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DEVELOPMENT AND DISEASE

Tbx5 is required for forelimb bud formation and continued outgrowth

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SUMMARY

***Tbx5* is a T-box transcription factor expressed exclusively in the developing forelimb but not in the developing hindlimb of vertebrates. *Tbx5* is first detected in the prospective forelimb mesenchyme prior to overt limb bud outgrowth and its expression is maintained throughout later limb development stages. Direct evidence for a role of *Tbx5* in forelimb development was provided by the discovery that mutations in human *TBX5* cause Holt-Oram Syndrome (HOS), a dominant disorder characterised predominantly by upper(fore) limb defects and heart abnormalities. Misexpression studies in the chick have demonstrated a role for this gene in limb-type specification. Using a conditional knockout strategy in the mouse to delete *Tbx5* gene function in the developing forelimb, we demonstrate that this gene is also required at early limb bud stages for forelimb bud development. In addition, by misexpressing dominant-negative and dominant-activated forms of *Tbx5* in the chick wing we provide evidence that this gene is also required at later stages of limb bud development for continued limb outgrowth. Our results provide a context to understand the defects observed in HOS caused by haploinsufficiency of *TBX5* in human. Moreover, our results also demonstrate that limb bud outgrowth and specification of limb identity are linked by a requirement for *Tbx5*.**

Key words: Limb development, Limb-type identity, *Tbx5*, T-box genes, Mouse, Chick

INTRODUCTION

The forelimb and hindlimb buds are derived from territories of the lateral plate mesoderm located at defined rostrocaudal levels along the main body axis (Capdevila and Izpisua Belmonte, 2001). Cells of the lateral plate mesoderm within these limb 'fields' respond to axial cues that initiate limb bud outgrowth and subsequently produce a morphologically distinct limb bud by stage 16 in the chick (Hamburger and Hamilton, 1951). Classical embryological experiments in the chick have demonstrated that limb-type specification occurs prior to the initiation of overt limb bud outgrowth. Grafts of lateral plate tissue from limb forming regions from as early as stage 8 (four-somite stage) will develop into limbs of the appropriate type – wing or leg – when grafted to the flank or coelomic cavity (Chaube, 1959; Rudnick, 1945; Wolff, 1934).

Several genes have been identified that are expressed exclusively in either the developing forelimb or hindlimb. The T-box transcription factor *Tbx5* is first detected in the prospective forelimb mesenchyme prior to overt limb bud outgrowth and this limb-type restricted expression pattern is maintained throughout limb development stages (Gibson-Brown et al., 1996; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). A closely related T-box gene, *Tbx4*, and a paired-like homeodomain factor, *Pitx1*, are both expressed in a reciprocal pattern in the developing hindlimb mesenchyme (Gibson-Brown et al., 1996; Isaac et al., 1998; Lanctot et al., 1997; Logan et al., 1998; Ohuchi et al., 1998). Misexpression experiments in the chick have demonstrated that ectopic expression of *Tbx5* in the leg bud is capable of transforming the hindlimb to a more forelimb character (Takeuchi et al., 1999). Conversely, misexpression of *Tbx4* or *Pitx1* in the developing wing bud is capable of transforming the forelimb to a more hindlimb character (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Direct evidence for a role of *Tbx5* in forelimb development has been provided by the discovery that mutations in human *TBX5* cause Holt-Oram Syndrome (HOS, OMIM 142900), a dominant disorder characterised predominantly by upper(fore) limb defects and heart abnormalities (Basson et al., 1997; Li et al., 1997). Targeted deletion of *Tbx5* in the mouse has demonstrated that this gene is essential for normal heart

development (Bruneau et al., 2001). *Tbx5*-null embryos die at or around embryonic day (E) 10 because of the severity of the heart defects. Diminished *TBX5* function in human, however, does not obviously affect limb-type identity but instead produces deletion deformities (Basson et al., 1994). Therefore, *Tbx5* may have roles related to growth and differentiation of the embryonic limbs that are distinct from, or intrinsically linked to, its role in defining limb-type identity.

To examine the role of *Tbx5* in forelimb development we have undertaken two strategies to disrupt its function in the developing limb bud. We have used a conditional knockout strategy to delete *Tbx5* function in the developing limbs while leaving the gene intact in other areas of the developing embryo. This approach avoids the complication of phenotypes arising from *Tbx5* loss-of-function in regions of the embryo other than the limb, in particular the heart. The second approach involves using avian retroviruses to misexpress dominant-negative *Tbx5* constructs to knock down *Tbx5* function in the developing wing bud. As a complementary strategy, we also misexpressed full-length and dominant-active forms of the gene.

MATERIALS AND METHODS

Embryos

Mouse embryos were staged according to Kaufman (Kaufman, 1992). Noon on the day a vaginal plug was observed was taken to be E0.5 days of development. The mouse lines carrying a conditional allele of *Tbx5* (Bruneau et al., 2001) and a Prx1Cre transgene (Logan et al., 2002) have been described previously. Fertilised chicken eggs (Needle's Farms, Winter's Farms) were incubated at 37°C and staged according to Hamburger Hamilton (HH) (Hamburger and Hamilton, 1951).

PCR

PCR analysis to genotype pup tail and embryonic material (E10, 30 somites) was carried out in a single reaction using three primers that identify the endogenous *Tbx5* allele, and both the conditional (floxed) and deleted (floxed-out) *Tbx5* allele (Bruneau et al., 2001).

Retrovirus production and infection

Cloning of retroviral constructs and production of concentrated retroviral supernatants were carried out as described previously (Logan and Tabin, 1998). The *Tbx5Δ* construct contains amino acids 1-274 of the full-length chick *Tbx5* clone (Accession Number AF069396). The $Tbx5^{en}$ construct contains amino acids 1-274 of the full-length *Tbx5* clone fused to amino acids 2-298 of the *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991). The $Tbx5^{vp16}$ contains amino acids 1-274 of *Tbx5* fused to a duplex of the lambda hinge region and VP16 (Ohashi et al., 1994). Cells of the prospective forelimb were infected between HH stages 8-10 with concentrated viral supernatants as previously described (Logan and Tabin, 1998).

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was carried out essentially as previously described (Riddle et al., 1993). A minimum of two mutant embryos were analysed at each stage described with each probe. Most probes have been described previously: chick *Shh* (Riddle et al., 1993), mouse *Shh* (Echelard et al., 1993), chick *Msx* (Ros et al., 1992), chick *Hoxc4* (Nelson et al., 1996), mouse *Fgf10* (Bellusci et al., 1997), mouse *Pea3* (Chotteau-Lelievre et al., 2001), mouse *Fgf8* (Crossley and Martin, 1995), mouse *Tbx4* (Bruneau et al., 2001), mouse *Pitx1* (Logan and Tabin, 1999) and chick *Fgf8* (Vogel et al., 1996). A fragment of the chick *Lhx9* sequence was isolated from a chick plasmid library (Logan et al., 1998) and its identity confirmed by sequencing and comparison with published sequences. A chick Groucho homologue *Grg4* was generously provided by Johan Ericson (Muhr et al., 2001). Section in situ hybridisation was performed on 20 μm paraffin wax-embedded sections of stage 21 chick embryos. Additional chick Groucho genes were cloned by degenerate PCR from chick limb cDNA prepared from HH stages 20-23. The degenerate PCR primers lie in the highly conserved Q domain and WD40 domains of *Drosophila*, mouse and human Groucho genes: 5′-AARACIGARATGCARMGICAY-3′, 5′-IGCYTCICCICCIACDATIAR-3′. PCR using a 45°C annealing temperature yielded multiple products, including a 1.1 kb fragment that was cloned into the pGEM-T vector (Promega). Sequencing of this clone revealed similarity to human TLE3.

Histology, TUNEL analysis and immunofluorescence assays

The cartilage and bone elements of newborn mouse pups were stained with Alcian Blue and Alizarin Red, respectively, essentially as described previously (Hogan et al., 1994). Apoptotic cell death was assayed with TdT-mediated dUTP nick end labelling (TUNEL). Mouse embryos were fixed overnight in 4% paraformaldehyde and then processed in whole mount using TUNEL reagents (Q-BIOgene) following the manufacturer's protocol. Chick embryos were fixed overnight in 4% paraformaldehyde, washed in PBT and embedded in OCT (BDH, Merck). Transverse sections (12 μm) were assayed by TUNEL according to the manufacturer's protocol. To detect cells in mitosis, a rabbit anti-phosphorylated histone H3 primary antibody (Upstate Biotechnology) and Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson Laboratory) were used following the protocol described previously (Yamada et al., 1993).

RESULTS

Forelimbs fail to form following deletion of Tbx5 in the cells of the developing forelimb

To examine the function of *Tbx5* in forelimb development, we genetically inactivated the gene in the limb buds by generating mice carrying the *Tbx5* conditional allele, $Tbx5^{lox/lox}$ (Bruneau et al., 2001) and a transgene that expresses *Cre* recombinase in the limb bud, *Prx1Cre* (Logan et al., 2002). Previous work using the Z/AP reporter indicated that the *Prx1Cre* transgene is capable of inducing recombination of floxed alleles in the forelimb by E9.5. Cre-catalysed recombination of the *Tbx5* conditional allele in $Tbx5^{lox/lox}$;*Prx1Cre* embryos was confirmed by PCR (Fig. 1D). The deleted allele was present in DNA samples prepared from hindlimb buds at E10 (30 somites). Mice in which *Tbx5* has been deleted in the developing limb die perinatally but it was not possible to identify the exact cause of mortality. Analysis of the gross anatomy indicated there were no obvious abnormalities of any of the internal organs. In all examples, however, the ribcage failed to fuse and the sternum was absent. This corresponds to regions of the embryo in which both *Tbx5* and the *Prx1-Cre* transgene are expressed at later embryonic stages (data not shown). It is likely that, as a result of these defects, the mutant pups cannot breathe independently and that this is the most probable cause of death. Survival of $Tbx5^{lox/lox}$;*Prx1Cre* pups throughout the entire gestation period extends the developmental time window for studying the loss-of function phenotype in the limb. The forelimbs of newborn (P0) pups are completely absent although the hindlimbs are unaffected (Fig. 1A,B). In some examples a small, rudimentary flap of skin is present on one side of the embryo where the forelimb would

have formed (Fig. 1B). In the majority of cases, however, the skin where the forelimb would normally form is uniform and indistinguishable from the rest of the inter-limb flank. All skeletal elements of the forelimb, including the elements of the pectoral girdle, the scapula and clavicle, are clearly identifiable in control littermates (Fig. 1E-G) but are absent in *Tbx5$^{lox/lox}$;Prx1Cre* P0 pups (Fig. 1H-J).

Scanning electron micrographs of control and *Tbx5$^{lox/lox}$;Prx1Cre* embryos indicate that the limb defect is manifest by embryonic day 10.5 (E10.5) (Fig. 2B,C). In wild-type E10.5 embryos, the limb bud is a prominent outgrowth from the flank of the embryo and the apical ectodermal ridge (AER) is clearly visible (Fig. 2A). In *Tbx5$^{lox/lox}$;Prx1Cre* embryos, no limb outgrowth is visible and no morphologically distinguishable AER is present (Fig. 2B). In most cases, the region where the forelimb would normally form is indistinguishable from other regions of the embryo flank. In a minority of examples, a small mass of tissue is present in the forelimb region (Fig. 2C). This vestige of the forelimb bud most probably forms in examples where the Cre recombinase has failed to delete *Tbx5* function completely from every cell of the prospective forelimb mesenchyme. This small mass of tissue may ultimately give rise to the small flap of tissue occasionally observed in *Tbx5$^{lox/lox}$;Prx1Cre* P0 pups (Fig. 1B).

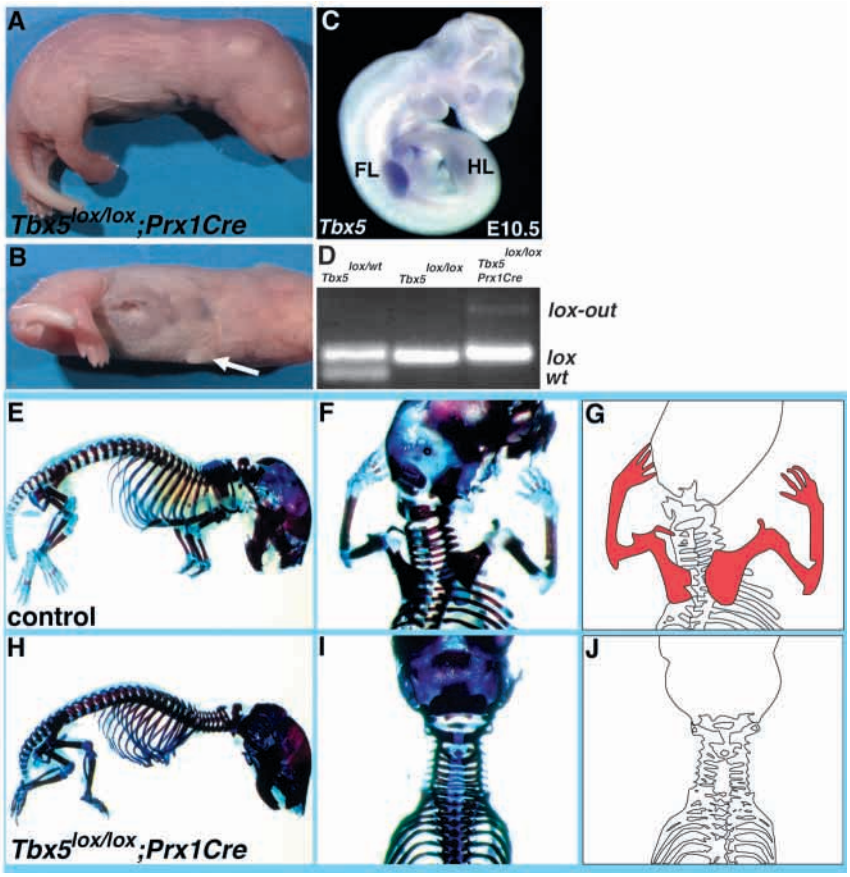

Fig. 1. Absence of forelimb skeletal elements in newborn *Tbx5$^{lox/lox}$;Prx1Cre* pups. (A) *Tbx5$^{lox/lox}$;Prx1Cre* pup viewed from the side. The forelimb has failed to form although the hindlimb has developed normally. (B) Ventral view of the pup shown in A. The arrow indicates a small flap of skin, present on one side of the embryo. (C) *Tbx5* expression in the developing forelimb but not the developing hindlimb of an E10.5 mouse embryo. (D) PCR analysis identifies the presence of the wild-type (wt) and conditional allele (*lox*) in heterozygous *Tbx5$^{lox/wt}$* embryos, the conditional allele (*lox*) in the homozygous *Tbx5$^{lox/lox}$* embryos and the *lox-out* deleted allele in *Tbx5$^{lox/lox}$;Prx1Cre* limb buds. (E) Skeletal preparation of a control littermate. (F) Dorsal view of the thoracic region of the embryo in E showing the pectoral girdle and forelimb skeletal elements. (G) An outline of the skeletal preparation shown in F. The forelimb skeletal elements are highlighted in red. (H) Skeletal preparation of a *Tbx5$^{lox/lox}$;Prx1Cre* pup. (I) Dorsal view of the thoracic region of the embryo shown in H. All the elements of the forelimb and pectoral girdle are absent. (J) An outline diagram of the skeleton shown in I. FL, forelimb; HL, hindlimb.

Tbx5 is required for establishing the signalling centres of the limb bud

Although analysis of E10.5 embryos clearly demonstrated a loss of a morphological forelimb in *Tbx5$^{lox/lox}$;Prx1Cre* embryos, we were curious to learn if molecular markers of the limb were expressed in the forelimb region. Limb bud formation involves the establishment of key signalling centres within the nascent bud. Classical embryological experiments in the chick have demonstrated the requirement of the AER for limb bud outgrowth. Members of the fibroblast growth factor (Fgf) family expressed in the AER have been shown to mediate the effects of this signalling centre (Fallon et al., 1994; Niswander et al., 1993). At E9.5, *Fgf8* is expressed in the nascent AER in both the forelimb and hindlimb and provides a sensitive molecular marker of this structure (Fig. 2D). In the same stage *Tbx5$^{lox/lox}$;Prx1Cre* embryo, *Fgf8* expression is not detected in the forelimb region (Fig. 2E). Expression of *Fgf8* is detected in the AER of the hindlimb bud, consistent with the normal development of the hindlimb in *Tbx5$^{lox/lox}$;Prx1Cre* pups (Fig. 2E). The secreted signalling molecule *Shh* expressed by cells of the zone of polarising activity (ZPA) is essential for normal limb patterning (Echelard et al., 1993; Riddle et al., 1993). At E10.5, *Shh* is expressed in the distal posterior of the limb buds, in the cells of the ZPA (Fig. 2F). In *Tbx5$^{lox/lox}$;Prx1Cre* embryos, however, *Shh* is not expressed in the forelimb region, although normal expression is detected in the hindlimb bud (Fig. 2G).

Hindlimb markers are not expressed in the forelimb region in the absence of Tbx5

Previous studies in the chick have suggested that *Tbx5* expressed in the prospective forelimb region may repress expression of the closely related gene *Tbx4* which is normally restricted to the hindlimb (Takeuchi et al., 1999). We were therefore interested to determine whether, in the absence of *Tbx5*, hindlimb markers would be ectopically expressed in the forelimb region. In *Tbx5$^{lox/lox}$;Prx1Cre* embryos, *Tbx4* expression is detectable in the hindlimb but is not detected in the forelimb region at E9.5 or E10.5 (Fig. 2I; data not shown).

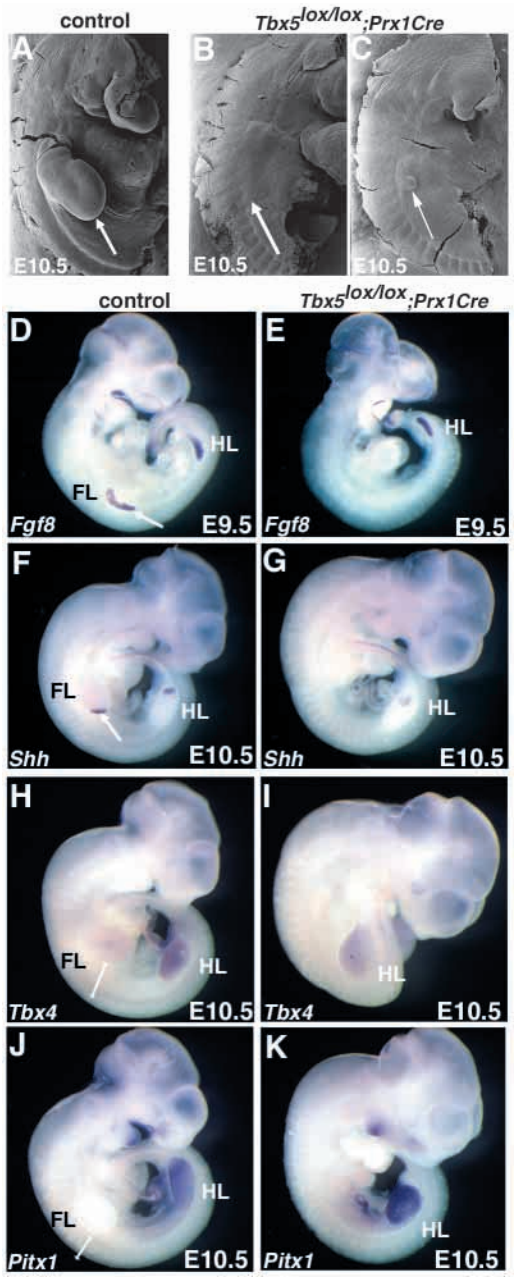

Fig. 2. Absence of a forelimb bud in *Tbx5$^{lox/lox}$;Prx1Cre* embryos. Scanning electron micrographs of (A) a control embryo and (B,C) *Tbx5$^{lox/lox}$;Prx1Cre* embryos at E10.5. In B, no morphological limb bud or AER is present in the forelimb region (arrowed). In C, a small outgrowth of tissue from the flank is present (arrowed). (D-G) Expression analysis of limb bud markers by whole-mount RNA in situ hybridisation. (D) *Fgf8* expression in the AER of the forelimb (arrowed) and hindlimb of a control embryo at E9.5. (E) *Fgf8* expression in the hindlimb AER but absence in the forelimb region of a *Tbx5$^{lox/lox}$;Prx1Cre* embryo. (F) *Shh* expression in the ZPA of the forelimb (arrowed) and hindlimb of a control embryo at E10.5. (G) *Shh* expression in the hindlimb ZPA but absence in the forelimb region of a *Tbx5$^{lox/lox}$;Prx1Cre* embryo. (H-K) Hindlimb markers are not upregulated in *Tbx5$^{lox/lox}$;Prx1Cre* embryos. (H) *Tbx4* expression in the hindlimb but not the forelimb (arrowed) of a control embryo at E10.5. (I) *Tbx4* is expressed normally in the hindlimb and is not upregulated in the forelimb region of an E10.5 *Tbx5$^{lox/lox}$;Prx1Cre* embryo. (J) *Pitx1* expression in the hindlimb but not the forelimb (arrowed) of a control embryo at E10.5. (K) *Pitx1* is expressed normally in the hindlimb but is not upregulated in the forelimb region of an E10.5 *Tbx5$^{lox/lox}$;Prx1Cre* embryo. FL, forelimb; HL, hindlimb.

Similarly, *Pitx1* expression is restricted to the hindlimb in the mouse (Fig. 2J). After deletion of *Tbx5* in the forelimb, *Pitx1* expression remains restricted to the hindlimb and is not expressed in the forelimb region at E9.5 or E10.5 (Fig. 2K; data not shown). Taken together, these data suggests that *Tbx5* does not normally function to repress the expression of hindlimb markers, *Tbx4* or *Pitx1*, in the forelimb region as, in the absence of *Tbx5*, the expression patterns of *Tbx4* and *Pitx1* are unaffected. These results are in agreement with similar studies in which *Tbx5* function has been deleted in all cells of the developing embryo (Agarwal et al., 2003).

Tbx5 is required for limb bud outgrowth

The absence of any morphological forelimb bud led us to suspect a defect at earlier stages of limb development. *Fgf10* is expressed in the lateral plate mesoderm (LPM) of the prospective limb field prior to the expression of *Fgf8* in the prospective AER (Ohuchi et al., 1997). *Fgf10* and other members of the Fgf family, are capable of inducing ectopic limb bud formation when applied to cells in the interlimb flank (Cohn et al., 1995; Martin, 1998). The functional importance of *Fgf10* in limb formation was further demonstrated by the observation that mice carrying a null mutation in *Fgf10* fail to form an AER and pups are born with severely truncated limbs (Min et al., 1998; Sekine et al., 1999). In the absence of *Tbx5* function, *Fgf10* is not expressed in the prospective forelimb bud mesenchyme by E9.5 (21 somites) (Fig. 3B,D). *Pea3* is an *Et*s-related transcription factor that is expressed in the prospective limb mesenchyme (Chotteau-Lelievre et al., 2001) in a complementary pattern to *Fgf10* (Fig. 3E). It has been proposed to mediate the nuclear response to Fgf signalling directly (Raible and Brand, 2001) and it therefore provides a molecular read-out of Fgf signalling. *Pea3* is not expressed in the forelimb region of *Tbx5$^{lox/lox}$;Prx1Cre* embryos by E9.5 (21 somites) (Fig. 3F) consistent with a failure of *Fgf* signalling. *Pea3* is also expressed in the intermediate mesoderm lateral and caudal to the forelimb (Fig. 3E). This expression domain is not affected in *Tbx5$^{lox/lox}$;Prx1Cre* embryos (Fig. 3F), indicating that the effect on *Pea3* is limited to the cells of the prospective forelimb and is not affected at other sites in the developing embryo. TdT-mediated dUTP nick end labelling (TUNEL) analysis demonstrated that by E9.5 (24 somites) cells in the prospective forelimb region of *Tbx5$^{lox/lox}$;Prx1Cre* embryos were first detected to be undergoing an increased extent of apoptotic cell death (Fig. 3H) when compared with control embryos at a similar stage (Fig. 3G). By E10.5 the domain of cell death in *Tbx5$^{lox/lox}$;Prx1Cre* embryos was extensive throughout the forelimb forming region (Fig. 3J) compared with control embryos (Fig. 3I). These results demonstrate that as a result of *Tbx5* inactivation, *Fgf10* is not expressed and cells of the prospective forelimb subsequently undergo extensive apoptosis.

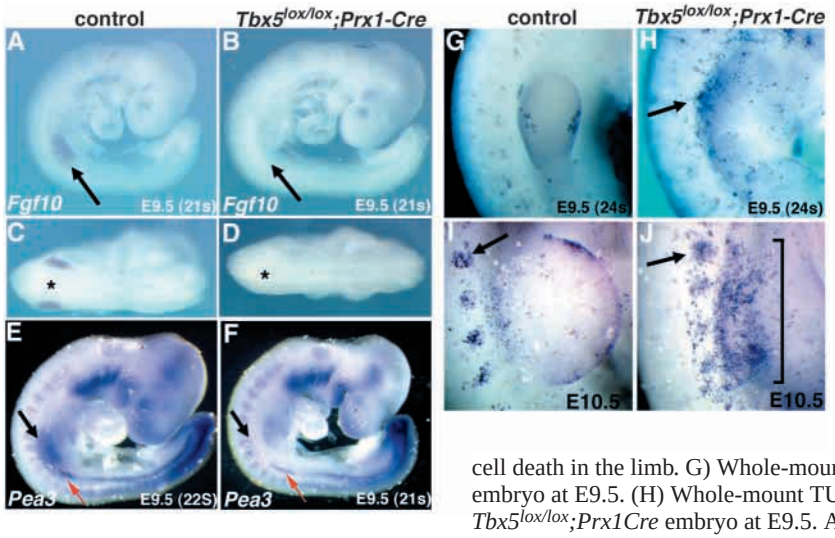

Fig. 3. Early markers of the prospective forelimb mesenchyme are absent in *Tbx5*$^{lox/lox}$*;Prx1Cre* embryos. (A-F) Expression analysis by whole-mount RNA in situ hybridisation. (A) Expression of *Fgf10* in the forelimb (arrowed) of a control embryo E9.5. (B) *Fgf10* is not expressed in the forelimb region of a *Tbx5*$^{lox/lox}$*;Prx1Cre* embryo at E9.5. (C) Dorsal view of the embryo shown in A. *Fgf10* expression in the forelimb buds is indicated by an asterisk. (D) Dorsal view of the embryo shown in B. No *Fgf10* expression is detected in the forelimb-forming region. (E) Expression of *Pea3* in the forelimb (black arrow) and intermediate mesoderm (red arrow) of a control embryo at E9.5. (D) *Pea3* is expressed in the intermediate mesoderm (red arrow) but not the forelimb region of *Tbx5*$^{lox/lox}$*;Prx1Cre* embryos at E9.5 (black arrow). (G-J) Analysis of cell death in the limb. G) Whole-mount TUNEL staining of the forelimb region of a control embryo at E9.5. (H) Whole-mount TUNEL staining of the forelimb region of a *Tbx5*$^{lox/lox}$*;Prx1Cre* embryo at E9.5. A zone of increased apoptotic cell death is present at the site where the forelimb would normally form (arrowed). (I) Whole-mount TUNEL staining of the forelimb region of a control embryo at E10.5. Some cell death is observed in the limb and foci of apoptotic cells are observed in the somites (arrowed). (J) Whole-mount TUNEL staining of the forelimb region of a *Tbx5*$^{lox/lox}$*;Prx1Cre* embryo at E10.5. A region of increased cell death is present in the forelimb-forming region (bracketed). Foci of apoptotic cells are also observed in the somites in a similar pattern to the control embryo (arrowed).

Tbx5 is required at later stages of limb development

During normal development, *Tbx5* is expressed throughout the limb mesenchyme from the earliest stages of forelimb development (Gibson-Brown et al., 1998; Logan et al., 1998) consistent with a role for *Tbx5* in forelimb bud initiation. This limb-type restricted expression pattern is also maintained during later limb bud development stages, suggesting *Tbx5* may have additional roles during subsequent stages of limb outgrowth and patterning. To examine these possible later roles of *Tbx5*, we injected avian retroviruses into chick embryos to misexpress dominant-negative and dominant-active forms of *Tbx5* in the developing forelimb. Misexpression of dominant-negative *Tbx5* using this method should lead to a disruption of *Tbx5* function at later limb bud stages than the genetic deletion approach we used in the mouse. *Tbx5* is a transcription factor that, by analogy to other members of the *Tbx* family, is thought to mediate its effects by binding to target sites on DNA via the conserved N-terminal T domain. The transcriptional effector domain resides in the C terminus of the protein (Fig. 4G). Mutations that lead to truncations of the C terminus of TBX5 have been identified in individuals with HOS, indicating that this region is essential for TBX5 protein function. We have generated two putative dominant-negative constructs of *Tbx5*: a truncated construct that contains residues encompassing the N terminus and T domain only (Tbx5Δ); and a construct that contains the N terminus and T domain fused directly to the transcriptional repressor domain of the *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991) (Tbx5^{en}) (Fig. 4G). Such fusion proteins have been demonstrated to function as dominant-negative constructs in a range of tissues and organisms (Markel et al., 2002; Yu et al., 2001). It should be noted however that the *engrailed* 'active' repressor domain has been shown to be Groucho dependent (Jimenez et al., 1997). We therefore examined the expression of Groucho homologues during chick and mouse limb development. We find that several, including *Groucho 1*, *Groucho3* and *Groucho4*, are widely expressed in the developing limb (Fig. 4H; data not shown) giving confidence that the *engrailed* 'active' repressor domain is likely to function in the limb. Both dominant-negative constructs would be expected to compete with endogenous Tbx5 protein for DNA-binding sites upstream of *Tbx5* target genes. The truncated construct would fail to activate expression of target genes, while the Engrailed-fusion protein would directly repress gene expression. In our experiments, misexpression of both dominant-negative constructs produced indistinguishable results (n=144), suggesting that both forms had similar effects of blocking endogenous *Tbx5* gene function. Scanning electron micrographs of wing buds injected with dominant-negative forms of *Tbx5* indicate that the injected limb is severely truncated when compared with an uninjected control wing bud (Fig. 4A,B; n=2/2, 100%). In contrast to the phenotype observed after deletion of *Tbx5* in the mouse, a limb bud does form in the chick. We presume that this difference is due to incomplete knockdown of gene function using this retroviral misexpression technique. We examined the relative rates of cell proliferation in control and dominant-negative *Tbx5* injected limb buds by assaying for phosphorylated histone H3 (PH3), a marker of mitotic cells. No differences were found in the frequency of mitotic cells of injected limb buds (Fig. 4D; n=3/4, 75%) compared with the contralateral uninjected control limb bud (Fig. 4C) when regions containing the same total cell number were compared. The extent of programmed cell death was examined using TUNEL staining. An increased frequency in cell death was observed in injected limb buds (Fig. 4F; n=3/4, 75%) compared with the contralateral uninjected control limb bud (Fig. 4E). Taken together, these results indicate that injected limb buds are smaller because of increased cell death and not because of a decrease in the rate of cell proliferation. These data are consistent with our observations in *Tbx5*$^{lox/lox}$*;Prx1Cre* mouse embryos and *Fgf4*/*Fgf8* double knockout embryos that have smaller limbs

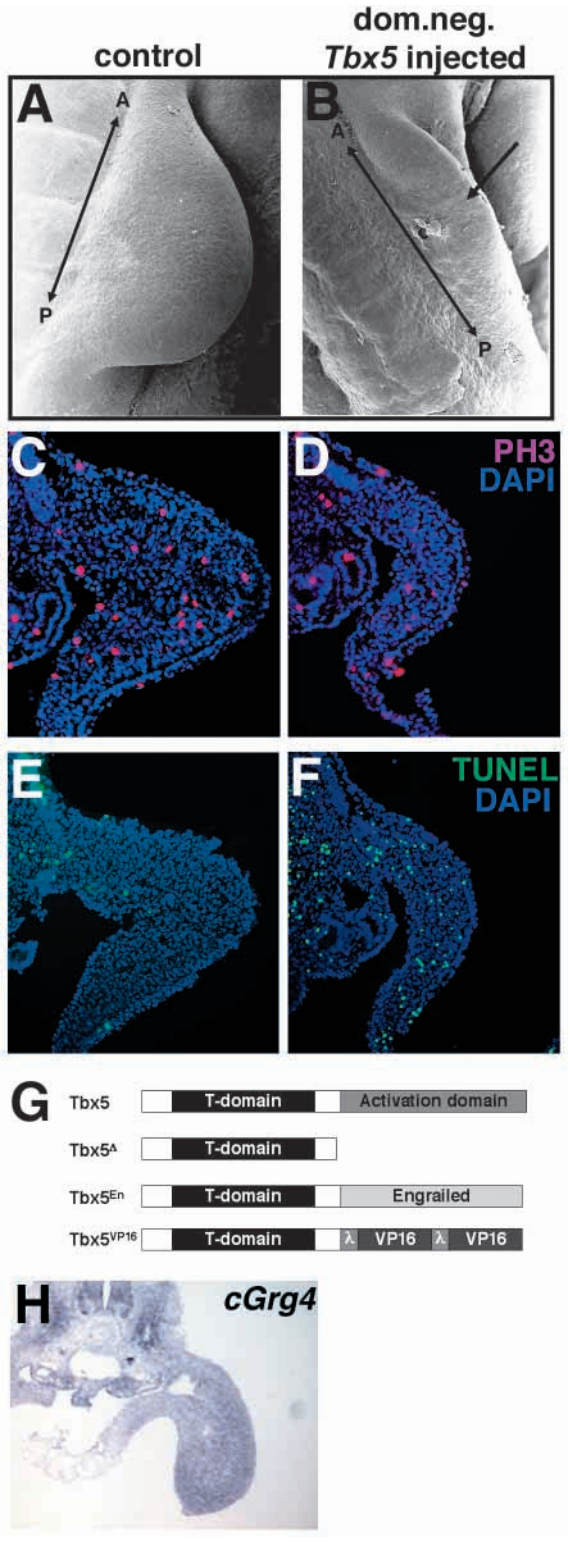

Fig. 4. Limb outgrowth is disrupted following misexpression of dominant-negative *Tbx5* in the chick wing bud. Scanning electron micrographs of (A) control and (B) dominant-negative *Tbx5* (*Tbx5Δ*) injected limb buds at stage 20 show a dramatic reduction in limb size after *Tbx5Δ* misexpression. A, anterior; P, posterior. Sections through (C) contralateral control and (D) dominant-negative *Tbx5* (*Tbx5Δ*) injected limb buds at stage 20 stained with an antibody against phosphohistone H3 to identify cells undergoing mitosis (pink). Sections through (E) contralateral control and (F) dominant-negative *Tbx5* (*Tbx5Δ*) injected limb buds at stage 20 stained with TUNEL reagents to identify cells undergoing apoptotic cell death (green). (G) Schematic representation of the *Tbx5* full-length, truncated and En/VP16-fusion retroviral constructs (see Materials and Methods for details). (H) Transverse section in situ hybridisation analysis of chick Grg4 expression in the chick limb at stage 22.

as a consequence of increased cell death, rather than as a result of a reduction in cell proliferation (Sun et al., 2002). A common feature in the generation of these similar phenotypes is the disruption of Fgf signalling.

To understand how normal patterning is affected in the truncated forelimbs after dominant-negative *Tbx5* misexpression, we analysed the expression patterns of two of the signalling centres of the limb. We observe that *Fgf8* expression is downregulated in the anterior AER and *Shh* is absent in the injected wing buds (Fig. 5B; *n*=2/9, 22%) compared with control embryos (Fig. 5A). If injection of the retrovirus is performed at later stages, lower-level infection of the wing bud results (Rallis and Logan, unpublished) (Logan and Tabin, 1998) and we observe more subtle effects on *Fgf8* and *Shh* expression (Fig. 5C,D). In these examples, *Fgf8*-expressing cells are still present in the distal, anterior of the limb but in a broader, flattened domain of the ectoderm that has not formed a ridge (Fig. 4D,F; *n*=4/18, 22%). This broad pattern of *Fgf*8 expression is reminiscent of cells of the immature, nascent AER present in earlier stage limb buds, suggesting a delay of AER formation or a failure to maintain the AER. We conclude that the effect on *Fgf8* expression in the ectoderm must be occurring as a secondary effect to the knock down of *Tbx5* function in the limb mesenchyme because *Tbx5* is expressed in the forelimb mesenchyme but not the ectoderm. Furthermore, the injection protocol leads to dominant-negative *Tbx5* misexpression in the limb mesenchyme that does not spread to the overlying ectoderm (data not shown) (Logan and Tabin, 1998). Indirect action of misexpressed, mesenchyme-restricted *Tbx5* on the ectoderm is consistent with the normal mesenchymal-restricted expression of *Tbx5* (Gibson-Brown et al., 1998; Logan et al., 1998). Strikingly, the effect on *Fgf8* expression in the AER was observed predominantly in the anterior of the limb bud (Fig. 5B,D).

To investigate the effect of the knock-down of *Tbx5* activity in the limb in more depth, we analysed the expression of other markers of the limb mesenchyme. *Msx1* is a homeobox gene expressed in the AER and in the distal-most mesenchyme cells that lie just under the AER at early limb bud stages (Fig. 6A) (Ros et al., 1992). *Msx1* has been considered a sensitive marker for the influence of the AER on the distal mesenchyme cells as *Msx* expression is rapidly lost after AER removal (Ros et al., 1992). This occurs more rapidly than the reported wave of apoptosis in the distal mesenchyme that follows AER removal (Dudley et al., 2002; Rowe et al., 1982), although some of the observed downregulation of *Msx1* may be attributable to cell death. In the dominant-negative *Tbx5*-injected wing bud, *Msx1* expression is downregulated in the anterior, whereas the expression pattern was not significantly affected in the posterior of the limb (Fig. 6B; *n*=2/5, 40%). To investigate the apparent anterior bias of the limb truncations produced by the dominant-negative *Tbx5* constructs, we analysed the effect

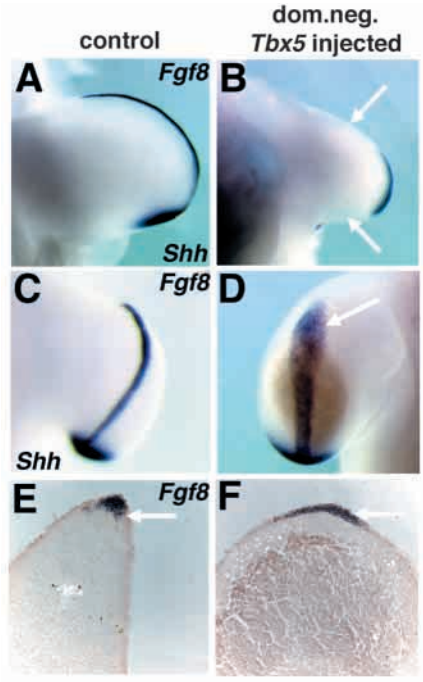

Fig. 5. RNA whole-mount in situ hybridisation analysis of limb buds injected with dominant-negative forms of *Tbx5*. (A) Dorsal view of the control, uninjected limb bud showing *Fgf8*, expressed in the AER, and *Shh*, expressed in the ZPA at stage 23. (B) Dorsal view of *Tbx5Δ*-injected limb. *Fgf8* is not expressed in the anterior of the injected limb and *Shh* expression is not detected in the ZPA. In this example, the injection was carried out at stage 8 to achieve high-level infection. In A, the image of the contralateral uninjected control limb has been reversed to provide a clearer comparison with the respective experimental, injected limb in B. (C) Distal view of an uninjected control limb showing *Fgf8* expression in the AER and *Shh* expression in the ZPA. (D) Distal view of the limb injected with *Tbx5Δ* virus at stage 10 and harvested at stage 23. Injection at the later stage produces lower-level infection and a weaker phenotype. Cells expressing *Fgf8* are present in the anterior of the limb in a broader region of flattened ectoderm and have not formed a ridge of cells (arrow). (E) A transverse section through the anterior of the limb shown in C. The *Fgf8*-expressing cells (arrow) form a ridge of cells at the distal tip of the limb. (F) Transverse section through the anterior of the limb shown in D. The *Fgf8*-expressing cells (arrow) are present in the flattened ectoderm and do not form a ridge. The effect of *Tbx5* misexpression is observed primarily in the anterior mesenchyme.

of expression of markers of the anterior limb mesenchyme. *Lhx9*, a homeodomain factor, is normally expressed in the anterior of the limb bud (Fig. 5C) (Retaux et al., 1999). A functional role of *Lhx9* in limb development has not been established (Birk et al., 2000) but for our purposes it serves as a marker of anterior limb mesenchyme. After misexpression of dominant-negative *Tbx5*, the expression domain of *Lhx9* is reduced in the anterior mesenchyme (Fig. 6D; *n*=5/9, 56%). The homeobox gene *Hoxc4* is also expressed in the anterior of the forelimb bud (Fig. 6E) (Nelson et al., 1996); however, this expression domain is lost after expression of dominant-negative *Tbx5* (Fig. 6F; *n*=4/6, 67%). By contrast, expression of *snail* is not affected (4/4, 100%; data not shown) after expression of dominant-negative *Tbx5*. These results confirm our earlier observations that knock down of *Tbx5* function after misexpression of *Tbx5* dominant-negative constructs predominantly affected the anterior mesenchyme of the limb.

Tbx5 acts as a transcriptional activator in the limb mesenchyme

As a complementary strategy to misexpressing dominant-negative forms of *Tbx5*, we also generated dominant-active constructs by fusing the N-terminal region of Tbx5, including the DNA-binding T domain, to the VP16 transcriptional activation domain (Fig. 4G) (Ohashi et al., 1994). This fusion construct would be expected to bind to the endogenous DNA-binding sites upstream of *Tbx5* target genes and to activate their expression. After misexpression of dominant-active *Tbx5*, the wing bud is enlarged and expanded anteriorly (data not shown, Fig. 6H,J). Analysis of *Fgf*8 expression indicates that the domain of *Fgf8*-expressing cells in the AER extends more anteriorly than in the uninfected wing bud, but is not expanded in the posterior of the bud (Fig. 6G,H; *n*=5/7, 71%). The domain of *Lhx9* expression is expanded anteriorly but not posteriorly in the wing bud (Fig. 6I,J; *n*=4/5, 80%). A similar anterior expansion of the expression domains of *Fgf10* (*n*=2/2; 100%) and *Msx* (*n*=3/3; 100%) was also observed (data not shown). Similar anterior expansions of the limb mesenchyme were also obtained after misexpression of full-length *Tbx5* constructs (*n*=17, data not shown). This finding and phenotypes observed with dominant-negative forms of *Tbx5* support the conclusion that *Tbx5* is acting as a transcriptional activator in the developing limb bud.

DISCUSSION

Tbx5 is required for forelimb bud development

*Tbx*5 is expressed in the prospective forelimb mesenchyme of the lateral plate prior to overt limb bud outgrowth and coincident with the time that these cells are specified to their limb-type fate (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). *Tbx5* expression precedes that of *Fgf10* (Agarwal et al., 2003) and *Tbx5* transcripts are present in the limb-forming region of *Fgf10* knockout mice (Min et al., 1998; Sekine et al., 1999). By contrast, when *Tbx5* is inactivated in all cells of the embryo prior to limb bud stages, *Fgf10* is never expressed demonstrating that *Tbx5* is required to initiate *Fgf10* expression in cells of the prospective forelimb (Agarwal et al., 2003). Moreover, inactivation of *Tbx5* in cells of the prospective forelimb at early limb induction stages, E9-E9.5, using the *Prx1Cre* transgenic leads to loss of *Fgf10* expression and the failure to form a morphological limb bud. Together, these data establish a genetic hierarchy required for forelimb initiation. *Tbx5* is genetically upstream of the *Fgf10* and is essential for the induction and maintenance of *Fgf10* that, in turn, is required for the initiation of forelimb bud formation. TUNEL staining of mutant embryos indicated that failure to establish the positive-feedback loop of *Fgf* signalling between the limb mesenchyme and overlying ectoderm cells leads to programmed cell death in the prospective forelimb-forming region. *Tbx5* is therefore the earliest known marker of the prospective forelimb mesenchyme and is essential for forelimb bud formation.

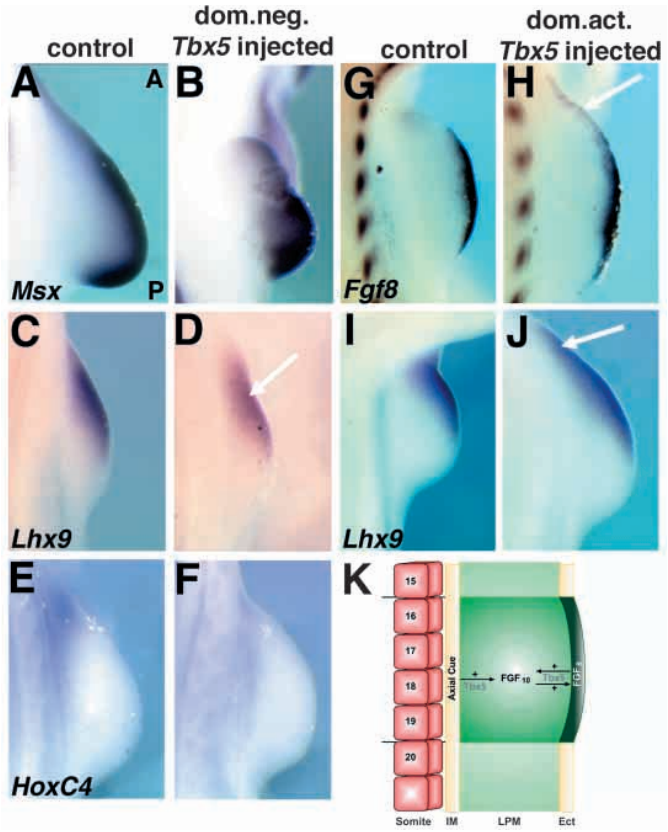

Fig. 6. (A-F) Misexpression of dominant-negative *Tbx5* leads to downregulation of anterior mesenchymal markers. (A) In the contralateral control, uninjected limb *Msx* is expressed in the distal mesenchyme of the limb just under the AER at stage 21. (B) In the dominant-negative *Tbx5*- (*Tbx5*^{en}) injected limb, *Msx* is downregulated in the distal mesenchyme, most obviously in the anterior of the limb. (C) *Lhx9* is expressed in the anterior limb mesenchyme in the control limb at stage 19 and (D) in the *Tbx5*^{en}-injected limb, expression is downregulated (arrowed). (E) *Hoxc4* is expressed in the anterior limb mesenchyme of a control limb (stage 20) and (F) is downregulated in the *Tbx5*^{en}-injected limb. (G-J) Misexpression of dominant-active forms of *Tbx5* (*Tbx5*^{vp16}) leads to an anterior expansion of the limb. (G) *Fgf8* expression in the uninjected control limb (stage 20). (H) After misexpression of *Tbx5*^{vp16}, the domain of cells expressing *Fgf8* in the AER is expanded anteriorly (arrow). (I) Expression of *Lhx9* in the anterior of the control limb at stage 20. (J) After misexpression of *Tbx5*^{vp16}, *Lhx9* expression is expanded anteriorly (arrow). In A,C,E,G,I, the image of the contralateral uninjected control limb has been reversed to provide a clearer comparison with the respective experimental, injected limbs in B,D,F,H,J. A, anterior; P, posterior. (K) A model for *Tbx5* function in the developing limb bud. *Tbx5* is first expressed as limb bud initiation commences in response to axial cues. *Tbx5* is acting genetically upstream of *Fgf10*, and is required for *Fgf10* expression in the limb mesenchyme. Later, *Tbx5* is also required for the positive inductive loop between *Fgf8*, which is expressed by cells in the AER, and *Fgf10*, which is expressed by cells of the distal mesenchyme.

A striking additional observation of conditional knockout of *Tbx5* in limb mesenchyme is the complete absence of all the elements of the appendicular skeleton. *Tbx5* is required for the formation of the clavicle and scapula of the pectoral girdle in addition to the skeletal elements of the limb proper. This phenotype is more severe than that observed in the forelimb of *Fgf10*-null mice. This indicates that *Tbx5* has a broader influence on forelimb development than *Fgf10* and is absolutely required for the formation of all elements derived from the forelimb lateral plate mesoderm. Although all skeletal elements of the limb proper and most of the pectoral girdle are formed from lateral plate tissue of the prospective forelimb region, the proximal portion of the scapula blade is derived from the somites that lie medial and adjacent to the forelimb (Burke, 2000; Huang et al., 2000). After limb ablation in the chick, the somite-derived hypaxial myoblasts that form the limb musculature, but do not express *Tbx5*, are never recruited to the limb field (Gumpel-Pinot et al., 1984). By extension, the most likely explanation for the loss of the entire scapula in *Tbx5*$^{lox/lox}$*;Prx1Cre* embryos is that, after deletion of *Tbx5* in the limb mesenchyme and the failure of early limb bud formation, the somite-derived (and at that stage non-*Tbx5*-expressing) scapula precursors are not recruited into the limb field.

Recently, the requirement of *tbx5* for the formation of the pectoral fin in zebrafish has been demonstrated using morpholino antisense oligonucleotides to knock down *tbx5* function (Ahn et al., 2002; Garrity et al., 2002). This observation is consistent with work in other species by ourselves and others (Agarwal et al., 2003; Ng et al., 2002). In one report (Ahn et al., 2002), the authors conclude that the function of *tbx5* in the development of the zebrafish forelimb involves the directed migration of lateral plate mesodermal cells to the future limb-bud-forming region. However, the results of our experiments in the chick and mouse do not support a model in which *Tbx5* is involved in directing migration of cells of the prospective forelimb. In higher vertebrates, cells of the prospective forelimb do not undergo a similar migration (Saunders et al., 1957; Saunders et al., 1959; Searls, 1967; Searls and Janners, 1969) and *Tbx5*-expressing cells detected in the prospective forelimb region are not migratory (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). Somite-derived cells that contribute to the limb do not express *Tbx5* (data not shown). Furthermore, in *Tbx5*-null embryos, patterning of the lateral plate mesoderm at limb levels is intact and only limb bud outgrowth is affected by constitutive loss of *Tbx5* (Agarwal et al., 2003). Although our results and those of Ahn et al. are phenotypically similar, our results demonstrate a different mechanism for *Tbx5* action. Although in lower vertebrates *tbx5* may have a role in migration of limb precursor cells, in higher vertebrates *Tbx5* is required to regulate inductive signalling interactions essential for limb bud initiation and continued limb outgrowth.

Tbx5 is required for continued limb outgrowth

Our misexpression experiments in the chick demonstrate that *Tbx5* is not only required for limb initiation but is also required at later stages of limb development for continued outgrowth. Knock down of *Tbx5* function by misexpression of dominant-negative forms of the protein leads to the disruption of the induction and maintenance of the AER. This role for Tbx5 can be placed within our current models of limb initiation and outgrowth (Fig. 6K). *Fgf10* expressed in the lateral plate mesoderm is initially required for the induction of *Fgf8* in cells

of the nascent AER (Fig. 6K) (Ohuchi et al., 1997; Yonei-Tamura et al., 1999). *Fgf8* expressed by cells of the AER is required, in turn, for the maintenance of *Fgf10* in distal mesenchyme underlying the AER. After AER formation, a positive feedback loop is established between *Fgf8*-expressing cells in the AER and cells of the distal mesenchyme expressing *Fgf10* (Fig. 6K) (Ohuchi et al., 1997). Our results are consistent with a requirement for *Tbx5* in the AER-mediated maintenance of *Fgf10* expression in the distal mesenchyme at later stages of development. Disruption of this epithelial-mesenchymal induction loop would lead to severe truncation of limb outgrowth and could explain the reduction deformities observed in HOS which, in the most severe cases, results in an almost complete absence of all elements of the limb (phocomelia). The more commonly observed characteristic features of HOS phenotypes are deformities of anterior elements of the limb, such as the thumb, thenar elements or radius. After knock down of *Tbx5* by misexpression of dominant-negative constructs we observed defects in AER formation and maintenance primarily in the anterior of the infected wing bud. Loss of *Fgf8* expression is observed in the anterior AER and a concomitant failure of *Fgf*-mediated signalling to the underlying mesenchyme is indicated by the loss of expression of various markers in the anterodistal mesenchyme. Our chick misexpression protocol generated a phenotype consistent with characteristic abnormalities presented in individuals with HOS. The downregulation of these anterior markers therefore provide a molecular context to understand the genesis of HOS deformities. Unfortunately, we are unable to analyse the skeletal deformities that would have resulted from the disruption of gene expression at early limb bud stages, because continued spread of the retrovirus produces heart defects that lead to embryonic lethality (data not shown). A direct, positive regulatory relationship between a T-box gene and an Fgf gene has previously been demonstrated between *brachyury* and *eFgf* during mesoderm induction in *Xenopus* (Schulte-Merker and Smith, 1995). Our results suggest that this regulatory relationship has been conserved and reused in the context of the limb. This is consistent with results demonstrating that *Tbx5* binding sites are present in the promoter of *Fgf10* (Agarwal et al., 2003; Ng et al., 2002). *Tbx4*, a closely related T-box family member (Agulnik et al., 1996), may play an analogous role to *Tbx5* in the hindlimb.

Tbx5 acts as a transcriptional activator

Misexpression of both types of dominant-negative *Tbx5* constructs produced essentially identical results and are consistent with defects observed because of haploinsufficiency of *TBX5* in HOS (Basson et al., 1997; Li et al., 1997) and defects in the *Tbx5* heterozygous knockout mouse (Bruneau et al., 2001). In the converse experiment, misexpression of full-length *Tbx5* or constructs containing the T-domain of Tbx5 fused to the VP16 transcriptional activation produced identical results. Moreover, the phenotypes observed complement those observed with dominant-negative constructs. Together, these observations support the conclusion that *Tbx5* is acting as a transcriptional activator in the forelimb. These results are consistent with reports demonstrating that *Tbx*5 can transactivate expression from constructs containing a region of the *Fgf10* promoter (Agarwal et al., 2003; Ng et al., 2002).

Dual functions for Tbx5

The results of loss-of-function studies in mouse, chick and zebrafish, combined with previous misexpression studies performed in the chick reveal dual roles for *Tbx5* in limb development. *Tbx5* is required to induce and maintain *Fgf10* expression that in turn is necessary for forelimb initiation and continued outgrowth (Fig. 6K). *Tbx5* also acts to specify forelimb identity by influencing the response of cells of the forelimb to common patterning cues (Takeuchi et al., 1999). As *Tbx5* is required for limb initiation and outgrowth, loss-of-function approaches have not been informative as to the role of *Tbx5* in specifying limb-type identity. Further studies will be required in which the requirement for *Tbx5* in limb outgrowth can be uncoupled from its role in specifying limb-type identity.

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