Regionalized \textit{Twist1} activity in the forelimb bud drives the morphogenesis of the proximal and preaxial skeleton

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\textbf{A B S T R A C T}

Development of the mouse forelimb bud depends on normal \textit{Twist1} activity. Global loss of \textit{Twist1} function before limb bud formation stops limb development and loss of \textit{Twist1} throughout the mesenchyme after limb bud initiation leads to polydactyly, the ulnarization or loss of the radius and malformations and reductions of the shoulder girdle. Here we show that conditional deletion of \textit{Twist1} by \textit{Mesp1-Cre} in the mesenderm that migrates into the anterior-proximal part of the forelimb bud results in the development of supernumerary digits and carpals, the acquisition of ulna-like characteristics by the radius and malformations of the humerus and scapula. The mirror-like duplications and posteriorization of pre-axial tissues are preceded by disruptions to anterior–posterior Shh, Bmp and Fgf signaling gradients and dysregulation of transcription factors that regulate anterior–posterior limb patterning.

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\textbf{Introduction}

Limb morphogenesis in the mouse embryo begins with the formation of a bud, followed by outgrowth, the shaping of the autopod and zeugopod segments, and the specification and formation of digits. Proximal–distal growth and patterning are controlled by Fgf10 and Bmp signaling in the mesenchyme and Fgf4/8 signals from the apical ectodermal ridge (AER) (Bénazet and Zeller, 2009). Specification of the number and identity of digits is controlled by their position in the gradient of Shh signaling from the zone of polarizing activity (ZPA) and the duration of exposure to the signal. Shh signaling is in turn regulated by feedback loops involving Fgf signals from the AER and Bmp in the mesenchyme (Robert, 2007; Sun et al., 2000). The characteristics of skeletal elements are specified by transcription factors including members of the Hoxd, Alx and Msx families that are subject to regulation by the Fgf, Shh and Bmp signaling pathways (Bensoussan-Trigano et al., 2011; Kuijper et al., 2005; Zakany et al., 2007).

\textit{Twist1}, a basic helix–loop–helix transcription factor, plays a key role in integrating the actions of signaling pathways and transcription factors during limb development (O’Rourke and Tam, 2002; Zuniga et al., 2002). In \textit{Twist1}\textsuperscript{−/−} mouse embryo outgrowth of the forelimb buds is impaired and they are smaller than wild-type limbs by E10 (Chen and Behringer, 1995). Poor forelimb outgrowth in \textit{Twist1}\textsuperscript{−/−} embryos is accompanied by reduced expression of Fgf genes in the AER, and weaker Bmp and Shh signaling in the mesenchyme (O’Rourke et al., 2002; Zuniga et al., 2002). Outgrowth of the hindlimb buds is affected to a lesser degree, and the disruptions to Fgf, Bmp and Shh signaling are less severe (O’Rourke et al., 2002). Further investigation into the morphogenetic outcome of these defects has been prevented by the death of \textit{Twist1}\textsuperscript{−/−} embryos at approximately E11.5, not long after the limb pattern emerges.

In contrast, partial reductions in \textit{Twist1} activity affect anterior–posterior patterning of the limb buds. In \textit{Twist1}\textsuperscript{−/−}\textsuperscript{-/+} heterozygous mice, this manifests as pre-axial polydactyly of the hindlimbs (O’Rourke et al., 2002). Homozygosity for a hypomorphic \textit{Twist1} point mutation affecting protein–protein interactions (\textit{Ska10}, also known as \textit{Charlie Chaplin}, CC) or a combination of \textit{Ska10} and null alleles results in loss of digits, and abnormal or missing long bones of the forelimb (Krawchuk et al., 2010). However, conditional loss of \textit{Twist1} function widely in the mesenchyme after the limb bud has begun to develop, driven by \textit{Prx1-Cre}, leads to a disruption of A–P patterning that causes polydactyly and ulnarization of the radius, as well as abnormalities of the humerus and scapula, (Krawchuk et al., 2010; Zhang et al., 2010). Genetic and biochemical studies suggest that \textit{Twist1} influences anterior–posterior patterning of the limbs through interactions with ETV-family proteins (Zhang et al., 2010) that influence Shh signaling.

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In addition, Twist1 interacts genetically with Gli3, which encodes a Shh signaling repressor (O'Rourke et al., 2002). These findings suggest that Twist1 function is critical for the acquisition of anterior limb characteristics and the formation of pre-axial limb structures. Whether Twist1 is required locally in the anterior tissues of the limb bud is not yet known.

Precursors of limb bud tissues are derived from the lateral plate mesoderm, which forms the skeletal elements (Koussoulakos, 2004) and the hypaxial dermomyotome, which contributes to the muscles (Buckingham et al., 2003). We have discovered that Cre recombinase expressed from a knock-in allele at the Mesp1 locus (Saga et al., 1999) leads to the loss of Twist1 early in the mesoderm that eventually populates the mesenchyme in the anterior and proximal regions of the forelimb bud, providing a unique experimental system in which to investigate the contribution of Twist1 in a specific region to the anterior–posterior patterning of the limb. We show that loss of Twist1 function in this restricted domain within the forelimb bud mesenchyme results in anterior–posterior patterning defects reminiscent of those that result from deletion of Twist1 in the entire limb bud mesenchyme (Krawchuk et al., 2010; Zhang et al., 2010), but less severe defects in the humerus and scapula. The patterning defects are likely to be consequences of reduced Gli3 and A64 expression and disruptions to Fgf, Bmp and Shh signaling during a critical phase of limb patterning.

Material and methods

Mouse strains and genotyping

Twist13loxPneo/3loxPneo and Twist1del/+ mice were maintained and genotyped as previously described (Bildsoe et al., 2009; Loebel et al., 2007). Mesp1-Cre mice (Saga et al., 1999) were maintained on a C57Bl/6 background and genotyped by PCR with the following primers:

5′-CTGACGTACACAAAAATTGGCTTC-3′ (CreF),
5′-GATAATCGGAACATCTCCGTTC-3′ (CreR).

We first crossed Mesp1-Cre mice (Saga et al., 1999) to Twist1del/+ mice to generate Twist1del/−; Mesp1cre/− mice. To generate embryos with a mesoderm-specific Twist1 deficiency, Twist1del/−; Mesp1cre/− mice were crossed with Twist13loxPneo/3loxPneo mice. Conditional knockout (CKO) embryos of Twist13loxPneo/3loxPneo; Mesp1cre/+ genotype were compared with Twist13loxPneo/3loxPneo; Mesp1cre/− (WT) embryos and Twist13loxPneo/3loxPneo; Mesp1cre/+ (heterozygous) embryos.

To trace the distribution of the descendants of Mesp1-Cre expressing cells, we initially crossed the Rosa26R line (Soriano, 1999) to the Twist13loxPneo/3loxPneo line generating Twist13loxPneo/−; Gt(Rosa)26Sor+ offsprings which were then crossed with Twist13loxPneo/3loxPneo, Gt(Rosa)26Sor− mice to generate β-galactosidase positive CKO and WT embryos for analysis. The Rosa26R allele was detected by PCR for lacZ with the following primers:

LacZ-F: 5′ TTATCGATGACCTACAAAATTGGCTTC-3′
LacZ-R: 5′GGCGGTACATCGGGAAAATATTC-3′

Bone and cartilage staining

Embryos were collected at E16.5 and E17.5 in PB1 (Kinder et al., 2000) and rinsed in cold PBS (without calcium and magnesium). Bone and cartilage were stained with alizarin red and alcian blue (Hogan et al., 1994). Stained specimens were washed, stored and photographed in aqueous 20% ethanol: 20% glycerol. Images were captured using a SPOT camera and Leica microscope.

β-galactosidase reporter staining

For whole mount staining to detect β-galactosidase activity, embryos were fixed for at least 2 h in glutaraldehyde solution (Igepal CA630 0.02%, Sodium Deoxycholate 0.01%, Glutaraldehyde, 0.2%, EGTA 5 mM, MgCl2 2 mM), washed in X-gal washing buffer briefly before being incubated in X-gal staining solution at 37 °C for 2–3 h for color development (Watson et al., 2008). The embryos were then washed twice in X-gal washing buffer and fixed in 4% PFA.

For β-galactosidase staining of cryosections, limbs were dissected from E13.5 embryos in PB1 medium, rinsed in PBS and fixed in 4% PFA overnight at 4 °C. The limbs were then infiltrated with 15% sucrose/ PBS overnight, followed by 25% sucrose/PBS overnight at 4 °C. Tissues were embedded in equal volumes of 25% sucrose/PBS and OCT embedding medium (Tissue Tek). Sections were cut at 5–8 μm on a cryostat (MI1900, Leica), collected on Super Frost Plus slides (Menzel–Glaser) and stored at −80 °C until needed. Sections were thawed at room temperature, rinsed in Lact Buffer 3 times and then incubated in X-gal staining solution at 37 °C overnight for color development followed by washing in X-gal washing buffer and water, counterstained with nuclear fast red and mounted in Ultra-mount No.4 (Fronine).

Immunofluorescence

Embryos between E9.5–10.5 were dissected in PB1 and rinsed in cold PBS. The limb buds were fixed in 4% PFA overnight at 4 °C and cryoembbed as previously described (Bildsoe et al., 2009). Cryosections were stored at −20 °C until use. Prior to staining, slides were allowed to reach room temperature, washed three times for 3 min. in washing solution (PBS plus 0.1% BSA, 0.02% triton X-100) and blocked with 3% BSA 0.02% triton in PBS for 1 h at room temperature. Slides were incubated overnight at 4 °C with mouse anti-Twist1 (Abcam, 1/50 dilution) and rabbit anti-Ki67 (Abcam, 1/100 dilution) blocking solution and washed three times in washing solution for 5 min at room temperature. Secondary antibodies (AlexaFluor-488 Donkey anti-mouse and AlexaFluor-594 goat anti-rabbit, Invitrogen) diluted 1/500 in PBS plus 0.2% triton, 1% donkey serum were added in washing solution for 1–2 h at room temperature. Slides were then washed three times for 5 min in washing solution, stained with DAPI for 10 min, washed again for 5 min in PBS before mounting in 70% glycerol/PBS. Ki67 positive and DAPI stained nuclei were counted in identically sized and shaped regions within the Twist1-deleted zone of CKO limb buds and equivalent regions of wild-type limb buds.

In situ hybridization

Riboprobes for whole mount in situ hybridization to E10.5 or E11.5 mouse embryos were made from plasmid clones containing fragments of the following cDNAs: Alx1, Alx4, Ems2, Fgf4, Fgf8, Fgf10, Gli3, Greml, Gsc, Hand2, Hoxd13, Msx1, Pbx1, Pch1, Shh and Twist1. To generate a riboprobe to detect Cre transcript, a fragment of the open reading from was amplified using the following primers:

Cre-F: 5′-CCGTACACAAAAATTGGCTTCATTG-3′
Cre-R: 5′-ACATGTTGCCCCGTGTCTACATCCA-3′

The amplified fragments were gel purified and re-amplified using primers identical to the first round of amplification, except that the reverse primer contained a T7 promoter sequence at the 5′ end. This product was gel purified and used for riboprobe generation. Digoxigenin-labeled riboprobes were synthesized using Ampliscribe (Epicentre Technologies). Automated whole mount in situ hybridization was carried out using an In situ Pro machine (Intavis AG) as described previously (Bildsoe et al., 2009; Loebel et al.,...
2004). Stained embryos were washed in 0.1% Tween20 in H2O, fixed in 4% PFA and photographed. Measurements were made using ImageJ and statistics calculated with Microsoft Excel.

Cell death analysis

Apoptotic cells were detected by whole mount TUNEL staining with the ApopTag Plus Peroxidase In situ Apoptosis kit (Millipore) following published methods (Martinez-Barbera et al., 2002). Briefly embryos were dissected in PB1, rinsed in cold PBS and fixed in 4% PFA overnight at 4 °C. For the detection step the embryos were incubated in blocking buffer containing 2% Blocking Reagent (Roche) and 20% heat inactivated fetal calf serum in PBS/0.1% Tween. After antibody labeling, the embryos were washed overnight (Roche) and 20% heat inactivated fetal calf serum in PBS/0.1%

Results

Mesp1-Cre labels cells in the anterior mesoderm

Cre transcript expressed from the Mesp1 locus was detected in the nascent mesoderm adjacent to the primitive streak of E7.5 embryos (Fig. 1A), but at E8.5 and E9.5 Cre expression was restricted to a stripe of tissue at the cranial end of the presomitic mesoderm (Figs. 1B, C). This expression pattern recapitulates that of the endogenous Mesp1 transcript (Saga et al., 1996; Saga et al., 1999).

In Mesp1Cre; Rosa26R embryos at E7.5, β-galactosidase labeled cells were found throughout the mesoderm, consistent with the anterior migration of mesoderm cells that had previously expressed Cre [Figs. 1D, D(i), E]. By E7.75, stained cells were found predominantly in the anterior half of the embryo, indicating that the mesoderm present at E7.5 contributes predominantly to these tissues (Fig. 1F). At E8.5, the heart and the majority of the cranial mesoderm strongly expressed β-galactosidase [Figs. 1G, G(i)]. Widespread labeling of the lateral plate mesoderm of the splanchnopleure and somatopleure continued into the trunk region, but the paraxial mesoderm became progressively more sparsely labeled caudally [Figs. 1G, G(ii)]. At the level of the hindgut, only the blood vessels, body wall and extraembryonic mesoderm expressed β-galactosidase [Fig. 1G (iv)]. At E9.0–E9.5, a similar rostral–caudal gradient of staining intensity was observed in the paraxial mesoderm, with the cranial, cervical and heart mesoderm cells being heavily labeled [Figs. 1I, I(i), J, J(i)], but progressively sparser labeling in caudal paraxial mesoderm [Figs. 1I (ii–iii), J (ii–iii)]. At E9.5 we observed β-galactosidase activity in the forelimb bud, contiguous with the stained lateral and ventral mesoderm populations [Fig. 1(ii)].

Mesp1-Cre mediates ablation of Twist1 in cells that populate the anterior-proximal limb bud mesenchyme

Closer examination of β-galactosidase staining in the forelimb buds at E9.5 revealed activity throughout the majority of the forelimb bud, with strongest staining toward the anterior and a patchy appearance in the posterior limb-bud mesenchyme (Figs. 2A, C, supplementary Fig. S1A). In the forelimb bud of E10.5 embryos, the distributions of β-galactosidase positive cells varied, but in most cases labeled cells were found predominantly in the mesenchyme of in the anterior two-thirds of the forelimb bud (Figs. 2E, G, supplementary Fig. S1B). By E13.5, marked cells in the autopod were predominantly found in the tissues of digits 1, 2 and 3 at a lower frequency in the postaxial digits (Figs. 2I, K). β-galactosidase positive cells contributed strongly to the radius, but patchily to the ulna, humerus and scapula (Fig. 2M).

In conditional Twist1 mutant embryos (Mesp1Cre/+; Rosa26R; Twist1flox/floxCKO), β-galactosidase positive cells had a more restricted distribution in the forelimb buds. At E9.5, the distribution of labeled cells contracted proximally and anteriorly (Figs. 2B, D, supplementary Fig. S1A). At E10.5 the distribution of labeled cells

Fig. 1. Mesp1-Cre is expressed in the anterior mesoderm. (A–C) Mesp1-Cre expression, revealed by in situ hybridization for Cre transcript in the nascent mesoderm near the primitive streak at E7.5 (A, embryo shown with anterior to the left) and in a restricted domain in the pre-somitic mesoderm at E8.5 (B) and E9.5 (C). (D–J) β-galactosidase from the Rosa26R reporter transgene detected widely in the mesoderm of embryos at E7.5 (D, E, shown with anterior to the left in D and D(i), and en face in E), and predominantly in anterior, cardiac and lateral mesoderm populations at E7.75 (F), E8.5 (G, H) E9.0 (I) and E9.5 (J); D(i), G [i–iv], I (i–iii), J [i–ii] show sections through embryos at the planes indicated in D, G, I and J respectively. Abbreviations: da, dorsal aorta; fig, foregut; h, heart; hg, hindgut; lb, forelimb bud; m, mesoderm; mg, midgut; ov, otic vesicle; pm, paraxial mesoderm; ps, primitive streak; so, somatopleure; sp, splanchnopleure; va, vitelline artery.

Fig. 2. β-Galactosidase positive cells are restricted to the anterior-proximal mesoderm of the forelimb bud. (A–C) Mesp1-Cre expression, revealed by in situ hybridization for Cre transcript in the nascent mesoderm near the primitive streak at E7.5 (A, embryo shown with anterior to the left) and in a restricted domain in the pre-somitic mesoderm at E8.5 (B) and E9.5 (C). (D–J) β-galactosidase from the Rosa26R reporter transgene detected widely in the mesoderm of embryos at E7.5 (D, E, shown with anterior to the left in D and D(i), and en face in E), and predominantly in anterior, cardiac and lateral mesoderm populations at E7.75 (F), E8.5 (G, H) E9.0 (I) and E9.5 (J); D(i), G [i–iv], I (i–iii), J [i–ii] show sections through embryos at the planes indicated in D, G, I and J respectively. Abbreviations: da, dorsal aorta; fig, foregut; h, heart; hg, hindgut; lb, forelimb bud; m, mesoderm; mg, midgut; ov, otic vesicle; pm, paraxial mesoderm; ps, primitive streak; so, somatopleure; sp, splanchnopleure; va, vitelline artery.

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was variable but, like the E9.5 embryo, occupied a territory that was generally more restricted toward the anterior and proximal regions of CKO forelimb buds (Figs. 2F, H, supplementary Fig. S1 B). However, some labeled cells were present in other parts of the limb bud mesenchyme. At E13.5, β-galactosidase positive cells congregated mainly in the two most pre-axial digits (Figs. 2J, L) in CKO embryos.

Fig. 2. Mesp1-Cre causes loss of Twist1 activity in a restricted region of the limb bud mesenchyme. (A–N) Staining for Rosa26R activity in E9.5 (A–D), E10.5 (E–H) and E13.5 forelimb buds (I–N). Rosa26R activity is detectable in a broader domain of anterior mesenchyme in the wild-type (A, C, E, G, I, K, M) than the Mesp1-Cre;Twist1 CKO embryos (B, D, F, H, J, L, N). (C, D) Sections through stained wild type and CKO limb buds at E9.5 and (G, H) at E10.5 reveal a more restricted distribution of labeled cells in CKO forelimbs. (K–N) Staining for Rosa26R activity on frozen sections of E13.5 limb buds shows that in wild-type embryos (K, M), labeled cells contribute strongly to digits 3, 4 and 5 and to the radius, and are found throughout the other digits and in the ulna, humerus and scapula. In CKO embryos labeled cells contribute mainly to digits 4 and 5, and to the radius, with weaker contributions to other digits and little contribution to the ulna, humerus or scapula (L, N). (O–P) Immunofluorescent staining for Twist1 and Ki67 on cryosections of wild-type (O) and CKO (P) forelimb buds at E9.5 revealing a region of Twist1-negative cells in the proximal mesenchyme with a greater concentration of these cells toward the anterior of the limb bud and a lower frequency of Ki67 in the CKO limb buds than in an equivalent region of the wild-type limb bud. The left panel shows Twist1 immunostaining, the middle panel shows merged DAPI and Ki67 and the right panel shows a merged image of all three. (Q–R) Immunofluorescent detection of Twist1 (left and merged with DAPI, right) at E10.5 in wild-type (Q) and CKO limb buds (R). Abbreviations: h, humerus; r, radius; s, scapula; u, ulna.
galactosidase positive cells colonized the radius of the CKO embryo as in the wild type embryo, but fewer were found in the ulna, humerus and scapula (Figs. 2M–N).

Examination of Twist1 expression by whole mount in situ hybridization confirmed the region-specific reduction in Twist1 expression at E9.5 and E10.5 (Supplementary Fig. S2). Anti-Twist1 immunostaining at E9.5 showed Twist1-deficient cells were present along most of the length of the limb bud, but more concentrated proximally and in the anterior two-thirds of the limb buds (Figs. 2O, P), reflecting the pattern of Rosa26R reporter activity in CKO limb buds. At E10.5, Twist-deficient cells were mostly localized to the proximal half of the forelimb bud, and mostly in the anterior sector (Figs. 2Q, R).

To test whether Twist1-deficient cells in CKO limb buds might be less proliferative we compared Ki67 immunostaining of the Twist1-deficient regions to equivalent regions in wild-type limb buds (Figs. 2O–P). At E9.5, the proportion of Ki67-positive Twist1-deficient cells in CKO limb buds (mean 2.1%±s.e.m. 0.20, n = 5) was significantly lower than in wild-type limb buds (3.2%±0.43, n = 4; p = 0.02 by a two-tailed t-test). At E10.5, there was a similar trend of reduced proliferation, though not statistically significant (wild-type, 3.10%±0.3, n = 4; CKO 1.94%±0.41, n = 4; p = 0.091). At E10.5, the cell density was significantly greater in Twist1-deficient mesenchyme than in corresponding regions of wild-type forelimb buds (wild-type, 3965.12 nuclei/mm², n = 4; CKO 4680.23 nuclei/mm², n = 4, p = 0.008). Analysis of apoptosis by whole mount TUNEL staining of CKO embryos revealed no increased cell death in the limb bud mesoderm at E9.5, or in the prospective limb field at E8.75, prior to limb bud initiation (Fig. S3. A–D). An increase in cell death was observed at E9.5 in the β-galactosidase positive paraxial mesoderm anterior to the forelimb bud, where Twist1 expression was lost (Fig. S3C–H).

Regionalized loss of Twist1 activity affects patterning of pre-axial structures

By E13.5, CKO forelimb buds were broader and contained more digit primordia than their wild-type counterparts (Figs. 2I, J). When examined at E17.5, the forelimbs of CKO embryos had 6–8 digits (Supplementary Fig. S4), ranging from an apparent duplication of digit 1 (Supplementary Fig. S4 A) to extensive polydactyly with bifurcated digits (Supplementary Fig. S4 E).

Skeletal preparations revealed that the major skeletal elements of the limbs were present, but malformed in CKO embryos (Figs. 3A, B). Anterior–posterior asymmetry was less pronounced in the autopod of CKO embryos than in wild-type embryos. The most pre-axial digit was often longer in CKO embryos than in wild-type embryos (Figs. 3C, D). While the phalanges were not yet ossified, the metacarpals of all the central digits of E17.5 CKO forelimbs were ossified as were the three central metacarpals (II, III and IV) of the wild type limb (Figs. 3E, F). The arrangement of carpal bones in the wrist of the CKO forelimb differed from the wild type limb. Supernumerary bones were present in the distal row of carpal (Figs. 3C–F) and the proximal carpals of the CKO limb were flattened and the pisiform was reduced (Figs. 3E, F). In some cases the appearance of the anterior carpals of the CKO limb was consistent with a mirror duplication of posterior elements (Figs. 3E, F), but not in others (e.g. Fig. 3D). In 11/12 limbs examined, the radius was abnormal, with some morphological characteristics of the ulna including an olecranon process, resulting in a more symmetrical elbow joint (Figs. 3A, B, G, H). The humerus was short, curved and lacking the deltoid tuberosity in CKO embryos (10/10 limbs examined). Its proximal end was often fused with the scapula to form an immobile shoulder joint (6/9 limbs; Figs. 3A, B, I, J). The scapula of CKO embryos was hypo plastic with a small or absent spine (7/7 limbs; Figs. 3I, J). The data show that loss of Twist1 in the anterior mesenchyme results in significant changes in the anterior–posterior patterning of the distal pre-axial structures and affects the morphogenesis of proximal skeletal elements of the limb.
confined to a smaller domain at the anterior margin (Figs. 4A–C; the proportion of anterior limb perimeter covered by Alx1 expression domain is shown as mean ± SEM: wild-type, 0.226 ± 0.009, n = 6; het, 0.242 ± 0.008 n = 2; CKO, 0.163 ± 0.011 n = 4; two-tailed t-test comparing wild-type and CKO, p = 0.0025). Expression of Emx2 and Pbx1, which act upstream of Alx1 in scapula development, (Capellini et al., 2010) was similar in wild-type, heterozygous and CKO embryos (Fig. S5 A–F). In contrast, the expression of Gsc, which is also required for normal development of the shoulder joint (Belo et al., 1998), was slightly reduced in the proximal mesenchyme of CKO and heterozygous forelimb buds (Fig. S5 G–I). Alx1 is normally expressed in the anterior limb bud mesenchyme and loss of function mutations in Alx1 result in polydactyly (Qu et al., 1998). Alx4 expression was reduced in 6/6 CKO limb buds but not in heterozygous buds (2/2, Figs. 4D–F). In heterozygous forelimb buds, the expression domain of the posterior transcription factor Hoxd13 expanded toward the distal limb region (2/2, Figs. 4G–H), suggesting that the overall reduction in Twist1 expression resulted in de-repression of Hoxd13 in some cells. In CKO limb buds the ectopic expression extended into the anterior rim of limb bud (6/6, Fig. 4I). In contrast, expression of Hand2, which interacts with Twist1 (Firulli et al., 2005), was unchanged the posterior mesenchyme in 2/2 heterozygous and 6/6 CKO limb buds (6/6, Fig. S5, J–L). These data indicate that Twist1 is required for maintaining the expression of genes in the anterior mesenchyme, as well repressing some genes that are normally expressed in the posterior mesenchyme.

**Abnormal signaling in CKO limb buds**

The Shh, Bmp and Fgf signaling pathways are essential for tissue outgrowth and patterning during limb development. At E10.5 Gli3, which encodes a transcription factor that represses the downstream response to Shh signaling, is robustly expressed in an anterior–posterior gradient in mesenchyme of the wild-type forelimb bud (Fig. 5A). Whereas no change in Gli3 expression was observed in Twist1 heterozygous buds (2/2), its expression was reduced in the anterior mesenchyme of 6/6 CKO buds (Figs. 5B, C). Ptc1, a repressor and transcriptional target of Shh signaling, was expressed in the posterior mesenchyme of the limb bud of wild-type and heterozygous embryos.
expressed broadly in the nascent mesoderm only during gastrulation, **Mesp1** including a restricted region of the forelimb bud. These cells contribute to anterior mesodermal populations in the embryo, remaining within a well-defined band in the distal limb bud indicating that there was no appreciable dorso-ventral expansion of the AER in CKO embryos. This is consistent with some of the labeled cells in the forelimb bud being derived from the **β-galactosidase-positive mesodermal anterior** to the forelimb bud. It is also likely that the **β-galactosidase-positive cell population medial to the forelimb bud contributes to the bud mesenchyme.** Wild-type forelimbs also contain unlabeled cells, predominantly toward the posterior part of the limb bud.

In this study we have used **Mesp1-Cre** to produce a conditional deletion of **Twist1** in the mesoderm during gastrulation. These cells contribute to anterior mesodermal populations in the embryo, including a restricted region of the forelimb bud. **Mesp1-Cre** is expressed broadly in the nascent mesoderm only during gastrulation, reflecting the expression pattern of the wild-type **Mesp1** transcript. (Saga et al., 1996). Descendants of this population populate the heart, cranial mesoderm, the paraxial mesoderm in the cervical region rostral to the forelimb bud and the lateral plate mesoderm that contributes to the body wall and forelimb. These results are in agreement with previous fate mapping data based on cell transplantation, showing that the mesoderm of late-streak stage embryos contributes primarily to heart, cranial, lateral plate and anterior somite mesoderm in the early somite-stage embryo. (Parameswaran and Tam, 1995).

Since Cre expression is not detectable in the forelimb bud, **β-galactosidase-labeled cells in the limb bud probably share ancestry with the Rosa26R expressing cells adjacent to the limb field.** A recent cell labeling study (Wynnaarden et al., 2010) showed that cells entering the limb bud move generally in a caudal direction. This is consistent with some of the labeled cells in the forelimb bud being derived from the **β-galactosidase-positive mesodermal anterior** to the forelimb bud. **β-galactosidase-positive cell population medial to the forelimb bud contributes to the bud mesenchyme.** Wild-type forelimbs also contain unlabeled cells, predominantly toward the posterior part of the limb bud.

Although we observed variations in the distribution of reporter labeled cells in **Twist1** CKO embryos at E10.5, in general they were more concentrated in the mesenchyme in the anterior and proximal parts of the limb bud. By E13.5, the labeled cells (which are likely to be lacking **Twist1**) in CKO embryos clearly contributed most strongly to pre-axial skeletal structures, notably the radius and the two most anterior digits. Since the radius and preaxial autopod elements are the most severely affected, having undergone duplications or loss of anterior identity, **Twist1** may be acting autonomously within the progenitor cells to regulate anterior–posterior identity. This may be due to reduced expression of transcription factors (such as Aristaless-related genes) in **Twist1-null cells.** However, **Twist1** may also be acting in a non-cell-autonomous fashion to affect patterning and identity in the limb. Consistent with this, the effects on skeletal patterning spread beyond the zones where **Twist1** expression has been lost and our results show that the signaling environment in the anterior half of the limb bud has been disrupted. Therefore, localized loss of **Twist1** can disrupt patterning non-cell-autonomously by perturbing signaling gradients across the limb (Fig. 6).

In both **Mesp1-Cre** and **Prx1-Cre** CKO embryos (Krawchuk et al., 2010; Zhang et al., 2010), supernumerary digits were formed and

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**Discussion**

In the wild-type (WT) limb bud, **Twist1** is required for normal expression of **Gli3** and **Alx1** in the anterior limb mesenchyme, consistent with previous limb bud expression studies. Gli binding sites are present upstream of **Alx1**, suggesting that **Gli3** might regulate **Alx1** expression. **Gli3 and Alx1** repress **Fgf10, Grem1 and Hoxd13**, restricting their expression to the posterior or distal mesenchyme. Expanded Grem1 expression in CKO limb buds causes a reduction in Bmp signaling in the anterior limb, in addition to alterations to Shh signaling downstream of **Gli3**. Ligand independent ectopic activity of the Shh pathway is augmented by ectopic Shh ligand expression in a minority of cases (indicated by the dotted arrow). In the posterior mesenchyme, which is populated by **Twist1** heterozygous cells, gene expression patterns do not change. Lighter shading in CKO shows the **Twist1**-deficient region. References: 1, (O'Rourke et al., 2002); 2, (Loebel et al., 2002); 3, (Te Welscher et al., 2002); 4, (Vokes et al., 2008); 5, (Kuijper et al., 2005); 6, (Zakany et al., 2007); 7, (Buscher et al., 1997); 8, (Khokha et al., 2003); 9, (Zakany et al., 2004); 10, (Bastida et al., 2009); 11, (Goodrich et al., 1996).
preaxial structures were posteriorized (Table 1). Proximal limb elements lost some anterior characteristics: the anterior tuberosity of the humerus and spine of the scapula were absent. Some Prx1-Cre CKO embryos lacked a radius altogether (Krawchuk et al., 2010), which was not seen in the Mesp1-Cre CKO embryos. Embryos in which the Ska10 missense mutant allele was the only functional Twist1 allele lacked a radius and had fewer digits, while showing signs of mirror-duplication (Krawchuk et al., 2010; Zhang et al., 2010).

Although similarities were observed between Mesp1-Cre and Prx1-Cre CKO embryos in distal limb development, there are differences in the degree of abnormality in the proximal limb (Table 1). Mesp1-Cre CKO embryos displayed reductions and abnormalities in the humerus and scapula. In Prx1-Cre CKO embryos, more dramatic reductions in scapula development were reported (Krawchuk et al., 2010; Zhang et al., 2010) (Table 1). The loss of the deltoid tuberosity and scapula spine in CKO embryos is consistent with the loss of anterior characteristics in the limb. The more pronounced abnormalities and reductions in the humerus and scapula in Prx1-Cre CKO embryos suggest that there are additional roles for Twist1 in other parts of the limb buds mesenchyme in growth and patterning of these structures.

We observed ectopic expression of Shh in a minority of CKO limb buds (Goodrich et al., 1996). This is perhaps surprising since Hoxd13 has previously been shown to activate detectable Shh transcription in most cases. Hindlimb polydactyly was significantly reduced in Twist1<sup>−/−</sup>; Ska10<sup>−/−</sup> embryos (Krawchuk et al., 2010), confirming that the Shh signaling pathway is involved in hindlimb polydactyly in Twist1 mutants, even though ectopic Shh expression was observed in only a small proportion of Twist1<sup>−/−</sup> or Twist1<sup>−/−</sup> hindlimb buds (Krawchuk et al., 2010; O’Rourke and Tam, 2002). Although it is possible that expression of Shh (and Ptch1) is below the level of detection in these assays, Shh ligand-independent polydactyly has been observed in Gli3<sup>−/−</sup>; Shh<sup>−/−</sup> embryos (Litingtung et al., 2002; Te Welscher et al., 2002) and ectopic Hoxd13 and Fgf4 expression occurs in Ax4<sup>−/−</sup>; Shh<sup>−/−</sup> embryos indicating an anterior–posterior patterning defect (Kuijper et al., 2005). Since both Ax4 and Gli3 are down-regulated in Twist1 CKO forelimb buds (Fig. 6) it is possible that ectopic Shh ligand expression is not a pre-requisite for limb anterior–posterior patterning defects. Rather, loss of Twist1 in the anterior–proximal forelimb bud mesenchyme might impact on Shh signaling at other points in the pathway, perhaps by reducing anterior Gli3 expression (Fig. 6). In a minority of cases, ectopic Shh expression, activated by anterior Hoxd13 expression and suppression of anterior Bmp signaling by Greml1 (Fig. 1) could reinforce the effects of Shh ligand-independent patterning defects, resulting in more pronounced mirror image-like polydactyly.

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