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The limb identity gene *Tbx5* promotes limb initiation by interacting with *Wnt2b* and *Fgf10*

Jennifer K. Ng[1,*], Yasuhiko Kawakami[1,*], Dirk Büscher[1,*], Ángel Raya[1,*], Tohru Itoh[1], Christopher M. Koth[1], Concepción Rodríguez Esteban[1], Joaquín Rodríguez-León[2], Deborah M. Garrity[3], Mark C. Fishman[3] and Juan Carlos Izpisúa Belmonte[1,†]

[1]The Salk Institute for Biological Studies, Gene Expression Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037-1099. USA
[2]Instituto Gulbenkian de Ciencia, Rua Da Quinta Grande n 6, 2780-901 Oeiras, Portugal
[3]Cardiovascular Research Center, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: belmonte@salk.edu)

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SUMMARY

A major gap in our knowledge of development is how the growth and identity of tissues and organs are linked during embryogenesis. The vertebrate limb is one of the best models to study these processes. Combining mutant analyses with gain- and loss-of-function approaches in zebrafish and chick embryos, we show that *Tbx5*, in addition to its role governing forelimb identity, is both necessary and sufficient for limb outgrowth. We find that *Tbx5* functions downstream of WNT signaling to regulate *Fgf10*, which, in turn, maintains *Tbx5* expression during limb outgrowth. Furthermore, our results indicate that *Tbx5* and *Wnt2b* function together to initiate and specify forelimb outgrowth and identity. The molecular interactions governed by members of the *T-box*, *Wnt* and *Fgf* gene families uncovered in this study provide a framework for understanding not only limb development, but how outgrowth and identity of other tissues and organs of the embryo may be regulated.

Key words: T-box, *Tbx5*, *Wnt2b*, *Fgf10*, Pectoral fin, Limb, Zebrafish, Chick, *heartstrings*

INTRODUCTION

Limb outgrowth and limb identity (forelimb versus hindlimb) begins very early in development with the establishment of a special group of cells termed the limb field (Harrison, 1918). As development proceeds, precisely positioned limb buds appear, opposite each other, and at very distinct trunk levels, as a result of the coordinated proliferation of cells derived from the lateral plate mesoderm (LPM) (reviewed by Johnson and Tabin, 1997; Tickle, 1999; Capdevila and Izpisua Belmonte, 2001). While the molecular mechanisms responsible for the initiation, positioning and identity of the limb field are still largely unknown, in the last few years several signaling molecules and transcription factors have been shown to be critical for these processes. Members of the fibroblast growth factor (FGF) family play central roles in limb initiation. When misexpressed in the LPM in a localized manner, several FGFs are capable of inducing an ectopic limb (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997). Furthermore, disruption of the mouse *Fgf10* gene, specifically expressed in the LPM before any visible outgrowth has occurred (Ohuchi et al., 1997), results in complete loss of limbs (Min et al., 1998; Sekine et al., 1999). In addition to FGF, we have recently demonstrated that different members of the WNT family are differentially expressed in the LPM before limb outgrowth. Specifically, *Wnt2b* is expressed in the presumptive forelimb region while *Wnt8c* is expressed in the presumptive hindlimb region. Both WNT2b and WNT8c are capable of inducing *Fgf10* and, subsequently, limb outgrowth (Kawakami et al., 2001).

Despite the orchestrated interactions needed between outgrowth and identity during development of tissues and organs, these phenomena, for ease of analysis, are often treated as distinct processes. The decision to become either a forelimb or a hindlimb is also made at the earliest stages of limb initiation. Several studies suggest the intriguing possibility that limb identity could be determined by the specific expression of a single transcription factor. Two members of the T-box family of transcription factors, *Tbx4* and *Tbx5* (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Tamura et al., 1999; Begemann and Ingham, 2000) (reviewed by Ohuchi and Noji, 1999), and a member of the OTX-related subclass of paired-type homeodomain proteins, *Pitx1* (Lanctot et al., 1997; Logan et al., 1998), show a restricted forelimb and hindlimb distribution in the LPM prior to limb budding. *Tbx5* transcripts are expressed in the presumptive forelimb area in all tetrapods studied so far, and conversely, *Tbx4* and *Pitx1* transcripts are restricted to the

presumptive hindlimb region. Furthermore, gain- and loss-of-function experiments in chick and mice lend support to the idea of limb identity being determined by a discrete set of molecular determinants (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999a; Szeto et al., 1999; Takeuchi et al., 1999).

The co-localization of factors shown to be capable of inducing limb outgrowth (WNT2b and WNT8c) and factors involved in limb identity (Tbx5 and Tbx4), raises the question of whether both processes are linked or take place independently of one another. In this study, we combine gain- and loss-of-function approaches in both zebrafish and chick embryos to analyze the molecular relationships occurring among *Fgf10*, *Wnt2b* and *Tbx5* during initiation and specification of forelimb identity. Our results demonstrate that, in addition to its role governing forelimb identity, *Tbx5* is both necessary and sufficient for forelimb initiation. We also demonstrate that *Tbx5* is directly upstream of *Fgf10* in the signaling cascade that directs limb outgrowth. In turn, a feedback loop is uncovered in which FGF signaling is required to maintain *Tbx5* expression. This study also extends our previous results in chick, showing that *wnt2b* is necessary for pectoral fin initiation in zebrafish. Finally, our results indicate that two key events of limb development, namely limb identity determination and limb initiation are not independent processes, and that *Tbx5* and *Wnt2b* function together to initiate and specify forelimb identity. Altogether, our results unveil the existence of a complex network of molecular interactions that establishes, propagates and maintains the expression of signaling molecules and transcription factors responsible for limb outgrowth and identity.

MATERIALS AND METHODS

Cloning of zebrafish *fgf10* and *wnt2b* genes

To isolate the zebrafish orthologue of *Fgf10*, we screened zebrafish genomic and 24 hpf cDNA λ phage libraries (Stratagene) under low stringency using chick *Fgf10* cDNA as a probe. Several independent clones were obtained and sequenced. The zebrafish *wnt2b* gene was similarly obtained except that chick *Wnt2b* cDNA was used as a probe.

Morpholino injections

Morpholino oligonucleotides were designed by and obtained from GeneTools LLC (Eugene, Oregon). The zebrafish *tbx5* morpholino lies from nucleotide position –5 to +18:

5′-GGTGCTTCACTGTCCGCCATGTCG-3′.

The zebrafish *wnt2b* morpholino sequence lies from nucleotide position –66 to –43 and targets the 5′ UTR:

5′-AAGTCACTAGATCATTGCAGTTCT-3′.

The standard control oligonucleotide available from Gene Tools was used. The morpholinos were solubilized in 1× Danieau's solution and injected into one-cell stage zebrafish embryos at a range of 2-10 ng/embryo.

heartstrings^{m21} (*hst*) mutant lines

The *hst* mutant was identified in an ENU-induced mutagenesis screen for perturbation of cardiac function in zebrafish. The mutation has been mapped to the *tbx5* gene and introduces a nonsense mutation at codon 316 (Garrity et al., 2002).

RNA injections

The open reading frame, excluding the 5′ and 3′ untranslated sequences, of zebrafish *fgf10*, *wnt2b* and *tbx5* were cloned into the pCS2 vector. Capped RNAs were synthesized from these constructs using the mMessage mMachine kit (Ambion). Seventy picogram of *fgf10* mRNA and 100 pg of *tbx5* and *wnt2b* mRNA was injected into one-cell stage zebrafish embryos.

Whole-mount in situ hybridization and Alcian Blue cartilage staining

Injected zebrafish embryos were scored for pectoral fin phenotypes at 30 hours post-fertilization (hpf) to 5 days post-fertilization (dpf) using a stereomicroscope. Further analysis was conducted at 24-48 hpf by whole-mount in situ hybridization, as described previously (Hammerschmidt et al., 1996), and at 5 dpf by Alcian blue staining as described previously (Schilling et al., 1996). Zebrafish riboprobes used were *fgf8* (Furthauer et al., 1997), *tbx5* (Tamura et al., 1999) and *shh* (Ekker et al., 1995). The zebrafish *fgf10* riboprobe spans the entire ORF, and the *wnt2b* riboprobe contains 900 bp of the coding sequence corresponding to the N-terminal region. Viral injected and bead implanted chick embryos were examined by whole-mount in situ hybridization and Alcian Blue cartilage staining as described by Vogel et al. (Vogel et al., 1996). For the zebrafish studies, a minimum of 100 embryos was examined for each in situ hybridization.

Viral production and injections into chick embryos

Adenovirus expressing the mouse *Axin* gene was produced and injected as previously described (Kawakami et al., 2001). The full-length mouse *Tbx5* cDNA and a truncated form of chick *Tbx5* (amino acids 62-521), lacking the N-terminal region upstream of the T-box, were cloned into an *RCAS* retroviral vector to produce *RCAS-Tbx5* and *RCAS-Tbx5ΔN* constructs, respectively. Subsequent transfection into chick embryonic fibroblasts and retroviral production were performed as described previously (Vogel et al., 1996). *RCAS-Tbx5* was injected into stage 5-8 chick embryos in the LPM. *RCAS-Tbx5ΔN* was injected into stage 8-10 chick in the LPM. Staging of chick embryos was according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). An *RCAS-alkaline phosphatase* virus was injected as a control and no phenotypic changes in gene expression or limb morphology were observed.

Bead implantation

Beads soaked with the FGF receptor tyrosine kinase inhibitor SU5402 (Calbiochem), used at 1 mg/ml in DMSO, were implanted into stage-14 to -18 chick embryos as described previously (Rodriguez Esteban et al., 1999b). Control beads were implanted at the same stage and no change in gene expression was observed.

RESULTS

Tbx5 is necessary for limb initiation

We, and others, have previously shown that the T-box genes *Tbx5* and *Tbx4* play key roles in specifying the identity of the vertebrate forelimb and hindlimb, respectively (Rodriguez-Esteban et al., 1999a; Takeuchi et al., 1999). The early onset of expression of *Tbx* genes in the LPM of the presumptive limb field suggests additional roles for these factors in early limb development. To further our understanding of the role of *Tbx* genes in limb development, we performed loss-of-function experiments in zebrafish by using morpholino antisense oligonucleotides injected into one-cell stage zebrafish embryos. We have focused on *tbx5* since the initiation of hindlimb structures, or pelvic fins, and concomitant *tbx4* expression in zebrafish embryos, does not take place until one month after fertilization (Tamura et al., 1999). While injection of a control morpholino at the one-cell stage gave no obvious phenotype (Fig. 1A,C), injection of a *tbx5* morpholino gave a range of

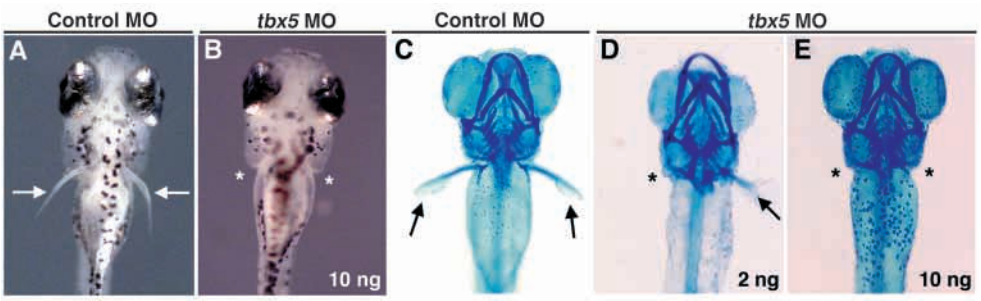

Fig. 1. Zebrafish *tbx5* is required for pectoral fin initiation and outgrowth. All panels show the dorsal view of zebrafish embryos with anterior to the top. (A-E) Five dpf zebrafish embryos injected with control (A,C) or *tbx5* (B,D,E) morpholinos. Embryos with 10 ng of *tbx5* morpholino lack pectoral fins (B, asterisk) and Alcian Blue cartilage staining (E, asterisk), unlike control-injected embryos (A,C, arrows). Injection of a lower dose (2 ng, as in D) results in a milder fin phenotype such as a reduced pectoral fin blade (arrow in D).

altered fin phenotypes depending on the amount injected. With the highest non-toxic levels of injected morpholino (10 ng), the most common phenotype was complete loss of pectoral fins (95%, *n*=250; Table 1; Fig. 1B). Cartilage staining confirmed that these embryos lacked pectoral fin structures and the shoulder girdle (Fig. 1E). Milder phenotypes, including shorter pectoral fins, reduced number of rays and sometimes upturned orientation of the pectoral fins, were obtained by decreasing the amount of injected morpholino (to a minimum of 2 ng; Fig. 1D). *tbx5* morphants also exhibited an enlarged pericardium and a stretched heart tube that failed to loop (data not shown).

The results of the *tbx5* loss-of-function experiments suggest that, in addition to its role in controlling limb identity, *tbx5* plays a role in limb initiation and outgrowth. The range of phenotypes obtained also indicates that the function of *tbx5* is dosage dependent. These results are in agreement with the recent Ahn et al. study which also showed that *tbx5* is required for pectoral fin formation (Ahn et al., 2002).

The FGF family of signaling molecules is known to be required and sufficient for limb initiation (reviewed by Martin, 1998; Martin, 2001). Given the known role of T-box proteins as transcriptional regulators, we reasoned that *tbx5* might function by regulating the expression of *fgf10*. To start addressing this, we screened several zebrafish cDNA libraries using chick *fgf10* as a probe and cloned zebrafish *fgf10* (Fig. 2A). As has been previously reported in mouse and chick embryos (Ohuchi et al., 1997), at 24 hpf, prior to initial budding of the pectoral fins, zebrafish *fgf10* can be detected in the LPM of the presumptive pectoral fin field, in a pattern temporally and spatially similar to *tbx5* (compare Fig. 2B,C). In later stages, zebrafish *fgf10* is observed in the mesenchyme of the pectoral fin buds, overlapping *tbx5* expression (compare Fig. 2D,F with 2E,G). Expression is also observed in the branchial arches, otic vesicle, heart primordium and tail bud (data not shown), as in the corresponding structures of other vertebrates (Ohuchi et al., 1997).

Given the aforementioned *tbx5* loss-of-function phenotypes, we decided to examine the expression of mesodermal [*fgf10* and *sonic hedgehog* (*shh*)] and ectodermal (*fgf8*) markers involved in the early stages of limb development. As shown in

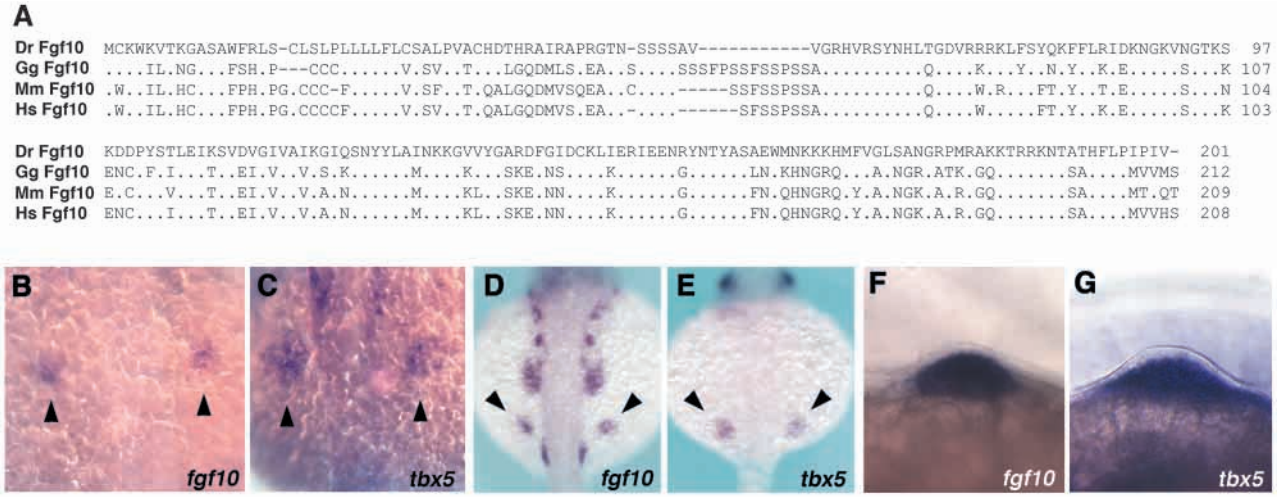

Fig. 2. Amino acid sequence alignment of FGF10 proteins and comparison of *fgf10* and *tbx5* expression in the zebrafish embryo. (A) Alignment of deduced FGF10 protein sequences from zebrafish (*Danio rerio*, Dr), chick (*Gallus gallus*, Gg), mouse (*Mus musculus*, Mm), and human (*Homo sapiens*, Hs). Identical amino acids are indicated by dots, and gaps by dashes. The zebrafish sequence shows homology to that of chick (44.7%), mouse (42.7%) and human (41.7%). The GenBank accession number for zebrafish *fgf10* is AF544025. (B-G) All panels show the dorsal view of zebrafish embryos with anterior to the top, excluding F and G. Whole-mount in situ hybridization staining of wild-type zebrafish embryos during pectoral fin development. *fgf10* (B,D,F) and *tbx5* (C,E,G) appear to be expressed in a similar spatial and temporal pattern during pectoral fin initiation and outgrowth. (B,C) *fgf10* and *tbx5* are expressed in the LPM of the presumptive pectoral fin bud (arrowheads) at 24 hpf. (D,E) At 30 hpf *fgf10* is expressed in the branchial arches, otic vesicle, and pectoral fin bud (arrowheads), while *tbx5* is expressed in the dorsal eye, heart tube and pectoral fin bud (arrowheads). (F,G) Lateral view of pectoral fin buds of 36 hpf embryos showing *fgf10* and *tbx5* expression throughout the mesenchyme. Anterior is to the left.

Table 1. Lack of pectoral fins in injected embryos

Injection sample (ng/embryo)	*n*	% of embryos lacking pectoral fins
tbx5 MO (10.0)	250	95
tbx5 MO (5.3)	60	82
tbx5 MO (2.0)	90	50
wnt2b MO (10.0)	230	75
wnt2b MO (5.0)	60	43
wnt2b MO (2.0)	80	30
wnt2b MO (10.0) +*wnt2b* RNA (0.1)	60	30
wnt2b MO (10.0) +*tbx5* RNA (0.1)	180	57

MO, morpholino.

Wild-type zebrafish embryos were injected with *tbx5* or *wnt2b* morpholinos and scored for the absence of pectoral fins at 5 dpf. In rescue experiments, morpholinos and capped RNA were co-injected into embryos and also scored for fin phenotypes.

Fig. 3A,B, injection of *tbx5* morpholino, but not control morpholino, resulted in complete loss of *fgf10* expression in the pectoral fin bud forming region of the LPM at 26 hpf, while expression in other regions remained unaffected. We could also not detect *shh* (data not shown) or *fgf8* (a marker for the apical fold; compare Fig. 3C with 3D).

To complement the morpholino studies, we made use of the novel zebrafish *heartstrings* (*hst*) mutant. The *hst* mutation has been very recently mapped to a point mutation in the open reading frame of zebrafish *tbx5*, and generates an early terminated protein (Garrity et al., 2002). As with *tbx5* morphant embryos, homozygous *hst* embryos do not develop pectoral fins. We examined the expression of *fgf10* in *hst* mutant embryos, at the early stages of fin development, and observed a complete loss of expression (Fig. 3E). We have also examined whether the *Fgf10* gene is a direct target of Tbx5 in vitro. A putative Brachyury/Tbx5 binding site is located approximately 400 base pairs upstream of the start site that is conserved in both human and mouse *Fgf10* genes. Deletion of this site results in complete loss of activation of the *fgf10* promoter by Tbx5, indicating that Tbx5 has the capacity to directly regulate *fgf10* expression (data not shown). Taken together, our data indicate that *tbx5* is required for fin initiation and suggest that it functions upstream of the FGF pathway that directs the early stages of fin outgrowth.

wnt2b is necessary for limb initiation and regulates *tbx5*

We recently reported that the WNT signaling pathway is required for normal limb development in chick embryos, regulating very early stages of limb induction (Kawakami et al., 2001). We demonstrated that *Wnt2b*, which is expressed in the LPM of the forelimb field, regulates limb initiation through induction of *Fgf10*. Given the lack of *Fgf10* expression in the *Tbx5* loss-of-function experiments, we hypothesized that *Tbx5* might interact with the WNT signaling pathway. To address this issue, we cloned the zebrafish *wnt2b* homologue and performed loss-of-function experiments.

We screened several zebrafish genomic and cDNA libraries using chick *Wnt2b* as a probe and obtained a putative *Wnt2b* clone with a full-length open reading frame. Sequence comparison demonstrated that our positive clones represented zebrafish *wnt2b* (Fig. 4A). As reported in chick embryos (Jasoni et al., 1999; Kawakami et al., 2001), at 22 hpf, zebrafish *wnt2b* is expressed in the developing eye, and a localized mesodermal region medial to the LPM at the somite level where the pectoral fin buds will form (Fig. 4B-D). In later stage embryos, when the fin bud started to form, we could no longer detect *wnt2b* (data not shown). Thus, the expression pattern of zebrafish *wnt2b* appears to be conserved between zebrafish and chick embryos.

wnt2b loss-of-function experiments were performed using a *wnt2b* morpholino to downregulate endogenous *wnt2b* (10 ng per injection). This loss-of-function study resulted in a large portion (75%, *n*=230; Table 1) of embryos that lacked pectoral fins (Fig. 5B), indicating that *wnt2b* plays an important role during pectoral fin development. Cartilage staining confirmed that these embryos lacked pectoral fin structures and the shoulder girdle (Fig. 5E). By reducing the amount of *wnt2b* morpholino injected (to a minimum of 2 ng), we were able to obtain a broad range of fin phenotypes very similar to those described for the *tbx5* morphants (data not shown). In 5 dpf *wnt2b* morphants, structures other than the pectoral fins developed similarly to wild type embryos (Fig. 5A,B,D,E). Thus, we exclude the possibility that the phenotypes observed were caused by general developmental arrest. These results indicate that the function of *wnt2b* is necessary for normal fin initiation, and is affected by gene dosage. Embryos injected with a control morpholino did not display any obvious abnormal phenotype (Fig. 5A,D).

To confirm the specificity of the morpholino antisense

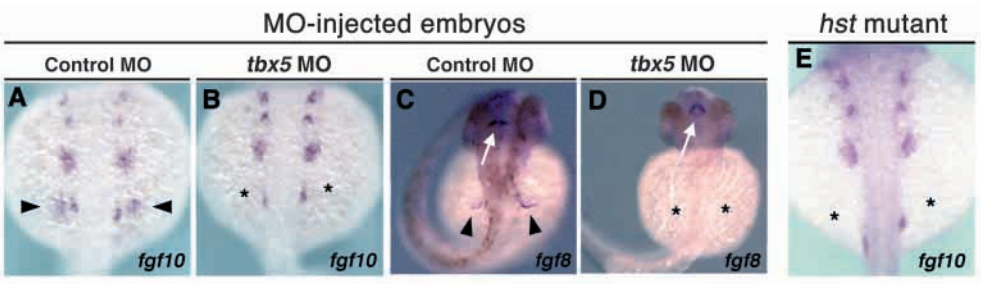

Fig. 3. Zebrafish *tbx5* is necessary for *fgf10* and *fgf8* expression in the pectoral fin budding region. All panels show the dorsal view of zebrafish embryos with anterior to the top. (A-D) Comparison of *fgf10* and *fgf8* expression patterns in embryos injected with 10 ng of control (A,C) or *tbx5* (B and D) morpholino. (A and B) *fgf10* expression in the pectoral fin bud region of 26 hpf (A, arrowheads) was not detected in the *tbx5*-morpholino injected embryo (B, asterisks). Note that expression remains unchanged in other regions of the embryo. (C and D) In 36 hpf *tbx5*-morpholino-injected embryos, *fgf8* expression could not be detected in the region where the pectoral fin buds develop (D, asterisks), as compared to the control embryos (C, arrowheads point to the expression in the apical fold). *fgf8* expression in the midbrain-hindbrain boundary remains unaltered in the injected embryos, as noted by the arrows. (E) *fgf10* was not detected in the pectoral fin bud region of 24 hpf hst mutants (asterisks), but remained unaltered in other regions.

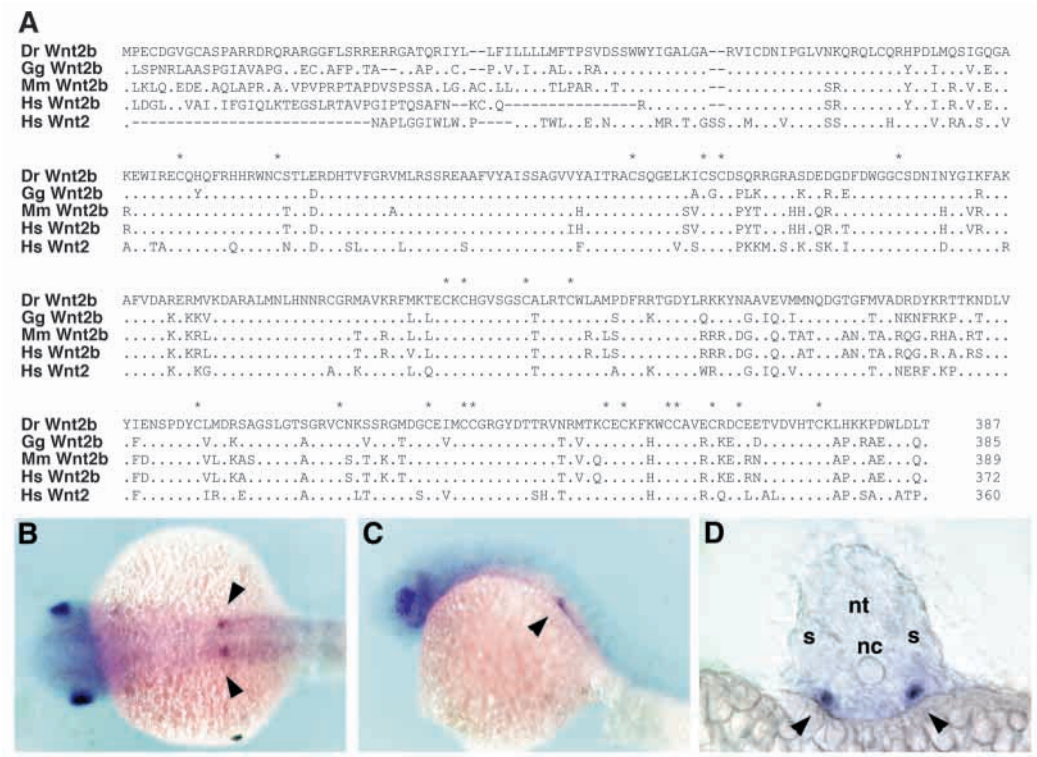

Fig. 4. Amino acid sequence alignment of WNT2b proteins and expression of zebrafish *wnt2b* in the zebrafish embryo. (A) Alignment of zebrafish (*Danio rerio*, Dr), chick (*Gallus gallus*, Gg), mouse (*Mus musculus*, Mm), and human (*Homo sapiens*, Hs) deduced WNT2b amino acid sequences, in addition to that of human WNT2. Identical amino acids are indicated by dots, and gaps by dashes. Asterisks indicate conserved cysteine residues among the WNT family members. The zebrafish Wnt2b sequence shows homology to that of chick (71.4%), mouse (61.2%), and human WNT2b (61.0%) and human WNT2 (63.0%). The presence of a 17 amino acid stretch just after the initiation methionine residue, which is characteristic of WNT2b but not WNT2, indicates that our clone encodes zebrafish *wnt2b*. The GenBank accession number for zebrafish *wnt2b* is AF544026. (B and C) Whole-mount in situ hybridization of 22 hpf zebrafish embryos showing *wnt2b* expression from a dorsal view (B) and a lateral view (C). Expression can be detected in the tissue medial to the LPM at the somite level where the pectoral fin buds will form (arrowheads) and the developing eye. Anterior to the left. (D) Cross section of 22 hpf zebrafish embryo at the pectoral fin bud forming level. *wnt2b* is expressed ventral to the somites and medial to the pectoral fin-budding region of the LPM. nt, neural tube; s, somite; nc, notochord.

experiments, we attempted to rescue the *wnt2b* loss-of-function phenotypes by co-injecting *wnt2b* morpholino with *wnt2b* RNA. As shown in Table 1, co-injection of the *wnt2b* RNA completely rescued the fin phenotypes in more than half of the *wnt2b* morphants (n=60).

Subsequent to morpholino injection, we analyzed the expression of early markers of limb development, as in the *tbx5* morphants. We could not detect *fgf10*, *fgf8* or *shh* expression in the fin field of embryos injected with the *wnt2b* morpholino (compare Fig. 5L with 5M; and data not shown). These results are consistent with the observed phenotypes and our previous studies in chick embryos (Kawakami et al., 2001).

The loss of *fgf* expression in both *tbx5* and *wnt2b* morphant experiments suggested that *tbx5* and *wnt2b* function in a common pathway with respect to limb development, and that this pathway lies upstream of the FGF signaling network that regulates limb initiation. The notion of a common pathway is further supported by the observation that *tbx5* expression was significantly downregulated in the *wnt2b* morphants (compare Fig. 5G,I with 5H,J,K). We reasoned that a key function of *wnt2b* in the limb initiation process might be to regulate *tbx5* expression. If this were the case, then forced expression of *tbx5* in the *wnt2b* morphants could rescue the fin phenotypes. To address this, we co-injected embryos with the *wnt2b* morpholino and *tbx5* RNA. As shown in Fig. 5C,F and Table 1, co-injection of the *tbx5* RNA could rescue the *wnt2b* morphant phenotypes, such that 18% fewer embryos had a no-fin phenotype (57% as compared with 75% of embryos injected with morpholino alone). Conversely, we could not rescue the *tbx5* morphant phenotypes with *wnt2b* RNA (data not shown). We note that LPM expression of *wnt2b* at the time of fin initiation was normal in the *tbx5* morphants (Fig. 5N). These results advocate a fin initiation pathway that is co-regulated by *wnt2b* and *tbx5*, and suggest that *tbx5* lies downstream of *wnt2b* in this process. We have also observed that a consensus binding site for Lef1, a transcription factor that interacts with β-catenin (Roose and Clevers, 1999), is conserved in both human and mouse *Tbx5* genomic sequences, approximately 7.3 kb upstream of the ATG. Deletion of this site results in a nearly 50% decrease in the activation of this promoter by *Wnt2b*, indicating that WNT signaling through the canonical β-catenin pathway has the capacity to activate the *Tbx5* promoter (data not shown).

Tbx5 is necessary and sufficient for limb induction

The above results indicate that *tbx5* and *wnt2b* function together to regulate limb initiation and outgrowth. We have previously shown that the WNT2b/β-catenin pathway is required and sufficient for limb initiation, and that downregulation of this pathway is able to inhibit limb formation (Kawakami et al., 2001). To further examine the relationship between the WNT signaling pathway and *Tbx5*, as well as the evolutionary conservation of this process, we made use of chick embryos, which permit misexpression experiments in a temporally and spatially restricted manner. An adenovirus expressing *Axin* was injected into the LPM of stage 8 chick embryos. Axin is a potent, well-characterized negative regulator of WNT/β-catenin pathway (Peifer and Polakis,

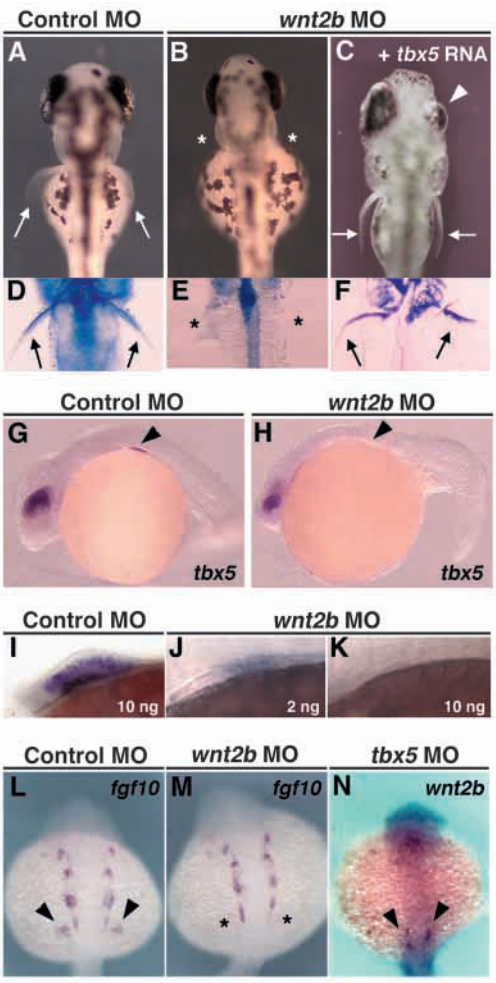

Fig. 5. Zebrafish *wnt2b* is required for *tbx5* and *tgf10* expression and pectoral fin initiation and outgrowth. (A-F) Zebrafish embryos injected at the one-cell stage with a control morpholino (A,D), *wnt2b* morpholino (B,E) or *wnt2b* morpholino + *tbx5* RNA (C,F), were allowed to develop for 5 days. Normal pectoral fin development (A, arrows) is abrogated after *wnt2b* morpholino injection (B, asterisks), and rescued by co-injection of *tbx5* RNA (C, arrows). Other defects caused by *tbx5* RNA injections were observed (arrowhead in C). (D-F) Cartilage staining confirms normal pectoral fin development in control injections (D, arrows) and after rescue with *tbx5* RNA (F, arrows), and lack of pectoral fins after *wnt2b* morpholino alone (E, asterisks). Dorsal view of zebrafish embryos, anterior to the top. (G-K) Effect of *wnt2b* morpholino injections on *tbx5* expression in whole embryos at 28 hpf (G,H) and in pectoral fin buds at 36 hpf (I-K). *tbx5* is expressed in control-injected embryos in the presumptive pectoral fin bud (arrowhead) and in the eye (G). After injection of *wnt2b* morpholino, *tbx5* expression was no longer detected in the presumptive fin field (arrowhead), but remained in the eye at 28 hpf (H). In 36 hpf embryos, expression was seen in the developing fin bud of control injected embryos (I), but was reduced after *wnt2b* morpholino injections of low dosage (2ng, J) or absent after injections of higher dosage (10 ng, K). Lateral view, anterior to the left. (L,M) Comparison of *fgf10* expression patterns in embryos injected with 10 ng of control (L) or *tbx5* (M) morpholino. After injection of *wnt2b* morpholino, expression of *fgf10* in the presumptive fin bud forming area at 24 hpf can no longer be detected (M, asterisks), compared to the control-injected embryo (L, arrowheads). Dorsal view, anterior to the top. (N) *wnt2b* expression in embryos injected with 10 ng of *tbx5* morpholino. In injected embryos, *wnt2b* expression remains unchanged in the LPM at 22 hpf (arrowheads). Dorsal view, anterior to the top. See Fig. 4B for comparison.

2000). An adenovirus expressing *EGFP* was co-injected to assess the spatial distribution of the adenovirus as well as tissue integrity. As shown in Fig. 6A, B, the regions of injected embryos expressing *Axin* displayed a significant downregulation of *Tbx5* (53%, *n*=60). Injection of control adenovirus expressing *EGFP* alone did not result in any obvious phenotypic alterations (data not shown). This result indicates that an active WNT/β-catenin pathway is required for normal expression of *Tbx5* in the LPM, and that this regulation is conserved between chick and fish.

To further investigate the conservation of *Tbx5* function, we generated a retrovirus expressing a truncated form of Tbx5 that maintains the DNA-binding T-box domain but lacks the amino terminus. Injection of this construct into the presumptive wing field of stage 8-10 embryos led to a significant truncation of the wings (87%, *n*=70). Embryos injected with a control retrovirus did not display any obvious phenotype. Examination of the morphology of the truncated wings after cartilage staining indicated limb elements were truncated at later stages of development. The most common phenotypes were hypoplasia or disappearance of zeugopodal elements, typically the radius, and the absence of some anterior digits (compare Fig. 6D with 6E,F). Importantly, the extent of the limb truncations observed was comparable to the zebrafish fin truncations obtained with low levels of the *tbx5* morpholino. We also observed that injection of the truncated *Tbx5* construct led to a downregulation of *Fgf10* expression in the treated embryos (Fig. 6C) (80%, *n*=50). Given these results, we postulate that since T-box transcription factors interact with other members of the transcription machinery to activate target genes (Bruneau et al., 2001; Hiroi et al., 2001), removal of the potential interaction domain, but not the DNA-binding domain, may have resulted in a dominant negative form of Tbx5. Taken together, these results suggest that, as in zebrafish, downregulation of *Tbx5* function in chick embryos inhibits limb outgrowth and that *Tbx5* functions upstream of *Fgf10*.

The combined results from our zebrafish and chick experiments indicate that normal *Tbx5* function is necessary for proper limb initiation. To determine if *Tbx5* is sufficient for this process, we injected a retrovirus expressing *Tbx5* in the LPM of chick embryos at stage 5-8. While embryos injected with a control virus did not display any obvious abnormal phenotype (data not shown), embryos injected with the *Tbx5* retrovirus had additional limb bud-like structures (40%, *n*=89). These embryos were left to develop further, and the morphological features of the ectopic limb-like structures were examined after cartilage staining. As shown in Fig. 6G,H, *Tbx5* overexpression induced additional cartilaginous elements. Stylopodal elements of the ectopic limb-like structure appear to be shared with the endogenous leg, whereas we obtained a range of extra zeugopodal and autopodal elements. The identity of the ectopic structures is difficult to determine because they appear as hybrid structures. However, our findings clearly demonstrate the inductive capacity of *Tbx5* during limb outgrowth. We also observed that during the process of ectopic induction, *Tbx5*, *Fgf10*, and *Wnt2b* are

induced (data not shown), suggesting a de novo deployment of the limb initiation program.

Fgf10 maintains *Tbx5* expression during limb initiation

The WNT and FGF signaling pathways interact and regulate each other to transfer inductive signals between tissues involved in limb initiation (Kawakami et al., 2001). Also, FGFs are capable of activating the expression of *Tbx5*, as demonstrated by the ability of FGF applied to the flank LPM to induce *Tbx5* expression (Isaac et al., 2000). To further our understanding of the regulatory network governing limb initiation, and the role *Tbx5* plays in that process, we blocked FGF signaling using a potent inhibitor of the FGF receptor tyrosine kinases (SU5402) (Mohammadi et al., 1997), and monitored the expression of *Tbx5*. Beads soaked in this inhibitor were applied to the LPM of stage 15-18 chick embryos. As shown in Fig. 7A, a significant downregulation of *Tbx5* was observed in a broad area of tissue surrounding the bead (88%, *n*=43). Beads soaked in DMSO did not result in any alterations (data not shown). This indicates that FGF signaling is required to maintain expression of *Tbx5* during limb outgrowth.

In agreement with the above results, the pattern of *tbx5* expression in zebrafish *hst* mutants is also altered at 26 hpf, when *fgf10* is normally expressed in the presumptive fin mesenchyme. In wild-type embryos, *tbx5* is expressed in the presumptive fin field of the LPM by 26 hpf, when fin outgrowth initiates (Fig. 7B), and continues in the mesenchyme of the developing fin. In *hst* mutant embryos, however, *tbx5* expression in the LPM is significantly decreased at 26 hpf (Fig. 7C) and is not detected by 48 hpf. Downregulation of *tbx5* was also seen in *tbx5* morphants (data not shown). Therefore, we examined the ability of *fgf10* to maintain *tbx5* expression, given the early loss of *tbx5* expression in *hst* mutants. As shown in Fig. 7D, injection of 70 pg of zebrafish *fgf10* mRNA into one-cell stage *hst* embryos resulted in a maintenance of *tbx5* expression in embryos examined at 36 hpf (100%, *n*=213). However, we were unable to rescue fin outgrowth (data not shown), suggesting that functional Tbx5 is required for continuous outgrowth of the pectoral fins. These data further support our hypothesis that *fgf10* is involved in maintaining *tbx5* expression.

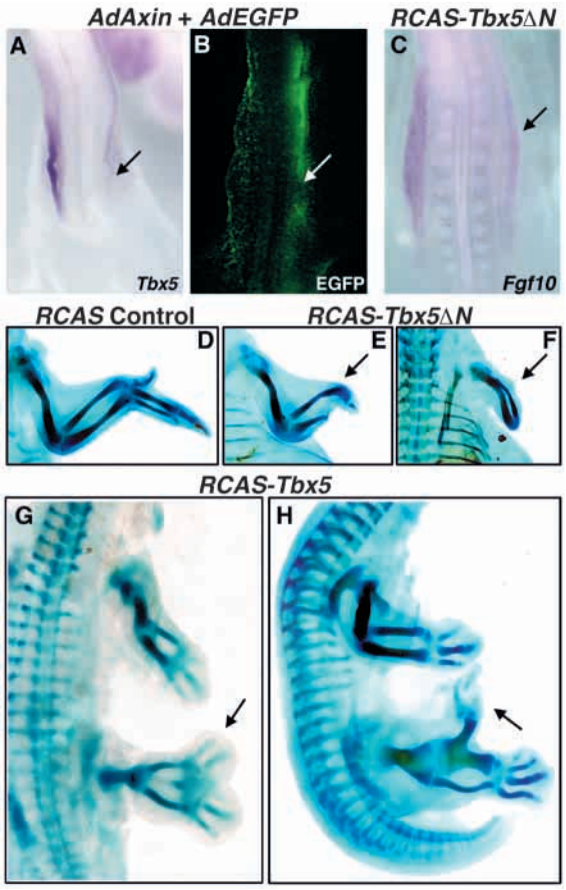

Fig. 6. *Tbx5* expression depends on WNT/β-catenin signaling and is sufficient for limb induction in chick embryos. All panels show anterior to the top. (A,B) *Axin*- and *EGFP*-expressing adenoviruses were co-injected into the LPM of stage 8 chick embryos. At stage 15, *Tbx5* expression was downregulated in the injected side (A, arrow). EGFP expression marks the spatial distribution of the adenovirus and the integrity of the injected tissue (B, arrow). A and B are images of the same embryo. (C-F) *tbx5* regulates *Fgf10* expression and mediates limb outgrowth in chick embryos. *RCAS* expressing an N-terminal truncated mutant of *Tbx5* (*Tbx5ΔN*; C,E,F) or control *RCAS* (D) was injected into the presumptive wing field of stage 8 embryos. In stage 16 embryos injected with *RCAS-Tbx5ΔN*, *Fgf10* expression is downregulated on the injected side (C, arrow). Cartilage staining of stage 36 injected embryos revealed that truncations occurred in the zeugopodal and autopodal structures of the wing, and consisted of hypoplasia of the radius and ulna as well as the complete absence of anterior digits (E,F, arrows). Control *RCAS* infection caused no obvious wing phenotypes (D). (G,H) Tbx5 is sufficient for limb induction in chick embryos. *RCAS* expressing full length *Tbx5* was injected into stage 7 chick embryos. Five days after injection, cartilagenous elements of the embryos were visualized by Alcian Blue staining. Ectopic limb-like structures were induced (arrows), and cartilage staining revealed additional autopod- and zeugopod-like elements.

DISCUSSION

Vertebrate limb initiation involves signaling mechanisms that mediate the directional transfer of signals between different tissues, such as the intermediate mesoderm, LPM and surface ectoderm. Among these tissues, signals from the LPM trigger the onset of limb outgrowth, by acting on the surface ectoderm. Implantation of the prospective limb field into host LPM can result in an ectopic limb by inducing the AER (Saunders and Reuss, 1974). A mesenchymal signal that is essential for limb outgrowth is the product of the *Fgf10* gene. *Fgf10* is initially widely expressed in the LPM, but at the time of limb induction, becomes restricted to the prospective limb regions (Ohuchi et al., 1997). Localized expression of *Fgf10* is a key factor for limb initiation. This is evidenced by the limbless phenotype of *Fgf10*$^{-/-}$ mice (Min et al., 1998; Sekine et al., 1999), and the ability of FGF10 to induce an ectopic limb in chick embryonic flank (Ohuchi et al., 1997).

In contrast to limb initiation, the process of limb identification appears to be regulated, at least in part, by the T-box family of transcription factors. *Tbx5* plays a role in determining the identity of forelimbs, while *Tbx4* determines the identity of hindlimbs (Rodriguez-Esteban et al., 1999a;

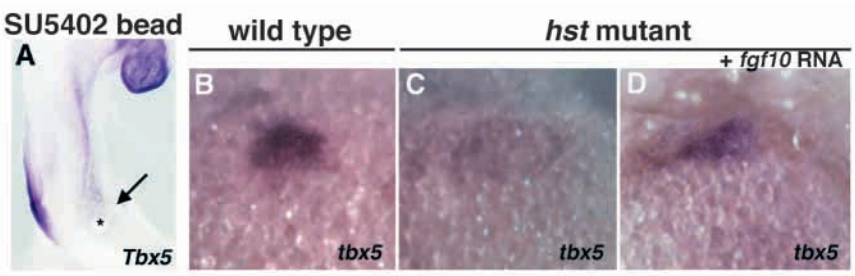

Fig. 7. *Fgf10* maintains *Tbx5* expression during limb initiation. (A) Beads soaked in SU5402 were applied to the LPM of stage 15 chick embryos. At stage 17, *Tbx5* expression was strongly reduced on the manipulated side (arrow). An asterisk indicates the position of the implanted bead. (B-D) *fgf10* maintains *tbx5* expression in the presumptive pectoral fin bud field of zebrafish. In the *hst* mutants, *tbx5* expression is downregulated at 26 hpf. In addition, the few remaining *tbx5*-expressing cells fail to condense into circular areas of the forming fin buds (C, compared to the wild-type embryo in panel B). The expression is no longer detected at 36 hpf (not shown). (D) *fgf10* RNA was injected into *hst* mutant embryos at the one-cell stage, resulting in the maintenance of *tbx5* expression in the pectoral fin bud region at 36 hpf, although the fin buds were not formed. Magnified view of the pectoral fin region, anterior to the left.

Takeuchi et al., 1999). Prior to this study, two key lines of evidence suggested that *Tbx5* might also be involved in the limb initiation process. First, the expression patterns of *Fgf10* and *Tbx5* partially overlap in the LPM at the time of limb initiation and before any morphological limb structures are visible. Second, *Tbx5* is quickly induced in ectopic limb induction experiments (Isaac et al., 2000). When beads soaked in FGF protein are implanted to induce an ectopic limb, *Tbx5* expression is detected in the LPM within 1 hour. This induction occurs earlier than that of other *Fgf* genes that are essential to establish limb buds (Isaac et al., 2000). These observations indicate that limb type specification and *Tbx* gene expression are very early events and also suggest that *Tbx5* may play a role in limb initiation. Here, through loss-of-function experiments in zebrafish, we demonstrate that *tbx5* is necessary for *fgf10* expression in the LPM. In agreement with its ability to activate *Fgf10* expression, we show that *Tbx5* is also sufficient to induce limb-like structures. Two key results also highlight a reciprocal regulation of *tbx5* by FGFs. First, inhibition of FGF receptor tyrosine kinase activity in the chick embryo resulted in downregulation of *tbx5* expression in the LPM at the initiation of limb budding. Second, injection of *fgf10* mRNA into the zebrafish *hst* mutant resulted in a maintenance of *tbx5* expression beyond what is normally observed in those embryos. It is worth noting that *fgf10*, although able to maintain the expression of *tbx5*, could not rescue the loss of pectoral fins in the *hst* mutant. This result further supports the notion that *tbx5* activity is necessary not only for limb initiation, but also to maintain outgrowth. Our molecular and genetic analyses with the *tbx5* morphants and the *hst* mutant extend the recent report by Ahn et al. (Ahn et al., 2002). The authors demonstrated that *tbx5* is required for pectoral fin formation and the cell movement in the LPM that contributes to pectoral fin budding.

Analysis of *Tbx5* expression in *Fgf10*$^{-/-}$ mice also suggests that *Fgf10* regulates *Tbx5* expression. In these mice, *Tbx5* expression is initially detected in the presumptive forelimb mesoderm, but later its expression is clearly downregulated (Sekine et al., 1999). Thus, *Fgf10* is not required for induction of *Tbx5* expression in the LPM, but does appear to play a role in the maintenance of its expression. Such a regulatory interaction between T-box genes and *Fgfs* also takes place in the *Xenopus* blastula. Here, Brachyury, the founding member of the T-box family, not only activates *eFgf* expression, but also forms a regulatory loop with eFGF, in which eFGF maintains *Brachyury* expression in isolated gastrula (Isaacs et al., 1994; Casey et al., 1998).

We have previously shown that *Wnt2b* functions upstream of *Fgf10* in the LPM. The results presented here are consistent with the notion that *wnt2b* also functions upstream of *tbx5*. First and foremost, *tbx5* mRNA can rescue the fin outgrowth phenotype of *wnt2b* loss-of-function morphants. In contrast, *wnt2b* mRNA cannot rescue the related phenotype of the *tbx5* loss-of-function morphants. Second, although *tbx5* expression is downregulated in the *wnt2b* morphants, *wnt2b* expression is unaffected in the *tbx5* morphants. Third, a requirement for WNT/β-catenin signaling for *Tbx5* expression was demonstrated in chick embryos. Specifically, Axin, an inhibitor of WNT/β-catenin signaling, blocks *Tbx5* expression in the LPM. Not surprisingly, we identified a highly conserved Lef1 binding site in the *Tbx5* promoter, a known element required for β-catenin-dependent transcription (Roose and Clevers, 1999) (data not shown).

Our data suggest that the roles of *Wnt2b*, *Tbx5* and *Fgf10* are conserved in chick and zebrafish during limb initiation and identity. Yet, recent findings in mouse suggest the existence of a more complex WNT signaling network mediating limb initiation. We have generated mice bearing loss-of-function mutations in the *Wnt2b* gene and have observed no alteration in limb patterning or outgrowth. Further, we could not detect *Wnt2b* expression in the LPM of mouse embryos at a time when limb initiation begins (A. R. and J. C. I. B., unpublished data). Additionally, results from others show that *Tcf1*$^{-/-}$*/Lef1*$^{-/-}$ mice lack a reduction in *Tbx5* expression (B. Bruneau, Hospital for Sick Children, Toronto, personal communication). However, it is possible that other Tcf family members could compensate for loss of the targeted genes. It should be noted that some other mouse *Wnt* genes are not expressed in comparable structures to those in chick and zebrafish. For example, *wnt3a*, a gene that is expressed in the apical ectoderma ridge (AER) during the process of limb budding in zebrafish (Y. K. and J. C. I. B., unpublished data) and chick (Kengaku et al., 1998), and whose function is necessary for limb outgrowth in those organisms, is not expressed in the AER of mice (Parr et al., 1993). Thus, it is apparent that although the programs of limb initiation and identification are conserved in tetrapods, the molecular action of specific *Wnts* has diverged. As such, a different, uncharacterized WNT molecule might be expressed in the LPM, comparable to the expression pattern of *Wnt2b* in chick and zebrafish. Efforts are currently underway to identify and characterize this signaling molecule in mouse.

The results presented here lead us to propose that WNT/β-catenin signaling controls *Tbx5* expression in the LPM. Tbx5,

in turn, regulates the expression of *Fgf10*, leading to limb initiation. Last, *Fgf10* appears to play a role in maintaining *Tbx5* expression, indicating the existence of a feedback loop between these two factors. We cannot exclude the possibility that *Tbx5* also regulates WNT signaling via a feedback loop. The interaction of *Tbx5* and *Fgf10* is well conserved in vertebrate limb development. In mice, loss of *Tbx5* function results in both loss of *Fgf10* expression and loss of forelimbs (B. Bruneau and M. Logan, National Institute for Medical Research, London, personal communications). While the forelimbs and hindlimbs of all tetrapods share many of the signaling pathways required for their outgrowth and patterning, so far no single transcription factor has been positioned in a molecular cascade that is specifically required for limb outgrowth. Our observations that *Tbx5*, currently regarded as a limb identity determination gene (Rodriguez-Esteban et al., 1999a; Takeuchi et al., 1999), is involved in the limb initiation process, provide significant insight into the tight linkage observed between limb initiation and limb identity. This may help us to further understand the orchestrated interactions needed during embryogenesis for the outgrowth and identity of other tissues and organs where T-box genes and the WNT and FGF signaling act in concert.

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