

FGF-Regulated Etv Genes Are Essential for Repressing *Shh* Expression in Mouse Limb Buds

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SUMMARY

Anterior-posterior (A-P) patterning of the vertebrate limb is controlled by sonic hedgehog (SHH) signaling, and the precise restriction of *Shh* expression to the posterior limb bud is essential for its polarizing effect. Fibroblast growth factor (FGF) signaling, a key control of proximal-distal (P-D) limb outgrowth, is known to promote *Shh* expression in the posterior limb bud. Here, we show that conditional knockout of the FGF-activated transcription factor genes *Etv4* and *Etv5* in mouse led to ectopic *Shh* expression in the anterior limb bud and a preaxial polydactyly (PPD) skeletal phenotype. These unexpected results suggest that ETV4 and ETV5 act downstream of FGF signaling to inhibit *Shh* expression in the anterior limb bud. This finding elucidates a novel aspect of the mechanism coordinating limb development along the A-P and P-D axes.

INTRODUCTION

Formation of the tetrapod limb serves as a paradigm for pattern formation in developmental biology. Along the anterior-posterior (A-P; thumb to little finger) axis of the limb, asymmetry in the skeletal pattern is executed by signals from the Zone of Polarizing Activity (ZPA), a region in the posterior mesenchyme of the limb bud (McGlinn and Tabin, 2006). The ZPA is defined by its ability to induce posterior limb skeletal elements when transplanted to the anterior limb bud. Cells in the ZPA can be distinguished by their expression of sonic hedgehog (SHH), a secreted signal that is necessary and sufficient for ZPA function. In addition to its role in polarizing the limb bud, SHH signaling is also essential for limb bud growth (Towers et al., 2008; Zhu et al., 2008). Thus, precise regulation of the amount and localization of *Shh* expression is critical for normal limb development.

Several mechanisms are known to control *Shh* expression in the limb bud. Antagonism between transcription factors HAND2 and GLI3 is essential for establishing the prepattern that instructs *Shh* expression in the posterior mesenchyme (te Welscher et al., 2002a). Fibroblast growth factors (FGFs) expressed in the apical ectodermal ridge (AER and AER-FGFs) are essential for inducing and maintaining *Shh* (Niswander,

2002). Repressors of *Shh* expression emerged from studies of mutants that exhibit extra digits on the anterior (thumb) side of the autopod, a phenotype known as preaxial polydactyly (PPD). In many of these mutants, including in *Twist1*, *Alx4*, and *Gli3*, *Shh* is ectopically expressed in the anterior limb bud, in addition to its expression in the ZPA (Bourgeois et al., 1998; Dunn et al., 1997; Qu et al., 1997). Thus, these PPD genes are required to restrict *Shh* expression to the ZPA.

In addition to promoting *Shh* expression, AER-FGFs act as key signals for development of the proximal-distal (P-D; shoulder to finger tip) axis of the limb (Mariani and Martin, 2003). Downstream mediators of FGF activity in either P-D development or *Shh* regulation remain to be identified. Here, we investigate the role of the PEA3 group of ETS-domain-containing transcription factors in mouse limb buds. We show that two of the three members of this group, *Etv4* (also termed *Pea3*) and *Etv5* (also termed *Ern*), are expressed under the control of AER-FGF signaling. Conditional inactivation of *Etv4* and *Etv5* led to ectopic *Shh* expression and a PPD skeletal phenotype. These observations reveal an unexpected mechanism by which AER-FGFs influence limb patterning along the A-P axis.

RESULTS

FGF Signaling Is Required to Maintain *Etv4* and *Etv5* Expression in Mouse Limb Buds

To identify genes that mediate FGF function in limb development, we searched for transcription factors that are regulated by FGF signaling. By using RNA in situ analysis, we found that two PEA3 group genes, *Etv4* and *Etv5* (*Etv4;5*), but not the third member, *Etv1*, are expressed in the distal portion of the limb bud mesenchyme adjacent to the AER (Figures 1A and 1B and data not shown). To address if their expression is controlled by AER-FGF signaling, we analyzed *Etv4;5* expression in FGF pathway mutants. In *Msx2-Cre;Fgf4^{-fl};Fgf8^{-fl}* (or *Msx2-Cre;4;8*) limb buds in which two of the four AER-Fgfs are inactivated (Sun et al., 2002), *Etv4;5* expression is reduced (Figures 1A–1D). Similarly, in *Prx1-Cre;Fgfr1^{co/co};Fgfr2^{cl/c}* (or *Prx1-Cre;Fgfr*) limb buds in which the principle FGF receptors (Fgfrs) expressed in the limb bud are inactivated in the entire mesenchyme (Eswarakumar et al., 2002; Logan et al., 2002; Xu et al., 2002), *Etv4;5* expression is also downregulated (Figures 1E–1H). To address whether Fgfrs are required cell autonomously for regulating *Etv4;5* expression, we analyzed *Shh^{cre/+};Fgfr1^{co/co};Fgfr2^{cl/c}* (or *Shh-Cre;Fgfr*) limb buds in which Fgfrs

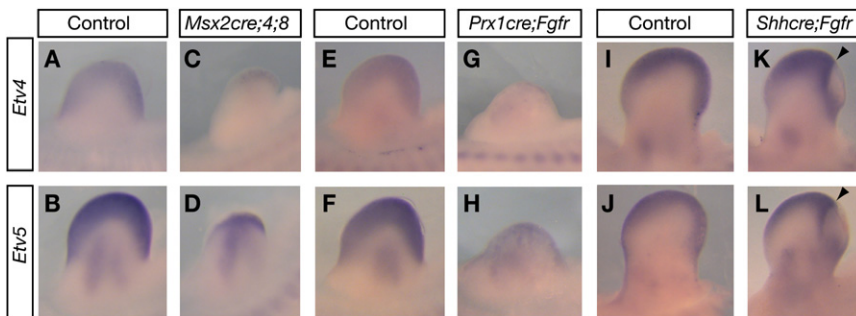


Figure 1. Inactivation of AER-Fgfs or FGF Receptors Leads to Downregulation of *Etv4;5* Expression in Limb Buds

(A–L) *Etv4* and *Etv5* expression in (A–H) E10.75 or (I–L) E11.5 limb buds. Arrowheads indicate the anterior boundary of the *Fgfr*-inactivated domain as identified both by downregulation of *Fgfr1* and FGF readout *Spry4* expression (data not shown) (Verheyden et al., 2005; Verheyden and Sun, 2008).

are inactivated in the posterior limb bud mesenchyme (Verheyden and Sun, 2008). We found that downregulation of *Etv4;5* expression is confined to the *Fgfr*-inactivated domain (Figures 1I–1L), indicating that AER-FGFs maintain *Etv4;5* expression without acting through additional secreted signals. These findings raised the possibility that *Etv4;5* act directly downstream of AER-FGF signaling.

Inactivating *Etv4;5* Function in Mouse Limb Buds

To address whether *Etv4;5* mediate FGF function in limb development, we inactivated both genes, as their shared expression domain indicates possible redundancy. Inactivation was achieved by generating a conditional allele of *Etv5* (*Etv5^{fl}*) and combining it with an existing null allele of *Etv4* (*Etv4⁻*) (Livet et al., 2002). In the *Etv5^{fl}* allele, *loxP* sites were engineered to flank exons that encode the N-terminal portion of the DNA-binding domain (Figure 2A). Excision of floxed exons led to *Etv5⁻*, predicted to encode a protein without the DNA-binding domain and the rest of the C-terminal region. A transcriptional assay shows that the remaining protein, if made, harbors no

residual transcriptional activity and does not interfere with full-length ETV5 function (Figure 2B). These results indicate that deletion of the floxed exons abolishes ETV5 activity.

Double homozygous mutants of *Etv4⁻* and *Etv5⁻* (in *Etv4^{-/-}; Etv5^{-/-}*) died prior to limb bud initiation (data not shown). To bypass early lethality, we used the *Brachyury* (*T*) Cre to inactivate *Etv5* in mesoderm-derived cells (Perantoni et al., 2005), and we combined this mutant with *Etv4⁻* (generating *Tcre;Etv4^{-/-}; Etv5^{-/fl}* or the *Tcre;Etv* mutant). Quantitative RT (qRT)-PCR analysis at embryonic day (E) 9.5 indicates that at the onset of limb development, ~2.5% of intact *Etv5* transcript was detected in the prospective hindlimb bud region in *Tcre;Etv* embryos (n = 3) (Figure 2C). This residual expression is diminished by E10.0 in both the forelimb and hindlimb buds (Figure 2D).

Inactivation of *Etv4;5* Leads to Preaxial Polydactyly

To investigate whether *Etv4;5* mediate FGF signaling, we first examined the expression of *Mkp3* and *Spry2*, common readouts of FGF activity (Tsang and Dawid, 2004). At ~E11, we detected no difference in their expression in *Tcre;Etv* mutants compared

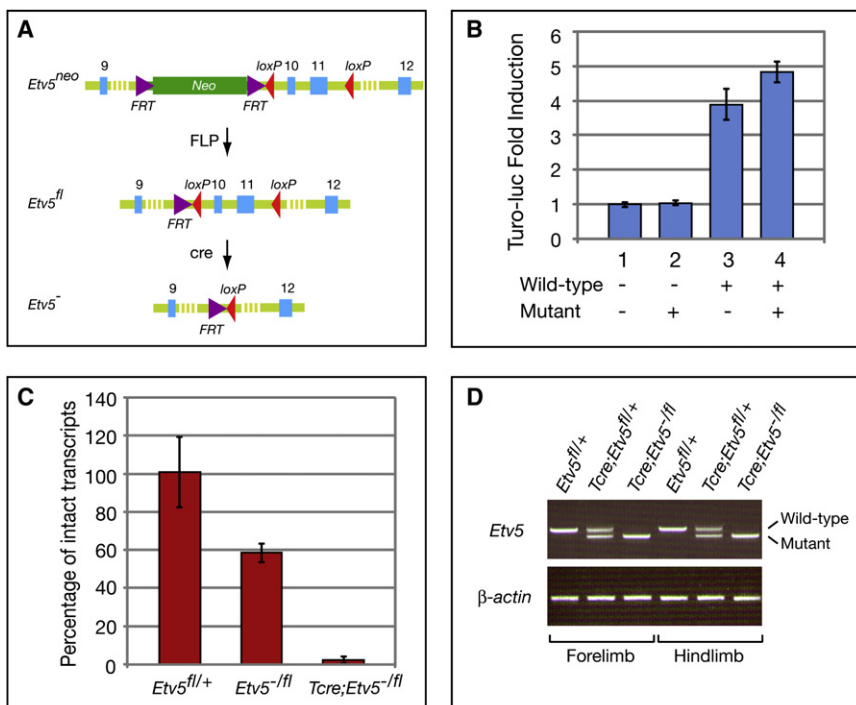


Figure 2. Deletion of Exons Encoding the ETV5 DNA-Binding Domain Leads to Inactivation of ETV5 Transcriptional Activity

(A) *Etv5* targeting strategy in which *loxP* sites flank exons 10 and 11. Germline *FLP* or *cre* mice were used to generate the *Etv5^{fl}* or *Etv5⁻* allele, respectively. The majority of *Etv5^{-/-}* animals die shortly after birth. This lethality was not reported in an existing mutant of *Etv5* (Chen et al., 2005), suggesting that our *Etv5⁻* allele is a more severe loss-of-function allele.

(B) Luciferase (Turo-luc) assay. Reporter fold induction is normalized to activity when empty vector is transfected (column 1). Expression of mutant ETV5 alone does not lead to a change (column 2 versus column 1, n = 3, p = 0.498). Expression of mutant ETV5 in the presence of wild-type ETV5 does not lead to downregulation of reporter activity compared to wild-type ETV5 alone (column 4 versus column 3, n = 3, p = 0.0483).

(C) qRT-PCR of *Etv5* in prospective hindlimb field of E9.5 embryos to detect intact, but not mutated, transcripts. The level of transcripts in mutant tissue is 2.5 ± 1.8 (SD)% (n = 3) of that in the *Etv5^{fl/+}* control (n = 3).

(D) RT-PCR of *Etv5* and β -actin in E10.0 limb buds. A two-tailed Student's t test was used, and error bars in all figures represent standard deviation (SD).

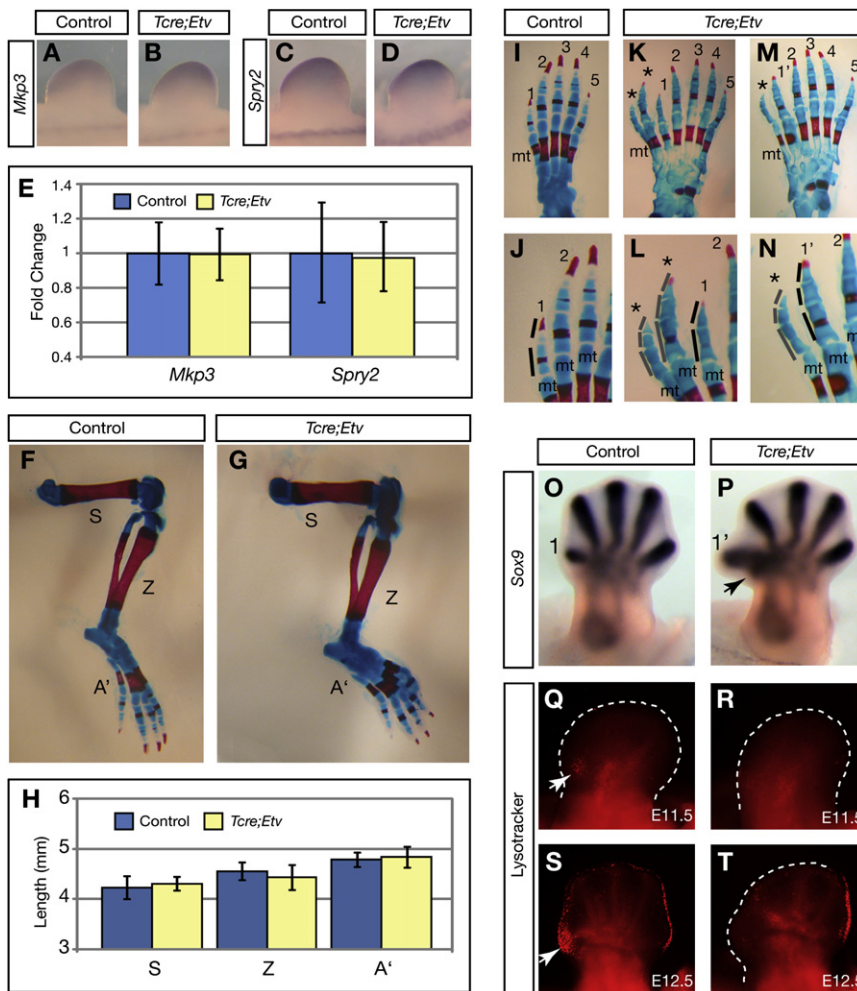


Figure 3. Inactivation of *Etv4/5* Leads to No Defects along the P-D Axis and a PPD Phenotype along the A-P Axis of Hindlimbs

(A–E) Expression of *Mkp3* and *Spry2* is normal in *Tcre;Etv* mutant limb buds, as assayed by (A–D) RNA in situ hybridization in E11 or (E) qRT-PCR in E10.75 (37- to 39-somite stage) hindlimb buds ($p = 0.49$ for *Mkp3*, $n = 3$; $p = 0.46$ for *Spry2*, $n = 3$). (F–H) Postnatal day 1 *Tcre;Etv* hindlimbs are normal along the P-D axis, as confirmed by measurements of the three segments ($n = 6$; $p = 0.24$, 0.17 , and 0.32 for S, Z, and A', respectively). (I–N) *Tcre;Etv* hindlimbs exhibit triphalangeal extra digits anteriorly (asterisks) and frequent digit 1 transformation from biphalangeal to triphalangeal (1', $n = 8/12$). Anterior digits in (I), (K), and (M) are magnified in (J), (L), and (N), respectively. (O and P) *Sox9* expression in E12.5 limb buds indicates an extra digit (arrow) and an enlarged digit 1' primordium. (Q–T) Lysotracker Red staining. Arrows indicate normal cell death. Autopod, A'; metatarsal, mt; stylopod, S; zeugopod, Z. Error bars represent standard deviations.

together are essential for establishing proper digit number and anterior digit patterning.

***Tcre;Etv* Mutant Limb Buds Exhibit Reduced Cell Death**

To uncover the cellular basis of the PPD phenotype, we examined the cell death pattern in *Tcre;Etv* mutants. In control limb buds, we detected a previously described pattern in the region termed foyer préaxial primaire (fpp), an area

to normal (Figures 3A–3E), indicating that *Etv4/5* are not essential for mediating FGF regulation of these genes in the limb bud.

Tcre;Etv embryos die shortly after birth due to internal organ defects. In newborn limbs along the P-D axis, *Tcre;Etv* embryos exhibit normal skeletal patterning (Figures 3F–3H). There is no upregulation of *Etv1* in the mutant limb bud that could compensate for the loss of *Etv4/5* function (data not shown). Thus, in limb P-D outgrowth, *Etv4/5* do not play a major role in mediating FGF function.

Along the A-P axis of the limb, however, all *Tcre;Etv* embryos exhibit a PPD phenotype in the hindlimbs, but not the forelimbs (Figures 3I–3N, $n = 9/9$ embryos; see Table S1 available online). Either one or two extra digits were observed, and a majority of them are triphalangeal, representing posterior digit identity. In over 50% of the limbs, digit 1 itself is transformed into a triphalangeal digit (Figures 3M and 3N). This transformation likely occurs early in autopod patterning, as suggested by longer digit 1 condensation outlined by *Sox9* expression at E12.5 (Figures 3O and 3P). Inactivation of both *Etv4* and *Etv5* contributes to the PPD phenotype, as *Etv4*^{-/-} animals exhibit no limb skeletal defects (Laing et al., 2000), and *Etv5*^{-/-} embryos exhibit a milder PPD defect with partial penetrance ($n = 12/17$, often only one extra digit, Table S1). These data demonstrate that *Etv4/5* genes

equivalent, but not identical, to the anterior necrotic zone observed in chick limb buds (Fernandez-Teran et al., 2006; Milaire, 1992). Cell death is reduced in this domain in *Tcre;Etv* limb buds (Figures 3Q–3T), similar to previous observations in other PPD mutants (Milaire, 1992). This observation offers a cellular mechanism for the presence of extra preaxial digits in *Tcre;Etv* mutants.

ETV4/5 Repress *Shh* Expression

Ectopic SHH activity is often observed in PPD mutants (Bourgeois et al., 1998; Dunn et al., 1997; Qu et al., 1997). In *Tcre;Etv* embryos starting from the 42-somite stage (~E11), we detected ectopic *Shh* expression in the anterior mesenchyme of hindlimb, but not forelimb, buds (Figures 4A–4D and data not shown). In all mutant limb buds, expression of *Shh* in the posterior mesenchyme remains normal. SHH-regulated genes, including *Gli1*, *Patched1* (*Ptch1*), *Hand2*, *Hoxd13*, and *Grem1*, are all ectopically expressed in E11.5 *Tcre;Etv* limb buds (Figures 4E–4N), consistent with upregulation of SHH activity. Furthermore, *Fgf4* expression in the AER, which is positively regulated by SHH, extends anteriorly after, but not prior to, ectopic *Shh* expression (Figures 4O–4R). These data indicate that *Etv4/5* are essential for repressing *Shh* expression in anterior limb bud mesenchyme.

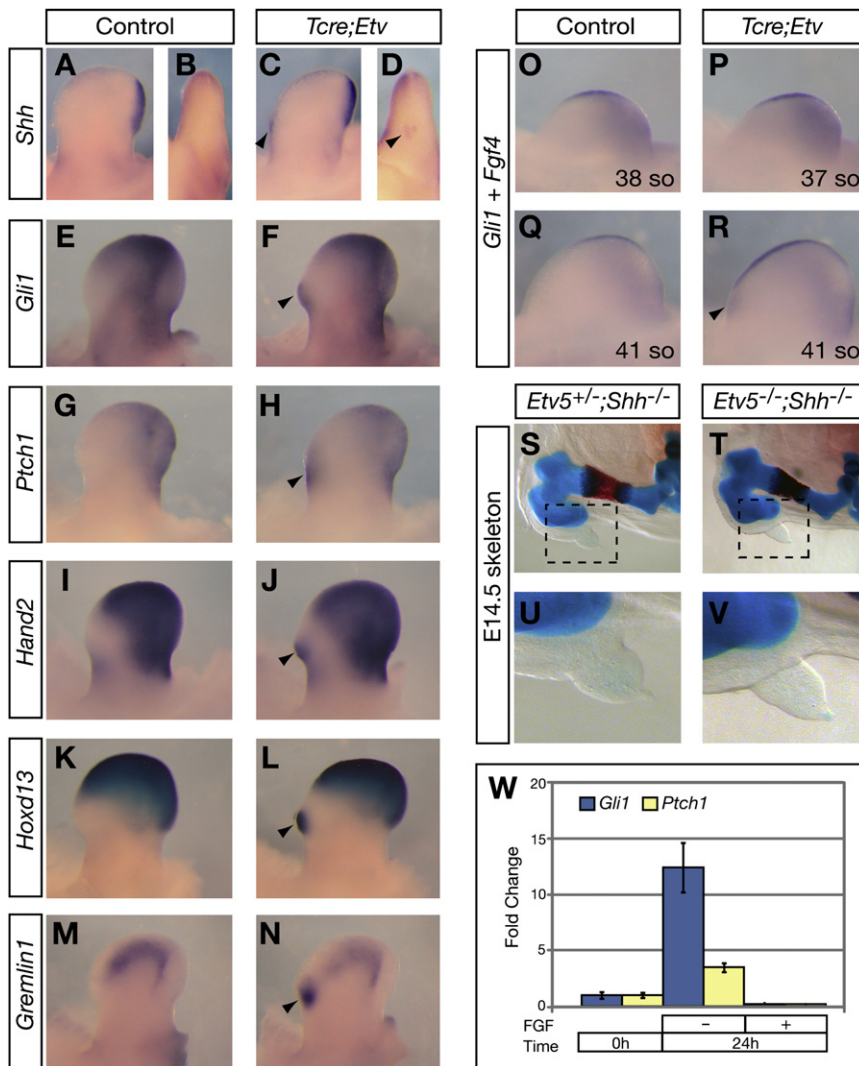


Figure 4. Inactivation of *Etv4;5* Leads to Ectopic *Shh* Expression in the Anterior Hindlimb Bud

(A–N) Gene expression in E11.5 hindlimb buds. Arrowheads indicate ectopic anterior expression. (B and D) Anterior views of limb buds in (A) and (C), respectively.

(O–R) Nonoverlapping *Fgf4* (AER) and *Gli1* (mesenchyme) expression at indicated somite (so) stages. The arrowhead indicates ectopic *Gli1*. (S–V) Hindlimb skeleton. Boxed regions in (S) and (T) are magnified in (U) and (V).

(W) Changes of *Gli1* and *Ptch1* expression in cultured anterior limb mesenchyme cells as assayed by qRT-PCR and normalized to their levels without culture ($n \geq 3$ for each time point and condition).

Error bars represent standard deviations.

qRT-PCR, no change in the expression of these regulators was observed (Figure S1 and data not shown). The results suggest that *Etv4;5* act either downstream of or in parallel with these factors to control *Shh* expression.

FGF Inhibits Polarizing Activity in the Anterior Limb Bud Mesenchyme

As FGFs are known positive regulators of *Shh*, the finding that the FGF-maintained genes *Etv4;5* inhibit *Shh* expression, and hence restrict polarizing activity, is unexpected. However, consistent with the general theme of our finding, a previous report shows that anterior mesenchymal cells can be converted to ZPA signaling cells when cultured in the absence of

Previous studies show that ectopic *Shh* accounts for the extra digit phenotype observed in some, but not all, PPD mutants (Litingtung et al., 2002; te Welscher et al., 2002b). To address this trait in our mutants, we introduced a null allele of *Shh* into the *Etv5*^{-/-} mutant background, which exhibits PPD in ~70% of the embryos ($n = 12/17$, Table S1). All *Etv5*^{-/-};*Shh*^{-/-} mutant embryos obtained ($n = 6$) show a limb skeletal phenotype that is indistinguishable from the phenotype of either *Etv5*^{+/-};*Shh*^{-/-} or *Shh*^{-/-} littermates (Figures 4S–4V and data not shown) (Chiang et al., 2001; Kraus et al., 2001). This result suggests that the PPD phenotype caused by disruption of *Etv4;5* function is dependent on SHH activity.

To investigate the relationship between *Etv4;5* and other regulators of *Shh* transcription, we examined the expression of *Hand2*, *Gli3*, *Alx4*, *Twist1*, *Hoxd11*, *Hoxd12*, and *Hoxd13* (Bourgeois et al., 1998; Charite et al., 2000; Dunn et al., 1997; Qu et al., 1997; Zakany et al., 2004). As SHH feeds back to control the expression of most of these genes (Fernandez-Teran et al., 2000; Laufer et al., 1994), we assayed *Tcre;Etv* mutant hindlimb buds at around the 39-somite stage, just before ectopic *Shh* expression is detected. By using either RNA in situ analysis or

the AER (Anderson et al., 1994). Furthermore, this conversion is prevented if cells are cultured in the presence of FGF2.

We repeated similar experiments and addressed whether the expression of *Gli1* and *Ptch1*, sensitive readouts of SHH signaling, is altered in these cells in accordance with the reported polarizing activity. We found that when anterior mesenchymal cells are placed in culture without FGF, there is an increase in *Gli1* and *Ptch1* expression (Figure 4W). This increase is inhibited by the presence of FGF. We speculate that the reason why a similar increase of SHH activity has not been observed in vivo after AER removal is because it is obscured by the massive cell death that occurs after this surgical manipulation. Taken together, the results presented in this study are consistent with the overall interpretation that FGF signaling, via its regulation of *Etv4;5*, inhibits polarizing activity in the anterior limb bud mesenchyme.

DISCUSSION

In this study, we show that in the limb bud, two PEA3 group genes, *Etv4;5*, are expressed under the control of AER-FGF

signaling. This finding is in agreement with similar regulations in other regions of the embryo (Bottcher and Niehrs, 2005), raising the possibility that *Etv4;5* genes function as general mediators of FGF function. Consistent with this possibility, in several developing tissues, including the kidney, conditional inactivation of both *Etv4;5* led to gross phenotypes that resemble those described in *Fgf* mutants (data not shown). However, in the limb, we show the surprising finding that instead of an apparent reduction of the P-D axis, a phenotype displayed by *Fgf* mutants, *Tcre;Etv* limbs exhibit an expansion of the A-P axis. A similar A-P phenotype is observed in the accompanying study after the expression of a constitutively repressor form of ETV4 (EtvEnR) (Mao et al., 2009 [this issue of *Developmental Cell*]). In *EtvEnR* limbs, although a slight decrease in P-D length is observed, this reduction resembles only the mild phenotypes exhibited by *Fgf* or *Fgfr* mutants (Boulet et al., 2004; Mariani et al., 2008; Sun et al., 2002; Verheyden et al., 2005; Yu and Ornitz, 2008). Thus, combined results from the two studies indicate that *Etv4;5* genes are not the principle mediators of FGF function in limb P-D outgrowth. Rather, the A-P axis defects reveal an unexpected role of FGF-regulated genes in PPD, a common birth defect.

We found that cell death is reduced in the *Tcre;Etv* limb buds, providing a plausible mechanism for the formation of extra digits. Preceding this reduction of cell death, anterior ectopic *Shh* and expansion of *Fgf4* were observed. Either one of these molecular changes was shown to result in decreased cell death (Lu et al., 2006; Sanz-Ezquerro and Tickle, 2000). Several lines of evidence suggest that altered *Shh*, rather than *Fgf4*, is the primary cause for the phenotypes in *Tcre;Etv* mutants. First, in these limbs, posterior fate has been imposed on anterior digits, an outcome associated with overexpression of SHH, but not FGFs (Riddle et al., 1993). Second, our finding that *Etv5*^{-/-};*Shh*^{-/-} limbs are indistinguishable from *Shh*^{-/-} limbs indicate that the extra anterior digits are dependent on SHH activity. Finally, whereas overexpression of *Fgf4* does not lead to ectopic anterior *Shh* expression, increase in SHH activity can lead to extension of *Fgf4* expression (Laufer et al., 1994; Lu et al., 2006; Niswander et al., 1994). Taken together, these findings support the conclusion that ETV4;5 control digit number and digit identity by inhibiting *Shh* expression in anterior limb bud mesenchyme.

Evidence suggests that *Etv4;5* are likely required at the beginning of limb bud development for *Shh* inhibition. In *Tcre;Etv* mutant limb buds, although complete *Etv5* inactivation is observed at E10.0 in all limb buds, this is an earlier time point in the course of hindlimb bud development than in forelimb bud development. Thus, the restriction of defects to the hindlimbs may be due to distinct recombination timing. Possible differences in inactivation efficiency may also contribute to the phenotype variations between *Tcre;Etv* and *EtvEnR* mutants (Mao et al., 2009). In *Tcre;Etv* limb buds, although *Tcre* is active early in limb bud development (Verheyden et al., 2005), loss of *Etv4;5* function is contingent upon degradation of wild-type *Etv5* transcripts/protein that are produced prior to Cre-mediated recombination. However, in *EtvEnR* mutants, *Etv4;5* function is inhibited as soon as the competing mutant protein is made. Thus, it is possible that disruption of *Etv4;5* function occurred earlier in *EtvEnR* mutants than in *Tcre;Etv* mutants. Despite these variations, results from the two studies support a common conclusion that *Etv4;5* repress *Shh* expression in the limb bud.

It remains to be determined whether this repression is achieved through direct binding of ETV4;5 to the *Shh* enhancer/promoter, or through ETV4;5 control of other *Shh* regulators. Among factors that are unaltered in *Tcre;Etv* limb buds are HAND2 and GLI3, proteins that establish the initial A-P polarity of the limb bud (te Welscher et al., 2002a). Additional studies will determine if *Etv4;5* expression is altered in mutants of these regulators, an indication that ETV4;5 act downstream. Alternatively, ETV4;5 may interact with these factors on the protein level, as has been demonstrated between HAND2 and TWIST1 (Firulli et al., 2005). Parallel protein-level interactions may also explain why ETV4;5 do not inhibit *Shh* expression in the ZPA, where positive regulators such as HAND2 are present at high levels (Charite et al., 2000), and may override the inhibitory effect of ETV4;5.

For A-P patterning of the limb, it is as important to prevent *Shh* expression in the anterior limb bud as it is to sustain its expression in the posterior limb bud (McGlenn and Tabin, 2006). Our findings indicate that in addition to the known function in promoting *Shh* expression, activities downstream of FGF signaling also play a role in inhibiting *Shh* expression. In this context, ETV4;5 are distinct from other inhibitors of *Shh* in that they are positively regulated by AER-FGF signaling. It appears that FGF signaling coordinates the positive and inhibitory regulator properties, providing an efficient mechanism to achieve polarization of the limb bud.

EXPERIMENTAL PROCEDURES

Phenotype Analyses

Tcre;Etv mutants were generated as described (Supplemental Experimental Procedures). Whole-mount in situ hybridization, skeletal preparations, and cell death analysis (with LysoTracker Red DND-99, Invitrogen) were performed by using standard or published protocols (Neubuser et al., 1997; Zucker et al., 1999). To examine the level of intact *Etv5* transcripts, cDNA was prepared from lateral plate mesoderm in the hindlimb bud field of embryos at the 25- to 27-somite stage (~E9.5) and limb buds of embryos at the 29- to 30-somite stage (~E10). To examine the expression of *Mkp3* and *Spry2*, cDNA was prepared from hindlimb buds of embryos at the 37- to 39-somite stage (Supplemental Experimental Procedures). A Student's t test was used for statistical analysis, and standard deviation was presented.

Cell Culture and Luciferase Assay

Each well of HeLa cells was transfected with a total of 800 ng DNA, including *TK-hRL* (Promega) as an internal control, *Turo-luc* as a ETV4;5-responsive reporter plasmid (Monte et al., 1995), and either empty vector alone, *pDEST-wtEtv5*, and/or *pDEST-mutEtv5* (Supplemental Experimental Procedures). Expression was assayed 36 hr later by using the Dual Luciferase kit (Promega).

Limb Bud Cell Culture

Following an established protocol (Anderson et al., 1994), FBS was pretreated with heparin beads to remove FGF prior to use in medium. Anterior limb bud mesenchyme was dissected from 40 E10.5 limb buds and pooled. Cells were dissociated and aliquoted into 16 wells and cultured in the presence or absence of FGF2 (100 ng/ml) (Peprotech). Expression of *Gli1* and *Ptch1* was assayed by qRT-PCR (Supplemental Experimental Procedures).

SUPPLEMENTAL DATA

Supplemental Data include one table, one figure, and Supplemental Experimental Procedures and can be found with this article online at [http://www.cell.com/developmental-cell/supplemental/S1534-5807\(09\)00082-3](http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00082-3).

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