The limb identity gene \textit{Tbx5} promotes limb initiation by interacting with \textit{Wnt2b} and \textit{Fgf10}

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\textbf{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 \textit{Tbx5}, in addition to its role governing forelimb identity, is both necessary and sufficient for limb outgrowth. We find that \textit{Tbx5} functions downstream of \textit{Wnt} signaling to regulate \textit{Fgf10}, which, in turn, maintains \textit{Tbx5} expression during limb outgrowth. Furthermore, our results indicate that \textit{Tbx5} and \textit{Wnt2b} function together to initiate and specify forelimb outgrowth and identity. The molecular interactions governed by members of the \textit{T-box}, \textit{Wnt} and \textit{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: \textit{T-box}, \textit{Tbx5}, \textit{Wnt2b}, \textit{Fgf10}, Pectoral fin, Limb, Zebrafish, Chick, heartstrings

\textbf{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 \textit{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, \textit{Wnt2b} is expressed in the presumptive forelimb region while \textit{Wnt8c} is expressed in the presumptive hindlimb region. Both \textit{Wnt2b} and \textit{Wnt8c} are capable of inducing \textit{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 \textit{T-box} family of transcription factors, \textit{Tbx4} and \textit{Tbx5} (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Ohuchi et al., 1999; 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, \textit{Pitx1} (Lanctot et al., 1997; Logan et al., 1998), show a restricted forelimb and hindlimb distribution in the LPM prior to limb budding. \textit{Tbx5} transcripts are expressed in the presumptive forelimb area in all tetrapods studied so far, and conversely, \textit{Tbx4} and \textit{Pitx1} transcripts are restricted to the
presumptive hindlimb field. 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 for perturbation of cardiac function in zebrafish. The mutation has been mapped to the codon 316 (Garrity et al., 2002).

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'-GGTGCTTACTGTCGACCACATCG-3'. The zebrafish wnt2b morpholino sequence lies from nucleotide position −66 to −43 and targets the 5' UTR: 5'-AAGTCACTAGTACCTCGAGGTCT-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.

heartsstrings<sup>21</sup> (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).

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...
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).

The results of the \( \text{tbx5} \) loss-of-function experiments suggest that, in addition to its role in controlling limb identity, \( \text{tbx5} \) plays a role in limb initiation and outgrowth. The range of phenotypes obtained also indicates that the function of \( \text{tbx5} \) is dosage dependent. These results are in agreement with the recent Ahn et al. study which also showed that \( \text{tbx5} \) 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 \( \text{tbx5} \) might function by regulating the expression of \( \text{fgf10} \). To start addressing this, we screened several zebrafish cDNA libraries using chick \( \text{fgf10} \) as a probe and cloned zebrafish \( \text{fgf10} \) (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 \( \text{fgf10} \) can be detected in the LPM of the presumptive pectoral fin field, in a pattern temporally and spatially similar to \( \text{tbx5} \) (compare Fig. 2B,C).

In later stages, zebrafish \( \text{fgf10} \) is observed in the mesenchyme of the pectoral fin buds, overlapping \( \text{tbx5} \) 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 \( \text{tbx5} \) loss-of-function phenotypes, we decided to examine the expression of mesodermal \( [\text{fgf10} \text{ and sonic hedgehog (shh)}] \) and ectodermal \( [\text{fgf8}] \) markers involved in the early stages of limb development. As shown in
Table 1. Lack of pectoral fins in injected embryos

<table>
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<th>Injection sample (ng/embryo)</th>
<th>n</th>
<th>% of embryos lacking pectoral fins</th>
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<tr>
<td>tbx5 MO (10.0)</td>
<td>250</td>
<td>95</td>
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<tr>
<td>tbx5 MO (5.3)</td>
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<td>82</td>
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<tr>
<td>tbx5 MO (2.0)</td>
<td>90</td>
<td>50</td>
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<tr>
<td>wnt2b MO (10.0)</td>
<td>230</td>
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<td>wnt2b MO (5.0)</td>
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<td>wnt2b MO (2.0)</td>
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<tr>
<td>wnt2b MO (10.0)</td>
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<td>+wnt2b RNA (0.1)</td>
<td>180</td>
<td>57</td>
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<tr>
<td>wnt2b MO (10.0) +tbx5 RNA (0.1)</td>
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<td>57</td>
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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.

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.

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

Subsequent to morpholin injection, we analyzed the expression of early markers of limb development, as in the \textit{tbx5} morphants. We could not detect \textit{fgf10}, \textit{fgf8} or \textit{shh} expression in the fin field of embryos injected with the \textit{wnt2b} morpholin (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 \textit{fgf} expression in both \textit{tbx5} and \textit{wnt2b} morphant experiments suggested that \textit{tbx5} and \textit{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 \textit{tbx5} expression was significantly downregulated in the \textit{wnt2b} morphants (compare Fig. 5G, I with 5H, J, K). We reasoned that a key function of \textit{wnt2b} in the limb initiation process might be to regulate \textit{tbx5} expression. If this were the case, then forced expression of \textit{tbx5} in the \textit{wnt2b} morphants could rescue the fin phenotypes. To address this, we co-injected embryos with the \textit{wnt2b} morpholin and \textit{tbx5} RNA. As shown in Fig. 5C, F and Table 1, co-injection of the \textit{tbx5} RNA could rescue the \textit{wnt2b} morphant phenotypes, such that 18% fewer embryos had a no-fin phenotype (57% as compared with 75% of embryos injected with morpholin alone). Conversely, we could not rescue the \textit{tbx5} morphant phenotypes with \textit{wnt2b} RNA (data not shown).

We note that LPM expression of \textit{wnt2b} at the time of fin initiation was normal in the \textit{tbx5} morphants (Fig. 5N). These results advocate a fin initiation pathway that is co-regulated by \textit{wnt2b} and \textit{tbx5}, and suggest that \textit{tbx5} lies downstream of \textit{wnt2b} in this process. We have also observed that a consensus binding site for LeF1, a transcription factor that interacts with \( \beta \)-catenin (Rooste and Clevers, 1999), is conserved in both human and mouse \textit{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 \textit{Wnt2b}, indicating that WNT signaling through the canonical \( \beta \)-catenin pathway has the capacity to activate the \textit{Tbx5} promoter (data not shown).

\textbf{\textit{tbx5} is necessary and sufficient for limb induction}

The above results indicate that \textit{tbx5} and \textit{wnt2b} function together to regulate limb initiation and outgrowth. We have previously shown that the \textit{Wnt2b}/\( \beta \)-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 \textit{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 \textit{Axin} was injected into the LPM of stage 8 chick embryos. Axin is a potent, well-characterized negative regulator of WNT/\( \beta \)-catenin pathway (Peifer and Polakis, 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. \(\text{Tbx5}\) was co-injected into the LPM of stage 8 chick embryos. At stage 15, \(\text{Tbx5}\) expression was downregulated in the injected side (A, arrow). EGFP expression marks the spatial distribution of the adenovirus and mediates limb outgrowth in chick embryos. RCAS expressing an N-terminal truncated mutant of \(\text{Tbx5}\) \((\text{RCAS-Tbx5}^{\Delta N})\) (D) was injected into the presumptive wing field of stage 8 embryos. In stage 16 embryos injected with \(\text{RCAS-Tbx5}^{\Delta N}\), \(\text{Fgf10}\) 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 \(\text{Tbx5}\) 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.

**Fig. 6.** \(\text{Tbx5}\) expression depends on WNT/\(\beta\)-catenin signaling and is sufficient for limb induction in chick embryos. All panels show anterior to the top. (A,B) \(\text{Axin-}\) and \(\text{EGFP}\)-expressing adenoviruses were co-injected into the LPM of stage 8 chick embryos. At stage 15, \(\text{Tbx5}\) 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) \(\text{tbx5}\) regulates \(\text{Fgf10}\) expression and mediates limb outgrowth in chick embryos. RCAS expressing an N-terminal truncated mutant of \(\text{Tbx5}\) \((\text{RCAS-Tbx5}^{\Delta N}; \text{C,E,F})\) or control RCAS \((\text{D})\) was injected into the presumptive wing field of stage 8 embryos. In stage 16 embryos injected with \(\text{RCAS-Tbx5}^{\Delta N}\), \(\text{Fgf10}\) 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 \(\text{Tbx5}\) 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.

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 \(\text{Fgf10}\) gene. \(\text{Fgf10}\) 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 \(\text{Fgf10}\) is a key factor for limb initiation. This is evidenced by the limbless phenotype of \(\text{Fgf10}^{-/-}\) mice (Min et al., 1998; Sekine et al., 1999), and the ability of \(\text{Fgf10}\) 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. \(\text{Tbx5}\) plays a role in determining the identity of forelimbs, while \(\text{Tbx4}\) determines the identity of hindlimbs (Rodriguez-Esteban et al., 1999a;
The maintain outgrowth. Our molecular and genetic analyses with activity is necessary not only for limb initiation, but also to movement in the LPM that contributes to pectoral fin budding. By Ahn et al. (Ahn et al., 2002). The authors demonstrated that the \( \text{tbx5} \), could not rescue the loss of pectoral fins in \( \text{It is worth noting that \( \text{zebrafish} \) of limb budding. Second, injection of \( \text{tbx5} \) expression in the presumptive limb initiation. Here, through loss-of-function experiments in \( \text{tbx5} \) is quickly induced in ectopic limb induction experiments (Isaac et al., 2000). When beads soaked in GFG protein are implanted to induce an ectopic limb, \( \text{tbx5} \) expression is detected in the LPM within 1 hour. This induction occurs earlier than that of other \( \text{Fgf} \) genes that are essential to establish limb buds (Isaac et al., 2000). These observations indicate that limb type specification and \( \text{Tbx} \) gene expression are very early events and also suggest that \( \text{Tbx5} \) may play a role in limb initiation. Here, through loss-of-function experiments in \( \text{zebrafish} \), we demonstrate that \( \text{tbx5} \) is necessary for \( \text{fgf10} \) expression in the LPM. In agreement with its ability to activate \( \text{Fgf10} \) expression, we show that \( \text{tbx5} \) is also sufficient to induce limb-like structures. Two key results also highlight a reciprocal regulation of \( \text{tbx5} \) by \( \text{Fgfs} \). First, inhibition of FGF receptor tyrosine kinase activity in the chick embryo resulted in downregulation of \( \text{tbx5} \) expression in the LPM at the initiation of limb budding. Second, injection of \( \text{fgf10} \) mRNA into the \( \text{zebrafish} \) \( \text{hst} \) mutant resulted in a maintenance of \( \text{tbx5} \) expression beyond what is normally observed in those embryos. It is worth noting that \( \text{fgf10} \), although able to maintain the expression of \( \text{tbx5} \), could not rescue the loss of pectoral fins in the \( \text{hst} \) mutant. This result further supports the notion that \( \text{tbx5} \) activity is necessary not only for limb initiation, but also to maintain outgrowth. Our molecular and genetic analyses with the \( \text{tbx5} \) morphants and the \( \text{hst} \) mutant extend the recent report by Ahn et al. (Ahn et al., 2002). The authors demonstrated that \( \text{tbx5} \) is required for pectoral fin formation and the cell movement in the LPM that contributes to pectoral fin budding.

Analysis of \( \text{tbx5} \) expression in \( \text{Fgf10}^{-/-} \) mice also suggests that \( \text{Fgf10} \) regulates \( \text{tbx5} \) expression. In these mice, \( \text{tbx5} \) expression is initially detected in the presumptive forelimb mesoderm, but later its expression is clearly downregulated (Sekine et al., 1999). Thus, \( \text{Fgf10} \) is not required for induction of \( \text{tbx5} \) expression in the LPM, but does appear to play a role in the maintenance of its expression. Such a regulatory interaction between \( \text{T-box} \) genes and \( \text{Fgfs} \) also takes place in the \( \text{Xenopus} \) blastula. Here, Brachury, the founding member of the \( \text{T-box} \) family, not only activates \( \text{eFGf} \) expression, but also forms a regulatory loop with \( \text{eFGf} \), in which \( \text{eFGf} \) maintains \( \text{Brachury} \) expression in isolated gastrula (Isaacs et al., 1994; Casey et al., 1998).

We have previously shown that \( \text{Wnt2b} \) functions upstream of \( \text{Fgf10} \) in the LPM. The results presented here are consistent with the notion that \( \text{wnt2b} \) also functions upstream of \( \text{tbx5} \). First and foremost, \( \text{tbx5} \) mRNA can rescue the fin outgrowth phenotype of \( \text{wnt2b} \) loss-of-function morphants. In contrast, \( \text{wnt2b} \) mRNA cannot rescue the related phenotype of the \( \text{tbx5} \) loss-of-function morphants. Second, although \( \text{tbx5} \) expression is downregulated in the \( \text{wnt2b} \) morphants, \( \text{wnt2b} \) expression is unaffected in the \( \text{tbx5} \) morphants. Third, a requirement for \( \text{Wnt/\beta-catenin} \) signaling for \( \text{tbx5} \) expression was demonstrated in chick embryos. Specifically, \( \text{Axin} \), an inhibitor of \( \text{Wnt/\beta-catenin} \) signaling, blocks \( \text{tbx5} \) expression in the LPM. Not surprisingly, we identified a highly conserved Lef1 binding site in the \( \text{TBx5} \) promoter, a known element required for \( \beta\text{-catenin-dependent transcription} \) (Roose and Clevers, 1999) (data not shown).

Our data suggest that the roles of \( \text{Wnt2b} \), \( \text{tbx5} \) and \( \text{Fgf10} \) are conserved in chick and \( \text{zebrafish} \) during limb initiation and identity. Yet, recent findings in mouse suggest the existence of a more complex \( \text{Wnt} \) signaling network mediating limb initiation. We have generated mice bearing loss-of-function mutations in the \( \text{Wnt2b} \) gene and have observed no alteration in limb patterning or outgrowth. Further, we could not detect \( \text{Wnt2b} \) 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 \( \text{Tcf1}^{-/-}\text{Lef1}^{-/-} \) mice lack a reduction in \( \text{tbx5} \) expression (B. Bruneau, Hospital for Sick Children, Toronto, personal communication). However, it is possible that other \( \text{Tcf} \) family members could compensate for loss of the targeted genes. It should be noted that some other mouse \( \text{Wnt} \) genes are not expressed in comparable structures to those in chick and \( \text{zebrafish} \). For example, \( \text{wnt3a} \), a gene that is expressed in the apical ectoderm ridge (AER) during the process of limb budding in \( \text{zebrafish} \) (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 \( \text{AER} \) 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 \( \text{Wnts} \) has diverged. As such, a different, uncharacterized \( \text{WNT} \) molecule might be expressed in the LPM, comparable to the expression pattern of \( \text{Wnt2b} \) in chick and \( \text{zebrafish} \). Efforts are currently underway to identify and characterize this signaling molecule in mouse.

The results presented here lead us to propose that \( \text{Wnt/\beta-catenin} \) signaling controls \( \text{tbx5} \) expression in the LPM. \( \text{Tbx5} \),
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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