**Tbx3 can alter limb position along the rostrocaudal axis of the developing embryo**

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**Summary**

The limbs of the vertebrate embryo form at precise locations along the body and these positions are fixed across different species. The mechanisms that control this process are not understood. Ectopic expression of *Tbx3*, a transcriptional repressor that belongs to the Tbx2/3/4/5 subfamily of T-box transcriptional regulators, in the forelimb results in a rostral shift in the position of the limb along the main body axis. By contrast, a transcriptional activator form of *Tbx3* shifts the limb to more caudal locations. We also show that *dHand* and *Gli3*, genes previously implicated in anteroposterior pre-patterning of the limb-forming region, are also involved in refining the position of the limbs. Our data suggest a new role for *Tbx3* in positioning the limb along the main body axis through a genetic interplay between *dHand* and *Gli3*.

Key words: Limb development, Limb position, *Tbx3*, T-box, *Gli3*, *dHand* (Hand2), Chick

**Introduction**

Vertebrate limbs develop as budding outgrowths from the lateral plate mesoderm (LPM) on either side of the main body axis. The forelimb and hindlimb fields are located at specific positions along the rostrocaudal axis of the embryo and this position is fixed across vertebrate species. The forelimb forms at the cervical-thoracic junction, while the hindlimb develops at the level of the lumbar-sacral junction (Burke et al., 1995). Hox genes are candidates to specify limb position. Many of these genes are expressed in nested patterns along the rostrocaudal axis of the embryo and may provide positional cues to cells of the LPM that will give rise to limb buds (Burke, 2000; Burke et al., 1995; Cohn et al., 1995; Cohn et al., 1997).

However, neither gene deletion nor gene misexpression experiments have provided direct evidence for a role of Hox genes in limb positioning.

*Tbx3* belongs to the Tbx2/3/4/5 subfamily of T-box genes that originated from a single ancestral gene through gene tandem duplication and cluster dispersion (Agulnik et al., 1996; Minguillon and Logan, 2003; Ruvinsky et al., 2000; Wilson and Conlon, 2002). *Tbx3* is expressed in the limb-forming territories prior to overt limb bud outgrowth. At later stages (st.24 chick, 11.5 dpc in the mouse), *Tbx3* is expressed in two stripes in the anterior and posterior limb mesenchyme (Gibson-Brown et al., 1998; Logan et al., 1998; Tumpel et al., 2002). *Tbx3* is required for normal limb development as mutations in human *TBX3* are associated with Ulnar-Mammary Syndrome (UMS, OMIM #181450), a dominant disorder characterized by upper(fore) limb deficiencies (Bamshad et al., 1997). Posterior structures of the limb, e.g. the ulna and fifth digit, are predominantly affected. *Tbx3* deletion studies in the mouse produce phenotypes consistent with the abnormalities observed in UMS (Davenport et al., 2003).

Experiments in the chick have shown that the posterior domain of *Tbx3* expression in the limb is positively regulated by *Shh* signalling, while the anterior expression domain is repressed by *Shh*, suggesting a potential role of *Tbx3* in the anteroposterior patterning of the vertebrate limb (Tumpel et al., 2002). Furthermore, recent misexpression experiments have suggested that *Tbx3* can alter the identity of posterior digits in the developing chick hindlimb (Suzuki et al., 2004).

Misexpression and gene deletion studies have implicated *Tbx3* in limb patterning during limb bud stages. However, *Tbx3* is expressed in the limb-forming region prior to overt limb bud outgrowth. To examine a potential early role of *Tbx3* in normal limb development, we have misexpressed transcriptional repressor and activator forms of *Tbx3* in the developing forelimb region using the avian retroviral system. We provide evidence for a new role for *Tbx3* in the genetic network that positions the limb along the rostrocaudal axis of the vertebrate embryo.

**Materials and methods**

**Embryos**

Eggs (Needle’s farm, Winter’s farm, The Poultry Farm) were incubated at 37°C and staged according to Hamburger-Hamilton (HH) (Hamburger and Hamilton, 1951).

**Retrovirus production and embryo infection**

Production of retroviral supernatants were carried out as described previously (Logan and Tabin, 1998). Two full-length *Tbx3* viruses were produced; one includes amino acid residues 1-732 the other amino acid 15-732 of the predicted protein (AF033669). Both forms produced identical results. *Tbx3* contains amino acids 15-289 of Tbx3, which spans the N terminus and DNA-binding T-domain, fused to the engrailed repressor domain (Jaynes and O’Farrell, 1991). *Tbx3* contains the same residues fused to two VP16 activation...
domains (Ohashi et al., 1994). The Gli3ZnF-Vp16 construct contains amino acids 471-636 of the human GLI3 (XP_004833) fused to two VP16 activation domains. The prospective forelimb territory on the right side of the embryo were infected between stages 8 and 10, as previously described (Logan and Tabin, 1998). The left limb served as a contralateral control. Each virus produced a limb shift phenotype in ~30% of infected embryos. For embryos analyzed before a limb shift phenotype was morphologically obvious, batches of infected embryos were analyzed. For embryos analyzed at later stages, embryos with a phenotype were selected.

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were carried out essentially as described (Riddle et al., 1993). Probes used were Shh (Riddle et al., 1993), Fgf8 (Nogel et al., 1996), MyoD, Pax3 (Pourquie et al., 1996), Hoxb8, Hoxb9, Hoxc5, Hoxd12 (Burke et al., 1995), Wnt26 (Kawakami et al., 2001), Tbx5, Tbx2, Tbx3 (Logan et al., 1998), dHand (also known as Hand2) (Fernandez-Teran et al., 2000), Gli3 (Schweitzer et al., 2000) and Bmp2 (Schlange et al., 2002). The Tbx15 probe was produced from a cDNA isolated from a limb cDNA library (M.P.O.L., unpublished).

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (Kardon, 1998). Axons were stained using the 3A10 monoclonal antibody (hybridoma supernatant diluted 1/100 from DSHB, Iowa, USA) and detected with a peroxidase-conjugated anti-mouse secondary (Jackson ImmunoResearch) diluted 1/250.

Cell lines, transfections and luciferase assays

Luciferase assays were performed using COS1 cells. Transfections were performed using Superfect transfection reagent (Qiagen) following the manufacturer’s protocol. For expression studies, full-length, Engrailed and VP16 fusion forms were cloned into pcDNA3.1(–) (Invitrogen). The reporter pGL3-promoter vector (Promega) containing a single Brachury binding site (Kispert et al., 1995) together with a basal SV40 promoter upstream of the Firefly Luciferase gene. Luciferase assays were carried out using the appropriate Reporter Assay System (Promega) according to the manufacturer’s protocol. Normalization of the results was carried out using β-Galactosidase Reporter Assay (Promega) according to the manufacturer’s protocol. As an internal control the reporter plasmid was co-transfected with the β-galactosidase reporter only. All experiments were performed in triplicate. Error bars represent the standard deviation over three experiments.

Dil injection

Dil crystals (Sigma) were diluted in 100% ethanol (5 mg/ml). A 10% working solution was prepared in 30% sucrose/PBS solution. Misexpression of retrovirus was performed at HH stage 8-10. Twenty-four hours after retrovirus infection (stage 14) Dil solution was injected into the embryos at several levels in the limb-forming region of the LPM and in the adjacent somites, to serve as axial reference. Equivalent Dil injections were performed in the injected and control side of the embryo.

Results

Analysis of Tbx3 expression in the developing chick embryo

To analyse the expression pattern of Tbx3 at stages prior to limb outgrowth, we performed whole-mount in situ hybridization between stages 12-16. Tbx3 expression is observed in the future forelimb and hindlimb domains at stages 12-13 (Fig. 1A), earlier than previously reported (Tumpel et al., 2002). Just prior to overt limb outgrowth (stage 16), Tbx3 expression is expressed in the presumptive forelimb and hindlimb areas (Fig. 1B) and expression appears more robust in the posterior region (Fig. 1B). In situ hybridization on sections of the forelimb region of stage 16 embryos (Fig. 1C,D) reveals that Tbx3 is present in the mesoderm of the presumptive wing region and in the lateral lip of dermomyotome. At stage 19, when a limb bud is clearly evident, expression is prominent in the posterior of the limb (Fig. 1E). At later stages (stage 25), expression is located in two stripes in the anterior and posterior limb mesenchyme (Fig. 1F).

Tbx3 is a transcriptional repressor in vitro

In vitro data suggest that human TBX3 is a transcriptional repressor (Carlson et al., 2001). We generated three constructs:
full-length Tbx3 (Tbx3); a construct that contains the N-terminus and T-domain of Tbx3 fused to the VP16 transcriptional activator domain (Ohashi et al., 1994) (Tbx3VP16); and a construct with the same residues fused directly to the repressor domain of the Drosophila engrailed gene (Jaynes and O’Farrell, 1991) (Tbx3EN) (Fig. 1G, see Materials and methods). The Tbx3VP16 construct is predicted to compete with endogenous Tbx3 protein for binding sites and activate target genes. The Tbx3 and Tbx3EN forms are predicted to repress target genes. Engrailed repressor activity is Groucho dependent (Jimenez et al., 1997) and Groucho homologues are present in the limb mesenchyme (Rallis et al., 2003). To test the transcriptional properties of these three Tbx3 constructs, we performed in vitro luciferase assays using a reporter plasmid containing a single T-box binding site. Both Tbx3 and Tbx3EN fusion constructs repress expression to an almost equal extent. By contrast, the Tbx3VP16 fusion construct activates expression (Fig. 1H). Co-transfection of Tbx3/Tbx3VP16 generates luciferase activity at intermediate levels between those achieved with Tbx3 or Tbx3VP16 alone. Together, the data demonstrate that Tbx3 functions as a transcriptional repressor in vitro.

Misexpression of Tbx3 can alter limb position

We have investigated the role of Tbx3 in normal limb development using the chicken retroviral system. Following our targeting strategy (Materials and methods), we could detect virus broadly in the limb-forming region from stage 14 onwards (data not shown). Strikingly, misexpression of full-length Tbx3 shifted the axial position of the injected limb rostrally (embryos that show rostral limb shift phenotype: n=142, phenotype frequency 25-30%). Identical results were produced with Tbx3EN (embryos with phenotype; n=82, phenotype frequency 25-30%), further suggesting that Tbx3 normally functions as a transcriptional repressor. Comparison with MyoD, which is expressed in the dermomyotomal compartment of each somite and serves as an axial reference, and Shh, which marks the zone of polarizing activity (ZPA) in the posterior limb, demonstrates the rostral shift in limb position following Tbx3 misexpression (limb on right), relative to contralateral control limbs (on left in all cases shown) (Fig. 2A, n=6/6, 100%). The shift in axial position can extend over the distance of one to three somites; however, the limb itself is otherwise morphologically normal. Pax3 is expressed in the dermomyotome of the developing somites and the migrating myoblasts (Williams and Ordahl, 1994). Following misexpression of Tbx3 and mislocation of the limb to a more rostral position along the embryo axis, myoblasts that migrate into the shifted limb are derived from somites at a more rostral level. Myoblasts at more caudal levels that normally migrate into the limb (Fig. 2B, black arrow), no longer contribute to the limb musculature and remain within the dermomyotome (Fig. 2B, red arrow) (n=7/7, 100%). Both delamination and migration of the myoblasts into the limb depend on the Met receptor and its ligand HGF, also called scatter factor, produced by non-somitic mesoderm (Dietrich et al., 1999). Following shift of the limb from its normal position, the source of HGF is presumably also shifted, leading to migration of myoblasts from somites at the incorrect axial level.

The neurons that innervate the wing and form the brachial plexus originate from the 16th to 19th spinal ganglia (Lillie, 1963), which marks the zone of polarizing activity (ZPA) in the limb mesenchyme. There is a one somite-level shift in limb position (compare level of left and right; Fig. 2A). Whole-mount in situ hybridization with MyoD marking somites and Shh marking the ZPA. There is a one somite-level shift in limb position (compare level of left and right bar). (B) Whole-mount in situ hybridization with Pax3 marking migrating myoblasts from the lateral lip of dermomyotome into the limb mesenchyme. Pax3 is not detected in the lateral lip of the dermomyotome in somites at levels that normally contribute to limb musculature (black arrowhead), as these cells have migrated into the limb. Following a limb shift on the injected side, these Pax3-expressing cells are still present in somites at the same axial level (red arrowhead). (C) Whole-mount immunohistochemistry for the neurofilament-associated antigen (3A10). Axons from axial levels that normally innervate the limb fail to send projections to the shifted limb (right, black arrowheads). At more rostral levels, following mislocation of the limb, axons that normally do not innervate the limb on the injected side, project into the limb mesenchyme (red arrowheads). (D-I) Genes expressed in regions of the limb ectoderm or mesenchyme. Fgf8 (D), Bmp2 (E), Hoxc5 (F), Tbx5 (G), Tbx15 (H) and Tbx2 (I) are all expressed in their normal pattern in the shifted limb. (J-O) Skeletal preparations of an embryo injected with Tbx3 in the wing (J-L) or in the leg (M-O). Although the wing and the leg are shifted rostrally (compare bar levels between left and right side in J and M), no alterations are observed in the vertebral column. Arrow in J indicates scapula, arrow in M indicates ischium. (K) Injected wing; (L) contralateral control wing. There is no alteration in the pattern of the limb elements. (N) Injected leg; (O) uninjected leg. No change is observed in digit patterning following Tbx3 misexpression. Chick stages: (A) 22, (B) 23, (C) 24, (D-I) 21, (J-O) 27.
In a previous study, misexpression of normally neurons from the 15th to 18th spinal ganglia (Fig. 2C, transplantation studies the shifted limb is innervated by limb innervation (Hamburger, 1939). Consistent with in the migration of neuron axons from the spine into the ectopic 1964

The limb shifted by Tbx3 misexpression is patterned normally

In a previous study, misexpression of Tbx3 and Tbx2 in the developing hindlimb, results in changes in anteroposterior patterning of digits, suggesting these genes have a role in specifying digit identity (Suzuki et al., 2004). To determine if patterning of the shifted limb is altered following our misexpression strategy, we analyzed the expression of genes normally regionally restricted within the limb. Fgf8 is expressed in the AER of the injected wing in a pattern indistinguishable from the control limb (Fig. 2D; n=5/5, 100%). Bmp2, which is expressed in the AER and in the posterior of the limb as a response to Shh signalling (Francis et al., 1994), is expressed in an identical pattern in injected and uninjected limbs (Fig. 2E; n=5/5, 100%) in contrast to other reports (Suzuki et al., 2004). This is consistent with the normal distribution of Shh in Tbx3-injected limbs (Fig. 2A).

Hoxc5, which is normally expressed in proximal regions of the limb mesenchyme (Burke et al., 1995), is unaffected in the injected limb (Fig. 2F; n=6/6, 100%). Expression of other T-box genes is also unaffected within the shifted limb despite the rostral mislocation: Tbx5 is expressed throughout the limb mesenchyme (Fig. 2G; n=7/7, 100%); Tbx15 is expressed in medial regions of the limb (Fig. 2H; n=6/6, 100%); and Tbx2 is expressed in anterior and posterior stripes, in a similar pattern to Tbx3 (Fig. 2I; n=6/6, 100% compare with Fig. 1H).

We also analyzed skeletal preparations of embryos with a shift phenotype that were allowed to develop to later stages (stage 27). In these examples, the vertebral column of the embryo is normal. However, the limb skeletal elements, including the scapula are mislocated rostrally, although there is no alteration in their morphology (Fig. 2J, n=10/10, 100%). Moreover, the morphology of the digits in the shifted wing (Fig. 2K) is normal compared with those in the contralateral control limb (Fig. 2L). No alterations in digit identity were observed. The same results were obtained following misexpression of Tbx3 in the developing hindlimbs (Fig. 2M; n=10/10, 100%). The axial position of the injected limb is shifted rostrally along the rostrocaudal axis, but hindlimb digit morphology (Fig. 2N) is indistinguishable from that in the contralateral control leg (Fig. 2O). In Tbx3-injected embryos with a shifted wing (n=8) or leg (n=7) analyzed at stage 27, Sox9 expression, which marks the skeletal progenitors, is normal in both forelimbs and hindlimbs, confirming the results obtained from skeletal preparations (data not shown).

Fig. 3. Early effects of Tbx3 misexpression at the forelimb level. In all panels, injected side is rightwards and control side is leftwards. Rostral is towards the top. (A) Levels of Tbx3 transcripts are unaffected at stage 13. (B) By stage 16, Tbx3 transcripts are detected throughout the limb field on the injected side. (C,D) There is no alteration in the rostral border (red arrowheads) of Hoxb8 (C) or Hoxb9 (D) following misexpression of Tbx3. (E) Wnt2b is expressed normally in the early forelimb-forming territory. The limb-forming region extends between somites 15 and 20 (numbered). (F) Tbx5 expression is expanded rostrally. The extent of rostral expansion is indicated with a broken yellow line. Red arrowheads indicate equivalent rostrocaudal levels. (G) Rostral expansion of Hoxc5 expression (indicated with a broken yellow line). Red arrowheads show equivalent rostrocaudal levels. (H) Dil labelling of the prospective limb-forming region and adjacent somites (for axial reference). In the uninjected limb, cells that normally contribute to the posterior limb stay in register with somites at the same axial level (left, arrowhead). On the injected side (right), cells at the same level instead contribute to the inter-limb flank but remain in register with adjacent somites (arrowhead). Somites 17 and 20 are numbered.
al., 1995; Cohn et al., 1997; Rancourt et al., 1995). Axial Hox gene expression is not altered in the Tbx3-injected forelimb area. The rostral expression boundary of Hoxb8 is not changed (Fig. 3C; n=30/30, 100%). In addition, the expression boundary of Hoxb9, which is caudal to the region of the LPM that will give rise to the posterior forelimb mesenchyme (Burke et al., 1995; Cohn et al., 1997), is at the same level on the rostrocaudal axis of the embryo in both the control and injected side (Fig. 3D; n=27/27, 100%). Further analysis of the expression of several other Hox genes (Hoxb4, Hoxc4, Hoxb5, Hoxc6, Hoxa9, Hoxc9 and Hoxd9) produced identical results (data not shown). These results indicate that the mechanism that shifts limb position in Tbx3-injected embryos lies downstream of any axial Hox code that may act to position the limbs.

Effects of Tbx3 misexpression on limb mesenchyme markers

Experiments in the mouse, chick and zebrafish have established that Tbx5 is required for forelimb initiation (Agarwal et al., 2003; Ahn et al., 2002; Ng et al., 2002; Rallis et al., 2003; Takeuchi et al., 2003). The secreted factor Wnt2b has also been implicated in limb initiation (Kawakami et al., 2001). Experiments in zebrafish have suggested that wnt2b acts upstream of tbx5 during forelimb initiation (Ng et al., 2002). Following misexpression of repressor forms of Tbx3, the domain of Wnt2b expression is unaffected (Fig. 3E; n=27/27, 100%); however, the domain of Tbx5 expression is expanded to more rostral regions of the LPM, whereas the more caudal expression domain, which would normally give rise to the posterior limb mesenchyme, is initially unaffected (Fig. 3F; n=6/19, 32%). These results would suggest that the effects of Tbx3 are mediated downstream or in parallel with Wnt2b. Expression of Hoxc5, which is also expressed in the early limb bud mesenchyme, is also expanded rostrally (Fig. 3G; n=7/20, 35%). These data demonstrate that the rostral shift in the limb is preceded by a rostral expansion of the limb mesenchyme, while their caudal domain is unchanged. At later stages, however, the expression domains of Hoxc5 and Tbx5 within the limbs are shifted rostrally (Fig. 2F,G). At this stage, Shh expression is not expanded but is present in a more rostral domain (Fig. 2A). In conclusion, after misexpression of Tbx5, there is an initial rostral expansion of the limb-forming region that is followed by a subsequent shift in axial limb position.

To investigate whether cell movement accounts for the phenotype, we performed Dil labelling experiments to follow the fates of cells of the presumptive limb-forming region. Following misexpression of Tbx3, cells that normally become part of the posterior limb mesenchyme are instead incorporated into the interlimb flank (Fig. 3H; n=5/5, 100%). Cells in more rostral locations that would not normally contribute to the limb, are recruited to form (anterior) limb. Therefore, the shift in limb position cannot be attributed to migration of cells in the LPM.

Tbx3 and positioning of the ZPA

A shift in limb position is demonstrated by the expression of Shh, in the posterior of the limb bud, at an inappropriate axial level (Fig. 2A). We therefore examined the expression of dHand and Gli3, genes which are involved in pre-patternning the anteroposterior axis of the limb and establishing the position of Shh expression in the ZPA (Charite et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002). During limb induction stages, dHand (also known as Hand2), a bHLH transcription factor, is expressed throughout the limb-forming region (Fig. 4A) (Charite et al., 2000; Fernandez-Teran et al., 2000). Subsequently, Gli3, a zinc-finger transcription factor, is expressed throughout almost the entire limb mesenchyme in an anterior-to-posterior graded fashion (Schweitzer et al., 2000). Genetic antagonism between Gli3 and dHand results in downregulation of dHand expression in the anterior limb mesenchyme (Fig. 4B). At later stages, dHand and Gli3 are expressed in the anterior and posterior limb mesenchyme, respectively, with an overlapping domain of co-expression in the medial limb (Fig. 4C). The interactions between dHand and Gli3 ultimately position the ZPA prior to Shh signalling (te Welscher et al., 2002; Zuniga and Zeller, 1999). Following misexpression of Tbx3, dHand is expressed throughout the limb-forming region, while in the control side dHand is restricted to the posterior limb mesenchyme (Fig. 4D; n=7/22, 32%). In addition, there is a downregulation of Gli3 throughout the injected limb and the caudal border of its graded expression domain shifts rostrally (Fig. 4G; n=8/22, 36%). At slightly later stages (stage 19), when the shift phenotype is already apparent, the domain of dHand expression is shifted to a more rostral location and is no longer expressed throughout the limb mesenchyme but is restricted to the posterior (Fig. 4E; n=7/7, 100%). Similarly, at stage 19, Gli3 is expressed in more rostral locations, in an anterior-posterior gradient in the limb mesenchyme of the shifted limb (Fig. 4H; n=8/8, 100%). At later stages (stage 21) when the shift phenotype is obvious, dHand is expressed normally in the posterior mesenchyme of the shifted limb (Fig. 4F; n=7/7, 100%) and Gli3 has a normal distribution in the anterior mesenchyme (Fig. 4I; n=8/8, 100%). To more accurately define the time course of the limb shift phenotype, we also analyzed expression of Shh and a downstream target of Shh, Hoxd12, in Tbx3-injected limbs at stage 19. Expression domains of both Shh (Fig. 4J; n=2/2, 100%) and Hoxd12 (Fig. 4L; n=2/2, 100%) were shifted to more rostral positions. At later stages (stage 21), Shh (Fig. 4K; n=2/2, 100%) and Hoxd12 (Fig. 4M; n=2/2, 100%) were expressed in the normal, posterior domains within the shifted limb. These data show that following misexpression of Tbx3, the normal restriction of dHand expression to the posterior limb mesenchyme and Gli3 to anterior is initially disrupted at early limb-forming stages prior to Shh expression. However, as soon as Shh is detectable, its expression domain is shifted to more rostral locations. The establishment of this altered expression domain serves as the first molecular evidence of a shift in the rostrocaudal location of the limb. After limb position has shifted, dHand and Gli3 expression is normal within the limb mesenchyme.

Misexpression of Tbx3VP16 can shift the limb caudally in axial position

As a complementary approach to misexpressing Tbx3 and Tbx3EN, we misexpressed Tbx3VP16 in the presumptive forelimb area. In contrast to Tbx3 and Tbx3EN, which act as repressors, Tbx3VP16 behaves as a transcriptional activator (Fig. 1F). Moreover, Tbx3VP16 has the ability to interfere with the repressor activity of Tbx3 in our in vitro luciferase system (Fig. 1F). We predict that Tbx3VP16 is able to compete with endogenous Tbx3 for binding sites, and targets will be activated instead of repressed. Following misexpression of Tbx3VP16, the injected limb is displaced caudally in axial
expression in the posterior limb mesenchyme owing to repression by Gli3, which is expressed at higher levels in the anterior. (C) At stage 18, Gli3 is expressed in the anterior limb mesenchyme, while dHand in the posterior is required for the establishment of Shh expression in the ZPA. In medial limb mesenchyme, dHand and Gli3 expression domains overlap. (D) At stage 17, dHand is restricted in the posterior limb mesenchyme in the control side (left, arrowhead shows rostral border of expression), following Tbx3 misexpression, dHand is expressed throughout the limb-forming region (right, arrowhead). At stage 19 (E) and stage 21 (F), when the alteration in limb position is apparent, normal expression of dHand is observed within the limb mesenchyme of the shifted limb. (G) At stage 17, following Tbx3 misexpression, Gli3 is downregulated in the injected side (right arrowhead) compared with the control side (left arrowhead). A rostral shift in the graded Gli3 expression domain is observed (compare left and right arrowhead levels). At stage 19 (H) and at later stages (stage 21 (I)), Gli3 is expressed normally within the mesenchyme of the shifted limb. Shh expressed in cells of the ZPA in the posterior of the limb is detected in more rostral locations in the shifted limb by stage 19 (J) and also at stage 21 (K). Hoxd12, a downstream target of Shh, is similarly expressed in more rostral locations in the Tbx3-injected limb by stage 19 (L) and also at stage 21 (M).

Fig. 4. Effects of Tbx3 and Tbx3\(^{EN}\) misexpression on dHand and Gli3 expression. (A-C) Schematics of the limb-forming region. (A) At stage 16, dHand is present throughout the limb forming region. (B) At stage 17, high levels of dHand expression are observed in the posterior limb mesenchyme owing to repression by Gli3, which is expressed at higher levels in the anterior. (C) At stage 18, Gli3 is expressed in the anterior limb mesenchyme, while dHand in the posterior is required for the establishment of Shh expression in the ZPA. In medial limb mesenchyme, dHand and Gli3 expression domains overlap. (D) At stage 17, dHand is restricted in the posterior limb mesenchyme in the control side (left, arrowhead shows rostral expression border). Following Tbx3 misexpression, dHand is restricted to the posterior limb mesenchyme in the control side (left, arrowhead shows rostral expression border), while in the injected side, dHand expression is displaced caudally (right, arrowhead). (E) Gli3 expression at stage 17 following Tbx3\(^{VP16}\) misexpression. Gli3 is upregulated in the injected side (right) compared with the control side (left). The graded domain of Gli3 expression is displaced caudally (compare left and right arrowheads).

Gli3 is implicated in positioning the limb
Following misexpression of Tbx3, Gli3 mRNA levels are decreased within the limb mesenchyme and the caudal border of its expression is shifted rostrally (Fig. 4G). Gli3 is a bifunctional zinc-finger transcription factor that undergoes default proteolysis to a truncated form (Gli3\(^T\)) that represses expression of Shh target genes. In the presence of Shh signalling, processing to produce the Gli3\(^R\) form is blocked and a full-length Gli3 protein is formed. Contradictory data, generated both in vitro and in vivo, suggest that the full-length Gli3 molecule is either an activator or a repressor (Bai et al., 2004; Litingtung et al., 2002; Sasaki et al., 1997; Wang et al., 2000). The effect of Tbx3 misexpression on Gli3 is observed at stages prior to Shh expression when Gli3 is acting as a repressor. To investigate whether Gli3 can directly alter limb positioning, we misexpressed a form of Gli3 that contains the DNA-binding domain of the protein fused with two VP16 activation domains (Gli3ZnF-VP16, Fig. 6A). Gli3ZnF-VP16 has the ability to bind Gli3 binding sites and activate transcription (D. Stamataki, F. Ulloa and J. Briscoe, personal communication). Misexpression of this form of Gli3 is
predicted to compete with the endogenous Gli3 repressor (GliR) for binding sites and to activate, rather than repress, target genes and thereby lower the repressor activity of endogenous Gli3. Misexpression of Gli3ZnF-VP16 generated a phenotypically similar result to that obtained following misexpression of Tbx3 repressor forms (embryos that show the rostral limb shift phenotype n=48; phenotype frequency 35-40%). A rostral shift in axial limb position is evident by comparing MyoD and Shh expression domains (Fig. 6B, n=7/7, 100%) and can extend from one to three somites. Following misexpression of Gli3ZnF-VP16, the Tbx3 expression domain is expanded rostrally in the injected side compared with the contralateral control side (Fig. 6C, n=6/16, 38%). A similar expansion is observed in dHand expression (Fig. 6D, n=5/14, 36%). By contrast, the endogenous expression of Gli3 is decreased following misexpression of Gli3ZnF-VP16 (Fig. 6E, n=5/13, 38%). These results suggest that Gli3 is a candidate to play a role in positioning the limb field and may do so through a genetic interaction between dHand and Tbx3.

Discussion

Tbx3 is a transcriptional repressor participating in mechanisms that position the limb

Our results, and those of others, demonstrate that Tbx3 can act as a transcriptional repressor in vitro (Fig. 1) (Carlson et al., 2001). Consistent with these observations, misexpression of Tbx3 and Tbx3EN in the forelimb region produces identical phenotypes, while misexpression of Tbx3VP16 generates the opposite phenotype. Data from in vitro and in vivo experiments suggest that Tbx3 acts as a transcriptional repressor in the developing limb bud.

Following misexpression of Tbx3, the expression domains of genes expressed in the limb are initially expanded rostrally and this is followed by a shift in limb position. Cells of the flank, rostral to the limb, which would not normally contribute to the limb, now become incorporated into the limb. Furthermore, cells that normally form posterior limb mesenchyme now no longer contribute to the limb. Strikingly, these cells had presumably initially expressed Tbx5, a gene required and apparently sufficient for limb initiation (Agarwal et al., 2003; Ahn et al., 2002; Ng et al., 2002; Rallis et al., 2003; Takeuchi et al., 2003). Fate mapping shows that the altered contribution to the limbs is not simply explained by migration of limb bud precursors. Our data suggest that once a ZPA is established in a more rostral position, the positive feedback loop between ZPA and AER now operates at altered axial levels. The absence of an effect of Tbx3 misexpression in the presumptive wing bud region, the anterior extent of the limb is expanded (Tanaka and Tickle, 2004). The wing bud is extended rather than shifted in position and this extension is only transient. At later stages, the limb appears to regulate its size and develops normally. Brachyury is expressed in the LPM at the onset of limb formation and at later stages of limb development, in the distal limb mesenchyme that lies underneath the AER. Misexpression of Brachyury in the chick wing results in anterior expansion of the AER and produces limb phenotypes consistent with augmented AER extent and function, including anterior digit duplications and, in rare cases, thickening of the anterior-most metatarsal (Liu et al., 2003).

Tbx3 misexpression does not affect the pattern of axial Hox gene expression. Hox genes have been implicated in limb position (Cohn et al., 1997) and a role for Hox genes in limb positioning is supported by Hoxb5 knockout mice, which exhibit a unilateral or bilateral rostral shift in axial forelimb position (Rancourt et al., 1995). However, the phenotype in Hoxb5−/− mice differs in several aspects from that obtained with Tbx3 or Tbx3EN misexpression. In Hoxb5−/− mice, homeotic transformations of the cervico-thoracic vertebrae from C6-T1 are observed. The clavicle retains a medial articulation with its normal target, the sternum, resulting in a V-shaped shoulder girdle. The alteration in limb position in the Hoxb5−/− mice is therefore associated with a transformation of the entire axial skeleton. The absence of an effect of Tbx3 on the expression
A genetic interplay between Tbx3, dHand and Gli3

At limb development stages, prior to Shh expression, genetic antagonism between dHand and Gli3 establishes an anteroposterior pre-pattern of the limb that is ultimately responsible for establishing the position of the ZPA in the posterior limb. At these stages, Gli3 is acting as a repressor (Wang et al., 2000), while dHand is shown to be a transcriptional activator (Dai and Cserjesi, 2002; Dai et al., 2002). Misexpression of Tbx3 leads to an expansion, or failure of repression, of dHand, potentially through an interaction between Tbx3 and Gli3 (Fig. 7). We predict that Tbx3 is acting to repress Gli3 expression in the future posterior limb mesenchyme. Repression by Tbx3 could be responsible for generating the anterior-to-posterior graded expression of Gli3 in the developing limb primordium. This model is consistent with the observation that in Tbx3 mutant mice, dHand is downregulated in the forelimbs and absent in the hindlimbs, which subsequently leads to a disruption in Shh expression. Our model would predict that downregulation of dHand is due to high Gli3 expression that, in the absence of Tbx3, is no longer restricted to the anterior limb and expands to the posterior.

Misexpression of Tbx3VP16 produces the opposite phenotype to that obtained with Tbx3 and Tbx3EN; the limb shifts to a more caudal position than normal. Gli3 expression is upregulated and expanded caudally in the Tbx3VP16-injected limbs, lending further support to a model in which Tbx3 normally functions to restrict Gli3 to the anterior mesenchyme. As a consequence of this Gli3 expansion, the dHand expression domain is shifted caudally. By contrast, in Tbx3- and Tbx3EN-injected embryos, disruption of the normal repression of dHand by Gli3 resets the rostrocaudal position of the ZPA to a more rostral position and this ultimately results in the limb shift phenotype. In Tbx3VP16-injected embryos, de-repression of Gli3 shifts the expression domain of dHand and this ultimately leads to the ZPA forming more caudally. Our data demonstrate that the signals required for pre-patterning the AP axis of the limb and setting the position of the ZPA can influence the position of the limb along the rostrocaudal axis of the embryo.

Gli3 and limb positioning

We predict that the effects of Gli3ZnF-VP16 on dHand and Tbx3 are caused by a disruption of endogenous Gli3 activity. Misexpression of Gli3ZnF-VP16 leads to the expansion of Tbx3 and dHand expression domains, rather than the induction of ectopic patches of expression. This may indicate a non-cell autonomous action of Gli3 on dHand and presumably also on Tbx3. A regulatory relationship between Gli3 and Tbx3 has been demonstrated during lung organogenesis (Li et al., 2004). Tbx3 is normally expressed in the mouse lung in the presence of Shh. In this environment, Gli3 acts as an activator of Tbx3 transcription. In Shh−/− animals, where Gli3 acts as a repressor,
Tbx3 transcripts are significantly reduced. However, in Shh<sup>−/−</sup>/Gli3<sup>−/−</sup> animals, de-repression of Tbx3 is observed and Tbx3 expression is, at least partially, restored (Li et al., 2004). These results, in combination with our own, suggest that regulatory relationships between Tbx3 and Gli3 may exist broadly during embryogenesis.

Our data implicate Gli3 in a genetic network that can influence limb position, yet mice mutant for Gli3 (Extra toes, X<sub>r</sub>), are not reported to exhibit any shift in axial limb position (Buscher et al., 1997; Buscher et al., 1998; Hui and Joyner, 1993; Litington et al., 2002). In X<sub>r</sub>/X<sub>r</sub> mice (that lack all Gli3 activity), dHand is expressed throughout the limb and subsequently Shh is expressed ectopically in the anterior limb mesenchyme. Misexpression of dHand alone, at limb bud stages, is capable of inducing Shh expression, but only in the anterior limb mesenchyme rather than medial locations (Charite et al., 2000; Fernandez-Teran et al., 2000). These results suggest that anterior and posterior limb mesenchyme may express a ‘licensing factor’ required together with dHand for the induction of Shh and that this factor is absent from the medial limb mesenchyme. Tbx3 is expressed in two stripes in the anterior and posterior limb mesenchyme at later stages of limb development (Fig. 1D) and may normally act as such a factor.

A requirement for Tbx3 to establish or re-set the domain of Shh-expressing cells in the ZPA may also explain why no limb shift phenotype is observed in X<sub>r</sub>/X<sub>r</sub> mice. Tbx3 expression is not expanded in X<sub>r</sub>/X<sub>r</sub> mice (Tumpel et al., 2002). Without the presence of both Tbx3 and dHand, the position of the limb is not altered. In Tbx3 misexpression experiments, however, Tbx3 can repress Gli3 and this leads to an expansion of dHand. The co-expression of Tbx3 and dHand can, in turn, ‘license’ ectopic Shh expression, which, in turn, alters the position of the limb (Fig. 7).

Mice in which Tbx3 is inactivated and humans with UMS who are haploinsufficient for TBX3 are not reported to exhibit any shift in axial limb position. This is consistent with the model we propose for the role of Tbx3 in the early limb bud. Although a limb shift phenotype is not observed in Tbx3<sup>−/−</sup> mice, the expression domains of dHand and Shh are downregulated or even eliminated (Davenport et al., 2003), consistent with Tbx3 being required for their normal expression. This finding supports our conclusions that, although not strictly required to fix limb position, Tbx3 is an important component of the signals establishing the position of the domain of Shh-expressing cells that comprise the ZPA in the posterior limb.

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