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Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb

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Abstract

Normal patterning of the developing limb requires a tight restriction of *Sonic hedgehog* (*Shh*) mRNA to the posterior margin of the limb bud. While several positive and negative regulatory factors have been identified which serve to position the *Shh* expression domain in the distal posterior limb, these factors cannot in themselves explain the tight restriction of *Shh* to the posterior margin, nor can they explain the similarly tight restriction of *Shh* to the anterior margin when the regulatory factors are disrupted or misexpressed. We suggest that the transcription factors Tbx2 and Tbx3 are excellent candidates for positively-acting factors responsible for limiting Shh expression to the margins of the limb bud. These closely related factors are indeed expressed at the anterior and posterior limb margins over a wide range of limb bud stages. Moreover, previous reports indicate that in addition, misexpression of *Tbx2* beyond the limb margin is sufficient to anteriorly expand *Shh*, and conversely, antagonizing *Tbx2* function leads to loss of *Shh*. In contrast to this idea, previous models have placed Tbx2 expression downstream of Shh and Bone Morphogenetic Protein (BMP) signaling. We find, however, that Tbx2 expression is neither affected by blocking Shh signaling with cyclopamine nor by genetic removal of several BMP activities in the limb bud. To understand the true source of the positional information responsible for limiting Tbx2, Tbx3 and Shh expression to the marginal mesenchyme of the limb bud, we undertook a series of grafting and extirpation experiments, which led to the identification of the dorsal–ventral (DV) border ectoderm exclusive of the apical ectodermal ridge (AER) as a new signaling center in the limb bud. We find that maintenance of *Tbx2* expression in the limb mesoderm requires proximity to the non-AER D–V border. Using chick-quail graft chimeras, we find that a graft of the non-AER D–V border ectoderm to a location on the surface of the middle of the limb bud is sufficient to induce ectopic expression of *Tbx2* in underlying mesoderm. These data demonstrate that the non-AER D–V border ectoderm is necessary and sufficient for *Tbx2* expression at the anterior and posterior limb margins. Similarly, we find that a graft of the non-AER D–V border can expand the domain of *Shh* anteriorly when grafted just anterior to the ZPA. It is notable that Tbx2 expression does not extend distally to the mesoderm underlying the AER. Moreover, we find that grafts of the AER to more proximal locations result in downregulation of Tbx2 expression, suggesting that the AER produces a negatively-acting signal opposing the activity of the non-AER DV border ectoderm. Indeed, implantation of beads soaked in fibroblast growth factor 8 (Fgf8), expressed in the AER, downregulates Tbx2 expression. The data presented here identify the non-AER border of dorsal–ventral ectoderm as a new signaling center in limb development that localizes the ZPA to the limb margin. This finding explains the tight restriction of *Shh* expression to the posterior margin throughout limb outgrowth as well as the tight restriction of *Shh* expression to the anterior margin in many mutants exhibiting preaxial polydactyly.

Keywords: Limb development; Tbx2; Shh; Tbx3; Fgh; Bmp; ZPA; Signaling center; AER

Introduction

Developmental fields are often organized by inductive cues emanating from distinct signaling centers. One important signaling center in limb development is the Zone of Polarizing Activity (ZPA). In a classical experiment by Saunders and Gasseling (1968), the ZPA was identified as a population of cells in the distal posterior margin of the chick limb bud capable of inducing mirror-image digit duplications when grafted into the anterior distal margin of another limb bud. We now know that limb bud cells with ZPA activity are congruent with cells that express the secreted factor *Sonic hedgehog (Shh)* (Riddle et al., 1993). Grafting cells that express *Shh* or a bead soaked in Shh protein into the anterior margin mimics the mirror-image

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digit duplications that ensue from ZPA grafts (Lopez-Martinez et al., 1995; Riddle et al., 1993; Yang et al., 1997).

These grafting experiments dramatically demonstrate the necessity for limiting Shh expression to the distal posterior margin of the limb bud if normal patterning is to emerge. Yet the localization of Shh expression at the distal posterior margin is not simply a passive result of the intrinsic properties of the ZPA cells themselves, but rather the Shh expression domain is continually reestablished along both the proximal distal and anterior–posterior axes. As the limb bud grows out, Shh expression is only maintained in the distal-most cells of the posterior margin, and is lost in more proximal cells even though they are descended from the ZPA (Vargesson et al., 1997). This distal shift in Shh expression is well understood. There is a second signaling center in the ectoderm at the distal tip of the limb bud called the Apical Ectodermal Ridge (AER). The AER, which forms at the border between the dorsal and ventral planes of ectoderm at the tip of the limb bud (hence forming a ridge running along the anterior–posterior axis of the future hand plate) is known to express members of the Fgf family which are required for maintenance of Shh expression (Laufer, 1994; Niswander, 1994). Thus, as the limb bud grows out only the ZPA cells towards the distal tip, within range of the Fgf signaling, are capable of continued Shh expression.

A similar phenomenon, albeit much less well understood, appears to continuously restrict Shh expression to the extreme posterior margin. Fate mapping studies have revealed that as the limb bud grows, descendants of *Shh*-expressing cells expand to populate most of the posterior third of the limb bud, a domain much larger than the region actively expressing *Shh* (Harfe et al., 2004). Thus, as this cell population proliferates, more anterior members of the population shut off *Shh* expression such that the *Shh* expression domain always remains tightly restricted to the posterior margin. While the factor(s) responsible for this tight posterior restriction are unknown, several factors (both negatively and positively acting) have been identified which together are responsible for broadly localizing Shh activity to the posterior and not anterior portions of the limb bud. Gli3 and Alx4 are negative regulators of Shh, expressed in the anterior of the limb bud and required to repress Shh in that domain.

Mice carrying mutations in these genes exhibit ectopic *Shh* in the anterior limb bud and subsequent preaxial polydactyly (Hui and Joyner, 1993; Masuya et al., 1995; Qu et al., 1998; Takahashi et al., 1998). Conversely, the transcription factors *Hoxb-8* (in the forelimb bud) and *Hand2* (previously called *dHand*) are broadly expressed in the posterior limb bud and positively regulate *Shh* expression. When these transcription factors are ectopically expressed in the anterior limb mesenchyme, they result in ectopic expression of *Shh* in the anterior (Charite et al., 1994; Charite et al., 2000; Fernandez-Teran et al., 2000).

While these factors localize the ZPA roughly to the posterior limb, their broad expression domains imply that an additional regulatory mechanism restricts *Shh* to the extreme posterior margin. Intriguingly, either disruption of the negative-acting regulatory factors, Gli3 or Alx4 (Hui and Joyner, 1993; Qu et al., 1998; Masuya et al., 1995) or global misexpression of the positive-acting regulating factors Hoxb8 in the forelimb or Hand2 (in either the fore or hindlimb) (Charite et al., 1994; Charite et al., 2000; Fernandez-Teran et al., 2000), result in ectopic anterior expression domains of Shh which are tightly restricted to the anterior margin. Together, these findings provide compelling evidence that an additional mechanism is responsible for the tight restriction of *Shh* to the posterior margin, and moreover, that the same mechanism is likely responsible for restricting *Shh* to both anterior and posterior margins.

Strikingly, the T-box transcription factors *Tbx2* and *Tbx3* are expressed similarly in tight stripes along the anterior and posterior margins of the developing limb bud (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998). These transcriptional repressors are related to the *Drosophila* gene *optomotor blind* (*omb*) and have been implicated in morphogenesis and organogenesis of a variety of tissues (Papaioannou, 2001; Papaioannou and Silver, 1998). Based on previous experiments in the chick limb bud, it has been postulated that Tbx3 is regulated downstream of signals which pattern the anterior–posterior axis such as *Shh* (Tumpel et al., 2002). However, reexamination of the literature suggests that the epistasis may actually be the reverse of this: in the human ulnar-mammary syndrome, haploinsufficiency of *TBX3* leads to a reduced or absent ulna and loss of digit V in patients, suggestive of a loss-of-function of *SHH* (Bamshad et al., 1997). Indeed, this phenotype is phenocopied in mice lacking *Tbx3* which exhibit a reduced or absent ulna/fibula, a reduced or absent digit V, and occasionally a reduced or absent digit IV, and these defects correspond with reduced or absent expression of *Shh* (Davenport et al., 2003). Similar data have been reported in the chick. Tbx2 and Tbx3 are believed to function exclusively as transcriptional repressors, hence fusing the DNA-binding domains of those proteins to the VP16 transcriptional activation domain, is predicted to yield a dominant-negative variant. As in the loss of function in mice, viral misexpression of *VP16-Tbx2* and *VP16-Tbx3* mutant genes, results in reduction or loss of posterior digits in the chick hindlimb (Suzuki et al., 2004). While these phenotypes were interpreted in terms of a direct role for *Tbx* genes in digit specification, the viral misexpression of *VP16-Tbx2* was also noted to reduce levels of *Shh* (Suzuki et al., 2004). Thus, a more parsimonious interpretation is that there is simply a loss of polarizing activity, and that Tbx2 and Tbx3 function are necessary for *Shh* expression. Conversely, viral misexpression of *Tbx2* anteriorly expands the endogenous domain of *Shh* in the chick limb (Suzuki et al., 2004), indicating that Tbx2 activity is also sufficient to induce or maintain Shh expression within the context of the posterior limb bud.

Given the restriction of *Tbx2* and *Tbx3* to the anterior and posterior margins and the apparent requirement of these genes for initiation and/or maintenance of *Shh*, it is likely that these genes are involved in the restriction of *Shh* to the limb margin. From the standpoint of understanding the spatial localization of *Shh* expression, however, this only begs the question, how are the *Tbx2* and *Tbx3* genes themselves restricted to the anterior and posterior limb margins? In this study, we identify the dorsal–ventral ectoderm border proximal to the AER as a new signaling center in limb development and show that it serves to regulate

the restricted expression of *Tbx2* and *Shh*. We demonstrate that *Tbx2* is not directly dependent on signals which pattern the anterior–posterior axis as previously postulated. We find that, instead, maintenance of *Tbx2* requires proximity to the dorsal–ventral (D–V) border ectoderm at the limb margins. Even more, the D–V border ectoderm when grafted to the mid-limb is sufficient to induce ectopic *Tbx2*. As would be predicted from expansion of *Tbx2*, the D–V border ectoderm when grafted in the posterior limb is capable of expanding the domain of *Shh* anteriorly. Finally, we investigate the exclusion of *Tbx2* from subapical mesoderm and show that the AER signal Fgf8 negatively regulates mesodermal expression of *Tbx2*. Our data provide evidence that the non-AER D–V border ectoderm is an important signaling center, responsible for maintaining *Shh* expression specifically at the posterior margin of the limb.

Materials and methods

Embryos

Experiments on wild-type chick embryos were performed on standard specific pathogen-free white Leghorn chick embryos provided by SPAFAS (Norwich, Connecticut). Fertile Japanese quail eggs were obtained from Truslow Farms. Eggs were incubated, windowed, and staged as described previously (Hamburger and Hamilton, 1951). Mouse *Bmp2* and *Bmp4* double mutants were generated as detailed elsewhere (Bandyopadhyay et al., submitted). Briefly, excision of floxed alleles of *Bmp2* and *Bmp4* was driven by a *Prx1*::*Cre* gene, resulting in removal of *Bmp2* prior to any endogenous expression and removal of *Bmp4* after only a few hours of very low expression, as judged by whole mount *in situ* hybridization.

D–V border ectoderm grafts

The ectoderm overlying the anterior or posterior limb margins was removed using an established technique for limb ectoderm removal (Yang and Niswander, 1995). Briefly, 1 μL of 1.0% Nile in PBS was pipetted over the limb margin. After 2 min, slight "blistering" of the ectoderm peeling off the mesoderm could be observed. The rectangle of ectoderm overlying the margin (both dorsal and ventral ectoderm as well as the dorsal–ventral ectoderm border) was cut using a tungsten needle. The rectangle of ectoderm was transferred to a host limb using a mouth pipet. The host limb was prepared by removing an equivalent rectangle of ectoderm from the dorsal surface of the mid-limb, and the dorsal–ventral border ectoderm was set in its place. Each ectoderm graft was held in place for 5–15 min to allow adequate sticking to the host mesoderm surface and hence to prevent loss of the ectoderm graft. A slight amount of bleeding from the host mesoderm surface greatly enhanced adhesion of the graft. Grafts from quail to chick limbs were performed in the same way.

Cyclopamine treatment

Cyclopamine (Toronto Research Chemicals) was added directly over the forelimb *in ovo* as previously described (Incardona et al., 1998). Briefly, 5 μl of 1.0 mg/mL cyclopamine in 45% HBC (Sigma) in PBS was added over the limb.

Mesoderm swapping experiment

A square of mesoderm under the anterior margin dorsal–ventral ectoderm border was cut cleanly using tungsten needles. Another square of mesoderm of equivalent size was cut in the mid-limb. These squares of tissue were swapped and grafted firmly into the square hole. To repeat this experiment with quail donor tissue, a square of anterior margin tissue was cut from a quail limb and transferred to a chick host limb using a mouth pipet. A square of chick tissue was removed from the mid-limb to allow grafting of the chick square tissue.

Whole mount in situ hybridization and quail antibody detection

Whole mount *in situ* hybridization was performed as previously described (Dietrich et al., 1997) with minor modifications. Briefly, embryos were fixed overnight in 4% paraformaldehyde, washed in PBS, dehydrated into methanol, and bleached for 1 h in 6% hydrogen peroxide in methanol. Embryos were then rehydrated into PBT and permeabilized with 10 μg/mL proteinase K in PBT for 20–30 min depending on embryonic stage. Embryos were then washed in PBT, post-fixed in 4% paraformaldehye/0.2% glutaraldehyde for 20 min, washed in PBT, and prehybridized for 1 h at 70 °C in hybridization buffer (50% formamide, 5× SSC pH 4.5, 2% SDS, 2% blocking reagent (Roche), 250 μg/mL tRNA, 100 μg/mL heparin). The embryos were then hybridized overnight at 70 °C in hybridization buffer with probes. After hybridization, the embryos were washed four times for 30 min each in 50% formamide/2× SSC ph 4.5/1% SDS and then washed in MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween-20). Embryos were blocked for 1 h in 2% blocking reagent/MABT followed by 1 h in 2% blocking reagent/20% heat inactivated goat serum/MABT, and then incubated overnight at 4 °C with secondary antibody (1:2500 anti-DIG AP, Roche). Embryo were then washed multiple times in MABT at room temperature and washed overnight at 4 °C. Embryos were then equilibrated in NTM (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$) and color detection was performed with NBT/BCIP (Sigma). DIG-labeled probes were generated for chick *Fgf8, Tbx2* (Suzuki et al., 2004), and *Shh* (Riddle et al., 1993).

For whole mount quail antibody detection, embryos fixed in 4% paraformalydehyde and dehydrated into Dent's fix (20% DMSO in methanol) and bleached. Embryos were then rehydrated into PBS+0.1% Triton-X (PBS-Tx) and incubated overnight at 4 °C with 1:10 dilution of QCPN antibody (Developmental Studies Hybridoma Bank) in 10% sheep serum/PBS-Tx. Embryos were washed several hours with PBS-Tx and then incubated overnight at 4 °C with 1:100 secondary antibody (goat-anti-mouse HRP-conjugated, Molecular Probes) in 1% goat serum/PBS-Tx. Embryos were washed several hours with PBS-TX and then signal was detected by DAB color reaction (Sigma).

Section in situ hybridization and quail antibody detection

Embryos were embedded in paraffin and 12 μm sections were collected. Section *in situ* hybridization was performed as previously described (Murtaugh et al., 1999) with minor modifications. Paraffin sections were dewaxed in xylenes, washing ethanol, and rehydrated into PBS, fixed for 10 min in 4% paraformaldehyde, washed in PBT, and permeabilized with 1.0 μg/mL proteinase K for 10 min. Sections were then washed in PBS, fixed in 4% paraformaldehyde for 5 min, and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed in PBS, rinsed in water, and air dried for 30 min. RNA probe was then added in 100 μL of hybridization buffer (10 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% Dextran Sulfate, 1× Denhardt's, 200 μg/mL yeast tRNA, 50% formamide). Slides were covered with coverslips cut from polypropylene bags, placed in chambers humidified with 1× SSC/50% formamide, and incubated overnight at 65 °C. The next day, coverslips were removed in 5× SSC and slides were washed for 30 min in 1× SSC/50% formamide at 65 °C. Slides were then transferred to TNE (10 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA) at 37 °C for 10 min, incubated in RNase A (20 μg/mL, Roche) in TNE for 30 min at 37 °C, and then washed in TNE for 10 min. Sections were then washed in 2× SSC for 20 min at 65 °C, then washed twice for 20 min each in 0.2× SSC, and then transferred into MABT. Slides were blocked in 2% blocking reagent (Roche)/20% heat inactivated goat serum/MABT for 1 h. Secondary antibody was added (anti-DIG AP antibody, 1:2500, Roche) in 2% blocking reagent/20% heat inactivated goat serum/MABT, and slides were incubated overnight at 4 °C. The next day, slides were washed in MABT and equilibrated in NTM for 10 min. Color detection was performed using Fast Red (Sigma). DIG-labeled probes were used for chick *Tbx2* (Suzuki et al., 2004).

Quail antibody was detected by rinsing slides in PBT, then incubating in 1:20 QCPN antibody in 5% heat inactivated goat serum/PBS, overnight at 4 °C. The next day, slides were washed in PBT and then incubated with secondary antibody (Cy2-conjugated anti-mouse antibody, Jackson Immunoresearch) overnight at 4 °C. The next day, slides were rinsed in PBT and visualized for immunofluorescence.

Bead implants

Affi-Gel Blue beads (BioRad) were used to administer Fgf8 protein to limbs. Beads were washed in PBS and incubated at room temperature for 1 h in 1.0 mg/mL human Fgf8 (PeproTech). Beads were then implanted into a small slit made in the anterior or posterior margin of the limb.

Results

Expression of Tbx2 at anterior and posterior margins of the limb

Tbx2 and *Tbx3* genes have been described as having very similar expression patterns during limb development, both restricted to the anterior and posterior margins of wing and leg buds, though Tbx3 in slightly wider domains (Gibson-Brown et al., 1998; Tumpel et al., 2002). For the purposes of this study, we chose to focus on *Tbx2*. To better understand how expression of *Tbx2* relates to limb bud formation and outgrowth, we first characterized mesodermal *Tbx2* expression at greater resolution than has been previously published, examining the wing bud from HH stages 15 through 27 (Fig. 1). Prior to wing bud formation at HH stage 15–16, *Tbx2* is expressed in a strip running the length of the lateral plate mesoderm encompassing the presumptive wing region and flank (Figs. 1A, B). Upon emergence of the wing bud at HH stage 17–18, *Tbx2* continues to be expressed in the flank and limb, but begins to disappear from the distal central limb (Figs. 1C, D). From HH stage 19–21, exclusion of *Tbx2* from the central limb becomes more pronounced while expression continues at the anterior and posterior margins of the limb as well as in the flank (Figs. 1E–G). By HH stage 22, the stripes of *Tbx2* expression at the anterior and posterior margins of the limb are clearly established (Fig. 1H). Shortly after this time, *Tbx2* expression is downregulated in the flank but maintained at high levels in the limb (Fig. 1K). This restriction to the anterior and posterior limb margins and flank continues through HH stage 27 and is apparent in both forelimbs and hindlimbs (Figs. 1I–L). At HH stage 27, *Tbx2* begins to also be expressed in the posterior interdigital mesoderm (arrowhead, Fig. 1J).

Relative epistasis of Shh and Tbx genes

Our reading of the literature led us to postulate that Tbx gene activity acts upstream to regulate Shh expression. There is evidence that *Tbx3* is necessary to initiate and/or maintain proper expression of *Shh* (Davenport et al., 2003; Suzuki et al., 2004) and ectopic misexpression of *Tbx2* is sufficient to expand the *Shh* expression domain (Suzuki et al., 2004). However, the reverse epistatic relationship has also been proposed. In particular, it has been suggested that the posterior stripe of Tbx3 expression is positively regulated by Shh and by BMP2 which is itself induced by Shh. In contrast, the anterior stripe of Tbx3 was proposed to be negatively regulated by Shh but positively regulated by BMP4. One difficulty in this model lies in the relative expression domains of these genes. While at HH stage 22, the limb expression of *Tbx2* encompasses expression of *Shh* in the posterior margin, extending more proximally and slightly more anteriorly than the *Shh* domain (Fig. 2A), by HH stage 25, the posterior Tbx2 domain can be seen to extend much further proximally than Shh (Fig. 2A). It is not immediately clear why Shh and/or BMP2 signaling would be able to maintain Tbx3 expression at such a long distance proximally, yet be unable to do so to an equivalent distance anteriorly. To directly examine whether *Tbx2* is regulated by Shh activity, we tested for changes in *Tbx2* expression after blocking the activity of Shh with cyclopamine in a HH stage 22 limb. Cyclopamine is a small steroidal alkaloid that blocks the cellular response to Shh (Incardona et al., 1998). Whereas genes upregulated directly in response to Shh such as *Ptc1, Ptc2, and Gli1* are

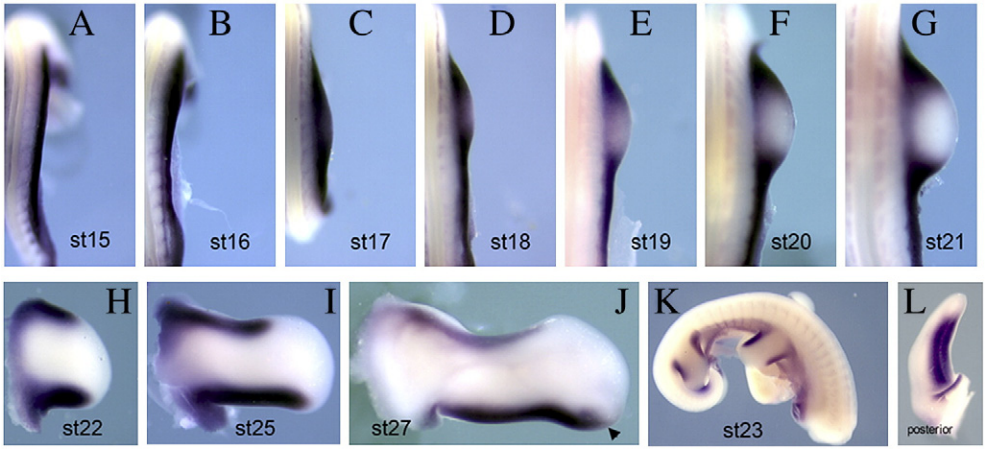

Fig. 1. Expression of *Tbx2* throughout limb bud development. (A–J) Dorsal view before and after limb bud formation from HH stages 15–27. (A–C) Prior to limb bud formation and just as the limb bud emerges, *Tbx2* is expressed throughout the lateral plate mesoderm in the presumptive wing region and flank. (D–G) Upon emergence of the wing bud, *Tbx2* begins to disappear from the mid-limb but continues to be expressed in the anterior and posterior of the limb as well as in the flank. (H–I, K) By HH stage 22, *Tbx2* expression in the limb is restricted to the anterior and posterior margins. (K) In the HH stage 23 embryo, *Tbx2* expression is restricted to the flank and the anterior and posterior margins of both forelimb and hindlimb. This restriction to the limb margins continues through HH stage 27 (J). At HH stage 27, *Tbx2* is also expressed in the posterior interdigital mesoderm (arrowhead). (L) Posterior view of a HH stage 24 limb. The domain of *Tbx2* tightly parallels a faint line that demarcates the non-AER D–V ectoderm border.

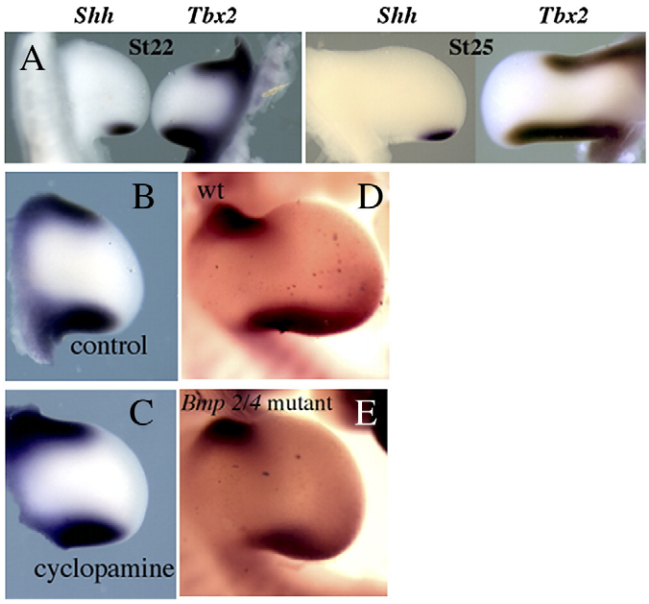

Fig. 2. Expression of *Tbx2* is not directly dependent on signals which pattern the anterior–posterior axis. (A) Comparison of *Shh* and *Tbx2* expression at HH stage 22 and HH stage 25. (B, C) Control limb and limb treated with cyclopamine and examined after 8 h for changes in *Tbx2* expression. (D, E) Comparison of *Tbx2* expression in wildtype mouse limb vs. limb lacking Bmp2 and Bmp4 activity.

downregulated as quickly as 4 h after cyclopamine is added to the limb (Scherz et al., submitted), *Tbx2* expression was maintained at the anterior and posterior margins 8 h after addition of cyclopamine ($n=6$) (Figs. 2B, C). To test the necessity for BMP signaling to attain proper Tbx gene regulation, we examined Tbx2 expression in mouse limbs in which both BMP2 and BMP4 had been genetically removed. *Tbx2* expression appears unchanged in these limbs compared to expression in wild-type limbs (Figs. 2D–E). Because BMP7 is, like BMP2, a target of Shh signaling in the posterior limb bud, it was possible that they might play redundant roles in regulating Tbx gene expression. We therefore, also analyzed limb buds devoid of both BMP2 and BMP7 activity, and again saw no alterations in Tbx2 expression (data not shown).

Regulation of Tbx2 by D–V border ectoderm

Signaling between the epithelial and mesenchymal tissues of the limb play many key roles in orchestrating limb development (for example, the signaling between the AER and ZPA discussed above). Thus, an attractive alternative hypothesis for explaining the regulation of *Tbx2* gene expression would be signals emanating from the overlying ectoderm rather than from the mesenchyme itself. The anterior and posterior margins of the limb mesenchyme lie underneath the location where the dorsal and ventral ectodermal surfaces meet (see posterior view of the edge of a HH stage 22 wing bud in Fig. 1L). The dorsal and ventral ectoderm have distinct properties. For example, the dorsal ectoderm expresses the secreted protein *Wnt7a* (Dealy et al., 1993; Parr et al., 1993), while the ventral ectoderm expresses members of the *Bmp* family (Lyons et al., 1990; Pizette et al., 2001). Moreover, at the distal tip of the limb bud, the border between the dorsal and ventral ectoderm forms a unique morphological structure with distinct signaling properties, the AER. These considerations suggested that either a combination of signals from the dorsal and ventral ectoderm only found in proximity to each other at the dorsal–ventral border or alternatively, a unique signal produced by the dorsal–ventral border ectoderm itself might be responsible for regulating expression of *Tbx* genes in the subjacent mesenchyme.

To test if signals present at the border between dorsal and ventral ectoderm regulate *Tbx2* expression in the anterior and posterior margins, we swapped a block of mesoderm at the anterior margin with a block of mesoderm in the mid-limb at HH stage 22/23 (Fig. 3A). This procedure effectively moves tissue that expresses *Tbx2* away from the D–V border and thereby tests the requirement of the D–V border to maintain *Tbx2* expression. At the same time, tissue that does not express *Tbx2* is moved under the D–V border, testing the sufficiency of the D–V border to upregulate *Tbx2* in underlying mesoderm. Importantly, the surgery was designed such that the D–V border ectoderm is kept intact during the operation (arrowhead). This swap can be appreciated by analyzing *Tbx2* expression 2 h after the operation (Fig. 3B). A block of limb tissue expressing *Tbx2* has been grafted into the mid-limb away from the D–V border, and a block of limb tissue not expressing *Tbx2* has been grafted under the D–V border. After 24 h, the block of tissue moved away from the D–V border into the mid-limb loses its *Tbx2* expression ($n=9/9$) (Fig. 3C). The same was observed when a block of tissue expressing *Tbx2* in the posterior limb was moved away from the posterior D–V border into the mid-limb ($n=2/3$) (Fig. 3F); we noted that *Tbx2* may be maintained in the posterior mesoderm grafts longer than in the anterior mesoderm grafts, as weak expression of *Tbx2* in a block of posterior limb tissue persisted at 24 h after grafting to the mid-limb ($n=1/3$), a finding consistent with similar persistence observed with *Tbx3* expression (Tumpel et al., 2002). The loss of *Tbx2* in mesoderm moved away from the D–V border suggests that the D–V border is necessary to maintain *Tbx2* expression. To verify that loss of *Tbx2* expression after 24 h is not simply due to loss of the graft, we performed a similar procedure grafting a piece of quail anterior margin tissue into the chick mid-limb. The quail tissue can be unambiguously identified using a specific antibody. As expected, 24 h ($n=2/2$) and even 48 h ($n=2/2$) after the operation, the quail graft is viable and appears to have integrated and grown with the host chick limb (Figs. 3D, E). Quail grafts remain viable in other regions of the limb as well, including the anterior and posterior margins (data not shown). These data suggest that the D–V border is necessary to maintain *Tbx2* expression in underlying mesoderm. To corroborate this conclusion, we examined the mesodermal expression of *Tbx2* at 0, 2, 6, and 10 h after removal of the D–V border ectoderm. While we expect the D–V border ectoderm to reconstitute after removal, we did observe a transitory downregulation of *Tbx2* occurring maximally at 6 h after removal of the overlying D–V border ectoderm ($n=5$) (Fig. 3G). By TUNEL analysis, we confirmed that this downregulation of *Tbx2* was not a result of

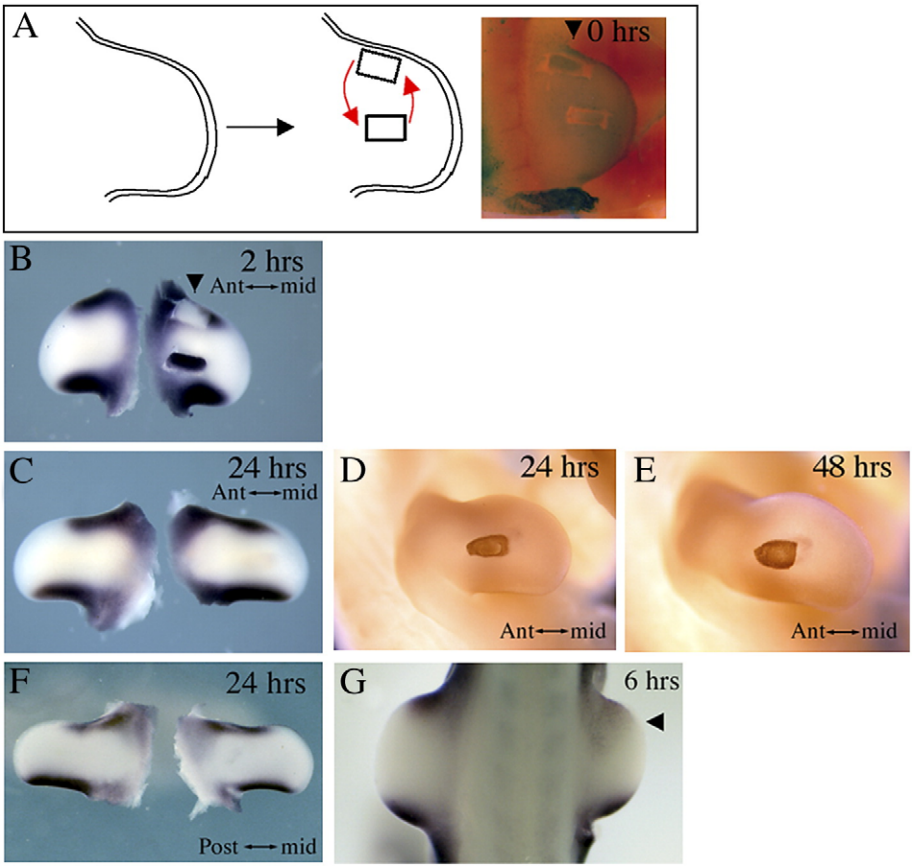

Fig. 3. Maintenance of *Tbx2* expression in the limb margin mesoderm requires proximity to the D–V ectoderm border. (A) Schematic representation and photograph of operated limb immediately after mesoderm swap in which mesoderm expressing *Tbx2* is swapped with mesoderm from mid-limb not expressing *Tbx2*. (B) Expression of *Tbx2* 2 h later confirms mesoderm swap. Note that the anterior D–V ectoderm border remains intact (arrowhead). (C) After 24 h, mesoderm moved to mid-limb has lost its *Tbx2* expression and mesoderm moved to anterior margin has gained *Tbx2* expression. (D, E) A graft of quail anterior margin mesoderm to the chick mid-limb after 24 or 48 h demonstrates that disappearance of *Tbx2* in such a graft (C) is not due to loss of the graft. (F) After 24 h, mesoderm moved from the posterior to the mid-limb has lost its *Tbx2* expression and mesoderm moved to the posterior margin has gained *Tbx2* expression. (G) Removal of the anterior D–V border ectoderm at stage 19 results in transitory downregulation of *Tbx2*, maximally observed at 6 h after removal of the D–V border ectoderm.

apoptosis. The transitory downregulation of *Tbx2* following removal of the overlying D–V ectoderm border is consistent with the specific requirement of the D–V border ectoderm to maintain mesodermal *Tbx2* expression.

In our experiments, the mesodermal grafts also included a reciprocal translocation of a block of tissue that does not express *Tbx2* from the mid-limb to a position under the D–V border ectoderm (Figs. 3A, B). After 24 h, this tissue appears to upregulate *Tbx2* expression, suggesting that the D–V border ectoderm is sufficient to induce *Tbx2* in underlying mesoderm (n=4/9) (Fig. 3C). We also observed upregulation of *Tbx2* when mid-limb tissue was placed in proximity of the posterior D–V border ectoderm (n=3/3) (Fig. 3F). In principle, the upregulation of *Tbx2* in mesoderm transplanted to the anterior margin could be explained by a combination of signals from the dorsal and ventral ectoderm or by unique signals emanating from the ectoderm at the dorsal–ventral border. To test the former possibility, we transplanted a patch of ventral ectoderm onto the dorsal surface, creating new regions of dorsal–ventral ectodermal juxtaposition over the central limb bud. Although the ectoderm healed and the transplants remained in place, we never saw upregulation of *Tbx2* in the underlying mesoderm (data not shown) (n=0/10). In control experiments, we also never saw ectopic AER induction following dorsal–ventral ectodermal juxtaposition at HH stage 22 (data not shown), consistent with previous reports that the ability to induce ectopic AERs in this manner is lost after HH stage 17 (Tanaka et al., 1997).

To test the alternative possibility that a unique *Tbx2*-inductive signal is produced by D–V border ectoderm, we grafted D–V ectoderm border from the anterior margin of one limb into an ectodermal gap cut in the dorsal surface of another limb at the level of the mid-limb where *Tbx2* is not normally expressed (Fig. 4A). We then examined whether this ectodermal border graft could induce *Tbx2* expression in the mid-limb. Indeed, when analyzed after 24 or 48 h, a clear ectopic band of *Tbx2* expression could be detected in the mid-limb (n=6/22) (Fig. 4B and data not shown). This ectopic band of *Tbx2* was observed with equivalent grafts of the D–V ectoderm border from the posterior margin as well (n=3/9, data not shown). The placement of the D–V ectoderm border on the host limb bud was somewhat variable, and indicated that virtually the entire limb bud mesenchyme is competent to respond to a D–V ectoderm border graft by inducing *Tbx2* expression. To

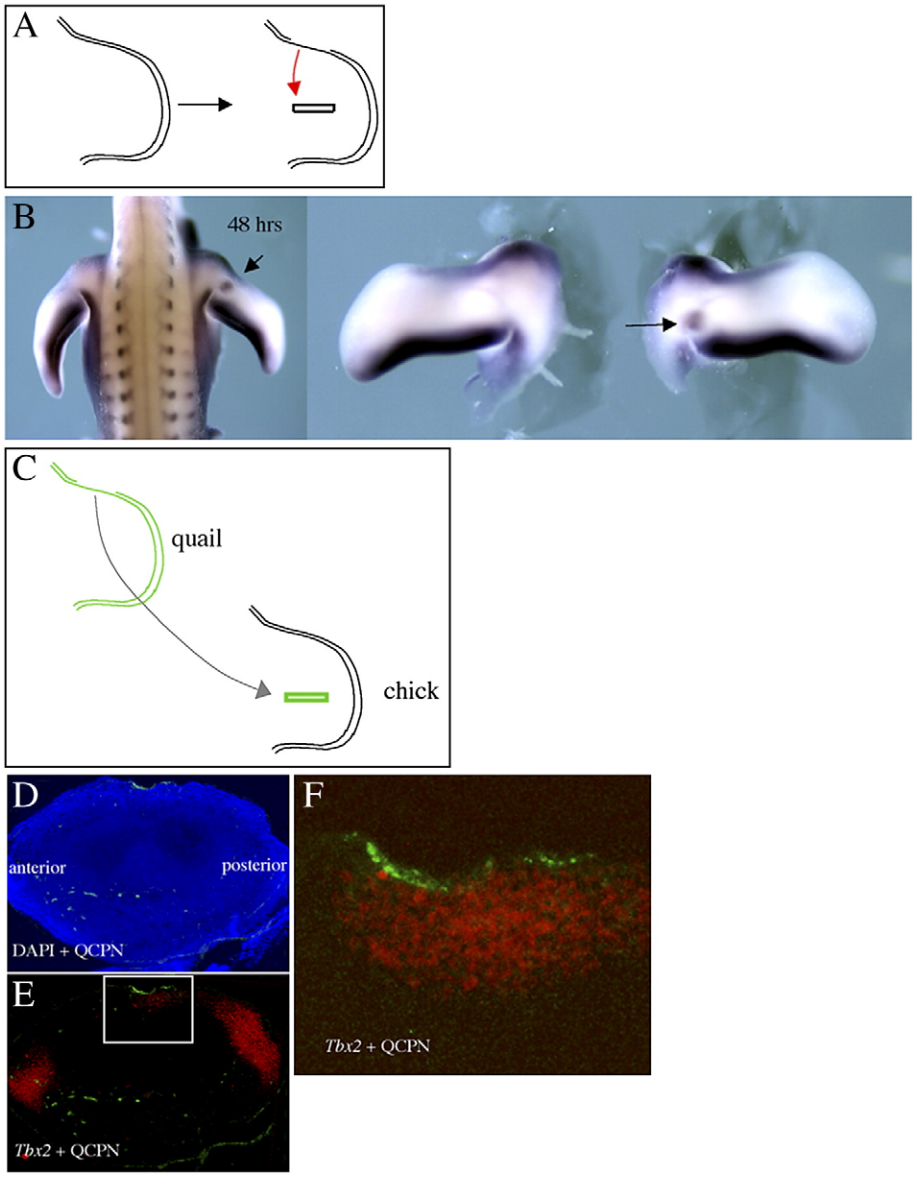

Fig. 4. The D–V ectoderm border grafted to the mid-limb is sufficient to induce ectopic *Tbx2* expression. (A) Schematic representation of the graft. (B) *Tbx2* expression 48 h after the graft. An ectopic domain of *Tbx2* can be seen in the mid-limb. (C) Schematic representation of quail anterior margin ectoderm grafted to a hole cut in the ectoderm of the chick mid-limb. (D, E) After 24 h, serial sections showing quail tissue (green)+DAPI nuclear stain (blue) (D) and quail tissue (green)+*Tbx2* expression (red) (E). (F) Higher magnification of inset in (E) showing quail ectoderm graft (green)+*Tbx2* expression (red) in underlying mesoderm.

rigorously demonstrate that a graft of D–V ectoderm border can non-autonomously induce *Tbx2* in underlying mesoderm, and that no marginal mesoderm was transplanted along with the ectodermal graft. We repeated the procedure grafting the anterior D–V ectoderm border from quail onto a chick mid-limb and visualized both grafted quail tissue and *Tbx2* expression (Figs. 4C–F). Indeed, no contaminating quail mesoderm is observed and the quail ectoderm graft can be seen overlying *Tbx2* induced in chick mesoderm. These data demonstrate that the D–V ectoderm border is sufficient to induce *Tbx2* in underlying mesoderm and establish the non-AER dorsal–ventral ectoderm border as a new signaling center in the limb.

In the flank, *Tbx2* is also expressed along the D–V ectoderm border before and after limb outgrowth (see Fig. 1). This expression is consistent with a role for the D–V ectoderm border in regulating *Tbx2* expression in the flank as well as in the limb. Because no morphologic landmarks visibly demarcate the D–V ectoderm border in the flank as in the limb bud, we did not test this possibility directly.

Regulation of Shh by D–V ectoderm border

Previous studies have demonstrated that the *Shh* expression domain is regulated by *Tbx2*. When *Tbx2* is virally mis-expressed in the limb, the domain of *Shh* expression is expanded anteriorly (Suzuki et al., 2004). This ectopic expansion of *Shh* did not extend across the entire limb bud, perhaps limited by other posteriorly restricted factors required for *Shh* expression such as *Hand2*. Our data shows that the D–

V ectoderm border regulates *Tbx2* at the limb margins, and a graft of the D–V ectoderm border is sufficient to induce ectopic *Tbx2*. Put together, these findings predict that a graft of D–V ectoderm border in the posterior limb bud should induce *Shh* expression in response to the ectopic *Tbx2* within the posterior domain where *Hand2* and other posteriorly acting factors are expressed.

To test this prediction, we grafted the D–V border ectoderm from the anterior margin of one limb to the dorsal surface of another limb at a position just anterior to the ZPA and monitored *Shh* expression (Fig. 5A). After 24 h, we found that an ectopic domain of *Shh* expression was indeed induced ($n=4/13$) (Figs. 5B, C). This result supports a model that a signal from the D–V ectoderm border regulates the expression of *Shh* at the posterior margin. Furthermore, the previous observation that misexpression of *Tbx2* expands expression of *Shh* (Suzuki et al., 2004) is consistent with the possibility that the effect of the D–V ectoderm border on *Shh* is mediated through expansion of the *Tbx2* domain, although a direct response of Shh to the non-AER dorsal–ventral ectodermal border signal is also possible.

The AER and non-AER D–V ectoderm border

We have identified the ectoderm at the dorsal–ventral border along the anterior and posterior margins of the limb bud as a new signaling center regulating Tbx gene expression. It is striking, however, that while Tbx2 is expressed along the entire anterior and posterior edges of the limb bud, it is completely absent from the distal mesenchyme, which also abuts a dorsal ventral ectodermal border, the AER. Indeed, direct comparison of mesodermal expression of *Tbx2* to expression of *Fgf8*, a marker of the AER shows that the domains are mutually exclusive in HH21 and HH23 limbs (Figs. 6A–D). The distal extent of *Tbx2* approaches the proximal extent of *Fgf8* in the AER, but is never found in mesenchyme under the AER. We also previously noted that mesodermal expression of *Tbx2* begins to be excluded from the distal tip as the limb bud emerges and the AER forms (see Fig. 1).

These observations are consistent with the dorsal–ventral border ectodermal signal, which we have shown is both able to induce *Tbx2* and is required for its maintenance, being turned off as the distinct AER signaling center is formed. It remains plausible, however, that mesodermal expression of *Tbx2* is also actively excluded from subapical mesoderm by a signal from the AER. To test this latter possibility, we grafted portions of the AER onto the dorsal–posterior surface of the limb at HH stage 22 and examined if this graft of AER changed the underlying *Tbx2* expression domain (Fig. 6E). As expected, the graft of AER induced ectopic outgrowth of the underlying mesoderm at the posterior edge of the limb (arrow, Figs. 6F, G), while a notch in the growth of the limb bud was evident anteriorly where the AER graft had been removed (Fig. 6G). Expression of *Tbx2* in the posterior margin within and around the ectopic outgrowth was downregulated ($n=5$). These data support the possibility that a signal from the AER actively inhibits expression of *Tbx2* in underlying mesenchyme. Members of the Fgf family are obvious candidates for such a signal emanating from the AER as several members of the Fgf family are specifically expressed in the AER and can substitute for the AER if it is surgically extirpated (Niswander et al., 1993; Fallon et al., 1994). To test this possibility, we implanted beads soaked in Fgf8, normally expressed throughout the AER, into the anterior or posterior margin mesoderm of HH stage 22 limbs and examined for changes in the expression of *Tbx2* after 24 h. We observed that *Tbx2* expression was downregulated immediately around the Fgf8 bead (Figs. 6H, I; $n=7/7$ anterior, $n=7/7$ posterior). This suggests that Fgf8 secreted from the AER excludes *Tbx2* expression from subapical mesoderm.

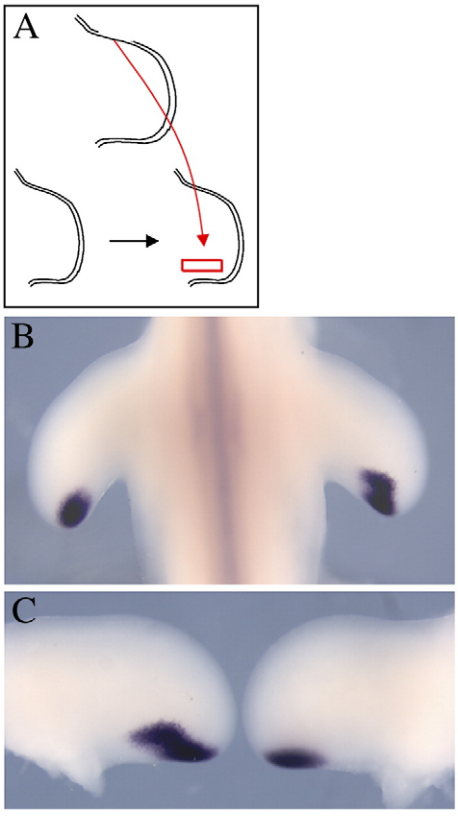

Fig. 5. Anterior margin D–V ectoderm border is sufficient to expand *Shh* expression domain anteriorly. (A) Schematic representation of graft in which anterior margin ectoderm is grafted slightly anterior to the ZPA. (B, C) Dorsal view of contralateral limbs showing anterior expansion of *Shh* expression in right operated limb.

Discussion

D–V ectodermal border as signaling center

Classical studies and more recent molecular analyses have identified the AER as a signaling center necessary for proper outgrowth of the limb. The AER coincides with the dorsal–ventral ectodermal border at the tip of the limb. The data presented in this study indicate that the dorsal–ventral border ectoderm proximal to the AER also serves as a signaling center, regulating expression of genes restricted to the anterior and posterior margins of the limb.

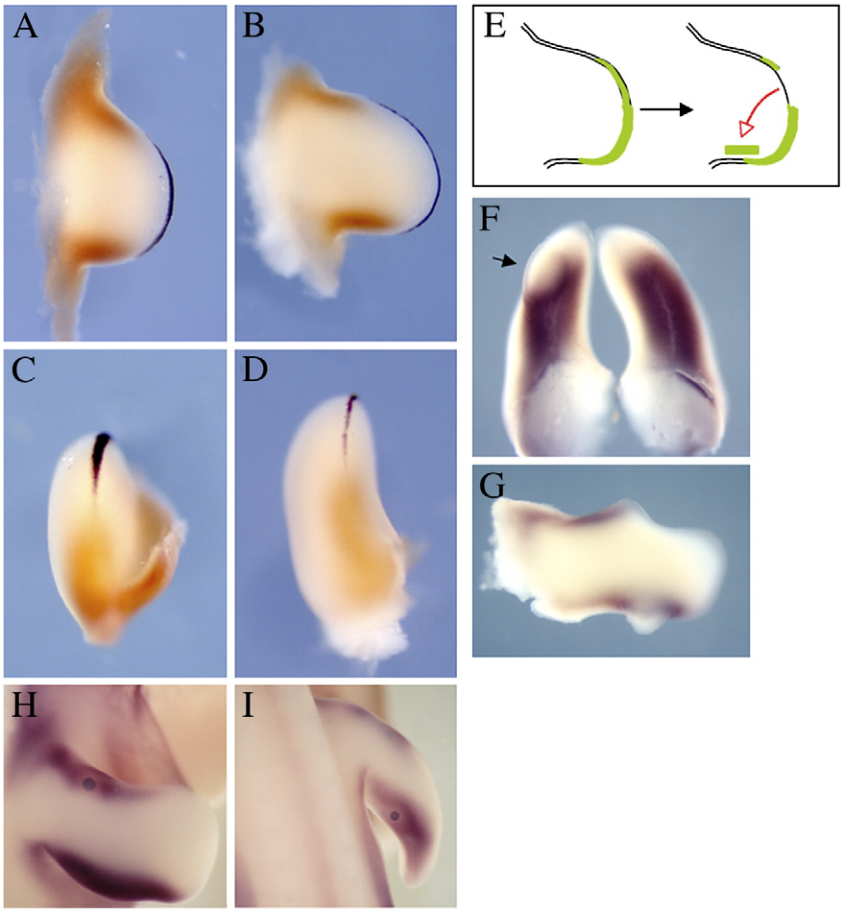

Fig. 6. *Tbx2* mesodermal expression domain in relation to AER. (A–D) *Tbx2* expression (brown) and *Fgf8* expression (black) at HH stage 21 (A, C) and stage 23 (B, D). Dorsal views (A, B) and posterior views (C, D) show that *Tbx2* expression abuts but does not extend under AER. (E–G) A graft of AER to the dorsal posterior surface causes ectopic proliferation and downregulation of *Tbx2* in underlying mesenchyme. A notch in the growth of the limb is apparent in the anterior limb where the AER was removed (G). A bead soaked in Fgf8 downregulates expression of *Tbx2* around the bead when implanted in the anterior (H) or posterior (I) margin of the limb.

Several lines of evidence support this model. The spatial expression pattern of *Tbx2* very closely relates to the non-AER D–V border ectoderm over a wide range of limb stages. When we moved mesoderm expressing *Tbx2* away from the D–V border ectoderm *Tbx2* expression was lost after 24 h, suggesting that proximity to the D–V border ectoderm is necessary to maintain mesodermal expression of *Tbx2*. Furthermore, grafts of the D–V border ectoderm from both the anterior and posterior margins were capable of inducing ectopic *Tbx2* in underlying mesoderm. This non-autonomous effect suggests that a signal from the non-AER D–V border ectoderm is sufficient to upregulate *Tbx2* in underlying mesoderm. The closely related transcription factor *Tbx3* is also expressed along the anterior and posterior limb margins, though in slightly larger domains. This suggests that *Tbx3* may also serve as a readout of signaling from the D–V border, but may require a lower level of this signal than *Tbx2*.

While the AER signaling center is characterized by distinct stratified (in the mouse) or pseudostratified (in the chick) epithelium, the non-AER border ectoderm is morphologically indistinguishable from adjacent dorsal and ventral ectoderm. This may partially explain why this signaling center escaped notice until now.

Regulation of Tbx2 gene expression

Our data suggest that *Tbx2* is regulated by the adjacent dorsal–ventral ectoderm border. A previous study has found that a bead soaked in Shh and placed in the mid-limb can expand the posterior domain of *Tbx3*, whereas a Shh bead placed in the anterior can cause downregulation of *Tbx3* (Tumpel et al., 2002). These observations were interpreted as evidence that the anterior and posterior domains of *Tbx3* are established by signals patterned along the anterior–posterior axis (Tumpel et al., 2002). This interpretation seems incomplete, given that *Tbx2* and *Tbx3* are maintained at the anterior and posterior margins all the way to the flank at considerable distances from the ZPA, even as late as HH stage 27 when *Shh* expression is quite distal. These anterior and posterior limb expression domains are continuous with flank expression, where regulation by Shh is very unlikely. Moreover, we have shown that *Tbx2* expression is not lost from the margins 8 h after blocking Shh activity nor in the context of limbs deficient in mesodermal expression of BMP2 and BMP7, suggesting that normal *Tbx2* expression is not directly regulated by signals which pattern the anterior–posterior axis. Conversely, there is considerable evidence that *Tbx2/3* act upstream of *Shh* in the

posterior margin of the limb. A mouse carrying a targeted deletion of *Tbx3* exhibits loss or absence of *Shh* (Davenport et al., 2003), and viral misexpression studies demonstrate that *Tbx2* is both necessary for normal *Shh* expression and sufficient to expand *Shh* expression in the posterior chick limb (Suzuki et al., 2004). Whereas loss-of-function of *Tbx2* or *Tbx3* in chick limbs and loss-of-function of *Tbx3* in humans leads to loss of posterior elements, as would be expected by downregulation of *Shh*, we note that *Tbx2* null mutant mice do not exhibit loss of posterior elements (Harrelson et al., 2004). Instead, these *Tbx2* mutant mice have a hindlimb-specific duplication of the most distal phalanx of digit IV at E14.5, and this likely reflects the interphalangeal expression of *Tbx2* where it may be involved in apoptosis. The lack of any effect on posterior patterning in *Tbx2* mutant mice may reflect redundancy with *Tbx3*.

The previous finding that a bead soaked in Shh and placed in the mid-limb can expand the posterior domain of *Tbx3* may be explained by a positive feedback interaction between *Shh* and *Tbx2/3*. It is possible that while *Tbx2/3* act upstream of *Shh*, *Shh* in turn can positively regulate *Tbx2/3*. Indeed, a pellet of cells expressing *Shh*, when implanted in the anterior limb from which the anterior AER has been removed, has been shown to upregulate *Tbx2* around the pellet (Gibson-Brown et al., 1998). A similar positive feedback loop has been demonstrated between *Shh* and *Hand2*, another transcription factor that is believed to regulate *Shh* expression in the posterior. The previous observation that ectopic Shh can downregulate Tbx3 in the anterior requires a different explanation. It has previously been demonstrated that ectopic placement of Shh in the anterior margin causes the AER to extend anteriorly (Laufer et al., 1994). In the current study, we have shown that *Tbx2* appears to be excluded from subapical mesoderm over a wide range of limb development (HH stage 19 to 27), and this pattern appears to be true for *Tbx3* as well (Tumpel et al., 2002), suggesting that the AER may negatively regulate *Tbx2/3* in subapical mesoderm and/or that the D–V border ectoderm signals required to maintain *Tbx2/3* are excluded from the AER (discussed below). We therefore speculate that the down-regulation of *Tbx3* observed when a Shh bead is placed in the anterior margin may reflect expansion of the AER over the Shh bead and consequent downregulation of *Tbx3*.

The signal from the D–V ectoderm border that regulates mesodermal *Tbx2* expression is currently unknown. While not a homologous structure, an interesting parallel exists in the regulation of the orthologous *Drosophila optomotor blind* (*omb*) gene in the wing disc. In the early wing disc, *omb* is expressed throughout the entire presumptive wing domain (del Alamo Rodriguez et al., 2004; Grimm and Pflugfelder, 1996). However, as the wing disc develops, *omb* disappears from the lateral-most regions of the disc and is most highly expressed in the central region of the disc (del Alamo Rodriguez et al., 2004). Strikingly, in this central region, *omb* becomes restricted to 3 domains: a stripe at the wing margin demarcating, as in the vertebrate limb ectoderm, the dorsal–ventral border of the wing disc and along 2 rings that surround the presumptive wing hinge and delimit the wing region (del Alamo Rodriguez et al., 2004). These 3 domains are marked by expression of *Wingless*, the *Drosophila* ortholog of the *Wnt* family. While *Wnt3a* is specifically expressed in the AER, no *Wnt* genes have yet been identified specifically in the non-AER D–V border ectoderm (Kengaku et al., 1998). How the signal from the D–V ectoderm border acts to regulate *Tbx2* expression also remains to be determined.

AER vs. non-AER D–V border ectoderm

From the time the limb bud emerges until the time digit rays begin to form, we observed that *Tbx2* is excluded from mesoderm under the AER. Interestingly, the appearance of *Tbx2* and *Tbx3* in the interdigital mesoderm at HH stage 27 correlates with degeneration of the AER overlying the interdigital mesoderm (Ganan et al., 1998). Furthermore, by using *Tbx2* expression as a readout of signaling from the D–V border ectoderm and *Fgf8* as a marker of the AER, we found that the domain of *Tbx2* approaches the AER but does not extend underneath it. This data suggests a compartmentalization of the D–V border ectoderm in which the AER specifically excludes *Tbx2* in underlying mesoderm while the non-AER D–V ectoderm border specifically upregulates *Tbx2* in underlying mesoderm. This model was supported by the finding that grafting an AER next to the non-AER border ectoderm resulted in a downregulation of underlying *Tbx2*. Indeed, we show that Fgf8, normally secreted from the AER, is capable of down-regulating mesodermal *Tbx2*. It is also known that Fgfs from the AER are required to maintain *Shh* expression in the ZPA. Put together, these data suggest that high levels of Fgf8 immediately under the AER (and immediately around an Fgf8 bead) repress *Tbx2* expression, while lower levels of Fgfs are sufficient to maintain *Shh* and do not act to downregulate the distal-most portion of the *Tbx2* domain along the posterior limb margin.

Fate mapping studies of the AER reveal that cells in the posterior 2/3 of the AER remain within the AER after 2 days but cells in the anterior 1/3 AER move out to the anterior margin ectoderm just proximal to the AER (Vargesson et al., 1997). Thus, it will be interesting to elucidate how distinct signaling from the AER vs. non-AER D–V border ectoderm is maintained as the distal border ectoderm first converts to an AER during limb bud initiation and then converts back to non-AER D–V border in the anterior.

The AER D–V ectodermal border manifests as a conspicuous ridge at the distal tip of the limb. The AER D–V ectodermal border is also characterized by gene expression boundaries and cell lineage boundaries which have functional significance in proper AER initiation and maintenance. For example, cells expressing *Engrailed1* (*En1*) contribute to the ventral ectoderm and ventral half of the AER (Kimmel et al., 2000), confirming a lineage compartment boundary in the mid-AER (Altabef et al., 1997). When this lineage boundary is disrupted by misexpressing *En1* in the dorsal AER half, the AER fails to form properly (Kimmel et al., 2000). It will therefore be interesting to characterize whether the non-AER D–V ectodermal border also has gene expression and cell lineage boundaries that are critical to its function.

Regulation of Shh at the limb margin

Placed in the context of prior work on various factors regulating *Shh* expression in the limb, our results allow us to formulate a model explaining the spatial control of *Shh* expression, and hence the localization of polarizing activity during limb development (Fig. 7). Prior to limb outgrowth or the expression of *Shh*, opposing gradients of negatively acting factors such as *Gli3* and positively acting factors such as *Hand2* (te Welscher et al., 2002) result in a posterior quadrant of the limb bud competent to express *Shh*. Of course, *Shh* regulation is complex and also requires the activity of *Hox* genes (Kmita et al., 2005). Signals from the non-AER D–V border ectoderm induce expression of *Tbx2*, required for *Shh* expression, along both the anterior and posterior margins of the limb mesoderm. However, *Shh* is only induced within the posterior margin due to the competence of that mesoderm conferred by *Hand2* expression. Indeed, the timing of ectopic *Shh* expression in response to a graft of the D–V border ectoderm (Fig. 5) is consistent with a requirement for *Tbx2* expression 24 h after placement of the graft, and the location of ectopic *Shh* expression is presumably limited to the posterior by the domain of *Hand2* expression. In addition, *Shh* expression requires Fgf signaling, provided from the distal tip as the distal dorsal–ventral ectoderm border is converted to the AER turning on *Fgf8* and other *Fgf* family members at the expense of the non-AER border signal. As the limb bud grows out, the AER, and hence the source of Fgf signaling, remains at the distal tip, thus assuring that only the most distal *Tbx2*-expressing domain continues to express *Shh*.

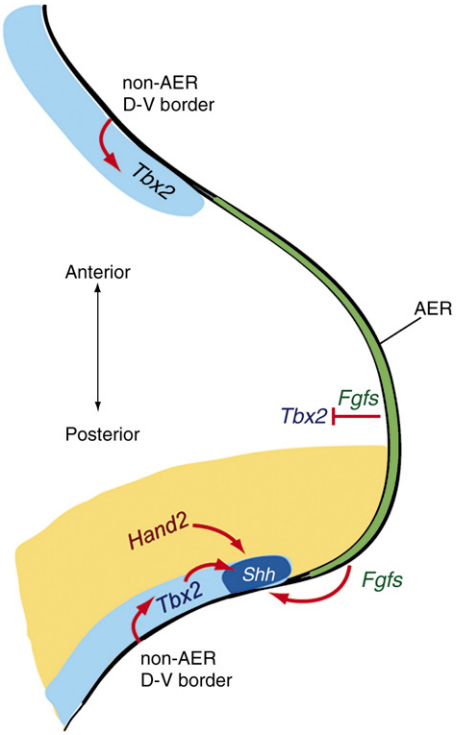

Fig. 7. Model for non-AER D–V ectoderm border function as a signaling center that localizes the ZPA to the limb margin. Expression of *Tbx2* serves as a readout for signaling from non-AER D–V border at the anterior and posterior limb margins. Signaling from non-AER D–V border restricts *Shh* expression to posterior margin, possibly via requirement of *Tbx2* for proper *Shh* expression. Other regulatory factors collaborate with the non-AER D–V border signal in positioning the ZPA. For example, *Hand2* restricts *Shh* to the posterior and not anterior margin, and *Fgfs* from the AER restrict *Shh* to the distal end of the posterior margin.

In support of this model, we find that the non-AER dorsal–ventral border ectoderm is both necessary and sufficient for the induction of *Tbx2* expression and is also capable of inducing *Shh* expression in the posterior limb mesenchyme. Moreover, previous studies have shown that viral misexpression of *Tbx2* expands *Shh* expression anteriorly (Suzuki et al., 2004). We noted, however, that the normal *Tbx2* expression domain extends slightly more anteriorly than the *Shh* domain. This could reflect a threshold requirement for levels of *Tbx2* gene expression not achieved at the edge of their expression domain. Alternatively, it could reflect an independent, direct requirement for the non-AER dorsal–ventral border ectoderm signal. In that case, *Shh* expression would be predicted to require a higher threshold level of that signal than *Tbx2*.

In addition to explaining the localization of *Shh* expression in the posterior-most distal mesenchyme, this model also provides an explanation for the limited ectopic expression of *Shh* at the extreme anterior limb margin seen in many mutants leading to preaxial polydactyly. When negative-acting factors are lost, the transcription factors providing competence for *Shh* expression, such as *Hand2*, can expand their expression across the entire limb bud mesenchyme (te Welscher et al., 2002). In this situation, the cells subjacent to the anterior non-AER dorsal–ventral ectoderm border are able to respond to the signal within the domain marked by anterior *Tbx2* expression, and turn on an ectopic domain of *Shh* expression. In addition to the AER and the non-AER dorsal–ventral border ectoderm, there is a third ectodermal signaling center in the limb bud; the entire dorsal ectodermal surface. The dorsal ectoderm plays an essential role in establishing the dorsal–ventral polarity of the limb bud (MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987, 1989). This activity is mediated by Wnt7a (Parr and McMahon, 1995); and interestingly Wnt7a also plays a role in supporting high levels of Shh expression in the ZPA (Parr and McMahon, 1995; Yang and Niswander, 1995). As a result, there is a dorsal bias in the expression domain of Shh within the ZPA, although the importance of this for limb patterning remains unclear.

In contrast, the restriction of *Shh* mRNA to the posterior margin is critical in establishing a proper spatial and temporal gradient of Shh activity. The cells initially expressing *Shh* proliferate and expand disproportionately, such that they end up encompassing most of the posterior half of the distal limb bud. As they do so, those cells that are forced further away from the posterior margin shut off *Shh* such that the domain of cells that continue to express *Shh* remains relatively quite small, although all these cells presumably are competent to express *Shh* (Harfe et al., 2004). Our current results explain this, as only the cells remaining along the extreme posterior margin during this expansion will still be within range of the non-AER dorsal–

ventral border signal and hence only in those cells will *Tbx2*, *Tbx3* and *Shh* expression be maintained.

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