Preaxial polydactyly: interactions among ETV, TWIST1 and HAND2 control anterior-posterior patterning of the limb

Zhen Zhang¹, Pengfei Sui², Aiwu Dong², John Hassell³, Peter Cserjesi⁴, You-Tzung Chen⁵, Richard R. Behringer⁵ and Xin Sun¹,*

SUMMARY
Preaxial polydactyly (PPD) is a common limb-associated birth defect characterized by extra digit(s) in the anterior autopod. It often results from ectopic sonic hedgehog (Shh) expression in the anterior limb bud. Although several transcription factors are known to restrict Shh expression to the posterior limb bud, how they function together remains unclear. Here we provide evidence from mouse conditional knockout limb buds that the bHLH family transcription factor gene Twist1 is required to inhibit Shh expression in the anterior limb bud mesenchyme. More importantly, we uncovered genetic synergism between Twist1 and the ETS family transcription factor genes Etv4 and Etv5 (collectively Etv), which also inhibit Shh expression. Biochemical data suggest that this genetic interaction is a result of direct association between TWIST1 and ETV proteins. Previous studies have shown that TWIST1 functions by forming homodimers or heterodimers with other bHLH factors including HAND2, a key positive regulator of Shh expression. We found that the PPD phenotype observed in Etv mutants is suppressed by a mutation in Hand2, indicative of genetic antagonism. Furthermore, overexpression of ETV proteins influences the dimerization of these bHLH factors. Together, our data suggest that through biochemical interactions, the Shh expression regulators ETV, TWIST1 and HAND2 attain a precise balance to establish anterior-posterior patterning of the limb.

KEY WORDS: Anterior-posterior patterning, Limb development, Sonic hedgehog signaling, Mouse

INTRODUCTION
The developing limb is an excellent model for studying pattern formation because construction of the limb skeleton follows a blueprint laid down in the early embryonic limb bud. Several signaling centers are responsible for precise formation of this three-dimensional structure (Niswander, 2002; Zeller et al., 2009). In particular, the anterior-posterior (A-P; thumb to little finger) axis of the limb is patterned by the zone of polarizing activity (ZPA), a group of mesenchymal cells near the posterior margin of the limb bud. These cells produce the secreted molecule sonic hedgehog (SHH), which is crucial for limb A-P patterning. Altering the level or location of Shh gene expression results in changes in the number and/or identity of digits. For example, ectopic expression of Shh in the anterior limb bud leads to preaxial polydactyly (PPD; the formation of extra digits anteriorly) in mice, which resembles a common limb-associated human birth defect of the same name. Thus, tight regulation of Shh expression is crucial for normal A-P patterning of the limb (McGilinn and Tabin, 2006).

Control of Shh expression in the limb bud is achieved through a cis-element called the ZPA regulatory sequence (ZRS), located ~1 Mb upstream of the Shh promoter (Lettice et al., 2003; Maas and Fallon, 2005; Sagai et al., 2005). The ZRS is necessary and sufficient to promote Shh expression specifically in the limb bud, but not elsewhere in the developing embryo. In addition, multiple point mutations scattered across the ZRS are individually linked to ectopic Shh expression in the anterior limb bud (Lettice et al., 2003; Maas and Fallon, 2005; Gurnett et al., 2007; Furniss et al., 2008), suggesting that the ZRS also mediates the repression that is essential for restricting Shh expression to the posterior limb bud. Although a number of transcription factors have been shown to regulate Shh expression in the limb bud (Buscher et al., 1997; Bourgeois et al., 1998; Qu et al., 1998; Zhang et al., 2009), few factors that control Shh expression in the limb bud can be characterized as either positive or negative regulators based on their mutant phenotypes. For example, loss-of-function mutations in Alx4, Gli3 and Twist1 display ectopic expression of Shh in the anterior limb bud, indicating that these genes normally inhibit Shh expression in this region (Buscher et al., 1997; Bourgeois et al., 1998; Qu et al., 1998). Loss-of-function mutants in Hand2, Hox10-13, Tbx3 and Pbx1;2 display reduced or absent Shh expression in the posterior limb bud, indicating that they normally activate Shh expression in this region (Charite et al., 2000; Davenport et al., 2003; Capellini et al., 2006; Tarchini et al., 2006). There is a substantial overlap in the expression patterns of many of these factors, raising the possibility that the normal posterior restriction of Shh expression may be achieved through compound interactions among these positive and negative regulators, acting either directly or indirectly on the ZRS.

The genetic and biochemical relationships among these Shh regulators are beginning to be elucidated (te Welscher et al., 2002; Chen et al., 2004; Firulli et al., 2005; Vokes et al., 2008). For example, HAND2 and TWIST1, two Shh regulators belonging to the basic helix-loop-helix (bHLH) family, antagonize each other by
forming protein heterodimers (Firulli et al., 2005). Data from knockout, overexpression and chromatin immunoprecipitation (ChiP) experiments are consistent with the conclusion that HAND2 acts as a key positive regulator of Shh expression in the limb bud (Charite et al., 2000; Fernandez-Teran et al., 2000; Galli et al., 2010). By contrast, there are conflicting data on the role of TWIST1 in regulating Shh expression. Twist1 heterozygous mutants exhibit hindlimb-specific PPD and ectopic Shh expression, suggesting that TWIST1 inhibits Shh expression (Bourgeois et al., 1998). However, Twist1 homozygous limb buds show overall diminished Shh expression, suggesting that TWIST1 is required for Shh expression (O’Rourke et al., 2002; Zuniga et al., 2002). This downregulation of Shh could be due to disruption of the apical ectodermal ridge (AER) observed in Twist1 homozygous limb buds. We investigate this possibility here by bypassing the requirement for Twist1 in AER formation. Our data from Twist1 conditional homozygous mutants suggest that, at a stage after the establishment of the AER, TWIST1 is required for inhibiting Shh expression in the anterior limb bud of both the forelimb and hindlimb.

Recent studies, including one from our laboratory, show that two PEA3 group ETS domain-containing transcription factors, ETV4 and ETV5 (hereafter ETV proteins), function as negative regulators of Shh expression (Mao et al., 2009; Zhang et al., 2009). Specifically, we found that limb-specific inactivation of both ETV genes led to ectopic Shh expression in the anterior limb bud and to PPD. We sought to address how they function together with the other known Shh regulators listed above. In this study, we report genetic and biochemical evidence suggesting that ETV inhibits Shh expression by regulating the dimerization of TWIST1/HAND2.

**MATERIALS AND METHODS**

**Mouse mutant phenotype analyses**

Embryos were dissected from time-mated mice, counting noon on the day the vaginal plug was found as E0.5. Mutant and transgenic alleles used in this study have been described previously: Twist1lox/lox (Chen et al., 2007), Twist1Cre/Cre (Bialek et al., 2004), Etv4-/- (Livet et al., 2002), Etv5-/- (Zhang et al., 2009), Hand2-/- (Morikawa et al., 2007), Prx1Cre (Logan et al., 2002) and Tcre (Perantoni et al., 2005). All mutants were generated in a mixed strain background. Although no genetic background effects were discerned, littermates were used to control for any possible differences due to background variations. Whenever possible, somite-matched mutant and littermates were used to control for any possible differences due to strain background. Although no genetic background effects were discerned, Shh mutants exhibit hindlimb-specific PPD and ectopic Shh expression.

**Generation of the ETV5 antibody**

An *E. coli* BL21(DE3) culture transformed by pET-Etv5 was used to produce His-tagged ETV5 (His-ETV5). The culture at OD600 0.6 was induced by 1 mM IPTG at 16°C for 3 hours. His-ETV5 protein was purified by Ni-NTA agarose (Qiagen) from the soluble fraction of the lysate and used to inject rabbits. The rabbit antiserum was commercially produced by Harlan Laboratories. Rabbits were boosted three times with freshly made His-ETV5 protein and the final bleed was used to purify the antibody against ETV5 by affinity chromatography using His-ETV5-coupled agarose beads. For pre-immune serum control, total IgG was purified using Protein A beads (Pharmacia) from a bleed prior to His-ETV5 injection. Equal amounts of the purified ETV5 antibody or pre-bled IgG were coupled to Affi-Gel 10 (BioRad) for immunoprecipitation (IP).

**Immunoprecipitation**

Protein lysates for IP were prepared from HEK293T cells or mouse E11 limb buds. To prepare samples from cultured cells, HEK293T cells were transfected with plasmids containing Flag-tagged full-length or truncated Etv5, Myc-tagged Hand2 or Twist1, or Flag-tagged Twist1 by Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Briefly, 3.6×10⁶ cells were plated per 100-mm dish 1 day before transfection. To test for ETV5 and TWIST1 interaction, Etv5 plasmids (8 μg) and Twist1 plasmids (8 μg) or empty vectors were mixed with 40 μl Lipofectamine 2000. To test for interaction between ETV5 and HAND2-TWIST1 dimers, Flag-tagged truncated Etv5 plasmid (8 μg), Flag-tagged Twist1 plasmid (4 μg), and Myc-tagged Hand2 or Twist1 (4 μg) were mixed with 40 μl Lipofectamine 2000. The mixture was added to the culture medium for 2 hours post-transfection. About 48 hours post-transfection, the cells were lysed in 1 ml IP buffer [50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, Protease Inhibitor Cocktail (1:100), Phosphatase Inhibitor Cocktail 1 (1:100)] per plate. For samples from limb buds, nuclear protein extract was prepared from 200 limb buds of E11 Swiss Webster mouse embryos using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce).

**For IP from cell lysates, we used Protein G beads (Pharmacia). Cell lysates were mixed with antibodies and rotated for 2 hours at 4°C, then mixed with Protein G beads for 1 hour at 4°C.** For IP from limb bud lysates, we used antibody-coupled Affi-Gel beads as described above. The lysates were diluted with IP buffer and split equally into two tubes. One was mixed with ETV5 antibody-coupled beads and the other with pre-immune IgG-coupled beads. The mixtures were rotated for 2 hours at 4°C. The beads were then washed four times with 1 ml wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20, 10% glycerol). Protein was eluted from the beads using 0.1 M glycine pH 2.5. Western blot analysis was performed using the precipitated products.

**Western blot analysis**

Protein samples were run on 8-15% SDS-PAGE gels and transferred onto PVDF membrane. The membrane was blocked with 5% dried milk powder in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 hour, incubated with primary antibody at 4°C overnight, and with secondary antibody for 1 hour at room temperature. Visualization was carried out using ECL Plus reagents (GE Healthcare). The following primary antibodies were used: antigen-purified anti-ETV5 1:2500 (see above), anti-TWIST1 1:1000 (sc-81417, Santa Cruz Biotechnology), anti-HAND2 1:1000 (sc-302, Santa Cruz), anti-Flag tag antibodies were used: antigen-purified anti-ETV5 1:2500 (see above), anti-Hand2 1:1000 (sc-302, Santa Cruz), anti-Flag tag 1:1000 (F1804, Sigma) and anti-c-Myc tag 1:1000 (C3956, Sigma).

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**Yeast two-hybrid analysis**

cDNAs encoding full-length or truncated ETV5, TWIST1 or HAND2 proteins without major activation domains were generated by RT-PCR (for primer sequences, see Table S1 in the supplementary material). The resulting PCR products were cloned into the pGEM-T vector (Promega) and sequence verified. These cDNAs were then subcloned into pGBK7T and pGADT7 vectors (Clontech). Protein interactions were analyzed according to the manufacturer’s recommendations (Clontech).
RESULTS
Bypassing the requirement for Twist1 in AER formation reveals a requirement for Twist1 in inhibiting Shh expression in the anterior limb bud
To investigate the relationship between Evt genes and other Shh regulators such as Twist1, we first clarified the role of Twist1 in regulating Shh expression in the limb bud. In Twist1 heterozygous mutants, Shh expression is not only present in the ZPA, but is also detected in the anterior mesenchyme of the hindlimb bud (O’Rourke et al., 2002). However, in Twist1 homozygous mutants, Shh expression is diminished in the entire limb bud (O’Rourke et al., 2002; Zuniga et al., 2002). This reduced expression in Twist1 homozygous mutants could be secondary to the reported failure to establish and maintain a normal AER. This role of Twist1 in AER formation is likely to be indirect as Twist1 is expressed in the limb bud mesenchyme. To bypass the requirement for Twist1 in AER formation, we inactivated Prx1cre with a floxed allele of Twist1 (Logan et al., 2002; Chen et al., 2007), generating Prx1cre;Twist1F/F (hereafter referred to as Prx1cre;Twist1) mutants. Western blot analysis indicated that Twist1 is present in mutant limb buds at a low level at E9.5, but is largely absent by E11 (Fig. 1A).

We found that in contrast to Twist1-null limb buds, the AER forms in Prx1cre;Twist1 limb buds, as assessed by Fgf8 expression (Fig. 1B-E). In Prx1cre;Twist1 mutants, consistent with the presence of FG in the AER, Mkpc3 (Dusp6 – Mouse Genome Informatics) and Spry4, two downstream targets of FG signaling, and Fgf1, a principal FG receptor, were expressed in the limb bud mesenchyme (see Fig. S1A-F in the supplementary material). Furthermore, Fgf10, a gene essential for AER formation, remained expressed in the mesenchyme of the Prx1cre;Twist1 mutant limb bud (see Fig. S1G,H in the supplementary material), in contrast to its downregulation in Twist1-null limb buds (O’Rourke et al., 2002; Zuniga et al., 2002). These results suggest that the transient presence of Twist1 at the beginning of limb bud initiation is sufficient to maintain the expression of key genes in the limb bud mesenchyme, which in turn allows for formation and maintenance of the AER in this conditional mutant.

In both the forelimb and hindlimb buds of Prx1cre;Twist1 mutants, Shh was ectopically expressed in the anterior mesenchyme in addition to its expression in the ZPA (Fig. 1F-M and data not shown). In the forelimb bud, for example, the ectopic Shh domain started as a dot beneath the anterior margin of the AER at E10.5 (Fig. 1F-I), and expanded proximally past the extent of the AER by E11 (Fig. 1J-M). Even though the mutant limb buds were smaller than those of the control at these stages due to an earlier role of Twist1 in cell survival (see Fig. S2 in the supplementary material), there was increased anterior regional growth by E12.5, possibly owing to the proliferative effect of ectopic SHH (data not shown). Also, as a consequence of the ectopic SHH, two downstream targets of SHH signaling, Gli1 and Ptch1, were upregulated in the anterior mesenchyme (Fig. 1N-Q). Furthermore, the expression domains of Fgf4 and gremlin 1 (Gremlin1), genes positively regulated by SHH signaling, were expanded anteriorly in both forelimb and hindlimb buds (Fig. 1R-U and data not shown). Consistent with the anterior expansion of Fgf4, the expression of Mkpc3 and Spry4 was also expanded anteriorly (see Fig. S1A-D in the supplementary material). Compared with other known Shh inhibitor mutants, including those that we have analyzed, such as Gli3+/- and Tcre;Etv4F/F;Etv5F/F (with inactivation of both Etv4 and Etv5 in the entire limb bud mesenchyme) (Buscher et al., 1997; Masuya et al., 1997; Qu et al., 1997; Zhang et al., 2009), Prx1cre;Twist1 forelimb buds show ectopic Shh expression at an earlier stage of development and this expression extends to a larger domain.

Anterior Shh expression and related changes in SHH signaling were also observed in the limb buds of a hypomorphic allele of Twist1, Twist1Fxa10Sxa10 (see Fig. S3A-F in the supplementary material) (Bialek et al., 2004). In addition, ectopic SHH activity was detected in the hindlimb buds of heterozygous Prx1cre;Twist1F/F mutants, although the domain was smaller than that in the homozygous Prx1cre;Twist1 mutants (see Fig. S3J-L in the supplementary material). Ectopic SHH activity was not detected in the forelimb buds of heterozygous Prx1cre;Twist1F/F mutants, although it was observed in the forelimb buds of
Genetic interaction between Etv genes and Twist1

Following identification of Twist1 as a negative regulator of Shh expression, we sought to address the relationship between Etv genes and Twist1 in their roles as negative regulators of Shh expression in mouse limb buds. We primarily used an in vivo approach because there is currently no established cell line that faithfully recapitulates the molecular context of the developing limb bud. To test for possible genetic interactions in vivo, we generated compound mutants, analyzed limb skeletal phenotypes in newborn pups and examined gene expression patterns in midgestation limb buds.

First, we tested for a possible interaction in Etv5+/–;Twist1Ska10+/– double-heterozygous mutants. Neither Etv5+/– nor Twist1Ska10+/– forelimbs or hindlimbs showed any zeugopod defects. In the double heterozygotes, whereas the forelimb zeugopod remained normal, 27% of double-heterozygous mutants (n=13/48) exhibited tibial agenesis in one or both hindlimbs (Fig. 2A–C). This phenotype is reminiscent of the zeugopod phenotype in Twist1Ska10/Ska10 and Prx1cre;Twist1 mutant hindlimbs (Fig. 2K,M). We also examined the autopod for genetic interactions. Whereas Twist1Ska10+/– heterozygous mutant hindlimbs exhibited a PPD phenotype, the forelimb autopods were normal. In ~9% (n=4/48) of the Etv5+/–;Twist1Ska10+/– double-heterozygous mutants, we observed extra preaxial digits in the forelimb (Fig. 3D–F). Even though this phenotype is found in a small number of samples, it is significant because extra digits are never detected in the forelimbs of Etv5+/– or Twist1Ska10+/– single-heterozygous mutants. Furthermore, extra digits are never observed in the forelimbs of Tcre;Etv4–/–;Etv5+/– or Twist1+/– mutants, in which the function of these genes is more severely disrupted (Bourgeois et al., 1998; Zhang et al., 2009). The manifestation of defects in the compound mutants indicates that mutations in Etv and Twist1 interact synergistically.

To address whether the genetic interaction between Etv genes and Twist1 represents a general phenomenon between Etv and other negative regulators of Shh expression, we generated Etv5+/–;Gli3Xt-J/+ double mutants. Gli3Xt-J/+ mutants have a loss-of-function allele of Gli3, offering a sensitive genetic background as it has been shown to genetically interact with several other mutants that exhibit PPD phenotypes (Dunn et al., 1997; O’Rourke et al., 2002; Panman et al., 2005). In contrast to Etv5+/–;Twist1Ska10+/– compound mutants, we observed an increased enhancement of defects in the zeugopod or autopod of Etv5+/–;Gli3Xt-J/+ mutants (n=37/13, Fig. 3G–L) compared with Gli3Xt-J/+ single mutants. This result therefore distinguishes Twist1 from Gli3 as a specific Etv cooperator.

To further address whether the Etv-Twist1 interaction revealed by the skeletal phenotypes reflects their cooperation in the control of Shh expression, we examined the pattern of Gli1 expression, a sensitive readout of SHH activity, in compound mutant forelimb buds. To increase the penetrance of the limb phenotypes, we inactivated both copies of Etv4 during limb bud initiation using Tcre (Zhang et al., 2009; Perantoni et al., 2005), and one copy of Etv4 and Twist1 using their null alleles. As predicted, in Tcre;Etv4–/–;Etv5+/–;Twist1+/– compound mutants, we observed an increased penetrance of the forelimb PPD skeletal phenotype (n=3/8) as compared with Etv5+/–;Twist1Ska10+/– mutants (n=4/48). At the molecular level, aberrant Gli1 expression was never observed in the forelimb buds of either Tcre;Etv4–/–;Etv5+/– (n=5)
Etv genes and Twist1 are likely to function in the same genetic pathway to inhibit Shh expression in the limb bud

The genetic interaction between Etv and Twist1 suggests that these genes might function either in parallel pathways or within a single pathway. To distinguish between these possibilities, we inactivated Twist1 and both Etv genes in the entire limb bud mesenchyme by generating the homozygous compound mutant Tcre;Etv4+/−;Etv5F/−;Twist1+/− (hereafter Tcre;Etv;Twist1) and analyzed the expression of Gli1 in these mutants. We reasoned that if Etv genes and Twist1 act in parallel pathways to repress Shh, severe disruption of both Etv and Twist1 should lead to more apparent ectopic SHH activity than in either mutant alone. However, if Etv genes and Twist1 act within the same pathway, disruption of both Etv and Twist1 should not lead to enhanced ectopic SHH activity.

Fig. 3. Genetic interaction between Etv genes and Twist1 in the limb. (A-L) Skeleton preparations of mouse P1 (A-F) or E16 (G-L) limbs of the indicated genotypes. Black arrowheads (B, G, H) indicate the presence of a normal tibia; white arrowheads (C, J) indicate truncated tibia. The hindlimb zeugopod phenotype in Etv5+/−;Twist1Ska10+/+ mutants (C) resembles that in Prx1cre;Twist1 and Twist1Ska10+/−;Twist1Ska10+/− mutants (see Fig. 2K, M), and is more severe than that in Etv5+/− (A) or Twist1Ska10+/− (B) mutants. The zeugopod remains normal in Etv5+/−;Gli3Xt-J+/− mutants (see Fig. 2K, M), and is more severe than that in Etv5+/− (A) or Gli3Xt-J+/− (B) mutants, but unlike that in Gli3Xt-J+/− limbs (I) in which the tibia is shortened. Asterisks (F, J, K) indicate extra digit elements. The forelimb phenotype in Etv5+/−;Twist1Ska10+/− mutants (D) resembles that in Prx1cre;Twist1 and Twist1Ska10+/−;Twist1Ska10+/− mutants (hereafter Twist1+/−;Twist1Ska10+/−) (E). This anterior spread is consistent with the findings that Etv genes and Twist1 act in parallel pathways to repress Shh. These results suggest that Etv genes and Twist1 do not act transcriptionally upstream or downstream of each other in A-P patterning.

Etv genes and Twist1 do not promote reciprocal expression in the limb bud

Recent data suggest that some Shh regulators act by controlling the expression of each other. For example, Hand2 expression is upregulated in Gli3 mutant limb buds, and GLI3 binds to the Hand2 regulatory region, suggesting that GLI3 protein directly represses the transcription of Hand2 (te Welscher et al., 2002; Vokes et al., 2008). Since our genetic data suggest that Etv genes and Twist1 function in the same pathway, we tested whether they promote reciprocal expression by addressing if there is downregulation of expression in mutant limb buds. We found that in Prx1cre;Twist1 mutant limb buds, the expression of Etv4 and Etv5 is not reduced, but rather slightly expanded anteriorly (Fig. 4E-H). This anterior spread is consistent with the findings that Etv genes serve as readouts of FGF signaling and that Fgf4 expression is expanded in Prx1cre;Twist1 mutant limb buds (Fig. 4S and see Fig. S1A-D in the supplementary material). Conversely, in Tcre;Etv limb bud limbs, the expression of Twist1 remained normal (Fig. 4I,J). These results suggest that Etv genes and Twist1 do not act transcriptionally upstream or downstream of each other in A-P patterning.

ETV5 associates with Twist1 in vitro and in vivo

We next tested the hypothesis that ETV and TWIST1 function by associating with one another at the protein level. First, we performed an in vitro pull-down assay to address whether ETV5 associates with TWIST1. Using His-tagged ETV5 expressed in E. coli, as bait, we were able to pull down TWIST1 protein from the lysate of HEK293T cells overexpressing Twist1 (Fig. 5A). Second,
we carried out a co-immunoprecipitation (CoIP) assay in HEK293T cells. In cells overexpressing Etv5 and Myc-tagged Twist1, we precipitated TWIST1 using an anti-Myc antibody. Western blot analysis of the immunoprecipitated product showed that ETV5 co-precipitates with TWIST1 (Fig. 5B). Third, we performed a yeast two-hybrid analysis. We found that ETV5 can interact with TWIST1 in this assay, consistent with the possibility that they can bind to each other directly (Fig. 5C). By contrast, ETV5 does not interact with itself, in agreement with previous data suggesting that PEA3 family transcription factors lack a homodimerization domain (Sharrocks, 2001).

Finally, to address whether the association between ETV5 and TWIST1 occurs in vivo, we carried out a CoIP assay using a polyclonal anti-ETV5 antibody that specifically recognizes both denatured and native ETV5 (Fig. 5D; see Fig. S4 in the supplementary material). We used this antibody to precipitate ETV5 from nuclear protein extracts of wild-type E11 limb buds. Compared with control pre-bleed IgG beads, the anti-ETV5 antibody-coupled beads pulled down ETV5, and together with it TWIST1 (Fig. 5D). By contrast, MYF5, another bHLH transcription factor expressed in limb buds, did not co-precipitate with ETV5. Together, these data indicate that ETV5 associates with TWIST1 in vitro and in vivo.

**Etv interaction with Hand2 in the limb bud**

Recent CoIP and genetic data demonstrate that TWIST1 impacts limb A-P patterning by forming a protein heterodimer with, and antagonizing the function of, another bHLH factor, HAND2, which is a positive regulator of Shh (Charite et al., 2000; Fernandez-Teran et al., 2000; Firulli et al., 2005). Our yeast two-hybrid data also support the conclusion that TWIST1 and HAND2 are capable of direct interaction (Fig. 5C). Given our finding that ETV5 binds TWIST1, we asked whether ETV5 might also interact with HAND2.
To address the genetic relationship between the two genes, we introduced a heterozygous loss-of-function mutant of Hand2 (Morikawa et al., 2007) into Tcre;Etv4–/–;Etv5F/–, a mutant background in which we have previously studied the requirements for Etv in the limb. Consistent with what was shown previously (Zhang et al., 2009), all Tcre;Etv4–/–;Etv5F/– mutants from this interaction mating exhibit a PPD phenotype in the hindlimb (Fig. 6A-C). However, the majority of Tcre;Etv4–/–;Etv5F/–;Hand2F/+ mutants that we examined showed normal hindlimbs (Fig. 6A,D,E). This reduction in penetrance of the PPD phenotype indicates that lowering Hand2 dosage attenuates the limb skeletal defect observed in Etv mutants.

We next addressed the biochemical relationship between ETV5 and HAND2. Yeast two-hybrid assays showed that ETV5 and HAND2 do not bind directly to each other (Fig. 5C), unlike ETV5 and TWIST1. In vivo CoIP tests showed that a small amount of HAND2 protein can be precipitated together with ETV5 (Fig. 5D). This result was obtained consistently from three CoIP experiments, whereas the negative control MYF5 was never detected in the pull-down. These data suggest that ETV5 might indirectly associate with HAND2 in the limb bud.

**ETV5 inhibits TWIST1-HAND2 heterodimerization**

Previous studies in the limb bud and other developmental settings have shown that different bHLH protein dimers exhibit distinct DNA binding specificities and transcriptional activities (Connerney et al., 2006; Firulli et al., 2007). Furthermore, in the limb bud, overexpression of TWIST1-HAND2 tethered dimers leads to a PPD phenotype, reminiscent of overexpression of the Shh-activator HAND2 alone (Firulli et al., 2007; Charite et al., 2000). By contrast, overexpression of TWIST1-TWIST1 tethered dimers leads to a phenotype that is reminiscent of Shh loss-of-function mutants (Chiang et al., 2001; Kraus et al., 2001; Lewis et al., 2001; Firulli et al., 2007). Point mutations in Twist1 that affect its dimerization choice alter the effects of the resulting protein on limb skeletal patterning, suggesting that maintaining a precise balance of dimer species is essential for normal development (Firulli et al., 2007).

To further probe the mechanism underlying the genetic interactions among Etv, Twist1 and Hand2, we addressed whether Etv regulates TWIST1 and HAND2 dimerization. In cultured cells, we found that a portion of the ETV5 protein containing the ETS DNA-binding domain (hereafter termed Flag-ETS) is capable of binding to TWIST1 (Fig. 7A,B). Binding was not observed between TWIST1 and the N-terminal portion of ETV5 that does not contain the ETS domain (hereafter termed Flag-EN).
Furthermore, we showed that the expression of Flag-ETS considerably reduces the amount of TWIST1 that is pulled down with HAND2 (Fig. 7C, columns 1, 2). A similar effect was not observed when Flag-EN is overexpressed (Fig. 7C, column 3). Finally, neither Flag-EST nor Flag-EN expression significantly altered the amount of Flag-TWIST1 that is pulled down with Myc-TWIST1 (Fig. 7C, columns 4-6). These data suggest that ETV5, probably through its ETS domain, interferes with TWIST1-HAND2 heterodimerization without having the same effect on TWIST1-TWIST1 homodimerization.

**DISCUSSION**

With the identification of multiple positive and inhibitory regulators of Shh expression in the limb bud, the current challenge is to decipher their functional relationship. In this study, we focused on three Shh expression regulators, Etv, Twist1 and Hand2, and addressed the mechanism of their interaction. Using a conditional knockout strategy, we have shown that Twist1 is required for preventing the expression of Shh in the anterior limb bud and for normal limb skeletal patterning. Data from compound mutants suggest that Twist1, Etv genes and Hand2 function in the same genetic pathway. Furthermore, data from biochemical assays are consistent with the notion that they function via protein-protein interactions. Our findings highlight the importance of a precise balance between positive and inhibitory regulators of Shh in the control of limb A-P patterning.

In the majority of limb mutants in which it is misexpressed, Shh is detected ectopically in the anterior limb bud, separate from the normal domain in the posterior mesenchyme (Buscher et al., 1997; Qu et al., 1997; Zhang et al., 2009). The prevalence of this aberrant pattern led to the hypothesis that a distinct subset of cells in the anterior mesenchyme is poised to express Shh. This possibility is underscored by the observation that several positive regulators of Shh expression, including Hand2, Tbx3 and Pbx1, are not only highly expressed in the posterior limb bud, but are also present, often in a separate domain, in the anterior proximal limb bud (Gibson-Brown et al., 1996; Charite et al., 2000; Capellini et al., 2006; Zakany and Duboule, 2007). We speculate that in a normal limb bud, these positive regulators are unable to activate Shh expression in the anterior mesenchyme because their function is inhibited by repressive machinery. Our findings suggest that the ETV-TWIST1 complex is a major component of this machinery, which in turn ensures the polarized posterior expression of Shh.

The question then arises as to why the presence of ETV and TWIST1 in the posterior limb bud is insufficient to inhibit Shh expression in the ZPA. This might be due in part to the fact that Hand2, a key positive regulator, is expressed much more intensely in the posterior than in the anterior mesenchyme (Charite et al., 2000; Galli et al., 2010). It is therefore plausible that although ETV and TWIST1 are present at a sufficient level to prevent HAND2 from activating Shh in the anterior limb bud, they are not abundant enough to do so in the posterior limb bud. In addition, there are negative regulators of Shh expression, such as ALX4, that are only present in the anterior mesenchyme (Qu et al., 1997). Their absence from the posterior mesenchyme might permit Shh expression in this domain.

Although Twist1 and Hand2 are the only bHLH genes that upon loss result in aberrant Shh expression, a number of other bHLH genes are expressed in the limb bud, including E2a (Tcfβ – Mouse Genome informatics) and the Id genes. Besides forming heterodimers with each other, TWIST1 and HAND2 are each capable of interacting with E12 and E47, two isoforms encoded by the E2a gene (Firulli et al., 2005; Firulli et al., 2007). ID proteins, which do not contain DNA-binding domains, can also compete with TWIST1 and HAND2 for binding to E12/47 (Jogi et al., 2002). In vitro evidence suggests that different homo- or heterodimer pairs exhibit distinct DNA binding specificities and affinities (McFadden et al., 2002; Firulli et al., 2007). Moreover, the limb skeletal phenotypes that result from in vivo overexpression of tethered dimers suggest that different dimers might have distinct effects on Shh expression (Chiang et al., 2001; Kraus et al., 2001; Lewis et al., 2001; Firulli et al., 2007). For example, the similarity of phenotypes between TWIST1-HAND2 transgensics and HAND2-only transgensics suggests that TWIST1-HAND2 might positively regulate Shh expression. Conversely, the similarity of phenotypes between TWIST1-TWIST1 transgensics and Shh loss-of-function mutants suggest that TWIST1-TWIST1 might negatively regulate Shh expression. A recent study has shown that there are multiple predicted bHLH factor binding sites (E-boxes) in the Shh limb enhancer ZRS, and that HAND2 is capable of activating the ZRS via direct binding to regions containing a subset of these E-boxes (Galli et al., 2010). These findings illustrate a plausible mechanism by which bHLH factors may directly regulate Shh expression in the limb bud, and that normal limb patterning is dependent on a proper combination of bHLH dimers.

Given the crucial role of bHLH dimers, it is important to understand the control of their pairing and function. Recent data demonstrate that phosphorylation of bHLH factors significantly influences both their dimerization potential and DNA binding affinity (Firulli et al., 2005; Firulli et al., 2007). Few non-bHLH transcription factors have been shown to influence bHLH dimer function through direct binding. In embryonic cardiomyocytes, biochemical data show that FHHL2, a LIM domain-containing transcription factor, binds HAND1 and influences HAND1-E12 dimer function (Hill and Riley, 2004). Data from our present study led us to propose that ETV proteins, probably through their ETS domain, bind directly to TWIST1. Furthermore, through the ETS domain, ETV might interfere with TWIST1 binding to HAND2. This model is not only supported by both genetic and biochemical results presented here, but is also compatible with previous findings that a similar phenotype (PPD) is observed in Etv mutants and in transgenics overexpressing TWIST1-HAND2 tethered dimers (Zhang et al., 2009; Firulli et al., 2007). In light of the evidence, it is plausible that ETV regulates limb patterning by modulating the bHLH dimer equilibrium.

The genetic and biochemical interactions between ETV and bHLH proteins, such as TWIST1 and HAND2, are likely to play important roles in other biological settings beyond limb development. Etv expression patterns overlap with Twist1 in several other developing tissues, such as the somites, cardiac mesenchyme, pharyngeal and branchial arches (Fuchtbauer, 1995; Chotteau-Lefevre et al., 2001; Brent and Tabin, 2004; Vincentz et al., 2008). Furthermore, the expression of both Etv and Twist1 is upregulated in breast cancer cells (Baert et al., 1997; Yang et al., 2004). Thus, the relationship revealed by the genetic and biochemical data shown in this study might represent a general mechanism of collaboration between these two classes of proteins.

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