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csal1 Is Controlled by a Combination of FGF and Wnt Signals in Developing Limb Buds

Elizabeth R. Farrell and Andrea E. Münsterberg[1]

Department of Anatomy and Physiology, Division of Cell and Developmental Biology, University of Dundee, Wellcome Trust Biocenter, Dow Street, Dundee DD1 5EH, United Kingdom

While some of the signaling molecules that govern establishment of the limb axis have been characterized, little is known about the downstream effector genes that interpret these signals. In *Drosophila,* the *spalt* gene is involved in cell fate determination and pattern formation in different tissues. We have cloned a chick homologue of *Drosophila spalt,* which we have termed *csal1,* and this study focuses on the regulation of *csal1* expression in the limb bud. *csal1* is expressed in limb buds from HH 17 to 26, in both the apical ectodermal ridge and the distal mesenchyme. Signals from the apical ridge are essential for *csal1* expression, while the dorsal ectoderm is required for *csal1* expression at a distance from the ridge. Our data indicate that both FGF and Wnt signals are required for the regulation of *csal1* expression in the limb. Mutations in the human homologue of *csal1,* termed *Hsal1/SALL1,* result in a condition known as Townes–Brocks syndrome (TBS), which is characterized by preaxial polydactyly. The developmental expression of *csal1* together with the digit phenotype in TBS patients suggests that *csal1* may play a role in some aspects of distal patterning.

Key Words: spalt; sal; **Townes–Brocks syndrome; limb; FGF; Wnt; Shh; BMP.**

INTRODUCTION

The vertebrate limb bud is an excellent model system for investigating the mechanism underlying establishment of positional information (Tickle, 1995; Johnson and Tabin, 1997; Schwabe *et al.,* 1998). The combined use of surgical ablation, transplantation, grafting, and molecular approaches has provided insight into the processes that define the anteroposterior, dorsoventral, and proximodistal axes of the developing limb.

Anteroposterior patterning is governed by a region of distal posterior limb mesenchyme, termed the zone of polarizing activity. The identity of digits formed in developing limb buds is determined by the degree of proximity to the polarizing region (Tickle *et al.,* 1975), and transplantation of this tissue into the anterior margin of a developing limb bud induces an ectopic set of digits with reverse anteroposterior polarity. Polarizing activity appears to be mediated by Sonic hedgehog (Shh; Echelard *et al.,* 1993; Krauss *et al.,* 1993; Riddle *et al.,* 1993) and ectopic application of Shh protein can induce mirror-image duplications (Chang *et al.,* 1994; Lopezmartinez *et al.,* 1995). Some effects of Shh may be indirect and mediated through other signaling molecules such as BMPs (Yang *et al.,* 1997; Drossopoulou *et al.,* 2000).

Dorsoventral limb patterning is controlled by *Wnt7a* expressed in dorsal ectoderm (Dealy *et al.,* 1993). *Wnt7a* confers dorsal identity to the limb through induction of *Lmx-1* in the mesenchyme. Ectopic expression of *Wnt7a* or *Lmx-1* dorsalizes ventral mesenchyme (Riddle *et al.,* 1995; Vogel *et al.,* 1995), while targeted disruption of *Wnt7a* leads to ventralization of the dorsal limb (Parr and McMahon, 1995).

Proximodistal patterning in the limb bud is closely linked to cell proliferation and outgrowth. Limb outgrowth is controlled by the apical ectodermal ridge (AER), a specialized structure of thickened epithelium localized at the dorsoventral boundary of the limb. The activity of the apical ridge is mediated by fibroblast growth factors (FGFs; Niswander *et al.,* 1993). In addition, *Wnt3a* is expressed in the ridge and has been implicated in regulating AER-specific genes, including *FGF4* and *FGF8* (Kengaku, 1998). Recent evidence suggests that regulatory interactions be-

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[1] To whom correspondence should be addressed. E-mail: a.e.munsterberg@dundee.ac.uk.

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tween BMPs and *Hoxd* genes (which are expressed distally) and the homeobox gene *Meis2* (expressed in proximal regions of the limb bud) are involved in determining proximal–distal patterning in the developing limb bud (Capdevila *et al.*, 1999). As the limb bud grows, different regions along the proximodistal axis arise sequentially (Summerbell *et al.*, 1973). Sequential specification of cell fate correlates well with *Hox* gene expression patterns in developing limb buds (Dollé *et al.*, 1989; Izpisúa-Belmonte *et al.*, 1991). Misexpression and gene ablation studies confirm that *Hox* genes control development of region-specific structures within the limb (Johnson and Tabin, 1997, for review and references therein).

This work focuses on expression and regulation of another gene that may also be involved in proximal–distal limb patterning, a chick homologue of *spalt*. *spalt* (*sal*) was first isolated in *Drosophila* and encodes a protein characterized by multiple double zinc-finger motifs of the C_2H_2 type, commonly found in transcription factors (Kühnlein *et al.*, 1994). *sal* was originally identified as a region-specific homeotic gene required for the specification of head and tail regions in *Drosophila* embryos (Jürgens, 1988). However, *sal* expression is found in various tissues in *Drosophila* embryos and larvae (Kühnlein *et al.*, 1994). At later stages of development *sal* does not determine segmental identity, but instead regulates pattern formation and cell fate (Chen *et al.*, 1998; deCelis *et al.*, 1996; Kühnlein and Schuh, 1996; Sturtevant *et al.*, 1997). For example, positioning of the L2 vein in the wing disc is determined by the anterior border of *sal* expression (Sturtevant *et al.*, 1997). Another *sal* expression domain in the thoracic region of the wing disc is required for development of two sensory organs and the positioning of two others, and *sal* downregulation is required for sensory organ differentiation (de Celis *et al.*, 1999). Furthermore, *sal* regulates pattern formation at three stages in tracheal development (Chen *et al.*, 1998; Kühnlein and Schuh, 1996).

Vertebrate *sal* homologues have been identified in mouse, *Xenopus*, and *Medaka* (Hollemann *et al.*, 1996; Koster *et al.*, 1997; Ott *et al.*, 1996), in which they are expressed in various tissues including brain, heart, pronephros, and limb/fin buds. In addition, two human homologues of *sal* have been isolated (Kohlhase *et al.*, 1998). Mutations in one of these genes, termed *Hsal1* or *SALL1*, cause Townes–Brocks syndrome (TBS), an autosomal dominant disorder characterized by anorectal, ear, and hand malformations, in particular preaxial polydactyly (Kohlhase *et al.*, 1998, 1999). These observations suggest that vertebrate *sal* homologues may be involved in limb patterning and digit formation.

In order to study the possible role of *sal* genes in limb development we utilized the chick embryo, which is particularly accessible to surgical manipulation and implantation of beads and/or cell pellets. In a previous study, Capdevila *et al.* (1999) reported the partial sequence and expression pattern of a chick *sal* homologue and began to address its regulation in the limb. Our study confirms and expands their observations. We report the full-length sequence of a chick homologue of *Hsal1/SALL1*, termed *csal1*, and give a more detailed characterization of its expression pattern in developing limb buds. In addition, we show that *csal1* expression depends on signals from the apical ectodermal ridge and is also regulated by signals from dorsal ectoderm. Finally, we present evidence that FGF and Wnt signals act together to activate and maintain normal expression of *csal1* in chick limb buds.

MATERIALS AND METHODS

Chick Embryos

Fertilized chick eggs were obtained from Muirfield Hatchery, Kinross, Scotland, and Needle Farms, Sussex, England, and incubated at 38.5°C in humidified incubators (Curfew, UK; Ehret, FRG). Embryos were staged according to Hamburger and Hamilton (1951).

Isolation of csal1

The region corresponding to the first double zinc-finger motif was amplified by degenerate RT-PCR from 3.5-day chick cDNA. RNA and cDNA were prepared as previously described (Münsterberg *et al.*, 1995), except that 5× transcription buffer and MoMuLV-reverse transcriptase from Promega were used. Primer sequences corresponded to the following amino acid residues: FCAKVFG (5′-TTYTGYGCIAARGTITTYGG-3′) and EKPVTTW (antisense, 5′-CCAIGTIGTIACIGGYTTYTC-3′). PCR products were cloned into pGEM-T Easy (Promega), sequenced, and used as a probe to screen a HH 12–15 chick cDNA library (a gift from Drs. Angela Nieto and David Wilkinson, NIMR). Nineteen positive clones were obtained. These fell into three classes by restriction analysis and contained overlapping regions of the same open reading frame but different 3′ UTR sequences. One clone was found to contain the complete open reading frame and was chosen for further analysis. All sequencing was done on an ABI 377 sequencer (Perkin–Elmer), and sequences were analyzed using DNA Star Software and the basic BLAST search facility at NCBI, http://www.ncbi.nlm.nih.gov/BLAST/.

Probe and in Situ Hybridization

The *csal1* probe was designed to prevent cross-hybridization with other *sal* homologues in chick. A DIG-labeled antisense RNA probe was synthesized from a 377-bp *Hin*dIII/*Pst*I fragment that lies between the first and the second double zinc-finger motifs and corresponds to amino acids 552–677. This probe does not contain any of the highly conserved zinc-finger motifs and specifically hybridizes with *csal1* transcripts. The *Hin*dIII/*Pst*I fragment was subcloned into pBluescript KS (Stratagene). This construct was linearized with *Hin*dIII and transcribed with RNA polymerase T7 (Promega). DIG–UTP (Roche) was included in the reaction mix. Whole-mount *in situ* hybridization was performed according to the protocol described by Henrique *et al.* (1995). For sections embryos were heavily stained, embedded in gelatin, and sectioned at 20 μm on a Leica CM 1900 Cryostat.

Microscopy and Photography

Whole-mount embryos were viewed using a Zeiss Stemi SV11 dissection microscope and photographed with a Yashica 108 multiprogram camera. Alternatively, they were viewed with a Leica MZ8 dissection microscope and photographed with an RS photometrics CoolSnap digital camera in conjunction with Openlab imaging software on a Macintosh computer. Sections were viewed with a Zeiss Axioplan 2 microscope and photographed with a Kodak DCS 420 digital camera.

Grafts and Ablations

Heparin acrylic beads (Sigma H5263) were soaked in FGF4 (700 μg/ml; R&D Systems), rhBMP-2 (1 μg/ml, 10 μg/ml, 100 μg/ml, or 1 mg/ml; Genetics Institute, Cambridge, MA), or rhBMP-4 (10 μg/ml; Genetics Institute) after the protocol described by Niswander *et al.* (1993). Beads were a gift from Dr. Cheryll Tickle, Department of Anatomy and Physiology, University of Dundee. Affi-Gel blue beads (Bio-Rad) were soaked in Shh (14 mg/ml; obtained from Ontogeny, USA) as described in Yang *et al.* (1997). The Wnt3a- and Wnt7a-producing rat B1a fibroblast cell lines were as described in Münsterberg *et al.* (1995). Preparation of rat B1a fibroblast pellets by centrifugation was essentially as described by Riddle *et al.* (1993). For apical ridge removal, the ectoderm of wing buds was stained with Nile blue sulfate (100 μg/ml) and the apical ridge was removed with a tungsten needle (Takahashi *et al.*, 1998). The same procedure was used to remove dorsal ectoderm. Beads/pellets were implanted in the distal part of stage 19/20 chick wing buds. When necessary, beads were held in place with platinum staples (Niswander *et al.*, 1993).

RESULTS

Cloning of a Chick spalt Homologue

Degenerate PCR primers, based on *Drosophila*, *Xenopus*, and mouse *sal* sequences (Hollemann *et al.*, 1996; Kühnlein *et al.*, 1994; Ott *et al.*, 1996), were used to amplify a 250-bp fragment from chick cDNA. Sequence analysis revealed that this fragment was highly homologous to *Drosophila* and other previously isolated vertebrate *sal* genes. The fragment was used as a probe to screen a chick HH 12–15 cDNA library; positive clones were isolated and characterized. We found one clone that contained the full open reading frame (3924 bp) of a chick *sal* homologue. This open reading frame also included a previously described 970-bp PCR fragment of a chick *sal* gene (Capdevila *et al.*, 1999). Since there is evidence for additional *sal* genes in chick (data not shown), we called this gene *csal1*. Sequence analysis revealed high homology to other known *sal* genes, especially within the conserved zinc-finger motifs. The predicted Csal1 protein was most closely (85.7%) related to the human Hsal1/SALL1 protein (Fig. 1).

The characteristic double zinc-finger motifs are highly conserved between Csal1 and Hsal1/SALL1 (Fig. 1, boxed regions). The so-called "SAL" box, an amino acid motif found in all spalt proteins within the zinc-finger motifs, is present in Csal1. Csal1 also contains a single amino-terminal zinc finger, which has been described in mouse, *Xenopus*, and human Sal proteins, but is not found in *Drosophila* or *Medaka* Sal (Fig. 1; Hollemann, 1996).

Expression of csal1 in Developing Limb Buds

Expression analysis of embryos from Hamburger and Hamilton (1951) stages 15 to 30 revealed a striking pattern in the developing wing and leg buds (Fig. 2). *csal1* was first detected in emerging limb buds at late HH stage 17 (Fig. 2A). At HH 20 transcript distribution appears slightly asymmetrical, with a broader *csal1* domain in posterior mesenchyme compared to anterior mesenchyme of both wing and leg bud. In contrast, *csal1* expression is even throughout the apical ectodermal ridge (Fig. 2B). As limb bud development proceeds, *csal1* is expressed symmetrically throughout the distal third of the wing and leg buds (HH 23, Fig. 2C). Cryosections of stained specimens at this stage showed that *csal1* is expressed in both the apical ridge and the limb mesenchyme (HH 23, Figs. 2E and 2F). We detected a strong hybridization signal in dorsal mesenchyme beneath the ectoderm compared with a weaker signal located ventrally (Figs. 2E and 2F). At HH 24/25, *csal1* begins to be downregulated in leg bud mesenchyme and subsequently in the mesenchyme of wing buds (Fig. 2D). Interestingly, *csal1* is downregulated in a distal-to-proximal direction in mesenchyme, while it continues to be expressed in the apical ridge until HH 26. We did not detect any *csal1* expression at later stages in limb development. A partial description of this expression pattern has previously been reported (Capdevila *et al.*, 1999).

csal1 is also expressed in the central nervous system as has been reported for *sal* genes in mouse, *Xenopus*, and *Medaka*.

Shh Can Upregulate csal1 Expression in Limb Buds in Concert with Signals from the Apical Ectodermal Ridge

In order to begin to understand the role of *csal1* during limb morphogenesis we investigated how *csal1* expression is regulated. In *Drosophila* wing imaginal discs *sal* is upregulated by *hedgehog* via *dpp* signaling (deCelis *et al.*, 1996; Lecuit *et al.*, 1996; Nellen *et al.*, 1996; reviewed in Smith, 1996). In *Medaka*, ectopic Sonic hedgehog activity expands the *sal* expression domain in the developing CNS and paraxial mesoderm (Koster *et al.*, 1997). These findings prompted us to test whether ectopic *csal1* expression could be induced by Shh. Beads soaked in Shh protein were implanted into limb buds at HH 19/20. Embryos were harvested 24 h later at HH 23/24. We found that a Shh bead leads to expanded *csal1* expression (Figs. 3A and 3B; $n = 13$). Ectopic *csal1* expression was seen, however, only in the region between the bead and the AER (Figs. 3A and 3B; $n = 13$), suggesting that Shh induction of *csal1* requires the apical ridge.

To test the role of the apical ridge in *csal1* expression we initially examined *csal1* expression 24 h after ridge removal

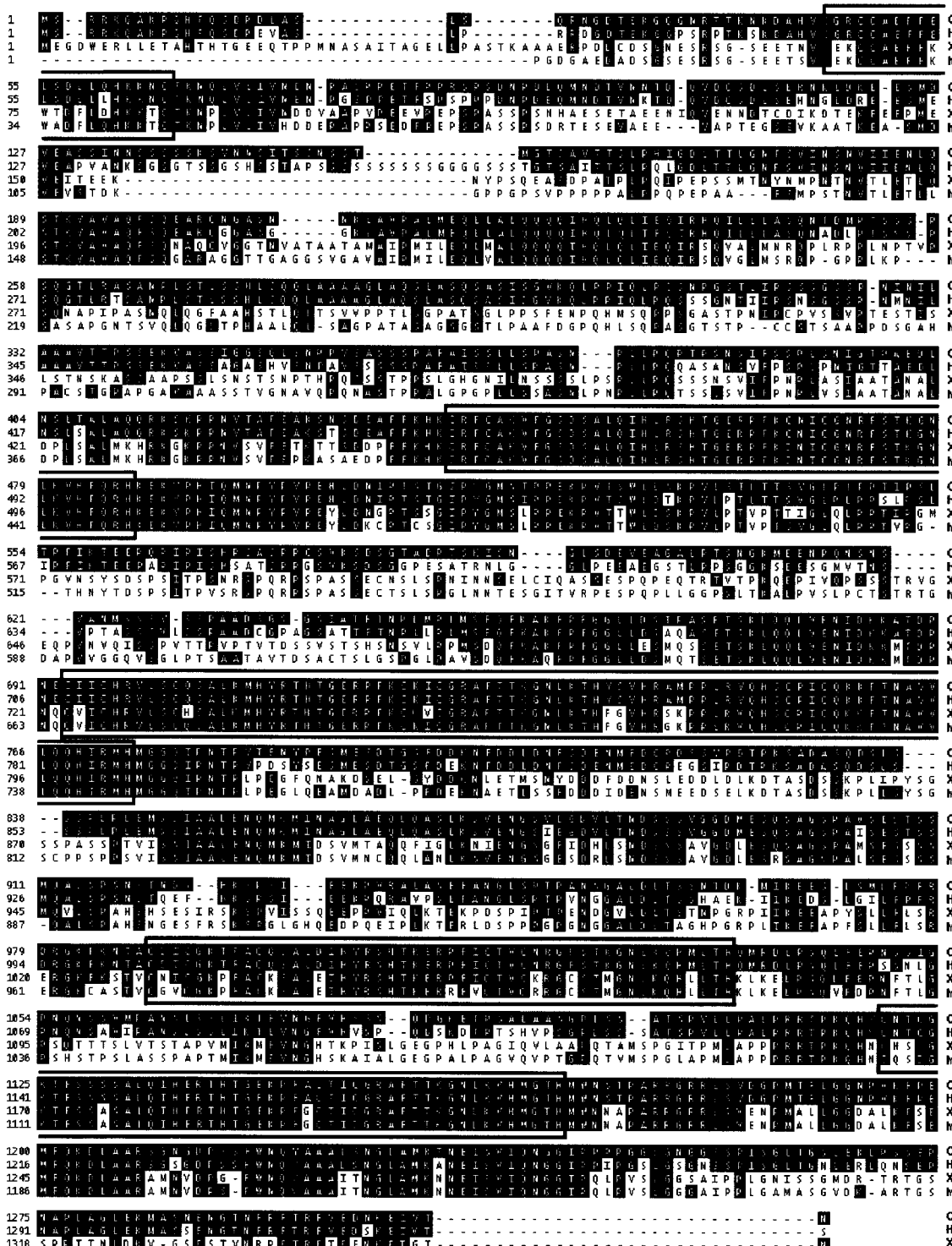
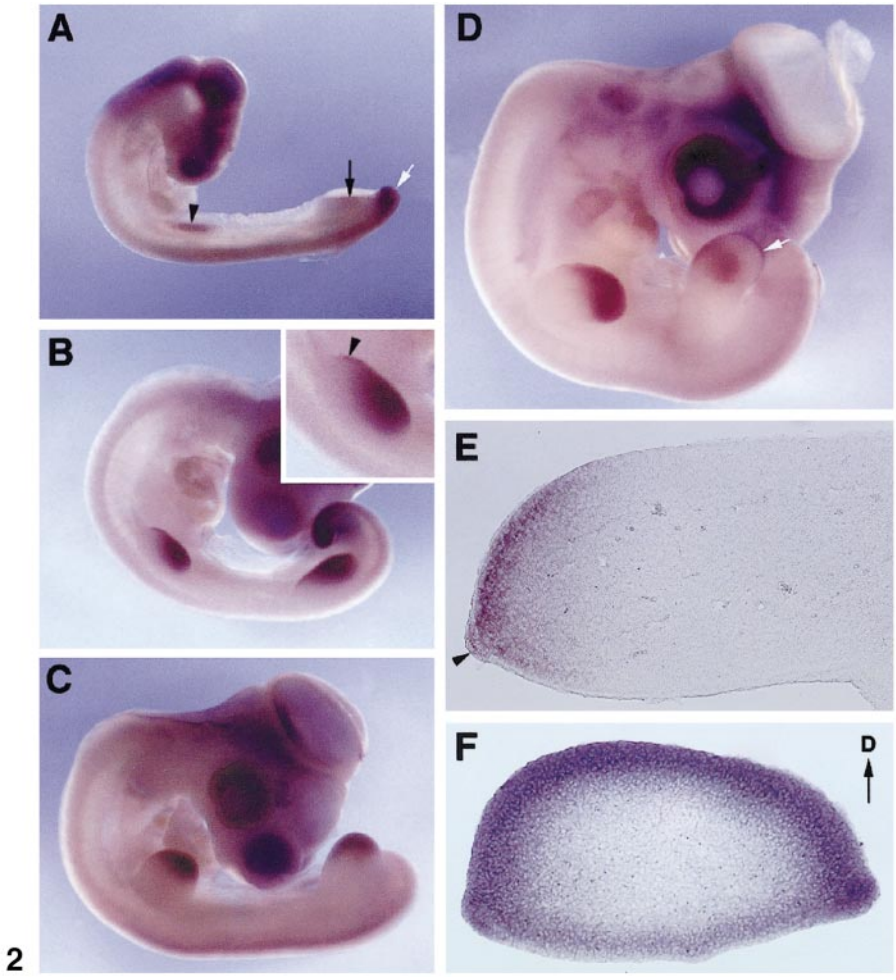


FIG. 1. (A) Comparison of Csal1 and Hsal1/SALL1, Msal, and Xsal1 proteins. The single amino-terminal zinc finger, which is not present in *Drosophila* Sal, is boxed. The next four double and triple zinc-finger motifs are also boxed. Phylogenetic analysis showed that Msal and Xsal1 are more closely related to each other than to Csal1 and Hsal1/SALL1, which are 85.7% identical. We found that Msal has 48.2% and Xsal1 has 47.7% homology to Csal1.



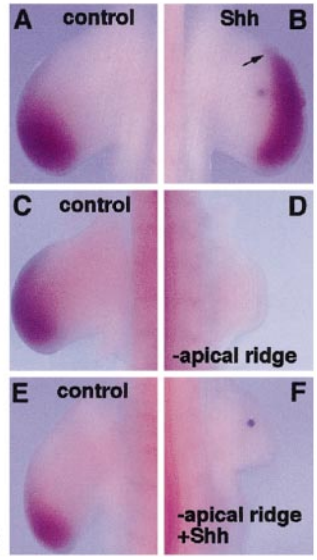


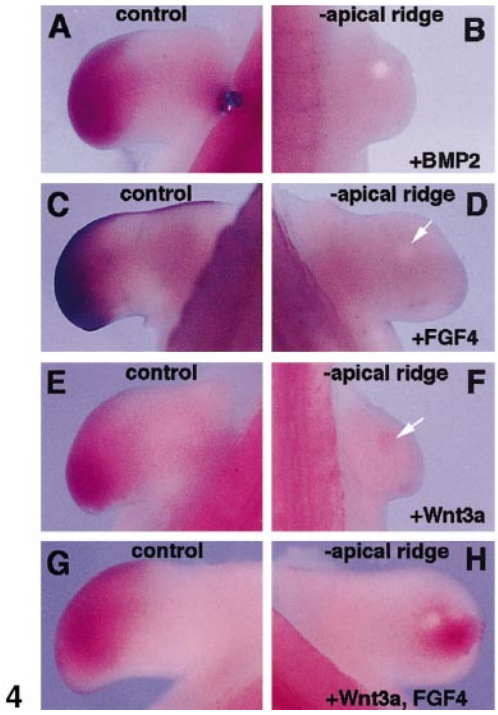
FIG. 2. Developmental expression of *csal1* during limb morphogenesis. (A) HH 17; black arrows indicate wing and leg buds, white arrow indicates tail bud. (B) HH 20. (C) HH 21. (D) HH 25; white arrow indicates expression in the AER; at this stage *csal1* begins to be downregulated in leg bud mesenchyme. (E, F) Sections of wing bud at HH 23, dorsal to top, ventral to bottom. (E) Longitudinal section; arrowhead indicates apical ridge, distal is to the left and proximal to right. (F) Transverse section.

FIG. 3. *csal1* expression in limb buds depends on dorsal ectoderm and signals from the AER. (A, B) Beads soaked in Shh (14 mg/ml) expand *csal1* expression in regions between the bead and the apical ridge. (A) Control limb from contralateral side showing the normal pattern of *csal1* expression. (B) Experimental limb with Shh bead; arrow indicates region of expanded expression. (C, D) Removal of the apical ridge from the wing bud results in complete loss of *csal1* expression. (C) Contralateral control limb showing normal pattern of *csal1* expression. (D) Experimental limb; embryo shown was harvested 24 h after apical ridge removal ($n = 10$). *csal1* expression has been completely abolished. (E, F) Shh does not rescue *csal1* expression following apical ridge removal. (E) Contralateral control limb showing normal *csal1* expression. (F) Experimental limb with Shh bead and no *csal1* expression.

from HH 19/20 wing buds ($n = 10$). We found that *csal1* expression was completely abolished in limb bud mesenchyme (Figs. 3C and 3D). The downregulation of *csal1* expression is quite rapid. *csal1* expression decreased within 4 h ($n = 6$) and was undetectable by 8 h ($n = 14$) after apical ridge removal. In addition, this downregulation cannot be rescued by application of a Shh-soaked bead alone (Figs. 3E and 3F; $n = 9$). These findings indicate that apical ridge-derived factors are required for *csal1* expression in developing limbs.

FGF4 and Wnt3a Together Maintain csal1 Expression after AER Removal

Signaling molecules expressed in the AER include FGFs, Wnts, and BMPs. We investigated whether any of these factors can maintain *csal1* expression after AER removal. We first implanted beads soaked in 10 μg/ml BMP2 in the distal mesenchyme of HH 19/20 wing buds following removal of the apical ridge. Embryos were subsequently harvested after 24 h. We found that BMP2 did not prevent



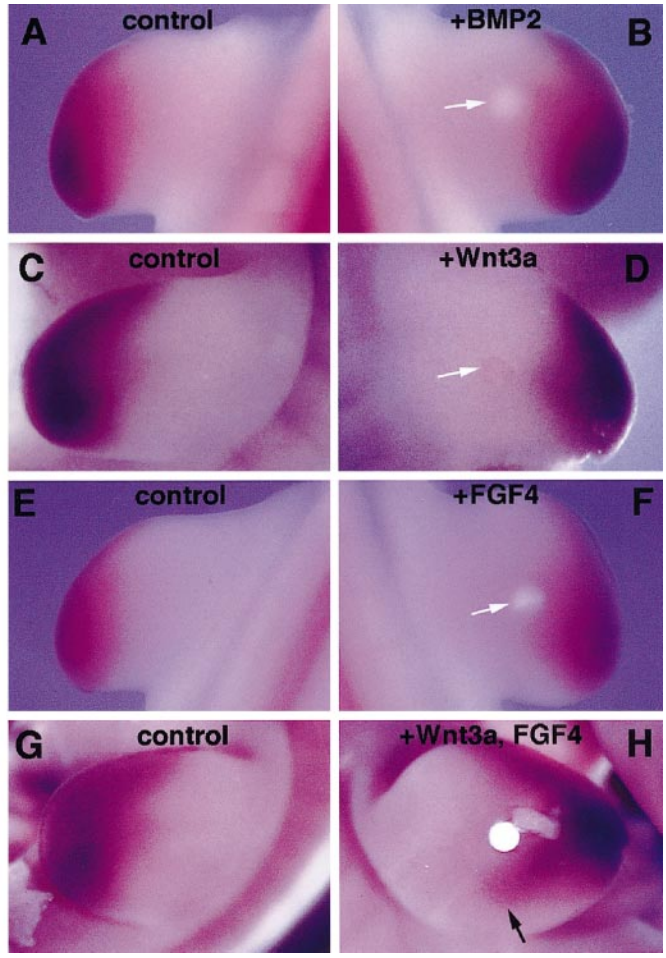


FIG. 4. *csal1* expression is maintained by a combination of FGF4 and Wnt3a after apical ridge removal. (A, B) Beads soaked in 10 μg/ml BMP2 cannot maintain expression of *csal1* after apical ridge removal. (A) Contralateral control limb showing normal expression of *csal1* (pin used for photography is visible in this frame). (B) Experimental limb; expression of *csal1* in the limb is abolished following apical ridge removal and is not maintained around the BMP2 bead. (C, D) FGF beads do not maintain expression of *csal1* after ridge removal. (C) Contralateral control limb showing normal *csal1* expression in the limb. (D) Experimental limb; *csal1* expression is abolished following apical ridge removal and is not maintained by the FGF4 bead (arrow). (E, F) Grafts of Wnt3a-expressing cells do not maintain expression of *csal1*. (E) Contralateral control limb showing normal expression of *csal1*. (F) Experimental limb; cell pellet indicated by arrow; *csal1* expression is abolished following apical ridge removal and is not maintained by the Wnt3a cell pellet. (G, H) FGF4 beads in combination with Wnt3a-expressing cells maintain *csal1* expression after apical ridge removal. (G) Contralateral control limb showing normal expression of *csal1*. (H) Experimental limb with FGF4 bead and Wnt3a cell pellet. Normal *csal1* expression is abolished by apical ridge removal, but expression is maintained around the double implant.

FIG. 5. Ectopic expression of *csal1* is seen in response to double implants of FGF4 beads in combination with Wnt3a cells. (A, B) Beads soaked in 10 μg/ml BMP2 placed in anterior regions of the wing bud do not result in ectopic expression of *csal1*. (A) Contralateral control limb showing normal expression of *csal1*. (B) Experimental limb with BMP2 bead (arrow); there is no significant expression beyond the normal domain of *csal1*. (C, D) Grafts of Wnt3a-expressing cells do not result in ectopic expression of *csal1*. (C) Contralateral control limb showing normal expression of *csal1*. (D) Experimental limb, arrow indicates cell pellet; there is no significant expression beyond the normal domain of *csal1*. (E, F) Beads soaked in FGF4 do not result in ectopic *csal1* expression. (E) Contralateral control limb showing the normal pattern of *csal1* expression. (F) Experimental limb with FGF bead (arrow); there is no significant expression beyond the normal domain of *csal1*. (G, H) Double implants of FGF4 beads with Wnt3a-expressing cells result in ectopic *csal1* expression. (G) Contralateral control limb showing the normal pattern of *csal1* expression. (H) Experimental limb with both FGF bead and Wnt3a cell pellet; there is ectopic expression of *csal1* (arrow) in the region adjacent to the double implant.

loss of *csal1* expression ($n = 4$; Figs. 4A and 4B). We also found that beads soaked in FGF4 could not maintain *csal1* expression in the absence of the apical ridge ($n = 5$; Figs. 4C and 4D). Similarly, a pellet of rat B1a fibroblasts expressing Wnt3a was not able to maintain *csal1* expression after apical ridge removal ($n = 8$; Figs. 4E and 4F). In contrast,

implanting a pellet of Wnt3a-expressing cells together with an FGF4 bead in the distal mesenchyme of the wing bud resulted in strong *csal1* expression in the area adjacent to the grafts ($n = 7$; Figs. 4G and 4H). This observation suggested that *csal1* expression in the distal limb might be regulated by a combination of FGF and Wnt signals.

Ectopic csal1 Expression in Response to FGF4 plus Wnt3a

We next investigated whether apical ridge-derived factors can produce ectopic *csal1* expression in the distal limb bud. Initially, beads soaked in BMP2 or BMP4 (10 μg/ml) were implanted into the mesenchyme of HH 19/20 wing buds; embryos were harvested 24 h later. Because effects of BMPs can be concentration dependent, beads were soaked in BMP2 concentrations ranging from 1 μg/ml to 1 mg/ml. We never observed ectopic *csal1* expression in response to BMP ($n = 26$; Figs. 5A and 5B). We next examined whether FGFs and Wnts, which are both expressed in the apical ridge, affect *csal1* expression in limb buds when applied ectopically. We implanted pellets of rat B1a fibroblasts expressing Wnt3a ($n = 5$; Figs. 5C and 5D) or beads soaked in FGF4 (700 μg/ml, $n = 8$; Figs. 5E and 5F) into the center of wing buds. As before, embryos were harvested 24 h after implantation. We did not observe ectopic *csal1* expression in response to these grafts. However, when we implanted Wnt3a-expressing cells and FGF4 beads together in the mesenchyme of wing buds, we observed strong ectopic *csal1* expression adjacent to the double implant after 24 h ($n = 7$; Figs. 5G and 5H). These findings indicate that a combination of FGF and Wnt signals can cause ectopic *csal1* expression in the distal limb bud.

A Combination of FGF4, Wnt3a, and BMP2 Is Required for Ectopic csal1 Expression in Proximal Limb Regions

Recently Capdevila and colleagues (1999) obtained ectopic *csal1* expression in the proximal limb by implanting BMP2 beads after infection with either Wnt3a- or FGF8-expressing retroviruses. In our hands *csal1* expression was not upregulated proximally when BMP2 beads were implanted with either pellets of Wnt3a-expressing cells or FGF4 beads ($n = 11$; Figs. 6A and 6B). Likewise, BMP2 implanted with either Wnt3a or FGF4 in the distal limb bud did not significantly rescue *csal1* expression following removal of the apical ridge (data not shown).

Surprisingly, we also did not find induction of *csal1* expression in response to a double implant of Wnt3a-expressing cells with FGF4 beads when placed in proximal regions ($n = 5$; Figs. 6C and 6D). This contrasted with our previous findings that ectopic *csal1* expression was observed in response to a double implant of FGF4 beads plus Wnt3a-expressing cells in more distal limb regions (Figs. 4H and 5H). However, we did find that implantation of Wnt3a-expressing cells with FGF4 and BMP2 beads resulted in ectopic *csal1* expression proximally ($n = 5$; Figs. 6E and 6F). These results agree with (1) the findings by Capdevila *et al.* (1999) that ectopic BMP2 is required for expression of distal markers such as *csal1* in proximal limb regions and (2) our observation that both FGF4 and Wnt3a are required to elicit *csal1* expression in the limb.

FGF4 Can Upregulate csal1 Expression in Conjunction with Wnt7a

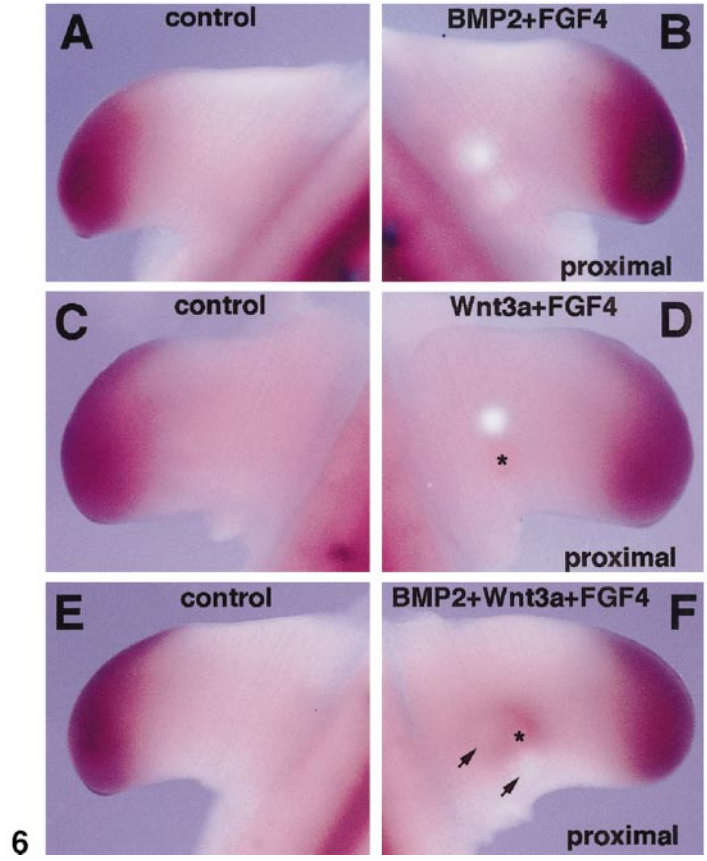
The fact that *csal1* expression is stronger in dorsal limb bud mesenchyme (Figs. 2E and 2F) suggests that dorsal signals are involved in *csal1* regulation. In order to test this hypothesis we removed dorsal ectoderm from HH 19/20 wing buds. Analysis of *csal1* expression 9 h after the operation showed that mesenchymal expression of *csal1* in operated wings had become confined to the extreme distal tip beneath the apical ridge ($n = 7$; Figs. 7A and 7B). This finding suggested that the extent of *csal1* expression in limb mesenchyme is modified by dorsal ectoderm signals.

Since Wnt7a is expressed in the dorsal ectoderm (Dealy *et al.*, 1993), we wanted to test whether Wnt7a could be involved in *csal1* regulation in the developing limb. To do this, we implanted pellets of rat B1a fibroblasts expressing Wnt7a into the mesenchyme of wing buds, as before. Embryos were harvested 24 h later. We found that Wnt7a by itself was not sufficient to cause ectopic *csal1* expression ($n = 10$; Figs. 7C and 7D). However, pellets of Wnt7a cells implanted together with FGF4 beads did cause ectopic expression of *csal1* ($n = 6$; Figs. 7E and 7F).

In addition, we demonstrate that FGF4 can interact with the dorsal ectoderm to cause *csal1* expression. FGF4 beads were grafted to the dorsal surface of HH 19/20 wing buds, and embryos were harvested 24 h later. We found that FGF4 was sufficient to cause ectopic *csal1* expression when placed in close proximity to the dorsal ectoderm ($n = 6$; Figs. 7G and 7H). However, FGF4 beads did not result in ectopic *csal1* expression if the dorsal ectoderm had been removed first ($n = 5$; Figs. 7I and 7J). Rather we observed downregulation of *csal1* in response to dorsal ectoderm removal as seen before (Figs. 7A and 7B). These results indicate that FGF4 can cooperate with the dorsal ectoderm to upregulate *csal1* expression and demonstrate that, like Wnt3a, Wnt7a can interact with FGF4 to cause ectopic expression of *csal1*.

Anterior Expansion of Ectopic csal1 Expression in Response to Shh and/or BMP2

We observed that ectopic *csal1* expression in distal regions was stronger posterior to the implants, where Shh and BMP2 are expressed (Figs. 4H, 5H, 7F, and 7H). In order to test whether Shh contributes to ectopic *csal1* expression in conjunction with FGF4 and Wnts, we performed triple implants of Shh, FGF4, and Wnt3a (Fig. 8). A bead of Shh was placed in the limb mesenchyme anterior to the double implant of FGF4 and Wnt3a. In this scenario, ectopic *csal1*



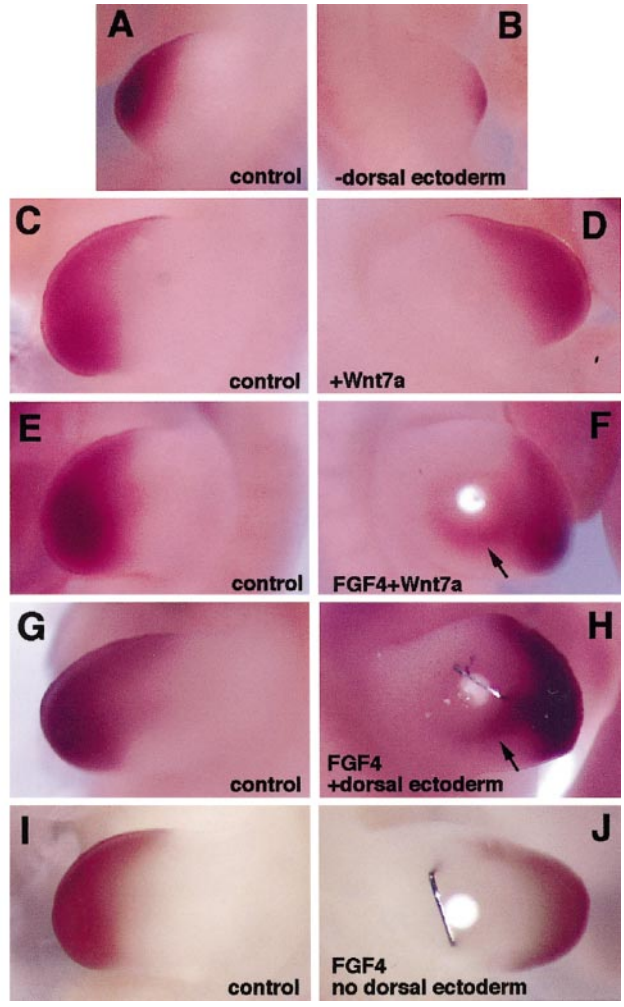


FIG. 6. BMP2 is required for proximal expression of *csal1* in response to FGF4 and Wnt3a. (A, B) Double implant of BMP2 and FGF4 does not result in ectopic upregulation of *csal1*. (A) Contralateral control limb showing normal *csal1* expression. (B) Experimental limb; embryo was harvested 24 h after implantation; beads have been placed in the proximal region of the limb bud, as in experiments described by Capdevila *et al.* (1999); *csal1* expression in the limb is normal and is not upregulated around the double implant. (C, D) FGF4 and Wnt3a do not upregulate *csal1* in proximal regions of the limb. (C) Contralateral control limb showing normal *csal1* expression. (D) Experimental limb showing double implant of FGF4 bead and Wnt3a pellet placed proximally; there is no *csal1* expression around the double implant. The Wnt3a pellet is indicated by an asterisk. (E, F) BMP2 together with FGF4 and Wnt3a induces ectopic *csal1* expression proximally. (E) Contralateral control limb showing normal *csal1* expression. (F) Experimental limb showing ectopic *csal1* expression around a proximal implant; arrows indicate beads; asterisk indicates Wnt3a pellet; staining is primarily around pellet.

FIG. 7. FGF4 interacts with Wnt7a from the dorsal ectoderm to cause *csal1* expression. (A, B) *csal1* expression 9 h after dorsal ectoderm removal is restricted to beneath the apical ridge. (A) Contralateral control wing bud showing normal expression. (B) Experimental limb; the domain of *csal1* expression has been severely reduced after removal of the dorsal ectoderm. (C, D) A pellet of Wnt7a-expressing cells does not cause ectopic *csal1* expression. (C) Contralateral control limb, showing normal *csal1* expression. (D) Experimental limb; arrow indicates pellet of Wnt7a-expressing cells; *csal1* expression is unaffected by the pellet. (E, F) A pellet of Wnt7a-expressing cells in combination with an FGF4 bead causes ectopic expression of *csal1*. (E) Contralateral control limb showing normal *csal1* expression. (F) Experimental limb; note ectopic expression of *csal1* (arrow) around the double implant. (G, H) FGF4 bead grafted to the dorsal ectoderm causes ectopic *csal1* expression. (G) Contralateral control limb showing normal *csal1* expression. (H) Experimental limb; FGF4 bead has been grafted to the dorsal ectoderm using a platinum staple (see Niswander *et al.*, 1993, for description of the use of platinum staples); arrow indicates ectopic *csal1* expression in response to graft. (I, J) Application of an FGF4 bead following removal of the dorsal ectoderm does not cause ectopic *csal1* expression. (I) Contralateral control limb showing normal *csal1* expression. (J) Experimental limb; FGF4 bead has been stapled to limb following removal of the dorsal ectoderm; *csal1* expression is not upregulated around the bead in the absence of dorsal ectoderm.

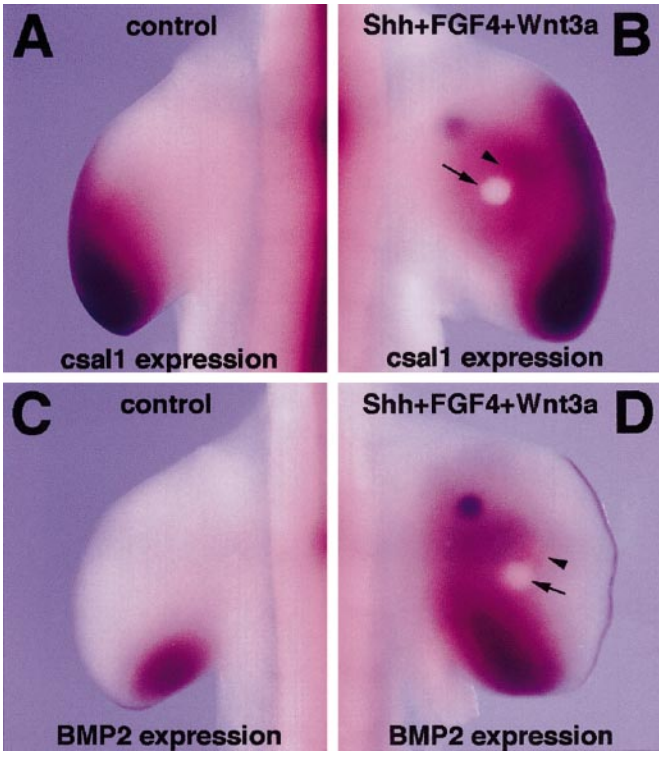


FIG. 8. Anterior expansion of ectopic *csal1* expression in response to Shh and/or BMP2. (A, B) *csal1* expression in response to ectopic Shh in the presence of FGF4 and Wnt3a. (A) Contralateral control limb showing normal *csal1* expression. (B) Experimental limb; Shh bead (blue) has been placed anterior to the double implant of FGF4 bead (arrow) and Wnt3a-expressing cells (arrowhead); ectopic expression of *csal1* is found both anterior and posterior to the double implant. Note that expression is somewhat stronger adjacent to the Wnt pellet. (C, D) BMP2 expression in response to ectopic Shh in the presence of FGF4 and Wnt3a. (C) Contralateral control limb showing normal *BMP2* expression. (D) Experimental limb; Shh bead (blue) has been placed anterior to the double implant of FGF4 bead (arrow) and Wnt3a-expressing cells (arrowhead); there is a significant expansion of the *BMP2* expression domain.

expression was found anterior as well as posterior to the FGF4–Wnt3a double implant ($n = 4$; Fig. 8B). The anterior expansion of *csal1* expression correlates with an increased domain of BMP2 expression ($n = 4$; Fig. 8D). These results indicate that Shh and/or BMP2 can contribute to the regulation of *csal1* in the limb. Interestingly, we observed that *csal1* expression was always slightly stronger in the area immediately adjacent to the Wnt3a pellet.

DISCUSSION

Shh Acts in Concert with the Apical Ridge to Regulate csal1 Expression

Hedgehog family members have been shown to be involved in regulating expression of *sal* genes in *Drosophila* and *Medaka* (Smith, 1996; Koster *et al.*, 1997). However, the data presented here indicate that *csal1* expression in chick limb buds is not regulated by Shh alone. (1) We showed that Shh upregulates *csal1* only in conjunction with the apical ridge. Ectopic expression was confined to regions between ridge and Shh bead (Fig. 3A). (2) Apical ridge removal significantly reduced *csal1* expression within 4 h, and expression was completely lost after 8 h. In contrast, Shh transcripts disappear within 10 h (Laufer *et al.*, 1994). (3) Beads soaked in Shh cannot rescue *csal1* expression following apical ridge removal (Fig. 3F). Taken together, these observations indicate that ectopic Shh can increase *csal1* expression (Figs. 3A and 3B) only in concert with the apical ridge. Shh has been shown to be required for maintenance and upregulation of a number of growth factors in the AER and distal mesenchyme (Johnson and Tabin, 1997; Schwabe *et al.*, 1998).

FGFs and Wnts Together Activate csal1 Expression in the Distal Limb Bud

Our results indicate that FGF4 in combination with either Wnt3a or Wnt7a can act to regulate *csal1* expression in the limb bud. Different members of the FGF family can substitute for each other to restore limb outgrowth after ridge removal (Martin, 1998). Since FGF4 starts to be expressed only after *csal1* expression begins (Niswander *et al.*, 1994; this paper), we propose that FGF8, which is expressed early (Crossley *et al.*, 1996), may be responsible for the initial activation of *csal1* expression at HH 17. It has been shown that Wnt3a can induce ectodermal expression of FGF8 (Kengaku *et al.*, 1998). However, we found that Wnt3a-expressing cells alone did not induce ectopic expression of *csal1* (Fig. 4D). It is possible that Wnt3a did not induce sufficient amounts of FGF8. Alternatively, upregulation of FGF8 may not have occurred in time to activate detectable *csal1* expression by HH 24.

Based on our findings, we propose a model in which Wnt3a and Wnt7a cooperate with FGFs to induce and maintain *csal1* expression in the distal limb bud. Interestingly, Wnt3a and Wnt7a have been shown to activate target genes via different signaling mechanisms (LEF-1 dependent and independent, respectively; Kengaku *et al.*, 1998), raising the possibility that the *csal1* gene may be downstream of different Wnt signaling pathways. Alternatively, it is possible that Wnt3a and Wnt7a may share the same signaling pathway for targeting some genes.

BMP2 Is Required for Ectopic csal1 Expression in Proximal Limb Regions

Application of BMP beads did not rescue *csal1* expression after apical ridge removal (Fig. 4B) or cause ectopic expression of *csal1* (Fig. 5B). This is consistent with findings reported by Capdevila and colleagues (1999). Given that *sal* is upregulated by *dpp* in *Drosophila* wing discs (deCelis *et al.*, 1996; Lecuit *et al.*, 1996; Smith, 1996), this result is

somewhat surprising. However, evidence from *Drosophila* shows that *sal* regulation is quite complex. For example in leg imaginal discs, *sal* is a target of the homeotic gene *Antennapedia* (Wagner-Bernholz *et al.,* 1991), while *sal* expression in developing tracheae is downstream of EGF receptor signaling (Kühnlein and Schuh, 1996). Thus, *sal* does not appear to be a direct target of *dpp* signaling in all tissues, but seems to be regulated by different signals depending on the developmental context.

Capdevila *et al.* (1999) report that BMP2 beads grafted to the proximal limb bud following viral overexpression of either FGF8 or Wnt3a result in ectopic *csal1* expression. According to their model, ectopic BMP2 relieves repression of distal identity mediated by *Meis2,* which allows proximal cells to respond to the distalizing influence of either FGF8 or Wnt3a. Our results are in agreement with this model in that ectopic BMP2 is required to induce *csal1* expression in extreme proximal regions. However, we find that both FGF and Wnt signals have to be present for ectopic *csal1* expression. It has been shown that viral overexpression of Wnt3a induces the ectopic expression of FGF8 (Kengaku *et al.,* 1998) and that Wnt7a can be ectopically upregulated by FGF4 (Akita *et al.,* 1996) (and therefore perhaps by FGF8). Thus, we would speculate that the ectopic expression of *csal1* seen in the experiments by Capdevila *et al.* (1999) may also involve cooperation between Wnts and FGFs.

Interestingly, while BMP2 is required in order to obtain *csal1* expression in the proximal limb bud, it is possible to observe *csal1* expression in the distal limb bud in response to FGF and Wnt without adding BMP2. This may be due to the fact that BMP2 is already expressed in the distal limb bud.

Shh and/or BMP2 Contributes to csal1 Regulation

We observed that ectopic *csal1* expression in distal regions was stronger posterior to the implants, where BMP2 and Shh are normally expressed (Figs. 4H, 5H, 7F, and 7H). We directly tested the role of Shh and/or BMP2 in the regulation of *csal1* by performing triple implants (Fig. 8). Based on our data, we propose that Shh and/or BMP2 may participate in the regulation of *csal1* in two ways. One possibility is that BMP2 induced by Shh may act to repress *Meis2* in the distal limb bud, thus allowing distal cells to respond to ridge-derived factors. Alternatively Shh and/or BMP2 may act in concert with the ridge-derived factors to regulate the expression of *csal1*.

Csal1 Function in the Developing Limb Bud

Our data suggest that the integration of signals from the distal–proximal, dorsoventral, and anterior–posterior axes dictate the endogenous expression of *csal1* in distal limb bud cells. Because *Drosophila sal* appears to mediate cell fate and pattern formation, it is tempting to speculate that *csal 1* is involved in fate determination of this population of cells. In this respect, *csal 1* may be similar to some of the *Hox* genes, which seem to specify different cell fates in the limb.

It has been shown that different regions along the proximodistal axis of the limb bud arise sequentially as the limb bud grows (Summerbell *et al.,* 1973). This observation led to the "progress zone" model, which states that proximal–distal identity is determined by the length of time cells reside in the progress zone, a region of proliferating mesenchymal cells located immediately beneath the apical ridge (Summerbell *et al.,* 1973). The progress zone model predicts that cells that exit the progress zone during the early stages of limb formation develop into more proximal structures, while those that are in the progress zone for a longer period of time are specified to give rise to more distal structures. In view of this hypothesis, the fact that the domain of *csal1* expression appears to overlap the progress zone is intriguing. One possibility is that *csal1* acts in the progress zone to prevent differentiation at the distal tip during early limb outgrowth. The subsequent downregulation of *csal1* at a later stage would then allow distal structures, such as digits, to form. This hypothesis is compatible with the observation that mutations in the human homologue, *Hsal1/SALL1,* result in the formation of extra digits seen in TBS (Kohlhase *et al.,* 1998, 1999). Consistent with this, in *Drosophila sal* must be downregulated in order for differentiation of sensory organ precursors to occur (deCelis *et al.,* 1999).

Now that *csal1* has been isolated, it will be interesting to investigate the molecular mechanisms underlying TBS in detail. Based on the human syndrome, we predict that disruption of *csal1* function in limb buds would lead to formation of extra digits, while overexpression of *csal1* would lead to loss of digits.

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