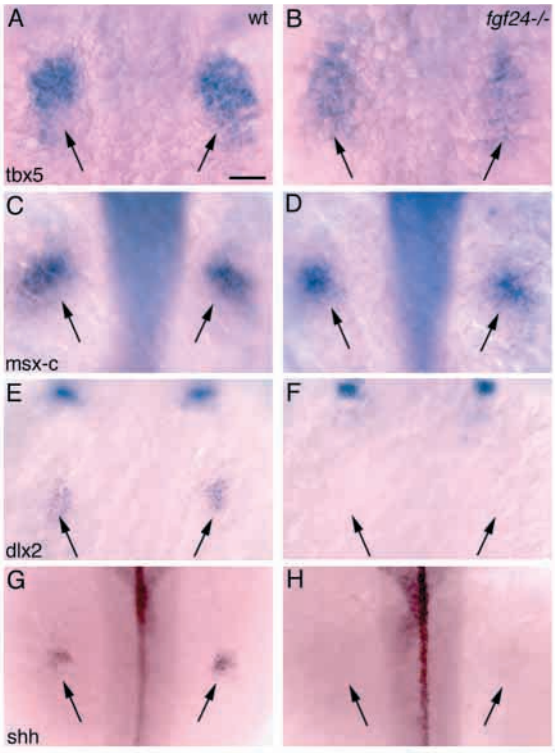

Fig. 4. Marker gene expression in the fin mesenchyme and ectoderm of 24 hpf wild-type and *fgf24*$^{-/-}$ embryos. (A) Wild-type *tbx5* expression in the pectoral fin primordia (arrows). (B) *tbx5* expression in the absence of *fgf24* activity. (C) Wild-type *msxc* expression in the pectoral fin primordia. (D) *msxc* expression in the absence of *fgf24* activity. (E) Wild-type *dlx2* expression in the pectoral fin primordia. (F) *dlx2* expression in the absence of *fgf24* activity. (G) Wild-type *shh* expression in the pectoral fin primordia. (H) *shh* expression in the absence of *fgf24* activity. Scale bar: 50 μm. All photos are at the same magnification.

tbx5 is the earliest markers for pectoral fin development, and is expressed in the mesenchymal compartment of the fin buds (Fig. 4A) (Begemann and Ingham, 2000; Ruvinsky et al., 2000). We find that it is also expressed in these cells at 24 hpf in *fgf24* mutants, although the expression appears weaker than in wild-type embryos (Fig. 4B).

msxc is expressed dynamically during pectoral fin development. At 24 hpf, it is expressed throughout the mesenchyme of the pectoral fin bud primordia (Fig. 4C and data not shown). The expression of *msxc* in *fgf24* mutants is indistinguishable from that in wild-type embryos at this stage (Fig. 4D). Between 28 and 32 hours, expression becomes weaker and then disappears completely in *fgf24* mutants (data not shown).

dlx2 is an early marker for the AER in zebrafish. We find that *dlx2* is activated in the ectoderm of wild-type pectoral fin buds already at 20-22 hpf (Fig. 4E; data not shown), which precedes formation of the AER (Grandel and Schulte-Merker, 1998). At early stages, *dlx2* appears to be expressed throughout the entire fin bud ectoderm, and later becomes restricted to the AER (data not shown). We find that *dlx2* expression is not

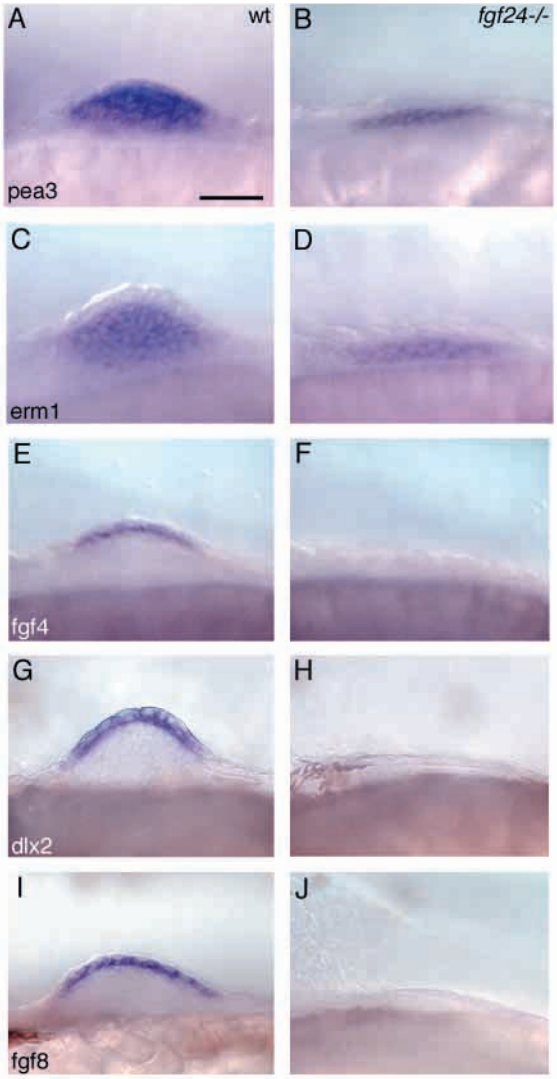

Fig. 5. Marker gene expression in the fin mesenchyme and ectoderm of 30 hpf wild-type and *fgf24*$^{-/-}$ embryos. (A) Wild-type *pea3* expression in the pectoral fin bud. Note expression both in the mesenchyme and the ectoderm. (B) *pea3* expression in the absence of *fgf24* activity. Note there is expression in the mesenchyme, but not the ectoderm. (C) Wild-type expression of *erm1* in the pectoral fin bud. (D) *erm1* expression in the absence of *fgf24* activity. (E) Wild-type *fgf4* expression in the pectoral fin bud. (F) *fgf4* expression in the absence of *fgf24* activity. (G) Wild-type *dlx2* expression in the pectoral fin bud. (H) *dlx2* expression in the absence of *fgf24* activity. (I) Wild-type *fgf8* expression in the pectoral fin bud. (J) *fgf8* expression in the absence of *fgf24* activity. Scale bar: 50 μm. All photos are at the same magnification.

detectable in the pectoral fin buds of *fgf24* mutants at any time (Fig. 4F; data not shown).

shh is first expressed in pectoral fins at 24 hpf. We detect no *shh* expression in the pectoral fins of *fgf24* mutants at 24 hpf (Fig. 4G,H), or at later stages (see Fig. 8A,B).

These data suggest that at least some mesenchymally expressed genes are activated in *fgf24* mutant pectoral fin buds, but not ectodermally expressed genes. We further addressed this possibility by examining marker gene expression at 30 hpf, when the mesenchymal and ectodermal compartments of the pectoral fin buds are easier to distinguish.

Table 1. Transplantation of wild-type cells into *fgf24* mutant fin buds

Location of wild-type cells	Rescue of growth	Rescue of *fgf10* expression	Rescue of apical fold
Fin bud mesenchyme (*n*=4)	+	+	+
	+	+	+
	+	+	–
	–	–	–
Fin bud ectoderm (*n*=6)	–	–	–
	–	–	–
	–	–	–
	–	–	–
	–	–	–
	–	–	–

+, present; –, absent.

erm1 and *pea3* encode transcription factors that have been shown to be dynamically expressed in many tissues during zebrafish development (Roehl and Nuesslein-Volhard, 2001). We find that both genes are expressed throughout the pectoral fin bud mesenchyme, and that *pea3* is additionally expressed in the ectoderm (Fig. 5A,C). Both *erm1* and *pea3* are expressed in the mesenchyme of *fgf24* mutants, but *pea3* fails to be activated in the ectoderm (Fig. 5B,D). Like *msxc*, *erm1* and *pea3* expression starts to fade around 30 hpf in *fgf24* mutants, and is no longer detectable at later stages (data not shown).

None of the ectodermal markers we tested are activated in the pectoral fin buds of *fgf24* mutants. These include *pea3*, *dlx2*, *fgf4*, and *fgf8* (Fig. 5A,B,E-J), *msxc* and *bmp2* (Fig. 7C-F), and *fgf24* (data not shown).

Taken together, these results suggest that activation of genes expressed in the ectoderm of the pectoral fin buds fails to occur in the absence of *fgf24* activity. The expression of some genes expressed in the fin bud mesenchyme, however, appears to be independent of *fgf24* activity.

fgf24 acts downstream of *wnt2b* and *tbx5* and upstream of *fgf10* during limb bud initiation

The *fgf24*$^{-/-}$ pectoral fin phenotype bears strong resemblance to that of zebrafish embryos lacking *wnt2b* or *tbx5* activity (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). To investigate the relationship of *fgf24* with these two genes, we examined the expression of *fgf24* in the absence of *wnt2b* or *tbx5* activity, by using a morpholino knockdown approach (Nasevicius and Ekker, 2002). We fail to detect *fgf24* expression at 20 hpf or 24 hpf in the pectoral fin bud primordia in the absence of either *wnt2b* or *tbx5* activity (Fig. 6A-C; data not shown). Injection of control morpholinos yielded embryos showing wild-type *fgf24* expression identical to that shown in Fig. 6A. Together with the observation that *tbx5* expression is initiated in *fgf24* mutants (Fig. 4B), these results indicate that *fgf24* acts downstream of *tbx5* in the genetic cascade initiating pectoral fin bud development. As *tbx5* fails to be activated in the absence of *wnt2b* activity (Ng et al., 2002), and as *fgf24* activation depends on *tbx5* (Fig. 6B), it is not surprising that *fgf24* also depends on *wnt2b* activity.

Like *fgf24*, the *fgf10* gene depends on *tbx5* for its activation. To clarify the regulatory relationship between *fgf24* and *fgf10*, we examined the expression of *fgf10* in *fgf24* mutants. We fail

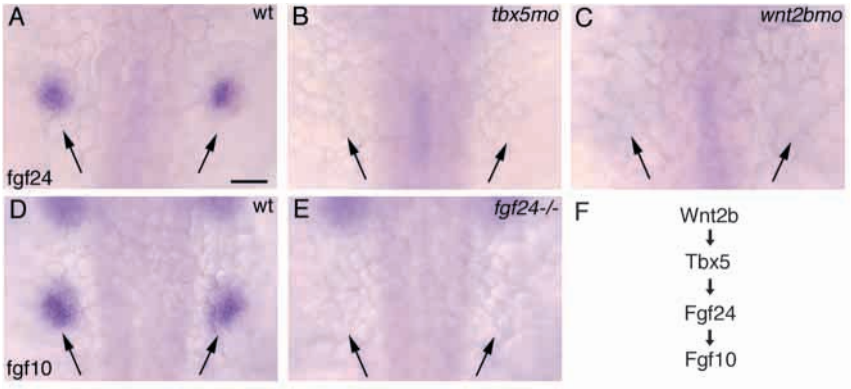

Fig. 6. *fgf24* acts downstream of *wnt2b* and *tbx5*, and upstream of *fgf10*. (A) Wild-type *fgf24* expression at 24 hpf. (B) *fgf24* expression in a *tbx5*-morpholino knockdown embryo (*tbx5mo*) at 24 hpf. (C) *fgf24* expression in a *wnt2b*-morpholino knockdown embryo (*wnt2bmo*) at 24 hpf. (D) Wild-type *fgf10* expression at 24 hpf. (E) *fgf10* expression in the absence of *fgf24* activity at 24 hpf. (F) Schematic representation of the hierarchy of genes involved in triggering limb development. Scale bar: 50 μm. All photos are at the same magnification.

to detect pectoral fin expression of *fgf10* at all stages in the absence of *fgf24* activity (Fig. 6D,E; data not shown).

Taken together, these results suggest that *fgf24* acts downstream of *tbx5* to activate *fgf10* expression during limb bud initiation (Fig. 6F).

fgf24 activity is required in the mesenchyme to activate *fgf10*

As *fgf24* is expressed both in the mesenchyme and in the AER, we wished to determine in which cells *fgf24* activity is required for *fgf10* activation. We therefore transplanted wild-type cells into *fgf24* mutant embryos. We observed rescue of fin bud outgrowth when wild-type cells were located in the fin bud mesenchyme (Table 1, *n*=3). These fin buds also showed an AER at 36 hpf, and *fgf10* expression. In one case, we did not observe rescue when wild-type cells were located in the lateral plate mesoderm at the level of the pectoral fin bud (data not shown). This may be due to the fact that not enough wild-type cells were present in this case. We never observed rescue of the *fgf24* phenotype when wild-type cells were located in the ectoderm at the pectoral fin level (Table 1, *n*=6).

These results suggest that *fgf24* activity is required in the mesenchyme, and not the ectoderm, for fin bud initiation and the activation of *fgf10*.

fgf24 activity is required for the migration of *tbx5*-expressing mesenchymal cells to the fin primordium

As *tbx5* is required for the movement of mesenchymal cells in the lateral plate mesoderm to the pectoral limb bud (Ahn et al., 2002), and as *fgf24* is activated downstream of *tbx5*, we asked whether *fgf24* might play a role in mediating this effect of *tbx5*. To this end, we compared the distribution of *tbx5*-expressing cells in the lateral plate mesoderm in wild-type and *fgf24* mutant embryos between 18 hpf and 32 hpf. At 18 hpf, *tbx5* expression is indistinguishable in wild-type and *fgf24* mutants (Fig. 7A,B). However, at 27 hpf, the *tbx5*-expressing cells have congregated towards the pectoral fin bud in wild-type embryos, but remain dispersed in *fgf24* mutants (Fig. 7C,D). This phenotype is even more striking at 32 hpf (Fig. 7E,F).

This observation indicates that *fgf24* activity is required for the correct movement of *tbx5*-expressing cells in the lateral plate mesoderm to the pectoral fin bud.

Anterior/posterior polarity in *fgf24* mutant pectoral fin buds

The activation of *shh* expression in the posterior mesenchyme of chick limb buds depends on Fgfs secreted from the AER (Laufer et al., 1994; Niswander et al., 1994; Sun et al., 2002). Because *fgf24* mutants do not show any sign of AER formation, we examined whether *shh* is activated in the absence of *fgf24* activity. We find that *shh* expression is not detectable in *fgf24* mutants at any stage, consistent with the absence of AER in these mutants (Fig. 4G,H; Fig. 8A,B). *msxc* is initially expressed throughout the mesenchyme of the pectoral fin buds, but expression becomes restricted to the anterior by 28-30 hpf in wild-type embryos (Fig. 4A; Fig. 8C). In *fgf24* mutant

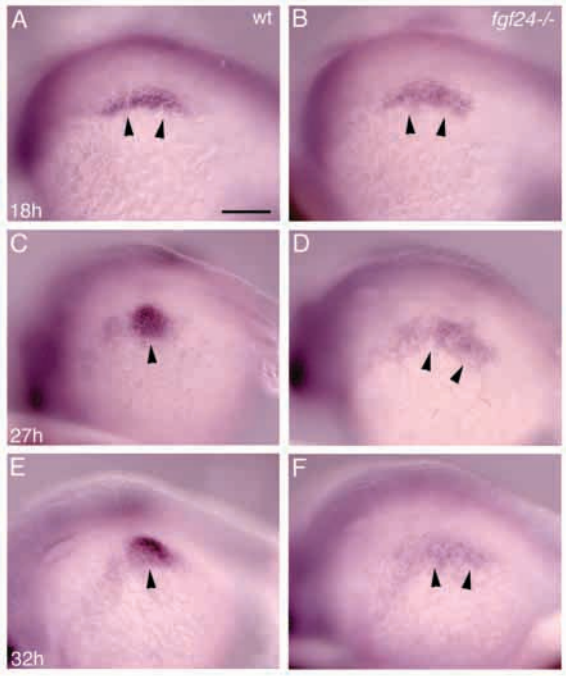

Fig. 7. Failure of *tbx5*-expressing cells to move towards the pectoral fin buds in the absence of *fgf24* activity. Shown is a time course of *tbx5* expression in the pectoral fin region (arrowheads) from 18 hpf to 32 hpf in wild-type and *fgf24* mutant embryos. Pictures were taken as oblique side views of embryos, with anterior towards the left. (A) Wild-type *tbx5* expression at 18 hpf. (B) *tbx5* expression at 18 hpf in the absence of *fgf24* activity. (C) Wild-type *tbx5* expression at 27 hpf. (D) *tbx5* expression at 27 hpf in the absence of *fgf24* activity. (E) Wild-type *tbx5* expression at 32 hpf. (F) *tbx5* expression at 32 hpf in the absence of *fgf24* activity. Scale bar: 100 μm. All photos are at the same magnification.

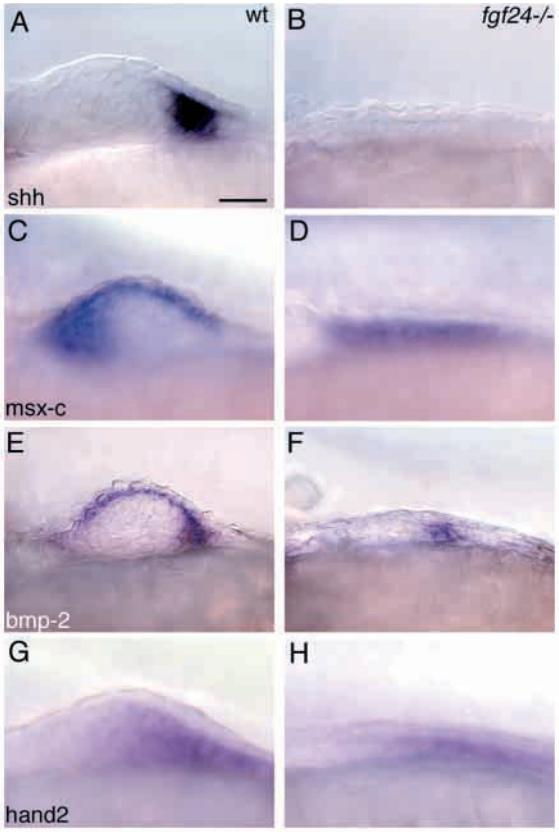

Fig. 8. Expression of marker genes in the fin anterior/posterior axis of 30 hpf wild-type and *fgf24*$^{-/-}$ embryos. Anterior is towards the left. (A) Wild-type *shh* expression in the pectoral fin bud. (B) *shh* expression in the absence of *fgf24* activity. (C) Wild-type *msxc* expression in the pectoral fin bud. (D) *msxc* expression in the absence of *fgf24* activity. Note that *msxc* is expressed in the mesenchyme, but not the ectoderm. Note also that *msxc* fails to be downregulated in the posterior mesenchyme. (E) Wild-type *bmp2* expression in the pectoral fin bud. (F) *bmp2* expression in the absence of *fgf24* activity. Note that *bmp2* is expressed in the posterior mesenchyme, but not in the ectoderm. (G) Wild-type *hand2* expression in the pectoral fin bud. (H) *hand2* expression in the absence of *fgf24* expression. Scale bar: 50 μm. All photos are at the same magnification.

pectoral fin buds, this repression in the posterior mesenchyme fails to occur (Fig. 8D). This is probably due to the absence of Shh, because the same phenotype is observed in *shh* mutant pectoral fin buds (Neumann et al., 1999). *hoxd13* and *hoxa13* also fail to be activated in *fgf24* mutant pectoral fin buds (data not shown). Again, this phenotype is similar to what is observed in the absence of *shh* activity (Neumann et al., 1999).

bmp2 is expressed both in the AER and in the posterior mesenchyme (Fig. 8E). We find that *bmp2* is activated in the posterior mesenchyme in *fgf24* mutants, but not in the ectoderm (Fig. 8F). This correlates with the observation that activation of *bmp2* in the posterior mesenchyme is independent of *shh* activity (Neumann et al., 1999). The *shh*-independent anterior/posterior (AP) polarity of pectoral fin buds has been shown to be directed by the *hand2* gene, which encodes a bHLH transcription factor (Yelon et al., 2000). Consistent with this observation, we find normal posterior activation of *hand2* in *fgf24* mutants (Fig. 8G,H), although *hand2* expression gradually fades after 30 hpf (data not shown).

These data suggest that the early aspects of AP polarity are established in the *fgf24* mutant pectoral fin primordium. Those aspects of AP polarity that are lost in *fgf24* mutants are ones which have been shown to depend on *shh* activity (Neumann et al., 1999), which correlates well with the failure to activate *shh* expression in *fgf24* mutant fin buds, while the *shh*-independent AP polarity is unaffected by the loss of *fgf24*.

DISCUSSION

Multiple layers of regulation control vertebrate limb initiation and the movement of mesenchymal cells to the forelimb bud

The initiation of vertebrate limb development involves the transfer of positional information from the intermediate mesoderm (IM) to the lateral plate mesoderm (LPM), and from there to the ectoderm. This process is based on a cascade of inductive events that depends on secreted signaling proteins sequentially activated in all three of these tissues. Fgf8 is thought to initiate this cascade at the level of the chicken forelimb by activating *Wnt2b* in the IM, which then signals to the LPM to activate *Tbx5* expression. *Tbx5* activates *Fgf10* in the LPM, which in turn signals to the overlying ectoderm to activate *Wnt3a*. Wnt3a completes the cascade by inducing *Fgf4* and *Fgf8* in the chicken AER.

In this study, we have shown that the zebrafish *fgf24* gene, which is disrupted by the *ikarus* mutation, acts downstream of *tbx5* to activate *fgf10* in the LPM, thus identifying another Fgf acting early in the cascade of limb induction. Consistent with this model, *fgf24* is expressed very early during pectoral fin development, and is activated in the LPM at a similar stage as *tbx5* (this study) (Begemann and Ingham, 2000; Ruvinsky et al., 2000). Together with the observation that *Tbx5* is activated within 1 hour of implanting an Fgf-soaked bead into the flank of a chick embryo (Isaac et al., 2000), these results suggest that Tbx5 and Fgf24 act early in the limb-inducing cascade. *Fgf10*, however, is not activated until 17 hours after Fgf application (Ohuchi et al., 1997), consistent with the proposal that its induction requires an additional signaling event. A more direct effect of mouse TBX5 on *Fgf10* transcription has been recently proposed, based on the finding that there is at least one potential TBX5-binding site upstream of the mouse *Fgf10* promoter, and that the *Fgf10* promoter can be upregulated by co-expressing *Tbx5* in cultured cells (Agarwal et al., 2003). However, this direct model does not explain why the activation of *Fgf10* by Fgf bead application takes so much longer than the activation of *Tbx5*, and it remains to be seen what the role of the TBX5-binding sites are in vivo. One possibility is that the *Fgf10* promoter integrates several different signals, and that direct binding by TBX5 is necessary, but not sufficient for activation in vivo. Our data indicate that one of the additional requirements for *Fgf10* activation in the zebrafish is the exposure of these cells to Fgf24.

The data presented here and elsewhere suggest that zebrafish Wnt2b, Tbx5, Fgf24 and Fgf10 act sequentially in a linear pathway in which Wnt2b induces Tbx5 expression, which then induces Fgf24, which in turn induces Fgf10 (Fig. 6F). In addition to its role in this linear cascade, zebrafish Tbx5 has

been shown to be required for the correct migration of lateral plate mesenchymal cells to the pectoral fin primordium (Ahn et al., 2002). Here we have shown that Fgf24 is also required for this process, as *tbx5*-expressing cells fail to congregate to the pectoral fin primordium in the absence of *fgf24* activity. The dispersal of *tbx5*-expressing cells is not as dramatic in the absence of *fgf24* activity as it is in the absence of *tbx5* activity, suggesting that Fgf24 does not mediate all effects of Tbx5 on this migratory event. This is consistent with the observation that *tbx5* activity is cell-autonomously required for the correct movement of cells to the fin bud (Ahn et al., 2002).

The pectoral fin phenotype of *fgf24* mutants is similar to that of zebrafish *raldh2* mutants (Begemann et al., 2001; Grandel et al., 2002). As *raldh2* has been shown to act upstream of *tbx5* activation in the fin bud (Begemann et al., 2001), it is also likely to act upstream of *fgf24* activation.

Fgf8-related Fgfs control several aspects of vertebrate limb induction

The Fgf24 protein is a new member of the Fgf8/17/18 subfamily of Fgf ligands (B.W.D., D. W. Stock and C. B. Kimmel, unpublished). Furthermore, Fgf24 also shows functional overlap with Fgf8, as both genes are expressed at early stages in the posterior mesoderm of the embryo, and *fgf8*/*fgf24* double mutants display developmental defects in this region that are not observed in either *fgf8* or *fgf24* single mutants, thus indicating that these two Fgfs have very similar activities (B.W.D., D. W. Stock and C. B. Kimmel, unpublished). Hence, the analysis of the *fgf24* mutant phenotype provides the first loss-of-function data demonstrating a role for an Fgf8-like gene in limb initiation, and complements the gain-of-function experiments which show that ectopic application of Fgf2, Fgf4 and Fgf8 can trigger the development of additional limbs (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996; Yonei-Tamura et al., 1999).

Members of the Fgf8 subclass of Fgfs appear to be expressed in all three tissues involved in limb initiation, and seem to have important functions at three distinct steps in early limb development. First, Fgf8, which is expressed in the chicken IM, initiates *WNT2B* expression in the IM. Secondly, Fgf24, which is expressed in the zebrafish LPM, activates *fgf10* expression in the LPM, and thirdly, Fgf4 and Fgf8, which are expressed in the chicken and mouse AER, direct outgrowth of the limb bud and maintain *Fgf10* expression in the mesenchyme. Interestingly, our data suggest that Fgf24 signals in an autocrine manner to activate *fgf10* in the LPM, as both genes appear to be co-expressed in the same region, although the activation of *fgf24* precedes that of *fgf10*. However, on the basis of these data, we cannot distinguish whether Fgf24 produced by an individual cell signals to the same cell, or whether there is signaling between cells in the same tissue. Our data also suggest that mesenchymally expressed Fgf24 activates mesenchymal *shh* expression at 24 hpf. This situation is clearly different from the chick and the mouse, where *Fgf8* secreted from the AER has been shown to activate *shh* in the mesenchyme (Laufer et al., 1994; Nieswander et al., 1994; Sun et al., 2002). Consistent with this observation, the zebrafish *fgf8* gene is activated in the AER at a much later stage than in tetrapods (12 hours after the activation of *shh* in the mesenchyme), and zebrafish *fgf8* mutants have no effect on fin development (Reifers et al., 1998), although this may be a hypomorphic mutation.

It is also noteworthy in this context that *fgf24* is activated in the AER after limb bud initiation has taken place, and at the same time is downregulated in the mesenchyme (Fig. 3C). This observation raises the possibility that Fgf24, together with the other Fgfs expressed in the AER, continues to direct *fgf10* expression in the mesenchyme during limb outgrowth, this time by a paracrine mechanism. This scenario is not supported by our transplantation results, however, because the *fgf24* mutant phenotype is rescued only by wild-type cells located in the fin mesenchyme, but not in the ectoderm, and rescue leads to restoration of *fgf10* expression even at 36 hpf, when *fgf24* is expressed in the AER, and not the mesenchyme. The possibility remains that at this stage, Fgf24 functions redundantly with other Fgfs expressed in the AER, such as Fgf4 and Fgf8.

Genetic differences between forelimb and hindlimb development

It is interesting to note that some genes are specifically required for the development of either forelimbs or hindlimbs, while other genes function in both types of limbs. Fgf24, for example, is only required for the development of pectoral fins, but not pelvic fins, and shares this characteristic with chicken Tbx5 and Wnt2b (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999; Kawakami et al., 2001). *Fgf10*, however, appears to play an equivalent role in both fore- and hindlimbs in mice and chicken (Min et al., 1998; Sekine et al., 1999). It is likely that chicken Tbx4 and Wnt8c, or related genes, play a role in hindlimb development that is similar to the role of Tbx5 and Wnt2b, respectively, in forelimb development (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999; Kawakami et al., 2001). Hence, our data suggest that a zebrafish Fgf closely related to Fgf24 fulfills a similar function during pelvic fin development.

Evolutionary conservation of genes involved in vertebrate limb induction

There appears to be strong evolutionary conservation of the developmental mechanism of limb bud initiation. For example, inactivation of the *Tbx5* gene leads to similar reductions of the forelimbs in chicken, zebrafish, human and mouse (Newbury-Ecob et al., 1996; Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002; Agarwal et al., 2003). In addition, the role of *Wnt2b* in limb initiation appears highly conserved in chicken and zebrafish (Kawakami et al., 2001; Ng et al., 2002). By contrast, however, there appears to be no role for *Wnt2b* in initiating mouse limb development (Ng et al., 2002), nor is there a role for *Wnt3a* in inducing the mouse AER (Barrow et al., 2003). Nevertheless, the phenotype of the *Lef1*/*Tcf1* double mutant mouse is consistent with WNT signaling being necessary for AER formation in the mouse (Galceran et al., 1999). These results suggest that different Wnt proteins, comparable in activity to the chicken Wnt2b and Wnt3a proteins, fulfill their respective roles in the mouse. Consistent with this proposal, it has recently been shown that the mouse *Wnt3* gene fulfills the function of chicken *WNT3A* during limb development (Barrow et al., 2003).

This idea is similar to the one that different genes of similar activity fulfill the same function during fore- and hindlimb

development, and suggests that the existence of gene families with similar activities has allowed the functional replacement of specific genes during evolution. By the same reasoning, our data suggest that an Fgf comparable in activity with Fgf24 occupies a similar position in the genetic cascade that initiates limb development in other vertebrate species. However, as both the mouse and human genomes have been sequenced, it is clear that they contain no ortholog of the zebrafish *fgf24* gene. Although it is possible that zebrafish Fgf24 fulfills the role attributed to tetrapod *Fgf8* in limb initiation, this is unlikely, because *Fgf8* is never expressed in limb mesenchyme in any vertebrate examined to date. An alternative possibility is that another tetrapod *Fgf* family member with similar activity to *Fgf8* is transiently expressed in the limb bud mesenchyme, and that this expression has so far gone undetected. Future experiments will hopefully resolve this issue.

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