Timed Deletion of Twist1 in the Limb Bud Reveals Age-Specific Impacts on Autopod and Zeugopod Patterning

David A. F. Loebel1,2*, Angelyn C. C. Hor1, Heidi K. Bildsoe1,2, Patrick P. L. Tam1,2

1 Embryology Unit, Children’s Medical Research Institute, Westmead, New South Wales, Australia, 2 Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia

Abstract

Twist1 encodes a transcription factor that plays a vital role in limb development. We have used a tamoxifen-inducible Cre transgene, Ubc-CreERT2, to generate time-specific deletions of Twist1 by inducing Cre activity in mouse embryos at different ages from embryonic (E) day 9.5 onwards. A novel forelimb phenotype of supernumerary pre-axial digits and enlargement or partial duplication of the distal radius was observed when Cre activity was induced at E9.5. Gene expression analysis revealed significant upregulation of Hoxd10, Hoxd11 and Grem1 in the anterior half of the forelimb bud at E11.5. There is also localized upregulation of Ptc1, Hand2 and Hoxd13 at the site of ectopic digit formation, indicating a posterior molecular identity for the supernumerary digits. The specific skeletal phenotypes, which include duplication of digits and distal zeugopods but no overt posteriorization, differ from those of other Twist1 conditional knockout mutants. This outcome may be attributed to the deferment of Twist1 ablation to a later time frame of limb morphogenesis, which leads to the ectopic activation of posterior genes in the anterior tissues after the establishment of anterior-posterior anatomical identities in the forelimb bud.

Introduction

The vertebrate limb is patterned by the interplay of the regionalized activities of secreted morphogens and transcription factors that drive tissue morphogenesis. Together they provide the positional information defining tissue patterns in the proximal-distal (PD), dorsal-ventral (DV), and anterior-posterior (AP) axes. AP patterning is influenced by the activity of the zone of polarizing activity (ZPA), located in the posterior-proximal limb bud. The ZPA expresses the signaling ligand sonic hedgehog (SHH) and imparts posterior characteristics to the limb tissues, controlling digit number and identity. Transplantation of the ZPA or ectopic presentation of SHH to the anterior limb bud results in the formation of duplicate post-axial limb elements (e.g. posterior digits) on the pre-axial side [1]. Loss of Shh function results in the loss of the ulna and most of the digits due to the absence of posteriorizing morphogenetic signals [2]. Maintenance of proper SHH signaling requires interactions with the BMP and FGF signaling pathways. Regulation of SHH signaling in the limb bud requires the activity of the BMP antagonist Grem1, and FGF signaling from the apical ectodermal ridge (AER). BMP signaling restricts the expression of Shh to the posterior domain by antagonizing FGF signaling. The antagonist, Grem1, is itself regulated via feedback inhibition from FGF signaling [3,4,5,6]. Within the signaling milieu, a network of transcription factors regulates the expression of pathway components and mediates the response to these signals. Hoxa and Hoxd genes are important for regulating the number and arrangement of digits, development of the radius and ulna [9], and for initiating the SHH-BMP-FGF signaling loop [10]. Deletion of Hoxd1-10 leads to ectopic Hoxd11-13 expression in the mesenchyme in the anterior part of the limb bud and consequently the loss of AP asymmetry [11]. Hoxa/d genes may therefore act combinatorially in the AP patterning of the limb.

The Twist1 gene, which encodes a basic helix-loop-helix (bHLH) transcription factor, is broadly expressed in the limb mesenchyme from early outgrowth of the limb bud. By E12.5, Twist1 expression is restricted to the proximal limb tissues, interdigital mesenchyme and interphalangeal joints [12]. Loss of Twist1 function curtails forelimb bud outgrowth, which is accompanied by reduced FGF, SHH and BMP signaling, but has less severe effects on the hindlimb buds [12]. Conditional loss of Twist1 function mediated by Pax1-Cre after the initiation of limb outgrowth [13,14] results in disrupted AP patterning of the limb leading to the development of supernumerary digits, loss of the radius and reductions in humerus, scapula and clavicle development [13,14]. Hand2 binds to and antagonizes the actions of Twist1, also triggering the formation of additional digits when it is ectopically expressed [7,8]. Ablation of Twist1 activity only in the anterior mesenchyme of the forelimb bud leads to posteriorization of anterior skeletal elements, including mirror-image digit duplications and the acquisition of an ulnar-like morphology by the radius [15]. This phenotype is associated with the down-regulation of Alx gene family members and Gli3 in the anterior...
tissue of the forelimb bud, and a concomitant expansion of Hoxd13 and Greml1 expression domains into the anterior region of the limb bud.

Here, we have examined the impact of loss of Twist1 on the patterning of limb tissues after the limb bud has initiated outgrowth. Following the induction of Cre activity at E9.5, the limb displayed a partial radius duplication and preaxial polydactyly, but without morphological posterior transformation. The dysmorphology of the autopod and zeugopod is accompanied by upregulation of SHH signaling. Greml1 and posterior transcription factors in the anterior limb bud, which is detectable at E11.5 but not E10.5.

Materials and Methods

Mouse strains and tamoxifen injection

Twist1<sup>del/−</sup>; Ubc-CreERT2 mice were generated by crossing Twist1ΔloxP/ΔloxP mice were obtained from the Jackson Laboratory. Mice and embryo yolk sacs were genotyped for Twist1<sup>Δ</sup> and Cre as previously described [16].

To produce conditional knockout embryos, Twist1<sup>Δl/Δ</sup>; Ubc-CreERT2 mice were crossed with Twist1<sup>Flox/Δ</sup> mice [18].

Pregnant females with injected intraperitoneally with 4 mg (E9.5 injection) or 8 mg (E10.5 or later injection) tamoxifen at embryonic day (E) 9.5–14.5. This study was carried out in accordance with the Australian Code for the Care and Use of Animals for Scientific Research and was approved by the Children’s Medical Research Institute/Children’s Hospital Westmead Animal Ethics Committee (project number C230). All efforts were made to minimize animal suffering.

Immunofluorescence

Staining for Twist1 protein was carried out on frozen sections of E11.5 forelimb buds using mouse monoclonal anti-Twist1 antibody 2C1A (Abcam ab50887, 1/50 dilution) and AlexaFluor 488 conjugated donkey anti-mouse secondary antibody (Life Technologies) for poly-A tailing, reverse transcription and amplification. qRT-PCR was performed on a Corbett RotorGene thermocycler. The following qRT-PCR primers were used:

- **Hoxd13**
  - **5′-tgaccagctgactctcctgtct-3′**
  - **5′-ctggccgtggctggtttgct-3′**
  - **5′-ggaaccggtacctgccctt-3′**

- **Ptch1**
  - **5′-gcaccaatctgacgctgccgc-3′**
  - **5′-aagtcctgacagccgctgccgc-3′**
  - **5′-cctgggctcctgagcagc-3′**
  - **5′-tttgcgccgctctcctcct-3′**

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Hand1 transcripts.

Results

Generation of embryos with Twist1 deleted at specific developmental ages

We utilized mice carrying a broadly expressed tamofoxen-activated Cre transgene, Ubc-CreERT2 [17] to generate timed deletions of Twist1 at specific stages of embryonic development. To reveal the age/stage-specific impact of loss of Twist1 activity on development, pregnant mice were injected with tamoxifen or oil at E9.5-14.5 and embryos were collected at E17.5.

Embryos from mothers injected with oil only and wild type embryos from tamoxifen injected mothers developed normally (Fig. S1 A, B). After injection at E9.5 (TAM E9.5) embryos were small and underdeveloped (Fig. S1 C). Consistent with previous studies, all TAM E9.5 embryos (7/7) displayed craniofacial defects including a cleft face and misshapen cranial region (Fig. S1 C, D) and TAM E10.5 embryos displayed a misshapen cranial and shortened snout (7/7 embryos, Fig. S1 E), whereas TAM E12.5 embryos showed no obvious head and face abnormalities (Fig. S1 F). Limbs of TAM E9.5 embryos appeared shorter than wild-type embryos and preaxial supernumerary digits were prominent in all embryos (Fig. S1 C', D'). Polydactyly was not observed in embryos from mothers injected at later stages, but embryos from E10.5-12.5 injected mothers (TAM E10.5, 7/7 embryos; TAM E11.5 3/4 embryos; TAM E12.5, 5/5 embryos) showed a curvature of the digits (Fig. S1 E, F; Table 1). The forelimb phenotypes observed in TAM E9.5 embryos differed from those previously observed in Twist1 conditional mutant limbs, prompting us to examine this unique phenotype further.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.

Whole mount in situ hybridization

Embryos were collected at E10.5 and E11.5 and fixed in 4% paraformaldehyde. In whole mount hybridization was carried out either manually or using a Intavis InSituPro robotic system according to methods previously described [16,20]. Digoxygenin labeled riboprobes were generated using Ampliscribe (Epicentre) from cDNA clones containing fragments of the Hand2, Hoxd13 and Pkhd1 transcripts.
### Table 1. Comparison of Twist1:Ubc-CreERT2 CKO forelimb skeletal phenotypes, from mothers injected at E9.5–E12.5 with Twist1: Mesp1-Cre and Prrx1-Cre CKO embryos and ska10 (charlie chaplin) mutant embryos.

|                         | TAM E9.5 | TAM E10.5 | TAM E11.5 | TAM E12.5 | Mesp1-Cre | Prrx1-Cre | ska10/ska10 | ska10/-  |
|-------------------------|----------|-----------|-----------|-----------|-----------|-----------|-------------|----------|
| Number of limbs analysed| 4        | 10        | 4         | 4         | N/A       | N/A       | N/A         | N/A      |
| Autopod                 |          |           |           |           |           |           |             |          |
| Supernumerary digits/fragments | Y         |           |           |           |           |           |             |          |
| Mirror transformation   | Y         | Y         | Y         |           |           |           |             |          |
| Bifid digits            | Y         | Y         | Y         | Y         |           |           |             |          |
| Fewer digits            |           |           |           |           |           |           | Y           |          |
| Extra carpals           | Y         |           | Y         |           |           |           |             |          |
| Curved digits           | Y         | Y         | Y         |           |           |           |             |          |
| Reduced/delayed ossification | Y         | Y         | Y         | Y         | Y         | Y         | Y           |          |
| Zeugopod and Stylopod   |          |           |           |           |           |           |             |          |
| radius: partial duplication or thickening | Y         |           |           |           |           |           |             |          |
| Radius: ulnarization    |           |           |           |           | Y         | Y         |             |          |
| Radius: loss            |           |           |           |           | Y         | Y         |             |          |
| Ulna: malformed         | Y         |           |           |           |           |           |             |          |
| Humerus: tuberosity reduced or lost | Y         | Y         |           |           |           |           |             |          |
| Humerus: short/malformed| fused to scapula |           |           |           |           |           |             |          |
| Scapula/clavicle: reduced/malformed | Y         | variable |           |           |           |           |             |          |

Y, presence of phenotype; N/A: published work, not applicable.

References: Mesp1-Cre data [15]; Prrx1-Cre, ska10 data [13,14].

doi:10.1371/journal.pone.0098945.t001
Conditional Twist1 deletion at E9.5–E12.5 causes different limb defects

The forelimb shoulder and stylopod phenotype. In TAM E9.5 embryos, the clavicle was absent and the scapula was malformed, consisting of two separate fragments (Fig. 2 B, compare with wild-type Fig. 2 A; 4/4 limbs). The scapula phenotype varied in TAM E10.5 embryos. In some limbs the scapula resembled that of TAM E9.5 embryos (Fig. 2 C, 4/10 limbs), but in others a more complete scapula was formed, but with reduced ossification (6/10 limbs, Table 1). The scapula and clavicle of TAM E11.5 and TAM E12.5 embryos were similar to wild-type embryos (Fig. 2 D, E). The scapula and clavicle abnormalities of TAM E9.5 and TAM E10.5 embryos was more dramatic than that seen when Twist1 was deleted specifically from the anterior limb bud by Mesp1-Cre (Table 1) [15]. The humerus of TAM E9.5 embryos lacked the deltoid tuberosity, which was also reduced in TAM E10.5 embryos (Fig. 2 A–C, 4/4 limbs). Loss of the deltoid tuberosity was also seen in Mesp1-Cre CKO embryos and ska10 point mutant embryos, but a more severe reduction in the humerus occurred in Prrx1-Cre CKO embryos (Table 1; [13–15]).

The forelimb zeugopod phenotype. The radius and ulna of TAM E9.5 embryos were bent and shorter than in normal embryos. Extra cartilage was formed at the distal end of the radius resulting in either a partial duplication (3/4 limbs), or broadening of the distal end of the radius (1/4 limbs; Fig. 2 B, Fig.3 C, D, compared with Fig. 3A). This was not previously observed in Mesp1-Cre or Prrx1-Cre Twist1 CKO embryos (Table 1; [13–15]) and there was no indication of the “ulnarization” of the radius that was observed in Mesp1-Cre Twist1 CKO embryos [15]. No extra radius cartilage was found in TAM E10.5–TAM E12.5 embryos (Fig. 2 C–E).

The forelimb autopod phenotype. Ossification of carpals of TAM E9.5 and TAM E10.5 embryos was delayed or reduced compared to control embryos (Fig. 3 A–D), a common feature of other Twist1 CKO limbs (Table 1). Digits displayed a curved morphology and the joints between phalanges were not well defined. One or two complete supernumerary digits with no clear AP identity were present on the pre-axial side in TAM E9.5 limbs (4/4 limbs), and additional metacarpal fragments were seen in 2/4 limbs (Fig. 2 B, Fig.3C, D). Supernumerary digits were not found in TAM E10.5 embryos (0/10 limbs; Fig. 3 B). One to four extra carpal elements, also of uncertain identity, were associated with the additional digits (Fig. 3 E, F). These forelimb autopod abnormalities differed from the evident posterior transformation observed in Mesp1-Cre and Prrx1-Cre Twist1 CKO limbs and the loss of digits seen in other mutant embryos (Table 1; [13–15]).

Hindlimb phenotype. In the hindlimb of TAM E9.5 embryos, the ischium was absent and the ilium was reduced (Fig. 2 F, G; 4/4 limbs). Ossification was retarded in the tibia, fibula and femur in TAM E9.5 and TAM E10.5 embryos (Fig. 2 F, H). Digits were curved in TAM E10.5–12.5 hindlimbs (Fig. 2 H, compare to Fig. 2 F). Hindlimb polydactyly with incomplete penetrance is observed in Twist1−/− mice [12,21] and therefore is not unique to the Ubc-CreERT2 CKO mutants. No exacerbation of this phenotype was observed in the hindlimbs of these CKO embryos.
Twist1 restricts posterior gene expression in forelimb buds

To further investigate the forelimb autopod and zeugopod defects in TAM E9.5 embryos, we examined the expression of genes that are normally differentially expressed in anterior and posterior parts of the forelimb bud by qRT-PCR (n = 4 embryos, collected at E11.5, for each genotype and transcript measured). In control embryos, Twist1 is preferentially expressed in the anterior half of the forelimb bud, and in TAM E9.5 embryos its expression was reduced to very low levels in both anterior and posterior fragments (Fig. S1 A).

Hoxd10 and Hoxd11 are normally expressed most strongly in the posterior part of forelimb bud. In forelimb buds from TAM E9.5 CKO embryos, both genes remained strongly expressed in the posterior half of the bud, but were significantly up-regulated in the anterior half of the limb bud (Fig 4 A, B). This is consistent with a previous observation that Hoxd11 is upregulated in compound Twist1ska10/- mutant limb buds [13]. In contrast, Hoxd13 was not significantly upregulated in CKO anterior limb bud tissues compared to controls (Fig 4 C).

Hand2, encoding a bHLH transcription factor normally expressed in the posterior limb bud also showed no significant upregulation (Fig. 4 D). Grem1, encoding a BMP antagonist that depends on Hox activity to initiate its expression [10] showed significantly higher expression in CKO anterior forelimb bud tissue than in control forelimb buds (Fig. 4 E). The expression of Ptch1, a target of SHH signaling, was not increased in CKO anterior relative to controls (Fig. 4 F) but was reduced in the posterior tissue of some CKO specimens. This suggested that there was a globally reduced level of SHH signaling in some TAM E9.5 CKO limb buds, which was also encountered in Twist1-null embryos [12].

Despite the lack of significant upregulation of Hoxd13, Hand2 or Ptch1 in anterior forelimb buds by qRT-PCR analysis, which indicated that there were no widespread differences in expression these genes between control and CKO forelimb buds,

Figure 2. Limb skeletal phenotypes of timed conditional knockout embryos at E17.5. (A–E) forelimbs, (F–J) hindlimbs. (A, F) Wild-type, (B–E, G–J) conditional knockout embryos harvested from mothers injected with tamoxifen at E9.5 (B, G), E10.5 (C, H), E11.5 (D, I) or E12.5 (E, J). Asterisk in B indicates additional cartilage attached to radius; arrowhead indicates supernumerary digits, arrows in (A–C) marks the deltoid tuberosity, which is absent in (B). Abbreviations: c, clavicle; fe, femur; fi, fibula; h, humerus; il, ilium; is, ischium; r, radius; t, tibia; u, ulna.
doi:10.1371/journal.pone.0098945.g002

Figure 3. Autopod and distal zeugopod phenotypes in TAM E9.5 embryos. (A, E) Wild-type, (B–D, F) CKO. Embryos were harvested at E17.5 from mothers injected with tamoxifen at E10.5 (B) or E9.5 (C, D, F) and stained with alcian blue or alizarin red. Digit identities are indicated with Roman numerals. Black asterisk indicates partial duplication of the distal radius, white asterisk indicates additional carpal elements. Black line shows the width of the distal radius in the TAM E9.5 embryo (D), superimposed on wild-type (A). Abbreviations: ca, capitate; hm, hamate; p, pisiform; r, radius; s, scaphula; sc-cn, scaphoid-centrale; tq, triquetral; tm, trapezium; tz, trapezoid; u, ulna.
doi:10.1371/journal.pone.0098945.g003

Twist1 and Forelimb Patterning

PLOS ONE | www.plosone.org 5 June 2014 | Volume 9 | Issue 6 | e98945
the range of expression values was greater in CKO anterior limb buds than in controls. This suggested that there may be subtle localized changes in gene expression. To test this possibility, we performed whole mount in situ hybridization of forelimb buds of TAM E9.5 embryos collected at E10.5 and E11.5. At E11.5, the Ptch1 hybridization signal was weaker in CKO limb buds (3/4) than in wild type limb buds, consistent with our qRT-PCR results (Fig. 5 A, B). Localized ectopic expression of Ptch1 (3/4 limb buds), Hand2 (4/4 limb buds) and Hoxd13 (4/4 limb buds) was found in the anterior tissue of the forelimb bud (Fig. 5 B, D, F, compared with Fig. 5 A, C, E). Ectopic expression was localized to a small anterior tissue swelling in CKO limb buds, possibly containing the precursors of the supernumerary digits. Ectopic expression of these genes was not seen at E10.5 (n = 2 limb buds for each; Fig. 5 G–L).

These observations suggest that, although there was no apparent morphological posterior transformation, the ectopic digits had acquired a posterior molecular identity. However, despite the widespread loss of Twist1 protein by 24 hours after tamoxifen injection (Fig. 1), detectable ectopic expression of these posterior tissue markers did not occur until after E10.5.

**Discussion**

**Stage-specific requirement for Twist1 in limb development**

In this study we have used a tamoxifen-inducible Cre recombinase to ablate Twist1 at different embryonic ages. The forelimb preaxial polydactyly and other skeletal defects observed in tamoxifen-treated embryos (Fig. 2, Fig. 3) differed from those previously reported in either Mesp1-Cre or Prrx1-Cre induced Twist1 CKOs (Table 1). Previous studies of Twist and limb development have used tissue-specific Cre transgenes to delete Twist1 in the newly formed anterior mesoderm, including the mesoderm that contributes to the head and anterior forelimb (Mesp1-Cre; [15,24]) or in the limb buds shortly after initiation of outgrowth (Prrx1-Cre; [13,14]). In this study, the ablation of Twist1 activity between E9.5 and E10.5 results in the formation of extra digits on the preaxial side of the forelimb and a partial duplication or enlargement of the distal part of the radius, without any apparent posterior anatomical transformations of skeletal elements. The consequences of deleting Twist1 after limb bud formation, which affects skeletal morphogenesis and differentiation but not AP patterning, are strikingly different from the mirror image duplications, bone loss and posterior transformations produced by the ablation of Twist1 with Mesp1-Cre, which acts in the nascent upper trunk mesoderm, or with Prrx1-Cre, which acts in the early limb bud mesenchyme (Table 1; [13–15]). Data obtained from tamoxifen injection at E9.5 show that Twist1 is no longer required for maintaining AP anatomical identity in the limb skeleton at this age. However, Twist1 may still have a role in regulating digit number and the morphogenesis of the distal zeugopod, and for restriction of posterior gene expression.

Twist1-dependent gene expression influences autopod and zeugopod patterning

The limb phenotypes seen in this study are reminiscent of those observed when Hand2 or Hand2-Twist1 dimers are mis-expressed during limb development, which differ from the consequences of Twist1 homodimer or Twist1-E2A heterodimer expression (Table 2). Hand2 encodes a bHLH protein that can dimerize with Twist1 and Forelimb Patterning
Twist1 and, when over-expressed in the limb bud, causes the formation of extra digits and the mirror image duplication of digits [25] similar to those observed when Twist1 was specifically deleted in the anterior compartment of the forelimb bud [15]. The formation of additional preaxial digits and malformations of the zeugopod do not require the ability of Hand2 to bind DNA [7], suggesting that Hand2 could exert these effects by interacting with another bHLH factor, such as Twist1. Consistent with this, enforced expression of tethered Twist1-Hand2 heterodimer results in the formation of supernumerary digits and additional radius structures (Table 2; [8]). Genetic interaction (in mice) and misexpression studies (in chick) indicates that Twist1 and Hand2 antagonize each other's actions in the limb bud [8]. Since Hand2 is normally expressed in the posterior forelimb bud, and Twist1 primarily functions in regulating gene expression in the anterior forelimb bud, ectopic expression of Hand2 could generate novel Twist1-Hand2 dimers that may compete with the endogenous Twist1-containing dimers for action, resulting in a loss of normal Twist1-dimer function. This contrasts with the effects of overexpression of Twist1 monomer or homodimer, which similarly

![Figure 5. Whole mount in situ hybridization of the forelimb bud. Expression of Ptch1 (A, B, G, H), Hand2 (C, D, I, J) and Hoxd13 (E, F, K, L) in wild-type (fl/wt) or heterozygous (fl/del) embryos (A, C, E, G, I, K) were compared to CKO embryos (B, D, F, H, J, L) collected at E11.5 (A–F) or E10.5 (G–L). Asterisk indicates ectopic anterior expression domains that may correspond to the site of formation of the extra digits. doi:10.1371/journal.pone.0098945.g005](image)

Table 2. Comparison of Twist1:Ubc-CreERT2 CKO embryo forelimb phenotype after tamoxifen injection at E9.5 with embryos that overexpress Twist1 monomer or Twist1-Hand2, Twist1-E12 or Twist1-Twist1 tethered dimers. [30].

|                      | TAM E9.5 | Twist1-Hand2 | Twist1 monomer | Twist1-Twist1 | Twist1-E12 |
|----------------------|----------|--------------|----------------|---------------|------------|
| Autopod              |          |              | Y              |               | Y          |
| Supernumerary digits/fragments | Y        |              | Y              |               | Y          |
| Fewer digits         |          |              |                |               |            |
| Extra carpals        |          |              | Y              |               |            |
| Curved digits        |          |              |                |               |            |
| Reduced/delayed ossification | Y        |              |                |               |            |
| Zeugopod and Stylopod|          |              |                |               |            |
| radius: partial duplication or thickening | Y        |              | Y              |               | Y          |
| Radius: loss/reduction |          |              | small          |               | Y          |
| Ulna: malformed      |          |              | Y              | small         | Y          |
| Humerus: tuberosity reduced or lost |          |              | Y              | Y             | Y          |
| Humerus: short/malformed |          |              | Y              |               | Y          |
| Scapula/clavicle: reduced/malformed |          |              | Y              |               | Y          |

doi:10.1371/journal.pone.0098945.t002
cause reductions and malformations of the zeugopod and stylopod and with the less drastic effects of the over-expression of Twist1-E12 heterodimer (Table 2). It has previously been noted that expression of Twist1 homodimers promotes Fgf8 expression and differentiation in calvarial osteoblasts, whereas Twist1-E2A heterodimers repress it [26]. Therefore, changes in dimer contents could account for the similar phenotype of heterodimer over-heterodimers repress it [26]. Therefore, changes in dimer contents 

Timing of Twist1 deletion influences the phenotypic response to downstream gene expression

In chick limbs, exposure of the anterior wing bud to SHH results in the formation of additional digits that mirror those on the posterior side. The induction of ectopic digits is sensitive to the time of exposure to SHH [28] with early, prolonged exposure leading to duplications of postaxial digits on the anterior side. In mouse embryos, individual digits show a differential sensitivity to timing of loss of Shh, with digit 2 only being lost when Shh is deleted early (E9.5 tamoxifen injection) and digit 3 affected when Shh deletion is initiated as late as E10.5 [29]. Together, these data suggest that the timing of onset and duration of exposure to SHH influences the formation of specific digits to different degrees and determines the morphological identity of the digits. In the present study, ectopic SHH signaling was detected at E11.5 but not E10.5. Ectopic SHH signaling commencing in this time window may cause the formation of additional preaxial digits and zeugopod elements but not posterior transformation. Therefore, although the molecular response to loss of Twist1 activity is similar when Twist1 is deleted at different stages [13–15], the timing of this response may determine the phenotypic impact on the skeletal elements.

Supporting Information

Figure S1 Timed deletion of Twist1 by tamoxifen inducible Cre recombinase. Embryos harvested at E17.5 from oil (A) or tamoxifen (B–F) injected mothers. (B) Twist1floxs/+ wild-type (WT) control. (C, D) Conditional knockout (CKO) embryos from mothers injected with tamoxifen at E9.5. (C’, D’) high magnification views showing supernumerary digits in TAM E9.5 embryos [white arrowheads]. (E, F) CKO embryos from mothers injected at E10.5 and E12.5. (F) Asterisk in (D) indicates cleft face; black arrowheads in (E, F), curved digits. Panels A, B, C, D, E, and F were photographed at the same magnification. (TIF)

Figure S2 miR10b is not significantly downregulated in conditional mutant limb buds. qRT-PCR for miR10b in anterior (A) and posterior (P) halves of E11.5 forelimb buds dissected from control (B/wt) and TAM E9.5 (cko) embryos. Reference gene was miR19a. N = 3 for each genotyoe. (TIF)

Acknowledgments

We thank the staff of the CMRI Biobioservice Unit for animal care and assistance, Ator Ashoti and Theressa Tang for technical assistance. We are grateful to R. Behringer for providing the Twist1-floxsPeho mice, and E. Olson (Hand2), P. Chambon (Hoxd13) and M. Scott (Pch1) for providing cDNA clones used to generate riboprobes.

Author Contributions

Conceived and designed the experiments: DAFL, PPLT, HKB. Analyzed the data: DAFL, PPLT. Wrote the paper: DAFL PPLT HKB. Checked and revised manuscript for intellectual content: ACCH HKB.

References

1. Yang Y, Dressopoulos G, Chauang PT, Deprez D, Marri E, et al. (1997) Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. Development 124: 4393–4404.
2. Chiang C, Liangying Y, Harris MP, Simandl BK, Li Y, et al. (2001) Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. Dev Biol 236: 421–435.
3. Lewandoski M, Sun X, Martin GR (2000) Fgf8 signalling from the AER is essential for normal limb development. Nat Genet 26: 460–463.
4. Bastida MF, Sheret R, Ros MA (2009) A BMP-Shh negative-feedback loop restricts Shh expression during limb development. Development 136: 3779–3789.
5. Khokha MK, Hsu D, Brunet LJ, Dionne MS, Harland RM (2003) Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. Nat Genet 34: 307–308.
6. Verheyden JM, Sun X (2008) An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. Nature 454: 638–641.
7. McFadden DG, McAnally J, Richardson JA, Charpie J, Olson EN (2002) Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. Dev Growth 129: 3077–3083.
8. Virshup DM, Fenoge-Nguyen C, Harwood HB, Gange PA, Jacobson EC, et al. (2002) Generation of a Twist1 conditional null allele in the mouse. Genesis 45: 588–592.
heterozygous mouse phenotype resemble those of human Saethre-Chotzen syndrome. Hum Mol Genet 7: 945–957.

22. Shen X, Fang J, Lv X, Pei Z, Wang Y, et al. (2011) Heparin impairs angiogenesis through inhibition of microRNA-10b. J Biol Chem 286: 26616–26627.

23. Li J, Zucker S, Pulko-Gross A, Kucu C, Karavaev M, Ju J, et al. (2012) Conversion of stationary to invasive tumor initiating cells (TICs): role of hypoxia in membrane type 1-matrix metalloproteinase (MT1-MMP) trafficking. PLoS One 7: e38463.

24. Bildsoe H, Loebel DA, Jones VJ, Hor AC, Braithwaite AW, et al. (2013) The mesenchymal architecture of the cranial mesoderm of mouse embryos is disrupted by the loss of Twist1 function. Dev Biol 374: 293–307.

25. Charite J, McFadden DG, Olson EN (2000) The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. Development 127: 2461–2470.

26. Connerney J, Andreeva V, Leshem Y, Mercado MA, Dowell K, et al. (2008) Twist1 homodimers enhance FGF responsiveness of the cranial sutures and promote suture closure. Dev Biol 318: 323–334.

27. Galli A, Robay D, Osterwalder M, Bao X, Benazet JD, et al. (2010) Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. PLoS Genet 6: e1000901.

28. Harfe BD, Scherz PJ, Nisim S, Tian H, McMahon AP, et al. (2004) Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 118: 517–528.

29. Zhu J, Nakamura E, Nguyen MT, Bao X, Akiyama H, et al. (2008) Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Dev Cell 14: 624–632.

30. Firulli BA, Redick BA, Conway SJ, Firulli AB (2007) Mutations within helix I of Twist1 result in distinct limb defects and variation of DNA binding affinities. J Biol Chem 282: 27536–27546.