Zebrafish *fgf24* functions with *fgf8* to promote posterior mesodermal development

Bruce W. Draper¹,∗,†, David W. Stock² and Charles B. Kimmel¹

¹Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA
²Department of Environmental, Population and Organismic Biology, University of Colorado, Boulder, CO 80309, USA

*Present address: Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

†Author for correspondence (e-mail: draper@fred.hfcc.org)

Accepted 12 June 2003

Development 130, 4639-4654

© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00671

Summary

Fibroblast growth factor (Fgf) signaling plays an important role during development of posterior mesoderm in vertebrate embryos. Blocking Fgf signaling by expressing a dominant-negative Fgf receptor inhibits posterior mesoderm development. In mice, *Fgf8* appears to be the principal ligand required for mesodermal development, as mouse *Fgf8* mutants do not form mesoderm. In zebrafish, Fgf8 is encoded by the *acerebellar* locus, and, similar to its mouse ortholog, is expressed in early mesodermal precursors during gastrulation. However, zebrafish *fgf8* mutants have only mild defects in posterior mesodermal development, suggesting that it is not the only Fgf ligand involved in the development of this tissue. We report here the identification of an *fgf8*-related gene in zebrafish, *fgf24*, that is co-expressed with *fgf8* in mesodermal precursors during gastrulation. Using morpholino-based gene inactivation, we have analyzed the function of *fgf24* during development. We found that inhibiting *fgf24* function alone has no affect on the formation of posterior mesoderm. Conversely, inhibiting *fgf24* function in embryos mutant for *fgf8* blocks the formation of most posterior mesoderm. Thus, *fgf8* and *fgf24* are together required to promote posterior mesodermal development. We provide both phenotypic and genetic evidence that these Fgf signaling components interact with *no tail* and *spadetail*, two zebrafish T-box transcription factors that are required for the development of all posterior mesoderm. Last, we show that *fgf24* is expressed in early fin bud mesenchyme and that inhibiting *fgf24* function results in viable fish that lack pectoral fins.

Supplementary data available online

Key words: Fibroblast growth factor, *fgf24*, *fgf8*, *acerebellar*, *no tail*, *spadetail*, Mesoderm, Posterior development, Limb development, Zebrafish

Introduction

In vertebrate embryos, the posterior body and tail develop in an anterior to posterior progression by the coordinated growth and morphogenesis of precursor cells located in the tail bud (Kanki and Ho, 1997; Davis and Kirschner, 2000). Studies in several organisms have established that the Fgf signaling pathway plays an essential role during the development of the posterior body, perhaps by maintaining a population of posterior precursors during embryogenesis. Inhibiting Fgf signaling in *Xenopus* or zebrafish embryos by overexpressing a dominant-negative Fgf receptor (dnFgfr) blocks the formation of posterior body structures, including all posterior mesoderm (Amaya et al., 1991; Amaya et al., 1993; Griffin et al., 1995). Similarly, mouse embryos mutant for the Fgf receptor 1 (*Fgfr1*), one of four known vertebrate Fgf receptors, produce limited amounts of posterior mesoderm (Yamaguchi et al., 1994; Deng et al., 1994). *Fgfr1* is cell autonomously required for posterior mesodermal development, as *Fgfr1* mutant cells transplanted into wild-type host embryos do not contribute to this tissue, and instead adopt neuronal fates (Ciruna et al., 1997; Ciruna and Rossant, 2001). Thus, the Fgf signaling pathway appears to play a conserved role during development of posterior mesoderm in vertebrates.

To date, 23 Fgf ligands (Fgf1-23) have been described in tetrapods (reviewed by Ornitz and Itoh, 2001) and several of these ligands are known to be expressed in early mesodermal progenitors in mice, including *Fgf3* (Wilkinson et al., 1988), *Fgf4* (Niswander and Martin, 1992; Drucker and Goldfarb, 1993), *Fgf5* (Haub and Goldfarb, 1991; Hébert et al., 1991) and *Fgf8* (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995). Mutational analyses in mice, however, suggest that not all of these ligands are required for the development of mesoderm. For example, embryos mutant for *Fgf3* and *Fgf5* have only slight (*Fgf3*) (Mansour et al., 1993) or no (*Fgf5*) (Hébert et al., 1994) defects in posterior development. Conversely, *Fgf8* mutant embryos do not form posterior mesoderm, indicating that *Fgf8* activity can account for the majority of Fgf signaling required for posterior development in mice. A role for *Fgf4* in posterior mesodermal development in mice has yet to be established, as *Fgf4* mutants die prior to mesoderm formation (Feldman et al., 1995).

In addition to Fgf signaling, T-box genes, which function as transcriptional regulators, are also required for formation of the posterior body during vertebrate embryogenesis. The founding member of the T-box gene family, mouse *T* or *Brachyury* is expressed early in mesodermal precursors and then in the
developing notochord (Herrmann et al., 1990). T is required for the development of these tissues, as T mutant embryos fail to form a notochord and lack posterior body structures (reviewed by Smith, 1999; Papaioannou, 2001). The role of T in mesodermal development appears to be evolutionarily conserved in vertebrates, as T orthologs in several organisms have been shown to have similar expression patterns and functions. For example, T orthologs in Xenopus and zebrafish, called Xbra and no tail (ntl), respectively are expressed in mesodermal precursors and in the developing notochord (Smith et al., 1991; Schulte-Merker et al., 1992), and are required (Halpern et al., 1993; Conlon et al., 1996) and sufficient (Culiff and Smith, 1992; O’Reilly et al., 1995) for notochord and posterior mesodermal development.

The T-box gene VegT/spt has also been implicated in mesodermal specification in vertebrate embryos. VegT in Xenopus is expressed in mesodermal precursors and in developing posterior paraxial mesoderm, and is also expressed maternally (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Inhibition of maternal VegT function results in embryos that fail to form both mesoderm and endoderm, showing that VegT has an early role in germ layer formation (Zang et al., 1998). The function of zygotically expressed VegT has not been determined. In zebrafish, spadetail (spt; tbx16 – Zebrafish Information Network) is an ortholog of VegT and is similarly expressed in mesodermal precursors and in developing paraxial mesoderm. In contrast to VegT, however, spt is not expressed maternally (Griffin et al., 1998). spt mutant embryos lack paraxial mesoderm in the trunk, but not in the tail, and form a relatively normal notochord (Kimmel et al., 1989; Amacher et al., 2002). Thus, spt mutants have a phenotype that is nearly reciprocal to that of ntl mutants. Although both spt and ntl mutants form lateral and ventral mesodermal cell types, spt;ntl double mutant embryos fail to form all posterior mesoderm (Amacher et al., 2002). These results suggest that spt and ntl have distinct roles in promoting the development of specific mesodermal subtypes, as well as a presumed earlier, and redundant role in the specification of all posterior mesodermal precursors.

A link between Fgf signaling and T-box gene function in posterior mesodermal development was revealed when it was shown that T-box gene expression in mesodermal precursors is dependent on Fgf signaling. In Xenopus and zebrafish, expression of Xbra/ntl is inhibited when Fgf signaling is blocked (Amaya et al., 1991; Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Griffin et al., 1995) and ectopic activation of the Fgf signaling pathway leads to ectopic Xbra/ntl expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Griffin et al., 1995). These and other results have led to the model that Fgf signaling and T-box genes form an auto-regulatory feedback loop during early mesodermal development, where the function of one component is necessary for the continued expression of the other. These interactions are thought to promote posterior development by maintaining and regulating the growth and morphogenesis of mesodermal precursors in the posterior region of the embryo (reviewed by Isaacs, 1997).

In zebrafish, inhibiting Fgf signaling leads to a phenotype that is strikingly similar to that of spt;ntl double mutant embryos (Griffin et al., 1995; Amacher et al., 2002). Because expression of both spt and ntl in mesodermal precursors is Fgf dependent (Griffin et al., 1995; Griffin et al., 1998), it is possible to explain the mesodermal defects associated with blocking Fgf signaling as a loss of spt and ntl function. Although spt and ntl are key regulators of posterior development in zebrafish, little is known about which Fgf signaling components are required to maintain their expression.

The zebrafish fgf8 gene is expressed in mesodermal precursors and is therefore a candidate Fgf ligand for regulating posterior development. A mutation in fgf8 (or acerebellar) (Reifers et al., 1998), has been identified, but unlike embryos injected with a dnFgfr (Griffin et al., 1995), fgf8 mutants (Reifers et al., 1998), or embryos in which fgf8 function has been inhibited with morpholino oligonucleotides (Araki and Brand, 2001; Draper et al., 2001) have relatively mild defects in posterior development. A hypothesis that we explore here is that additional Fgf ligands function together with Fgf8 during development of the posterior body in zebrafish.

We have identified and characterized a second Fgf ligand-encoding gene in zebrafish that is expressed in mesodermal precursors. This ligand is a new, but distinct, member of the fgf8/17/18 subclass of Fgf ligands, for which there is no ortholog among the 23 known Fgfs in tetrapods. We therefore designate this gene fgf24. We show that fgf24 is expressed in a domain that overlaps extensively with that of fgf8, ntl and spt in mesodermal precursors during gastrulation, and that fgf8 and fgf24 are together required for the formation of most posterior mesoderm. Furthermore, we present both gene expression and genetic data showing that interactions between the Fgf signaling pathway and the ntl and spt T-box genes are essential for posterior mesoderm development in zebrafish. Last, we show that fgf24 is also required for initiation of the pectoral fin bud, a role that appears similar to that of Fgf10 in mice (reviewed by Martin, 1998).

Materials and methods

Isolation and characterization of fgf18 and fgf24 cDNAs

Degenerate primers for RT-PCR of fgf8-related genes (5’-GCCGGATCCACNAGYGGNAARCAYGTNCA-3’ and 5’-GCCCAGATTCGNARCKNCTTCATRAARTG-3’, where the underlined sequences represent restriction sites added for cloning) were designed from an alignment of tetrapod Fgf8 sequences. PCR was carried out on cDNA produced from mRNA isolated from 5-day-old larvae. PCR products were cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and 34 independent clones were sequenced. Of these, seven were identified as fgf18, two as fgf17, seventeen as fgf18 and eight as fgf24, based on phylogenetic analyses of the 105-106 encoded amino acids. An fgf24 cDNA was isolated by using one of the cloned RT-PCR fragments as a probe to screen a gastrula-stage cDNA library (a gift from T. Lepage and D. Kimelman). Additional fgf18 cDNA sequence was isolated by 3’ and 5’ RACE using the First Choice RLM-RACE kit (Ambion, Austin, TX) following the manufacturer’s instructions. The fgf24 gene structure was determined by partial sequencing of a PAC clone containing the fgf24 gene. This clone was identified by screening a PAC library (Amemiya and Zon, 1999) by PCR with the gene specific primers 5’-CAGGGATCTGTGCTTCGTGGAG-3’ and 5’-GTGCCCTCTCTGCTTCTCTCTCTGCTC-3’ (231 bp fragment). Temporal expression profiles were determined by RT-PCR, as previously described (Draper et al., 2001) using the following primers (5’ primer/3’ primer): fgf8, CACATTTGGGAGTCGAGTTGCGTGCTTGCCATTTGCGTGTC-288 bp (fragment);
fgf24, GCAAGAGATTATCGGCAACTGGAATG; TTGATGTCGACCCCATCCTTCG (272 bp fragment); fgf8, GACACGACCTGTTGCAACC/ CGTACGCTCTTGTTGTTGGAAA (221 bp fragment); and odc, ACAT- TATGGACGCTGTTGACCG/CCTACTGACTGACGATTG (309 bp fragment). GenBank Accession Numbers for the cDNA sequences are: fgf24, AY243514; fgf24, AY204859.

**Sequence alignment and phylogenetic analysis**

Phylogenetic relatedness of fgf24 and fgf18 were determined by aligning sequences with the ClustalX program, and constructing trees from the alignments using the neighbor-joining method. Prior to alignment, we used the Signal IP program (Nielsen et al., 1997) to identify the most probable cleavage site of the signal peptide that comprises the N-terminal 25-30 amino acids of the proteins. The tree was then constructed using an alignment that contained only those sequences that were predicted to be present in the mature Fgf proteins. As an outgroup, the distantly related zebrafish Fgf10 protein sequence was used.

**Mapping**

The positions of fgf18, fgf24 and the EST fi43fi7 (GenBank Accession Number AW174476; M. Clark and S. Johnson, WUZGR; http://zfish.wustl.edu) in the zebrafish genome were determined by mapping on the Goodfellow T51 radiation hybrid (RH) panel (Kwok et al., 1998) (Research Genomics) using the following primers pairs (5’ primer/3’ primer): fgf18, CCCTGACTCAACCCGGGACC/ GTCTGCTGTCCCTGTAGC (411 bp fragment); fgf24, same primers as those used for PAC isolation (see above); fi43fi7, GTCACCCAGGGTTTTCCATTTCA/GTCTGATCCTTTAGCGC (199 bp fragment). Following PCR, fragments were separated by electrophoresis and scored as described by Geisler et al. (Geisler et al., 1999). The RH data was converted to a map position using the Instant Mapping program (http://134.174.23.167/zonrhmapper/instantMapping.htm).

**Fish stocks and maintenance**

Adult zebrafish stocks and embryos were maintained at 28.5°C as described previously (Westerfield, 1995). Embryos were produced by natural matings of the appropriate adult fish. Embryos were collected and sorted at early cleavage stages and maintained in embryo medium (Westerfield et al., 1995) at 28.5°C until the desired developmental stages according to Kimmel et al. (Kimmel et al., 1995). The following alleles were used for this study: fgf8–/fgf8–, AY243514; fgf24–/fgf24–, AY204859; and ntl–/ntl– double heterozygotes and +/– single heterozygotes of the following alleles were used for this study: fgf8–/fgf8–, AY243514; fgf24–/fgf24–, AY204859; and ntl–/ntl–; fgf8–/fgf8–, short tail, n=382, x²=0.196, P>0.90.

**Results**

Identification and molecular characterization of zebrafish fgf18 and fgf24

In zebrafish, the mesodermal and endodermal germ layers form from precursor cells at the margin of the early gastrula embryo (Kimmel et al., 1990). During gastrulation, these cells involute at the margin to form the hypoblast layer under the overlying epiblast layer, which contains ectodermal precursors (Warga and Kimmel, 1990; Warga and Nusslein-Volhard, 1999). fgf8 has previously been shown to be expressed in mesendodermal precursor cells during gastrulation in zebrafish (Fürthauer et al., 1997; Reifers et al., 1998). We sought to identify additional Fgf ligands that are expressed in mesendodermal precursors during gastrulation and focused on identifying fgf8-related genes. Using degenerate oligonucleotide primers that were designed to amplify genes closely related to fgf8, we isolated four distinct cDNA fragments. In addition to fragments corresponding to fgf8 (Fürthauer et al., 1997; Reifers et al., 1998) and fgf17 (Reifers et al., 2000), we identified fragments from two genes whose

**Tissue labeling**

Riboprobes for in situ hybridization were synthesized using the MaxiScript kit according to the manufacturer’s instructions (Ambion, Austin, TX). With the exception of fgf24 (this paper), the probes used have been described previously as follows: pcr2.1 (Krauss et al., 1991), krx20 (egr2 – Zebrafish Information Network) (Oxtoby and Jowett, 1993), myod (Weinberg et al., 1996), ntl (Schulte-Merker et al., 1992), spr (Griffin et al., 1998), fgf8 (Fürthauer et al., 1997; Reifers et al., 1998) and shh (Krauss et al., 1993). The fgf24 in situ probe was transcribed from the full length cDNA. Immunohistochemical staining with anti-Ntl (Schulte-Merker et al., 1992) and anti-Spt (Amacher et al., 2002) were preformed as detailed by Amacher et al. (Amacher et al., 2002). For in situ hybridization experiments using embryos older than 24 hpf, melanogenesis was inhibited by raising embryos in embryo medium containing 0.003% PTU (1-phenyl-2-thiourea) (Westerfield, 1995). For sectioning, embryos were embedded in epon and 7.5 μm sections were cut.

**Morpholino injection and RNase protection assays**

The splice-site targeted morpholino oligonucleotide (MO) fgf24-E3I3 was obtained from GeneTools (Corvalis, OR) and has the following sequence: 5’-AGGAGACCTCGGTACGTTGCG-3’. MO injections were performed as previously described (Draper et al., 2001). RT-PCR analysis shown in Fig. 3 was performed essentially as described above, but using the fgf24 specific PCR primer pair (5’ primer/3’ primer): CGGCAACGTTGGAACAGG/GTCTCTGCTGCTGGTTGGGAAGCG (411 bp fragment); fgf24, same primer pair (this paper), the probes used in the fgf24 cDNA using the primer pair ATGTCTGTTCGGCTCAAGG/ GTCTCTGCTGCTCCACACAGG, and cloning into the pCRII-TOPO TA vector (Invitrogen, Carlsbad, CA).

**Skeletal staining**

One-month-old fish were cleared and stained for bone (with Alizarin Red) and cartilage (with Alcian Green) as described by Grandel and Schulte-Merker (Grandel and Schulte-Merker, 1998).
sequence appeared most closely related to tetrapod Fgf18 (Ohbayashi et al., 1998; Hu et al., 1999).

To further characterize these two genes, we identified and sequenced cDNAs, and used their conceptually translated protein sequences to construct a phylogenetic tree (Fig. 1A,B). Sequence comparison to the 22 known human FGF ligands confirmed that these two genes are members of the FGF8/17/18 subfamily (henceforth referred to as ‘Fgf8 subfamily’) (reviewed by Ornitz and Itoh, 2001) and are most closely related to FGF18 (not shown). Of the two zebrafish proteins, one shared 73% amino acid identity with human FGF18, while the other shared only 65% identity (Fig. 1A,B).

For simplicity, we shall refer to these genes as fgf18 and fgf24 respectively. To better determine the relationship of fgf18 and fgf24 to known genes, we mapped them using the T51 radiation hybrid panel (Kwok et al., 1998) and found that they both localized to LG14, ~18 cM apart (Fig. 1C). Human FGF18 has been mapped to chromosome 5q34 (Whitmore et al., 2000), and previous studies have found that LG14 contains regions with conserved synteny to human 5q31-5q35 (Woods et al., 2000). Using these data, together with the map positions of additional zebrafish orthologs of human genes known to map in the 5q31-5q35 interval, we determined that there was significant conserved synteny between regions containing zebrafish fgf18 and human FGF18. For example, the human gene encoding the F-box WD40 protein FBXWB1, and its

Fig. 1. fgf24 is a member of the Fgf8/17/18 subfamily and is expressed during gastrulation. (A) Sequence comparison of the predicted amino acid sequences of zebrafish (Dr) Fgf24, Fgf18, Fgf8 and Fgf17, with human (Hs) FGF18, FGF8 and FGF17. Periods indicate identical residues; dashes indicate introduced gaps; arrows indicate exon boundaries. Only partial sequence for zebrafish Fgf18 is shown as the 5' end of the gene has not been identified. (B) Phylogenetic tree comparing the relatedness of zebrafish Fgf18 and Fgf24 with other members of the Fgf8 subfamily. Zebrafish Fgf10 is distantly related to the Fgf8 subfamily, and was included as an outgroup. Numbers indicate bootstrap support for the nodes. (C) fgf18 and fgf24 map to LG 14. Map position of fgf18 and fgf24, as determined by screening the T51 radiation hybrid panel is shown relative to representative zmarkers (left) and ESTs (right, listed by GenBank Accession Numbers). The entire linkage group is not shown. Zebrafish genes that are closely linked to zebrafish fgf18 have human homologs that are closely linked to human FGF18. (D) Temporal expression profiles for zebrafish fgf18 and fgf24 in comparison with fgf8 as determined by RT-PCR. The zebrafish ornithinedecarboxylase (odc) gene was used as an internal control (see Draper et al., 2001). cR, centiRay; MWM, molecular weight marker; hpf, hours post fertilization.
zebrafish ortholog, referred to by its GenBank Accession Number AW174476, are closely linked to fgf18 (Fig. 1C). By contrast, we found no significant syntenic conservation between the map location of fgf24 and any region of the human genome. Because Fgf24 protein sequence is as distantly related to Fgf18 orthologs as Fgf8 orthologs are to Fgf17 (Fig. 1B), we propose that Fgf24 defines a new clade in the Fgf8/17/18 subfamily, and for which a tetrapod ortholog has not been described.

We used RT-PCR to compare the temporal expression profiles of fgf18 and fgf24 with that of fgf8. Similar to fgf8, we found that fgf18 and fgf24 transcripts could be detected in one-cell stage embryos (Fig. 1D), indicating that these genes are maternally expressed. By contrast, fgf24, but not fgf18, is also expressed throughout gastrulation (6-10 hpf; Fig. 1D) during the period of mesoderm specification and involution. For the remainder of this study, we focus only on characterizing the expression and function of fgf24. The expression and function of fgf18 will be reported elsewhere (B.W.D. and D.W.S., unpublished).

Analysis of the Fgf24 protein sequence using the SignalP program (Nielsen et al., 1997) indicates that the C-terminal 30 amino acids encode a probable signal sequence, arguing that fgf24 is a secreted protein. We determined the intron/exon boundaries of the fgf24 gene by partial sequencing of the genomic locus and found that they are in positions that are conserved within the Fgf8 subfamily (Xu et al., 1999) (Fig. 1A).

**fgf8 and fgf24 are co-expressed in mesodermal progenitors during gastrulation**

We determined the expression pattern of fgf24 transcripts in gastrula-stage embryos by whole-mount in situ hybridization. We first detected localized fgf24 transcripts at the beginning of epiboly (6 hpf) in the dorsalmost cells of the blastula margin (not shown) and, soon after, expression extends completely around the margin with no obvious dorsoventral bias (Fig. 2A,B). fgf24 expression continues in marginal cells throughout gastrulation (Fig. 2C-F) and by the end of gastrulation is localized to the tail bud (Fig. 2G). Thus, fgf24 has a similar expression pattern to that of fgf8 in early embryos (Fürthauer et al., 1997; Reifers et al., 1998).

We characterized in more detail the expression of fgf24 in gastrula-stage embryos by whole-mount in situ hybridization. We first detected localized fgf24 transcripts at the beginning of epiboly (6 hpf) in the dorsalmost cells of the blastula margin (not shown) and, soon after, expression extends completely around the margin with no obvious dorsoventral bias (Fig. 2A,B). fgf24 expression continues in marginal cells throughout gastrulation (Fig. 2C-F) and by the end of gastrulation is localized to the tail bud (Fig. 2G). Thus, fgf24 has a similar expression pattern to that of fgf8 in early embryos (Fürthauer et al., 1997; Reifers et al., 1998).

We characterized in more detail the expression of fgf24 in gastrula-stage embryos by whole-mount in situ hybridization. We first detected localized fgf24 transcripts at the beginning of epiboly (6 hpf) in the dorsalmost cells of the blastula margin (not shown) and, soon after, expression extends completely around the margin with no obvious dorsoventral bias (Fig. 2A,B). fgf24 expression continues in marginal cells throughout gastrulation (Fig. 2C-F) and by the end of gastrulation is localized to the tail bud (Fig. 2G). Thus, fgf24 has a similar expression pattern to that of fgf8 in early embryos (Fürthauer et al., 1997; Reifers et al., 1998).

In addition to the germ ring, fgf8 is also expressed in the presumptive brain at 8 hpf in a domain that spans from the future midhindbrain junction (MHJ) posteriorly to rhombomere 4 (Fig. 2I,M) (Reifers et al., 1998; Maves et al., 2002). At this stage of development, fgf24 is not expressed in the presumptive brain, though weak staining can be seen in dispersed cells within the presumptive spinal cord (Fig. 2E). Thus, the co-expression of fgf8 and fgf24 in the germ ring, but...
not in the presumptive brain, supports the hypothesis that *fgf24* functions with *fgf8* during posterior mesoderm production and can readily explain why *fgf8* mutant embryos have only mild defects in the development of posterior mesoderm, yet have significant defects in the development of the MHB (Reifers et al., 1998).

**fgf24** splice-blocking morpholino oligos knock-down *fgf24* gene function

We directly tested the hypothesis that *fgf24* and *fgf8* function redundantly during the development of posterior mesoderm by knocking down *fgf24* gene function with antisense morpholino oligonucleotides (MOs) (Nasevicicius and Ekker, 2000) targeted to a splice junction site in the *fgf24* pre-mRNA. Splice site-targeted MOs have been shown to alter pre-mRNA splicing when injected into zebrafish embryos, and have the advantage that their efficacy can be quantified by ribonuclease protection (Draper et al., 2001). We obtained a MO targeted to the splice donor site located at the junction of exon 3 and intron 3 (henceforth referred to as *fgf24*-E3I3; Fig. 3A). We first asked if *fgf24*-E3I3 could alter splicing of *fgf24* pre-mRNA using RT-PCR. We injected 5 ng of *fgf24*-E3I3 into one- to four-cell stage embryos and harvested RNA at 24 hpf. Using primers that span exon 3 (Fig. 3A), we found that injection of *fgf24*-E3I3 results in two aberrant splice forms, one of which causes an ~100 bp deletion in the *fgf24* cDNA when compared with cDNA amplified from control embryos (Fig. 3B). We sequenced this RT-PCR product and found that the deletion results from the aberrant use of a cryptic splice donor site located 98 bp upstream of the correct exon 3 splice donor (Fig. 3C). Splicing at this cryptic splice donor shifts the reading frame of *fgf24* mRNA such that only 19 of the 178 amino acids that are predicted to form the secreted Fgf24 protein are encoded. This severely truncated form of Fgf24 is predicted to be non-functional (Fig. 3C).

We next quantified the ability of *fgf24*-E3I3 to reduce the amount of correctly spliced *fgf24* mRNA by ribonuclease protection. We injected *fgf24*-E3I3 into one- to four-cell stage embryos at doses ranging from 1.3-5.0 ng MO/embryo and harvested RNA at 24 hpf. Using a riboprobe that detects *fgf24* mRNA splice junctions (vertical lines). (E) The amount of wild-type *fgf24* mRNA in MO injected and control embryos was determined after amounts were normalized using the odc control.
myod (Weinberg et al., 1996) and pax2.1 (Krause et al., 1991), respectively (Fig. 4A). We found that we could not detect differences in marker gene expression when comparing fgf24MO embryos with wild-type control embryos (compare Fig. 4A with 4B). In addition, we compared the morphology of live fgf24MO embryos and wild-type embryos at 24 hpf and again could not detect any significant differences (compare Fig. 4E with 4F). Thus, reducing the level of fgf24 mRNA to undetectable levels in early zebrafish embryos appears to have no detectable effect on the development of posterior mesoderm under our assay conditions.

To test the possibility that lack of fgf24 function in fgf24MO embryos is compensated for by fgf8 function, we injected fgf24-E3I3 into fgf8 mutant embryos. We will refer to fgf8-embryos that have been injected with fgf24-E3I3 MO as fgf8:fgf24MO embryos. At the 12 somite stage, fgf8-embryos can be identified by their reduced expression of pax2.1. In this respect, fgf8:fgf24MO embryos more closely resembled embryos in which Fgf signaling had been inhibited by expression of a dnFgf (Griffin et al., 1995) or spt:ntl double mutant embryos (Amacher et al., 2002).

fgf8 and fgf24 are together required for maintaining ntl and spt expression in posterior mesoderm

Because the phenotype of fgf8:fgf24MO embryos is similar to that of spt:ntl double mutants, we asked if the defects in posterior mesoderm development were associated with defects in the expression of ntl and spt. We first compared the expression patterns of ntl transcripts and Spt protein in eight-somite stage fgf8:fgf24MO embryos with those of wild-type, fgf8- and fgf24MO embryos. We found that in comparison with wild-type embryos (Fig. 5A), fgf8- (Fig. 5B), but not fgf24MO embryos (data not shown), had reduced numbers of Spt protein-expressing cells in the presomitic mesoderm, and nearly 1/3 of the embryos had gaps in the axial mesodermal expression domain of ntl (Fig. 5B). Thus, loss of fgf8 function alone, but not fgf24, is sufficient to cause reduced levels of spt and ntl expression in developing posterior mesoderm, an observation that could explain why fgf8 single mutants have defects in somitogenesis (Fig. 4C) (Reifers et al., 1998). In contrast to single mutant embryos, we found that all fgf8:fgf24MO embryos had severe defects in spt and ntl expression in posterior mesoderm. Although all of fgf8:fgf24MO embryos had expression of ntl in anterior notochord cells (Fig. 4D, Fig. 5C), we could not detect expression of either ntl or Spt in more posterior regions (Fig. 5C) (see also supplemental Fig. S1 at http://dev.biologists.org/supplemental/). These data together suggest that fgf8:fgf24MO embryos at the 10-somite stage do not contain mesodermal precursors in the tail bud.

We next asked at what stage expression of ntl and spt become dependent on the function of fgf8 and fgf24. We analyzed the expression of ntl and spt at the beginning of gastrulation, and then again in mid-gastrula stage (8 hpf) embryos. At the beginning of gastrulation, we could not distinguish differences in the expression of the T-box genes in fgf8:fgf24MO embryos relative to wild-type embryos (data not shown). In mid-gastrula stage embryos, however, we found that fgf8:fgf24MO embryos had markedly reduced expression of ntl relative to wild-type embryos, with the ventral germ ring having the most dramatic reduction (compare Fig. 5D with 5E).
Similarly, we found that \(fgf8^{-}\cdot fgf24^{MO}\) embryos had markedly reduced expression of \(spt\) in both the germ ring and presomitic mesoderm (compare Fig. 5F with 5G). Reducing the activity of \(fgf8\) or \(fgf24\) alone did not result in significant decreases in \(ntl\) or \(spt\) expression at the embryonic stages analyzed here (data not shown). Thus, cooperative function of \(fgf8\) and \(fgf24\) in the germ ring is required for continued high level expression of \(ntl\) and \(spt\) in mesodermal precursors, but they are not required for the initial expression of the T-box genes at early gastrula stages.

**\(ntl\) and \(spt\) are required for some, but not all, of the expression of \(fgf8\) and \(fgf24\) in the germ ring**

It had been proposed that Fgf signaling and T-box genes form an auto-regulatory feedback loop, where the expression of one maintains the expression of the other (reviewed by Smith, 1999). We therefore asked what effect loss of \(spt\) and \(ntl\) function had on the expression of \(fgf8\) and \(fgf24\) during gastrulation. We found that mid-gastrula-stage (8 hpf) \(ntl\) mutants had reduced expression of both \(fgf8\) (Fig. 5I) and \(fgf24\) (Fig. 5M) in axial, but not ventral mesoderm compared with wild-type embryos (Fig. 5H and 5L, respectively). Surprisingly, \(spt\) mutant embryos also failed to express \(fgf8\) in axial mesoderm, but had apparently normal expression of \(fgf8\) in non-axial domains (Fig. 5J). By contrast, expression of \(fgf24\) in \(spt\) mutant embryos was reduced ventrally, but not dorsally (Fig. 5N). Finally, we examined the expression of \(fgf8\) and \(fgf24\) in \(spt;ntl\) double mutant embryos, and found that expression levels of \(fgf8\) were further reduced in the dorsal and lateral but not the ventral, germ ring (Fig. 5K). By contrast, we found that expression of \(fgf24\) was reduced both dorsally and ventrally, but not laterally in \(spt;ntl\) double mutants (Fig. 5O). These data show that wild-type function of \(spt\) and \(ntl\) are required for some, but not all, \(fgf8\) and \(fgf24\) expression in mesodermal precursors.

**\(fgf8\) interacts with \(ntl\) and \(spt\) in vivo**

We have so far provided only indirect evidence based on phenotypic analysis and gene expression that interactions between the Fgf ligands \(Fgf8\) and \(Fgf24\), and the T-box genes \(spt\) and \(ntl\) are required for posterior mesoderm development in zebrafish. We tested this hypothesis more directly by asking if we could detect genetic interactions between the Fgf ligands and the T-box genes. We therefore constructed and analyzed \(fgf8\);\(ntl\) and \(spt;fgf8\) double mutants and used \(fgf24^{E3L}\) MO to create \(fgf24^{E3L};ntl\) and \(fgf24^{E3L};spt\) mutant embryos. In comparison with wild-type embryos (Fig. 6A), embryos single mutant for either \(fgf8\) (Fig. 6B) or \(ntl\) (Fig. 6D) produce significant amounts of paraxial mesoderm, as revealed by the expression of \(myod\) at the 12-somite stage (Reifers et al., 1998; Halpern et al., 1993). By contrast, we found that \(fgf8;ntl\) double mutants produced significantly less paraxial mesoderm than would have been expected from simple addition of their single mutant phenotypes (Fig. 6E). Similarly, in comparison with wild-type embryos (Fig. 6K).

*Fig. 5. Fgfs and T-box genes interact during posterior mesoderm development. Expression of \(ntl\) (purple) and \(Spt\) (brown) in 10-somite stage wild-type (A), \(fgf8\) mutant (B) and \(fgf8\);\(fgf24^{MO}\) embryos (C) reveals that \(fgf8\);\(fgf24^{MO}\) embryos no longer have mesodermal precursors that in wild-type (A) and \(fgf8\) mutants (B) are located in the tail bud (white asterisks) and presomitic mesoderm (arrows). In addition, analysis of these markers reveals that, at this stage, the tail buds of \(fgf8\) mutant embryos (B) contain significantly less presomitic mesoderm precursors (Spt-expressing cells) in comparison with wild-type embryos (A; see also supplemental Fig. S1 at http://dev.biologists.org/supplemental/), and in the posterior notochord have a gap in the \(ntl\) expression domain (arrowhead). Dorsal (upper) and vegetal (lower) views showing expression of \(ntl\) (D,E), \(Spt\) (F,G), \(fgf8\) (H-K) and \(fgf24\) (L-O) in mid-gastrula-stage (75-80% epiboly; 8.5 hpf) wild-type and mutant embryos (asterrisks and arrows indicate dorsal and ventral tissues, respectively). \(fgf8\);\(fgf24^{MO}\) embryos (E,G) have reduced expression of \(ntl\) and \(Spt\) in mesodermal precursors relative to wild-type embryos (D,F). Expression of \(fgf8\) in dorsal mesoderm is reduced in \(ntl\) (I), \(Spt\) (J) and \(spt;ntl\) (K) mutant embryos relative to wild-type embryos (H). \(fgf24\) expression is reduced dorsally in \(ntl\) embryos (M) ventrally in \(spt\) embryos (N) and dorsally and ventrally, but not laterally in \(spt;ntl\) embryos (O), relative to wild-type embryos (L). Scale bars: in A, 50 \(\mu\)m for A-C; in D, 100 \(\mu\)m for D-O.*
embryos mutant for either \(fgf8\) (Fig. 6L) or \(spt\) (Fig. 6M) produce significant amounts of axial mesoderm, as revealed by the expression of \(Ntl\) protein in the nuclei of notochord cells (Fig. 6I-K). By contrast, we found that \(spt;fgf8\) double mutants produce significantly less axial mesoderm than would have been expected from simple addition of their single mutant phenotypes (Fig. 6N). Fig. 6F-J,O-R give representative examples of live embryos at 24 hpf for each genotypic class (see Materials and methods for segregation frequencies). We did not observe significant differences in the amount of mesoderm produced by either \(fgf24^{MO};ntl\) or \(spt;fgf24^{MO}\) embryos when compared with \(ntl\) or \(spt\) single mutants, respectively (see supplemental Fig. S2 at http://dev.biologists.org supplemental/). These data provide direct evidence that \(fgf8\) genetically interacts with \(ntl\) and \(spt\) during the development of posterior mesoderm.

### ntl is a dominant enhancer of \(fgf8\)

When a pair of fish heterozygous for both \(ntl\) and \(fgf8\) (i.e. \(ntl^{+/−};fgf8^{+/−}\)), are mated, four phenotypic classes are expected [wild type (Fig. 6F), \(fgf8\) single mutant (Fig. 6G), \(ntl\) single mutant (Fig. 6I) and \(ntl;fgf8\) double mutant (Fig. 6J)] that segregate in the ratio of 9:3:3:1, respectively. In this cross, however, we observed that the \(fgf8\) single mutant class, which was distinguished by lacking the MHB but producing somites and a notochord, could be further sorted into two phenotypic subclasses based on tail length at 24 hpf, or by the amount of notochord produced when assayed for marker gene expression at the 12-somite stage; 1/3 of these embryos were indistinguishable from \(fgf8\) single mutants (Fig. 6B,G) while 2/3 produced only anterior notochord and had tails that were intermediate in length between \(fgf8\) single mutants and \(fgf8;ntl\) double mutants (Fig. 6C,H). Based on these segregation frequencies and the fact that both phenotypic classes produced notochord, we reasoned that the \(fgf8\) homozygotes that had short tails and reduced notochord development were heterozygous for the \(ntl\) mutation (i.e. \(fgf8^{+/−};ntl^{+/−}\)), whereas those with long tails were \(ntl\) homozygous wild type (i.e. \(fgf8^{+/−};ntl^{+/+}\)). We tested this hypothesis by crossing \(fgf8^{+/−};ntl^{+/−}\) double heterozygous animals to \(fgf8^{+/−}\) single heterozygotes. In this cross, 1/2 of the \(fgf8^{−/−}\) embryos will also be genotypically \(ntl^{+/−}\). Again, we found that we could divide the \(fgf8\) homozygotes into two phenotypic classes: 1/2 of the \(fgf8\) mutants segregating in this manner.
Fig. 7. fgf24 expression during later embryonic and larval development. In all panels, anterior is towards the left unless specified, and fgf24 expression is visualized in purple. (A-D) 12 hpf (six-somite stage), dorsal views. (A) fgf24 is expressed in the nasal placode (asterisk), otic placode (arrow), lateral mesoderm (arrowhead) and tail bud mesenchyme surrounding Kupffer’s vesicle. Expression of fgf24 in the otic placode was confirmed by co-labeling embryos with either krr20 (red), which labels rhombomere 3 (r3) and r5 (B) or pax2.1 (red), which labels the otic placode and the midhindbrain boundary (C). (D) fgf24 expression in lateral mesoderm (arrowhead) is in cells that lie adjacent and medial to those expressing pax2.1 (red). (E,F) 18 hpf. (E) fgf24 is expressed in nasal ectoderm and in a discrete domain of the retina (arrow, dorsal view). (F) Lateral view of fgf24-expressing cells in the posterior gut (arrow), in tail bud mesenchyme and in the caudal fin primordium (arrowhead). (G) 20 hpf, dorsal view. fgf24 expression in early pectoral fin bud mesenchyme (arrow), (H-J) 24 hpf. (H) fgf24 expression persists in the posterior gut (arrow) and caudal fin primordium (arrowhead), but is no longer detected in the tail bud (lateral view, yolk extension removed). (I) fgf24 is expressed in the pharyngeal endoderm (arrowheads) and in the pectoral fin bud mesenchyme (arrow), and in a posterior domain of the otic epithelium (not in focus). Inset in I shows sagittal section through the otic vesicle (outlined), showing more clearly the expression of fgf24 in the posterior otic epithelium (arrow) and pharyngeal endoderm (arrowheads). (J) Transverse section (dorsal upwards) showing fgf24 expression in fin bud mesenchyme (arrow) and gut (arrowhead). (K-O) 52 hpf. (K) At this stage, fgf24 is no longer expressed in pectoral fin bud mesenchyme, but instead is strongly expressed in the apical ectodermal ridge. (L) Lateral view of head showing fgf24 expression in the first and second pharyngeal pouches (pp1, pp2), and the posterior ectodermal margin (pem, arrow) of the second pharyngeal arch. A ventral view (M) shows fgf24 expression in all pharyngeal pouches (pp1 and pp2-6, small arrows), and the olfactory bulb. Additionally, fgf24 is expressed in tooth germs, which develop on only the most posterior (seventh) pharyngeal arch. (N) A close-up ventral view shows fgf24 expression in bilateral domains (arrowheads) adjacent to the lateral edges of the mouth. (O) fgf24 is expressed in the olfactory organ and the olfactory bulb (dorsal view, anterior is upwards), dis, distal; e, eye; kv, Kupffer’s vesicle; mhb, midhindbrain boundary; mo, mouth; nec, nasal ectoderm; nc, notochord; nt, neural tube; ob, olfactory bulb; olf, olfactory organ; op, otic placode; ov, otic vesicle; pem, posterior ectodermal margin; pem, presomitic mesoderm; pro, proximal; ret, retina; tb, tail bud; tg, tooth germ; ye, yolk extension. Scale bars: 100 μm in A,G; 50 μm in B-F,H-O.

cross were indistinguishable from fgf8 single mutants, while the other 1/2 had short tails and patchy notochord, identical to the animals in Fig. 6C,H (see Materials and methods for segregation frequencies). These data show that a loss-of-function ntl allele can dominantly enhance the phenotype of fgf8 mutant embryos, providing further support that ntl and fgf8 interact genetically.

fgf24 expression in later development

After the completion of gastrulation, fgf24 expression can be detected in a variety of tissues during somitogenesis and larval development. Expression of fgf24 in the tail bud mesenchyme can be detected in 12-18 hpf embryos (Fig. 7A,D,F), but it is no longer expressed in this domain at 24 hpf (Fig. 7H). fgf24 is expressed in the otic placode beginning around the two-somite stage (10.5 hpf, not shown) and is clearly visible at 12 hpf (Fig. 7A) as bilateral patches adjacent to rhombomere 5 (Fig. 7B). Co-labeling with the otic placode marker pax2.1 (Krauss et al., 1991) indicates that fgf24 is uniformly expressed in the otic placode at 12 hpf (Fig. 7C). Expression of fgf24 in the developing ear is dynamic and by 24 hpf is localized to a discrete domain in the posterior otic epithelium (Fig. 7I). In addition to the otic placode, fgf24 is expressed in anterior neuroectoderm at 12 hpf, in a location that has been fate mapped to form the olfactory placode (Fig. 7A) (Whitlock and Westerfield, 2000) and in 18 hpf embryos, expression can be seen in the forming nasal organs (Fig. 7E). Expression of fgf24 persists in the nasal organ through 52 hpf (the latest time point analyzed), at which point expression can also be detected in the olfactory bulbs (Fig. 7M,O). Last, at 12 hpf, fgf24
expression is detected in bilateral stripes of cells that appear to be located in lateral mesoderm (Fig. 7A).

We investigated the pectoral fin phenotype in more detail by analyzing skeletal preparations of 1-month-old wild-type and fgf24\textsuperscript{MO} fish stained with Alizarin Red and Alcian Green to visualize bone and cartilage, respectively. The skeleton of the paired pectoral fins in zebrafish consist of fin rays or lepidotrichia, that support the visible part of the fin, and a pectoral girdle located internally that provides support for the fin rays as well as articulation with the skull (Grandel and Schulte-Merker, 1998). We analyzed pectoral skeletal morphology in wild-type (Fig. 8C,D) and fgf24\textsuperscript{E3I3} morpholino-injected fish (E,F) shown in lateral (C,E) and ventral (D,F) views. Bone is stained red and cartilage blue. In wild-type fish, exoskeletal (cleithrum and postcleithrum) and endoskeletal (scapula, distal radials and lepidotrichs) components of the pectoral fin are visible (C). By contrast, only exoskeletal components can be identified in fgf24\textsuperscript{E3I3} injected fish (E), cl, cleithrum; dr, distal radials; lep, lepidotrichs; pcl, postcleithrum; sc, scapula. Scale bar: in A, 50 μm for A,B.

fgf24 is required for pectoral fin formation

We used the fgf24\textsuperscript{E3I3} MO to address the function of fgf24 in later development. As shown previously, fgf24\textsuperscript{MO} embryos at 24 hpf are morphologically indistinguishable from their control siblings (Fig. 3E,F). We therefore allowed fgf24\textsuperscript{MO} embryos to develop to various stages past 24 hpf, and assayed for morphological phenotypes. We found that at 33 hpf, fgf24\textsuperscript{MO} embryos were indistinguishable form their control sibling embryos, with the exception that they did not have visible pectoral fin buds, which are easily scored at this stage of development as discrete epidermal bumps on the dorsal yolk (not shown) (Kimmel et al., 1995; Grandel and Schulte-Merker, 1998), or by their expression of shh (Fig. 8A,B) (Krauss et al., 1993). Surprisingly, we found that injected embryos could survive to adulthood, but they never develop pectoral fins.
identified in wild-type (Fig. 8C), but not in \textit{fgf24}\textsuperscript{MO} fish (Fig. 8E). Thus, loss of \textit{fgf24} function appears to affect a very early stage of pectoral fin development. A more detailed analysis of the role of \textit{fgf24} in pectoral fin development is presented elsewhere (Fischer et al., 2003).

**Discussion**

We have described the identification and function of zebrafish \textit{fgf24}, a new member of the fibroblast growth factor (Fgf) 8/17/18 subfamily of signaling molecules. Our results show that \textit{fgf24} is expressed in posterior mesodermal precursors during gastrulation where it functions cooperatively with \textit{fgf8} to promote mesodermal development, in part by maintaining the expression of the mesodermal T-box genes \textit{ntl} and \textit{spt}. We have presented double mutant analyses that reveal genetic interactions between the T-box genes and Fgf signaling. These results provide compelling evidence that these genes function in a genetic pathway that promotes posterior mesodermal development in zebrafish. Last, we have shown that \textit{fgf24} is expressed in a wide variety of tissues after gastrulation, including the early fin bud mesenchyme, and is required for an early stage of pectoral fin bud development.

\textit{fgf24} and its relationship to the \textit{fgf8/17/18} subfamily of Fgf ligands

With the addition of \textit{fgf24}, at least 22 distinct Fgf-encoding genes have been identified in vertebrates (human \textit{FGF19} and mouse \textit{Fgf15} may be orthologous genes). Based on sequence relatedness, the Fgf superfamily can be subdivided into seven subfamilies of more closely related genes (reviewed by Ornitz and Itoh, 2001). The genes encoding the ligands \textit{Fgf8}, \textit{Fgf17}, \textit{Fgf18}, and \textit{Fgf24} define one such subfamily and mouse members of this subfamily have been shown to have very similar Fgf receptor specificity profiles (Xu et al., 2000). It is therefore likely that in zebrafish \textit{Fgf8} and \textit{Fgf24} have similar activities.

Because Fgf24 so far appears to be unique to zebrafish, it is necessary to consider its origin. There is increasing evidence that a whole-genome duplication event occurred in the ray-finned fish lineage after it diverged from the terrestrial vertebrate lineage (Amores et al., 1998; Prince et al., 1998; Postlethwait et al., 2000). It is therefore possible that a single ancestral gene, following two sequential duplication events, gave rise to the four members of the \textit{Fgf8} subfamily. A similar hypothesis has been proposed for the origin of the four tetrapod Hox clusters (discussed by Furlong and Holland, 2001). In support of this model, a probable \textit{fgf24} ortholog has been identified in a shark (D.W.S., unpublished), arguing that \textit{fgf24} arose early in gnathostome (jawed vertebrate) evolution. It is therefore likely that an \textit{fgf24} ortholog was lost at some point in the terrestrial vertebrate lineage after its divergence from ray-finned fishes. Similar examples of lineage-specific gene loss have already been described, including the loss of functional copies of the \textit{hox} paralogs \textit{hoxb10}, \textit{hoxc1} and \textit{hoxc3} in the mammalian lineage, but not in zebrafish (Amores et al., 1998; Prince et al., 1998; Postlethwait et al., 1998).

**Fgf8 and Fgf24 are components of the Fgf signaling pathway that is required for posterior mesoderm development in zebrafish**

Our results show that \textit{fgf8} and \textit{fgf24} are components of the Fgf signaling pathway that regulates posterior mesoderm development in zebrafish. We found that \textit{fgf8} and \textit{fgf24} are expressed in mesodermal precursors and that \textit{fgf8} : \textit{fgf24}\textsuperscript{MO} embryos produce very little posterior mesoderm. Although the function of \textit{fgf8} and \textit{fgf24} can account for much of the Fgf signaling activity that is known to be required for posterior mesoderm development in zebrafish, we observed that \textit{fgf8} - \textit{fgf24}\textsuperscript{MO} embryos produce significantly more mesoderm than do embryos overexpressing the dn\textit{Fgfr} (Griffin et al., 1995). Because the dn\textit{Fgfr} is likely to block all Fgf signaling in early embryos (Ueno et al., 1992), ligands in addition to \textit{Fgf8} and \textit{Fgf24} are likely to contribute to early mesoderm formation in zebrafish. In addition to \textit{fgf8} and \textit{fgf24}, \textit{fgf3} is the only other Fgf gene in zebrafish that is known to be expressed in mesodermal precursors during gastrulation (Fürthauer et al., 2001). Although \textit{fgf3} may account for some of the Fgf activity present in \textit{fgf8} - \textit{fgf24}\textsuperscript{MO} embryos, it is not likely to account for all; injection of \textit{fgf3 MO} (Maves et al., 2002) into \textit{fgf8} - \textit{fgf24}\textsuperscript{MO} embryos does not appear to decrease the amount of posterior mesoderm produced relative to \textit{fgf8} - \textit{fgf24}\textsuperscript{MO} embryos alone (L. Maves and B.W.D., unpublished).

We cannot rule out the possibility that the mesoderm produced by \textit{fgf8} - \textit{fgf24} \textsuperscript{MO} embryos is due to residual activity of \textit{fgf8} and/or \textit{fgf24} in these embryos. The single \textit{fgf8} allele that has been isolated, \textit{fgf8}\textsuperscript{1282} (Reifers et al., 1998), is likely to be a hypomorph (Draper et al., 2001). However, using \textit{fgf8} MOs, which reduce the expression of functional \textit{fgf8} below the level produced by the \textit{fgf8} mutation (Draper et al., 2001), in combination with the \textit{fgf24} MO, does not appear to increase the severity of the phenotype relative to the \textit{fgf8} - \textit{fgf24}\textsuperscript{MO} embryos (B.W.D., unpublished). Similarly, it is possible that our \textit{fgf24} MO does not completely eliminate \textit{fgf24} function, although our RNase protection results argue against this. Last, \textit{fgf8} (Reifers et al., 1998; Draper et al., 2001), \textit{fgf24} and \textit{fgf18} (this study) are expressed maternally and these maternal mRNAs persist for several hours after
It is therefore possible that sufficient amounts of Fgf protein are produced from wild-type maternal transcripts to allow partial mesoderm development in the absence of zygotic fgf8 and fgf24 function. As only a few orthologs of the known vertebrate Fgf ligands have been identified in zebrafish, it remains to be seen how many other ligands participate in posterior mesodermal development.

**Fgf8 and Fgf24 maintain spt and ntl expression during posterior development**

Current models for how Fgfs and T-box genes interact during mesodermal development have proposed that they form an auto-regulatory feedback loop, where the function of one component maintains the expression of the other (reviewed by Isaacs, 1997). Although it is not yet clear how Fgf signaling regulates T-box gene expression, there is evidence in *Xenopus* that *Xbra*, the ortholog of *ntl*, can directly regulate the expression of embryonic (e)Fgf, an Fgf4 ortholog (Casey et al., 1998). This model predicts that wild-type expression patterns of *fgf8* and *fgf24* should require *ntl* and *spt* function, and indeed we found this to be true. However, *ntl* and *spt* can not be the only regulators of *fgf8* and *fgf24* expression during early mesoderm formation, as expression of *fgf8* and *fgf24* persist in the germ ring of early *spt*;*ntl* double mutant embryos. In addition to *spt* and *ntl*, the *spt*-related gene *tbx6* is also expressed in mesodermal precursors during gastrulation (Hug et al., 1997). *tbx6* is unlikely to contribute to Fgf regulation in the absence of *spt* and *ntl* function, however, because it is not expressed in *spt*;*ntl* double mutants (Griffin et al., 1998).

The expression patterns we observed for *fgf8* and *fgf24* in wild-type embryos and in embryos mutant for either *ntl* or *spt* suggest that their expression in the germ ring is not regulated by an identical genetic network. First, the expression patterns of *fgf8* and *fgf24* in wild-type embryos, while overlapping, are not identical. We found that cells expressing the highest levels of *fgf8* localize to the epiblast layer (similar to *ntl*), whereas those expressing the highest levels of *fgf24* localize to the hypoblast layer (similar to *spt*). As might be expected from these expression patterns, expression of *fgf8* and *fgf24* also have non-identical requirements for *ntl* and *spt* function. However, we did not observe a simple one-to-one correlation between an Fgf expression domain and a T-box gene. Instead, we found that the expression of *fgf8* in dorsal mesodermal precursors requires both *ntl* and *spt* function, while neither gene was required for *fgf8* expression in ventral precursors. By contrast, expression of *fgf24* in dorsal mesodermal precursors requires *ntl*, but not *spt*, whereas ventral expression requires *spt*, but not *ntl*. Although it is not possible at present to derive an accurate pathway that explains the regulatory relationships that exist between these Fgfs and T-box genes, our data are consistent with the proposed feedback loop because we have found that reduction of Fgf signaling leads to a reduction of T-box gene expression and vice versa.

**fgf8 genetically interacts with ntl and spt**

Data supporting the model that posterior development is promoted by a regulatory network between Fgfs and T-box genes has come largely from analyzing gene expression defects in single mutant embryos (e.g. Yamaguchi et al., 1994; Deng et al., 1994; Sun et al., 1999) or in embryos overexpressing single network components (e.g. Isaacs et al., 1994; Schulte-Merker and Smith, 1995). We have provided genetic evidence that directly links Fgf signaling and T-box gene function in a genetic pathway that promotes posterior development. We have shown that *fgf8*;*ntl* and *spt*;*fgf8* double mutants had phenotypes that were more severe than would be expected from the simple addition of either single mutant phenotype. For example, neither *fgf8* nor *ntl* has severe defects in trunk somite formation, as assayed by myod expression, whereas *fgf8*;*ntl* double mutants produce few myod-positive cells. Because trunk somite formation is known to require *spt* function cell-autonomously (Ho and Kane, 1990), we propose that the muscle phenotype observed in *fgf8*;*ntl* embryos results from attenuated *spt* function. Similarly, we found that *spt*;*fgf8* double mutant embryos appear to have attenuated *ntl* function as notochord development was reduced in double mutant embryos, but not in *spt* or *fgf8* single mutant embryos. These results indicate that *fgf8* cooperates with *ntl* to maintain *spt* function, and similarly with *spt* to maintain *ntl* function.

It is interesting that the expression of *pax2.1*, which marks the developing pronephric tubules (Krauss et al., 1991), is largely unaffected in either *fgf8*;*ntl* or *spt*;*fgf8* mutants. Pronephric tubules develop from intermediate mesoderm and *spt* and *ntl* are redundantly required for their formation (Amacher et al., 2002). It is possible that pronephric development requires lower levels of T-box gene activity relative to that required for the development of the notochord and somites. Alternatively, Fgf8 signaling may promote the expression of dorsal-specific factors that function in combination with *ntl* and *spt* to promote the development of dorsal mesodermal derivatives, such as notochord and somites, but not the development of more intermediate derivatives, such as pronephros. In support of this, *fgf8* is expressed at higher levels in dorsal mesoderm than ventral mesoderm and *fgf8* overexpression can strongly dorsalize early zebrafish embryos (Fürthauer et al., 1997).

In addition to the interactions described above, we found that *ntl* mutations dominantly enhance the phenotype of *fgf8* homozygotes: *fgf8*;−/−;*ntl*+/- embryos produced less posterior mesoderm than *fgf8*;−/−;*ntl*+/- embryos. Because *ntl* heterozygotes alone are phenotypically wild type, this result suggests that *ntl* function is attenuated in *fgf8* single mutants, and consistent with this, we found that a third of the *fgf8* single mutants have reduced *ntl* expression in axial mesoderm. These results imply that in the absence of *fgf8* function, *fgf24* function is not sufficient to maintain wild-type levels of *ntl* activity. Interestingly, *T* null mutations in mice, but not in zebrafish, are semi-dominant as *T*+/- heterozygotes have shorter tails than do wild-type mice (Dobrovolskaïa-Zavadskaï, 1927). Because wild-type expression of *T* in mouse mesodermal precursors is known to be dependent on Fgf8 function (Sun et al., 1999), it is possible that the apparent differences between the phenotypes of *T*/*ntl* heterozygotes in mice and zebrafish are due simply to differences in the quantitative levels of Fgf signaling in posterior tissue between these two organisms. In contrast to dominant interactions between *ntl* and *fgf8*, we could not find evidence that reduction of *spt* function could dominantly enhance the phenotype of *fgf8* mutant embryos, suggesting that the interactions between *fgf8* and *ntl* are stronger than those between *fgf8* and *spt*. Genetic interactions between the Fgf signaling pathway and T-box transcription factors is becoming a common theme in...
vertebrate development, as similar interactions have been proposed to play key roles in development of limbs (Ng et al., 2002) the cardiovascular system (Vitelli et al., 2002) and lungs (Cebra-Thomas et al., 2003).

**Fgf24 is required for pectoral fin development**

Last, we have shown that fgf24 expression is not restricted to developing posterior mesoderm, but is also expressed in a wide variety of tissues during larval growth. However, the only defect we could identify in fgf24 MO embryos was in the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).

We thank Thierry Lepage and David Kimelman for generously sharing their cDNA library; Karen Larison and Jewel Parker for expert sectioning assistance; Brian Summers for excellent technical assistance; Angel Amores for help with PAC library screening; Craig Miller for assistance identifying expression domains in larva and for assistance; Angel Amores for help with PAC library screening; Craig Miller for sectioning assistance; Brian Summers for excellent technical phenotype of skeletal elements of the external pectoral fin. Thus, the development of pectoral fins. We found that fgf24 MO fish never produced a morphological fin bud, nor did they produce any skeletal elements of the external pectoral fin. Thus, the phenotype of fgf24 appears similar to that of mice mutant for either fgf10 (Min et al., 1998; Sekine et al., 1998) or its likely receptor, Fgfr2 (Xu et al., 1998).


