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# Medaka *spalt* acts as a target gene of *hedgehog* signaling

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## SUMMARY

**In vertebrates, pattern formation in the eye, central nervous system, somites, and limb depends on *hedgehog* activity, but a general target gene controlled by *hedgehog* in all these signaling centers has remained largely elusive. The medaka fish gene *spalt* encodes a zinc-finger transcription factor, which is expressed in all known *hedgehog* signaling centers of the embryo and in the organizer region at the midbrain-hindbrain boundary. We show that the *spalt* expression domains expand in response to ectopic *hedgehog* activity and narrow in the presence of protein kinase A activity, an antagonist of *hedgehog* signaling, indicating that *spalt* is a *hedgehog* target gene. Our results also suggest a signaling mechanism for anterior-posterior patterning of the vertebrate brain that controls *spalt* expression at the midbrain-hindbrain boundary in a protein kinase A dependent manner likely to involve an unknown member of the *hedgehog* family.**

Key words: *spalt*, *hedgehog* target gene, medaka, patterning, mid-hindbrain boundary

## INTRODUCTION

Pattern formation in multicellular organisms depends on positional information generated by transient gradients of secreted signaling molecules (reviewed by Lawrence and Struhl, 1996). This graded activity is then permanently established by the differential expression of target genes. In *Drosophila*, one of the diffusible patterning activities is encoded by the segment polarity gene *hedgehog* (*hh*) which mediates pattern formation during segmentation (Lee et al., 1992) and during imaginal disc determination (Tabata and Kornberg, 1994).

Recently several homologues of the *Drosophila hedgehog* gene have been identified in various vertebrate species, revealing the existence of a *hedgehog* gene family of secreted signaling molecules (Zardoya et al., 1996). Best characterized is *sonic hedgehog* (*shh*), whose spatiotemporal expression is conserved throughout vertebrates. Initially, *shh* is expressed in the embryonic shield or node during gastrulation, then becomes localized to the ventral midline during axis formation and subsequently to the notochord, floorplate and anteriorly to the prechordal mesoderm. Later *shh* expression is found in the outgrowing posterior distal limb and the excretory system (reviewed by Hammerschmidt et al., 1997).

Diffusible HEDGEHOG protein emanates from localized sources, referred to as *hh* signaling centers, and mediates pattern formation over short and long distances. The eyes, the neural tube, the somites, the excretory system and limbs are structures that are organized through the patterning activity of SHH. Patterning in the vertebrate eyes is mediated by SHH emanating from the underlying prechordal mesoderm in the embryonic midline. The finding that *shh* deficient mice suffer phenotypically from cyclopia (Chiang et al., 1996) indicates that midline signaling by SHH is necessary for a proper separation of the anterior eye domain. Consistently, cyclopia was also observed when midline signaling was antagonized by overexpression of protein kinase A (PKA), which efficiently inhibits signaling of several members of the *hedgehog* family (Hammerschmidt et al., 1996). Conversely, ectopic overexpression of *shh* leads to the expansion of proximal eye structures at the expense of distal structures as shown by the expansion of proximal *Pax2* expression and suppression of the distal eye marker *Pax6* (Ekker et al., 1995b; Macdonald et al., 1995). In the notochord, *shh* expression leads to the contact dependent induction of floorplate cells in the neural tube which themselves start to secret SHH and subsequently induce the differentiation of motor neurons (Ericson et al., 1996 and references therein). In addition, lateral diffusion of SHH from midline structures was shown to control the patterning of the paraxial mesoderm (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995). In the excretory system *shh* is expressed at different sites (Bitgood and McMahon, 1995) and mediates patterning processes and induction events which are currently analyzed in *shh* deficient mice (Chiang et al., 1996). Finally, during limb development SHH is generated in the posterior part of the outgrowing limbs, the zone-of-polarizing activity (ZPA), which mediates anteroposterior (AP) polarity of the limb. An ectopic source of diffusible SHH in the anterior part of the limb bud mimics a ZPA and leads to mirror-image duplication of digits (Riddle et al., 1993; Chang, 1994; López-Martinez et al., 1995).

Although the important role of *shh* in patterning processes of vertebrate development is well established, little is known about the transmission of the HEDGEHOG signal. Recently, the transmembrane protein PATCHED (PTC) was discovered to function as SHH receptor releasing the bound

SMOOTHENED (SMO) protein from its inactive state by binding to SHH (Stone et al., 1996; Marigo et al., 1996a). General target genes of HH that mediate *hh* signaling at the level of transcription, and are activated in all *hh* signaling centers, remain largely elusive.

Here we describe the cloning of such a target gene, the medaka (*Oryzias latipes*) homologue of the *Drosophila* zinc finger transcription factor *spalt* (*sal*). In the *Drosophila* wing imaginal disc *sal* was shown to be activated by *hh* expression (Sturtevant et al., 1997) and to mediate patterning (de Celis et al., 1996; Sturtevant et al., 1997). In agreement with this, we find medaka *spalt* (*sal*) expressed in all *hh* signaling centers and in the organizer at the mid-hindbrain boundary (MHB). Medaka *sal* is activated by ectopic SHH activity. Suppression of *hh* signaling by overexpression of protein kinase A (PKA) results in suppression of *sal* expression. Our results indicate that *sal* is a *hh* target gene which is positively regulated by HH activity in all known signaling centers. In addition, the *sal* expression domain at the MHB expands in response to ectopic HH and it narrows in response to PKA activity. Our results are consistent with the proposal that AP patterning of the dorsal vertebrate brain is mediated by *hh* signaling, although a corresponding member of the HH family that is expressed in the relevant region of the brain has not yet been identified.

## MATERIALS AND METHODS

### Medaka stocks

Fish were maintained in a constant recirculating system at 28°C on a 14 hours light/10 hours dark cycle. Wild-type *Oryzias latipes* from a closed stock were originally obtained from Carolina Biological, North Carolina, USA. Pairwise mating was performed and collected embryos were kept at 28°C.

### Isolation and sequencing of medaka *sal*, *Pax2* and *shh*

Medaka *sal* was isolated by PCR using degenerate oligonucleotides [5′: TGYGCNGARTTYTTYAARTGGAC, 3′: CTDATCATYTTCATYTGRTTYTC, 40 cycles: 94°C, 1 minute (first: 3 minutes); 49°C, 1.5 minutes; 72°C, 1.5 minutes (final 10 minutes)] specific for conserved regions of the known *spalt* genes (Hollemann et al., 1996; Ott et al., 1996). A 2.5 kb fragment of coding region was amplified from genomic DNA and subsequently used to screen a neurula stage (stage 18) cDNA library (stages refer to Iwamatsu, 1994). The longest clone contained a 4.5 kb cDNA insert which was sequenced with internal primers (Applied Biosystems 373A DNA Sequencer). The coding region of this cDNA clone could be extended in the 5′ direction by sequencing genomic DNA (accession #U77376).

A 900 bp medaka *Pax2* fragment [5′ GCIGAYCCITTYWSIGCIATGCA, 3′: GGRTTISWRAAICKCCAIGCYTCRTTRTA, 35 cycles: 94°C, 1 minute (first: 5 minutes); 5×48°C + 30×53°C, 2 minutes; 72°C, 4 minutes (final 9 minutes)] and a 297 bp medaka *shh* fragment [5′: CCIAAYTAYAAYCCIGAYATHATITTYAA, 3′: YTCRTARTAIACCCARTCRAAICCIG, 35 cycles: 94°C, 1 minute (first: 5 minutes); 5×48°C + 30×53°C, 2 minutes; 72°C, 4 minutes (final: 9 minutes)] were isolated by RT-PCR with degenerate primers using RNA from the anterior part of the axis of early neurula stage (stage 17) embryos and TA cloned into the pCRII vector (Invitrogen). Identity of the cloned fragments was confirmed by sequencing and comparison to the known vertebrate homologues (EMBL accession numbers are *Pax2*, Z97020 and *shh*, Z97019).

### In situ hybridization and immunohistochemistry

In situ hybridization was performed as described by Oliver et al. (1996). When double staining was carried out medaka *sal* in situ hybridization was performed first. Acetylated tubulin was detected using a monoclonal antibody (Sigma #T6793 1:1000 dilution). Secondary anti-mouse antibody coupled to alkaline phosphatase (Jackson ImmunoResearch, 1:200 dilution) was preabsorbed to medaka embryos. Phosphatase activity was detected using Fast Red (Boehringer Mannheim) as substrate. Stained embryos were briefly rinsed in methanol and mounted in 87% glycerol. Intensely stained embryos were embedded in Technovit 7100 (Kulzer), sectioned (6 μm) and mounted in Entellan (Merck).

### RNA injections

Capped RNA for injections was transcribed from linearized pSP64T (Krieg and Melton, 1984) plasmids using the SP6 mMESSAGE mMACHINE Kit (Ambion #1340). Individual blastomeres of 2- to 4-cell stage embryos were injected as described by Wittbrodt and Rosa (1994). Initially different dilutions of mRNA were injected at concentrations of 50, 125 and 250 ng/μl (*shh*) and 2.5, 5, 10 and 20 ng/μl (PKA*) respectively. Concentration dependent effects observed in injected embryos were comparable to those described previously in that they became increasingly severe with rising mRNA concentrations in the injection solution (Ekker et al., 1995b; Hammerschmidt et al., 1996). Due to moderate morphological effects in injected embryos, concentrations of 125ng/μl (*shh*) and 2.5 ng/μl (PKA*) respectively were chosen for the injection experiments shown in Figs 5 and 6. Control injections using *Xenopus* EF1α (Ambion) mRNA were performed at identical concentrations. All injections were monitored with RNA encoding β-galactosidase (5 ng/μl), and subsequently a number of embryos were stained for *lacZ* activity to visualize distribution and functional integrity of injected RNA (Joore et al., 1996).

## RESULTS

### Isolation and analysis of the medaka *sal* gene

Based on the comparison of known vertebrate (Hollemann et al., 1996; Ott et al., 1996) and *Drosophila* (Kühnlein et al., 1994) *spalt* sequences we designed degenerate oligonucleotides specific for regions conserved in these *spalt* homologues. PCR amplification from medaka genomic DNA resulted in a 2.5 kb fragment. This fragment was subsequently used to screen a late neurula stage (stage 18) medaka cDNA library. Sequence analysis of the longest cDNA clone of 4.5 kb revealed that it contained the complete 3′ untranslated region including a polyadenylation consensus but lacked an inframe AUG at the 5′ end. The sequence could be extended in the 5′ direction using sequence information from genomic DNA. So far we have not been able to further extend the known sequence by RACE-PCR. Sequence comparison with the known vertebrate *spalt* genes indicates that this clone lacks approximately 70 residues at the amino terminus.

The deduced amino acid sequence of medaka *sal* shows high overall similarity to the known vertebrate *sal* genes (68% in comparison to mouse and 66% in comparison to *Xenopus*, respectively). Medaka *sal* encodes a protein that contains the three diagnostic double zinc finger domains and a single zinc finger of the C2H2-motif, located adjacent to the second pair of zinc fingers, which are characteristic for all SAL homologues. The entire domain containing the zinc fingers of SAL is highly conserved between vertebrates and *Drosophila* respectively, and shows the typical H/C-link of SPALT zinc fingers as well as the diagnostic SAL-box (Kühnlein et al., 1994) in each second finger of the three double zinc finger regions (Fig. 1B). The high degree of conservation in the SAL-box and the zinc fingers, which are required for sequence

specific DNA binding (Barrio et al., 1996), suggest that all *sal* homologues recognize a highly conserved binding site. At the amino terminus SAL contains an additional single zinc finger of the C2HC-type characteristic for vertebrate SAL proteins (Hollemann et al., 1996). In summary these features identify medaka *sal* as a homologue of the *Drosophila* and the known vertebrate *spalt* genes (Kühnlein et al., 1994; Hollemann et al., 1996; Ott et al., 1996; Kohlhase et al., 1996).

## Spatiotemporal expression pattern of *sal* during embryogenesis

In *Drosophila*, *spalt* functions downstream of the *hh* signaling pathway (Sturtevant et al., 1997). To investigate whether medaka *sal* plays a similar role in vertebrates we first examined *sal* expression during early medaka embryogenesis at the time when *shh* is known to exert its patterning activity. Localization of medaka *sal* was examined by whole-mount in situ hybridization. Transcripts of *sal* are detected during development of the medaka embryo in the eye, the central nervous system, the excretory system, the somites and the developing fins.

Initially, *sal* expression is apparent at neurula stages (stage 18; for stages refer to Iwamatsu, 1994). Expression is detected in the anteroproximal part of the optic vesicle, in the optic stalk and the rostral optic vesicle prior to the formation of the multilayered retina (Fig. 2A,D). *sal* expression levels fade when lens embedding and invagination of the optic vesicle occur (stage 21, not shown).

From neurula stages onwards, *sal* is expressed in the neural tube along the entire anteroposterior (AP) axis of the developing central nervous system (CNS) (stage 18) (Fig. 2D,E). Later, during organogenesis (stage 26), expression in the CNS becomes restricted to single cell clusters in the trunk region of the neural tube, whereas anteriorly in the rhombencephalon *sal* transcripts are found bilaterally in each rhombomere (Fig. 2C,F). In rhombomere four *sal* is expressed in commissural cells which cross the midline.

In the dorsal part of the midbrain, *sal* is expressed in a sharp stripe (*sal* stripe) of two to three cell diameters width in the mid-hindbrain boundary (MHB). This expression domain is first seen at early neurula stages (stage 17) and persists in the isthmus until the constriction between mid- and hindbrain has formed and separates mes- and metencephalon (stage 26; Fig. 2).

Strong *sal* expression is detected in pro- and opistonephros from neurula stages onwards (Fig. 2). In the pronephros, the progenitor of the head nephros, high expression levels of *sal* are detected throughout embryogenesis. Concomitant with the development of the head nephros to a lymphatic organ, *sal* expression disappears (not shown). In the opistonephros, expression is restricted to the posteriormost somites and declines from posterior to anterior in a graded manner suggesting a transient requirement of *sal* expression in the formation of the opistonephros.

## Expression of *sal* in the trunk is restricted to somitic cells and neurons

To analyze the expression of *sal* in the trunk in more detail transverse sections of whole-mount stained embryos where performed. Lower levels of *sal* expression were detected

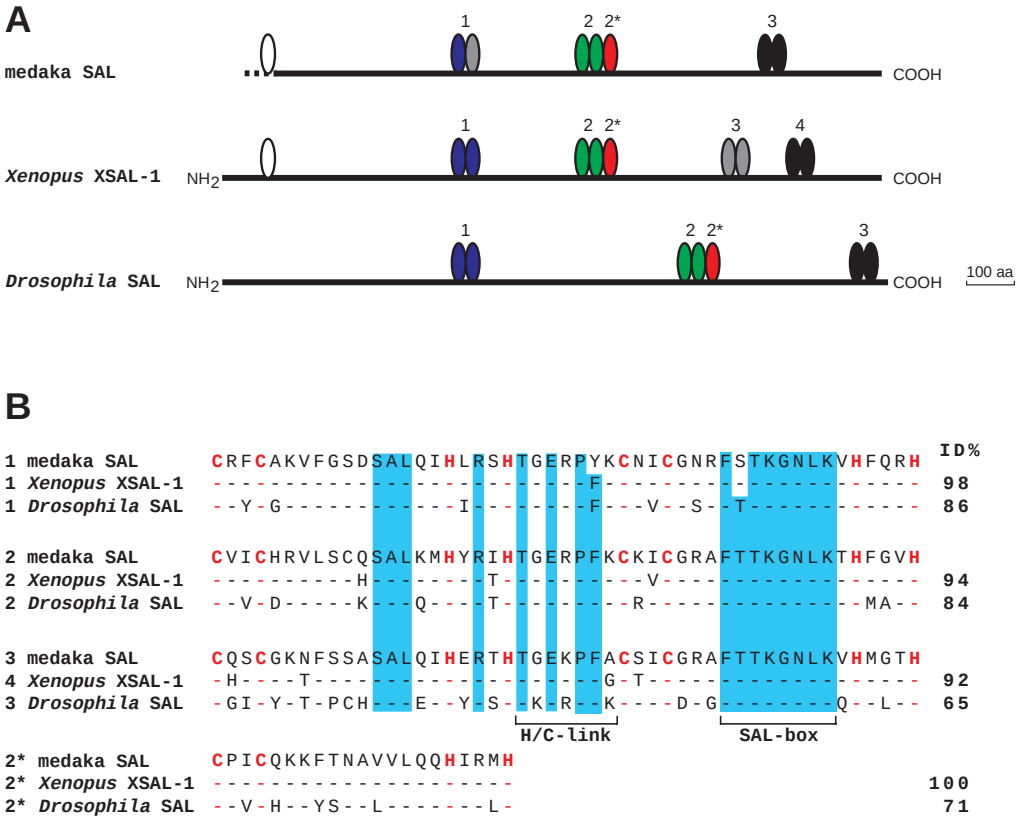


**Fig. 1.** Medaka *sal* is a vertebrate homologue of *Drosophila spalt*. (A) Diagrammatic representation of SAL proteins from medaka, *Xenopus* and *Drosophila* comparing relative positions of zinc finger motifs (ovals). Homologous fingers are in the same color; the single N-terminal C2HC motif, characteristic for vertebrate SAL proteins is in white. 2* marks the single C2H2 finger motif adjacent to the 2nd double finger present in all SAL-type proteins. For the alternatively spliced 3rd double finger (grey) see Hollemann et al. (1996). (B) Sequence comparison of homologous zinc finger motifs. Zinc complexing cysteine and histidine residues are in red. Identical amino acid residues are shown as dashes, positions identical in all double finger motifs are highlighted in blue, the conserved H/C-link and the SAL-box are underlined. Sequence identity values correspond to the medaka fingers 1, 2, 3, and 2* respectively. Numbering corresponds to fingers in A.

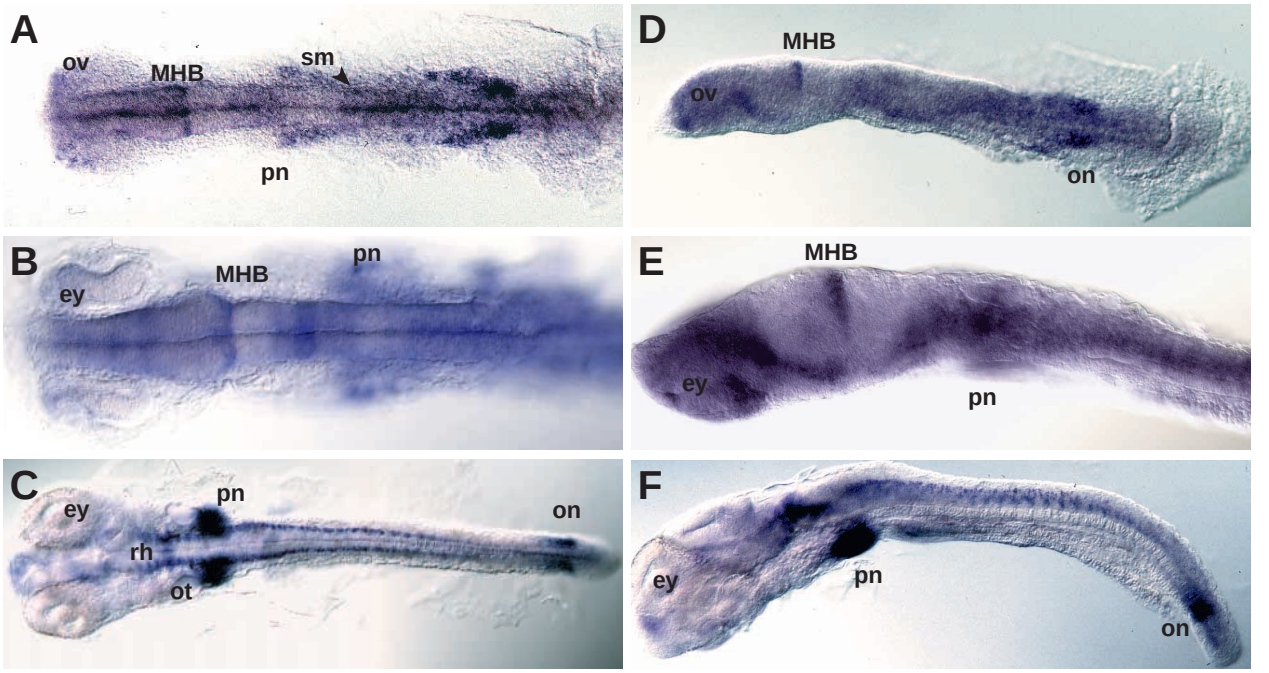


**Fig. 2.** Whole-mount in situ analysis during neurula stages and somitogenesis, anterior left, dorsal views (A-C), lateral views (D-F). (A,D) *sal* expression during neurula stages (stage 18) *sal* is expressed in the proximal part of the optic vesicle, in the neural tube along the anteroposterior axis and in the pro- and opistonephros. Note sharp expression domain in the dorsal part of the MHB (*sal* stripe). (B,E) Stage 24, intensity of *sal* expression in the *sal* stripe is increasing. Expression in the neural tube is confined to the ventralmost part. (C,F) Early organogenesis stages (stage 26). In rhombencephalon and spinal cord transcripts are detected in repeated clusters of 8-10 cells. In the rhombencephalon *sal* clusters are located in rhombomers 2-7. Prominent *sal* expression in pronephros and opistonephros. Note that expression in the opistonephros is confined to tubules in newly forming somites. ey, eye; MHB, mid-hindbrain boundary; on, opistonephros; ov, otic vesicle; pn, pronephros; rh, rhombencephalon.

medially in the forming somites, the region of the prospective sclerotome (Fig. 3A) which has been shown to be induced by long range signaling of a secreted HH family member (Fan and Tessier-Lavigne, 1994).

In neural structures *sal* expression is detected at neurula stages in the ventral third of the forming neural tube (stage 19), but is excluded from the ventralmost floorplate cells (Fig. 3). As neuronal differentiation proceeds during somitogenesis (stage 24), small bilateral clusters of *sal*-expressing cells are found in the ventral part of the neural tube (Fig. 3B). Here *sal* expression reflects the time course of neuronal differentiation, suggesting that *sal* transcripts are confined to differentiating neurons. To verify this, double labeling experiments were performed which combined in situ hybridization for *sal* and immunostaining of the neuronal axons (Chitnis and Kuwada, 1990) with an antibody directed against acetylated tubulin (Piperno and Fuller, 1985). *sal*-positive cells located ventrally in the neural tube project their tubulin-positive axons to the underlying somites, identifying these cells as motor neurons (Fig. 3C,D). Later, during organogenesis stages (stage 27), *sal*-expressing cell clusters are detected in intermediate and then in dorsal regions of the neural tube (Fig. 3E, 4E) representing interneurons and sensory neurons repectively. Taken together this shows that *sal* is expressed in neuronal cells, where its expression identifies differentiating neurons rather than a specific neuronal cell type.

### *sal* expression is confined to *hedgehog* signaling centers

In vertebrates, *hedgehog* signaling is required for the proper patterning of the eye, neural tube, kidneys, somites and limb buds. Secreted SHH protein, diffusing from localized sources, mediates pattern formation within the *hh* signaling center over short and long distances. In medaka, as reported for other vertebrates (Krauss et al., 1993; Riddle et al., 1993; Echelard et al., 1993; Roelink et al., 1994), *shh* is expressed in the notochord and subsequently in the ventral midline of the neural tube (Fig. 4A). Comparison of medaka *sal* and *shh* expression shows that in the *hh* signaling centers, the eye, the ventral part of the neural tube, the kidneys and somites, *sal* transcripts are found adjacent to *shh* expression (Fig. 4). In outbudding fins the expression domains of *sal* and *shh* are partially overlapping (not shown). At late neurula stages *sal* transcripts are found in the proximal part of the outbudding optic vesicle (Fig. 4C), which is known to be under the control of *hh* signaling in vertebrates (Ekker et al., 1995b; Macdonald et al., 1995; Chiang et al., 1996). Transverse sections through the rostral head region reveal *sal* expression in the optic chiasm (Fig. 4D) and a faint expression in the more distal part of the optic vesicle (Fig. 4D). In the early neural tube *sal* expression is limited to the ventral region (Figs 3A, 4D) but excluded from the floorplate cells expressing *shh* (Figs 3A, 4A). This expression of *sal* is subsequently refined to differentiating neurons and reflects the patterning activity of SHH which mediates at least two phases of patterning. First, medial neural plate cells are converted to ventralized progenitors (expressing *sal*; Fig. 3A), which are then, in a second step induced directly by SHH to differentiate into (*sal* positive; Fig. 3B-D) motor neurons (Marti et al., 1995; Tanabe et al., 1995; Ericson et al., 1996). The absence of the second induction step leads to the

induction of interneurons (Fig. 4E), located more dorsally, indicating that SHH acts as a morphogen in the neural tube (Ericson et al., 1996). The finding of *sal* expression at organogenesis stages in dorsally located sensory neurons (Fig. 3E) might be due to long range diffusion of an *HH* signal through the cavity of the neural tube as suggested for *patched* in chicken (Marigo and Tabin, 1996).

The pronephros shows abundant *sal* expression throughout embryonic development (Figs 2, 4E), which is consistent with the suggested role for *shh* in kidney formation in higher vertebrates (Fig. 4E; Bitgood and McMahon, 1995; Chiang et al., 1996).

In the pectoral fin buds (not shown) and subsequently in the outgrowing pectoral fins, *sal* transcripts are localized in the posterior half of the distal progress zone (Fig. 4F), an area analogous to the zone of polarizing activity (ZPA) of higher vertebrates. Here *shh* is required for proper AP-patterning of the limb (Riddle et al., 1993; Chang, 1994).

Thus, in all tissues known to be patterned by HH activity, expression of *sal* and *shh* coincide temporally and spatially, suggesting that *sal* expression is activated in response to HH activity.

## Ectopic HH activity expands *sal* expression domains

We have tested this inference using two complementary experimental strategies. First, we have examined the *sal*-inducing activity of SHH by injecting different amounts of *shh* mRNA (Krauss et al., 1993) into one blastomere at the 2-4 cell stage. Due to the limited stability of the RNA injected (data not shown) we focused our analysis on early effects and therefore analyzed embryos up to stage 22. Functional expression of ectopic *shh* was monitored by the altered size and shape of the developing eyes (Fig. 5C,H) (Ekker et al., 1995b; Macdonald et al., 1995). Already moderate levels of ectopic SHH activity expanded the *sal* expression domains significantly. We did not, however, observe ectopic de novo expression isolated from the wild-type *sal* expression domains. Fig. 5C shows that in *shh* injected embryos *sal* expression covers the entire eye, which is consistent with the observation that *hh* overexpression causes an expansion of proximal eye fates (Ekker et al., 1995b; Macdonald et al., 1995). In the neural tube and in the brain of injected embryos the ventral wild-type *sal* expression domain (Fig. 5B,D) expands towards the dorsal side (Fig. 5C,E), however, *sal* expression was never detected in the dorsal part of the mesencephalon (Fig. 5C). In the somites (Fig. 5D) and the pronephros (Fig. 5B), the mesodermal *sal* expression domains, *shh* injection causes a significant enlargement of the *sal* domains (Fig. 5C,E).

These results show that *sal* expression, which expands in *hh* signaling centers in response to increased HH activity, can indeed be activated by *hh* signaling. The finding that no de novo *sal* expression domains are observed, indicates that additional competence factors determine the response to ectopic HH activity which, in the case of *sal*, allow only an expansion of the wild-type expression domains.

## Ectopic PKA* activity reduces the size of the *sal* expression domains

To further investigate the regulation of *sal* expression through the *hh* signaling pathway we have assayed the effects of reduced HH activity. In *Drosophila* it was shown that protein

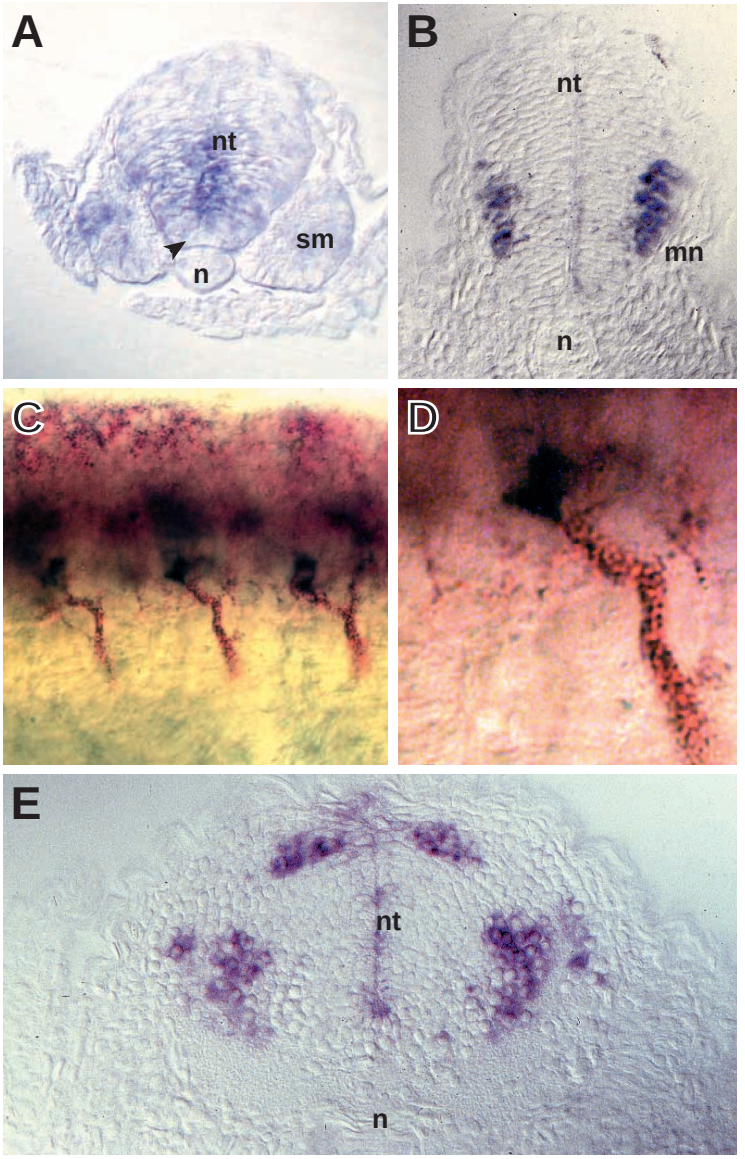


**Fig. 3.** Medaka *sal* expression is in the neural tube is confined to neurons. (A, B) Transverse sections through the trunk region of whole-mount stained embryos. (A) *sal* expression is restricted to the ventral third of the neural tube (stage 19). No *sal* expression is detected in the floorplate (arrowhead). In somites flanking the notochord faint *sal* expression is detected. (B) Ventral *sal* expression in the neural tube is restricted to ventral, bilateral clusters of 8-10 cells (stage 25). (C,D) Axons of primary motoneurons (red), visualized by an antibody directed against acetylated tubulin, project from *sal*-positive cell bodies (blue) to the underlying somites and identify *sal*-positive cells (in B) as motoneurons. (E) *sal* staining in a transverse section through the trunk region of an embryo at organogenesis stages (stage 27). In addition to ventral clusters of *sal*-positive cells in the neural tube staining becomes apparent at intermediate positions. mn, motoneurons; n, notochord; nt, neural tube; sm, somite; fp, floorplate

kinase A (PKA) acts to suppress the expression of *hh* target genes (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995). To interfere with *hh* signaling we made use of a constitutively active form of the catalytic subunit of protein kinase A (Orellana and McKnight, 1992; PKA*) previously shown to antagonize known *hh* signaling activities in vertebrates (Hammerschmidt et al., 1996; Concordet et al., 1996).

The injection of PKA* mRNA causes fusion of the eyes (Fig. 6B,D), indicating that PKA* activity is functional in antagonizing midline signaling mediated by HH activity as reported previously (Hammerschmidt et al., 1996; Concordet et al., 1996). While ectopic SHH was expanding the *sal* expression domains, the expression of PKA* had the opposite effect. We observed a concentration dependent, strong reduction of *sal* expression in all *hh* signaling centers. In the eye, minute traces of *sal* expression are detected (Fig. 6B). *sal* expression in the ventral CNS is almost eliminated upon PKA* injection (Fig. 6B). Only weak residual *sal* transcription is detected in the pronephros (Fig. 6B) and somites. Thus, *sal* expression is reduced in all *sal* expression domains confined to *hh* signaling centers, when *hh* signaling is antagonized by the expression of PKA*.

In addition to its expression in the known *hh* signaling centers described so far, *sal* is also detected at organogenesis stages in dorsally located neurons within the neural tube and already at the neurula stage in a stripe at the midbrain hindbrain boundary (MHB; Fig. 4B). While the limited stability of the mRNA injected did not allow the investigation of the effect of ectopic *shh* and its antagonist PKA* respectively on dorsal *sal* expessing neurons, this issue could be addressed for the *sal* expression at the MHB.

### *sal* expression at the MHB responds to ectopic *shh* and its antagonist PKA*

The MHB was previously identified as a patterning center required for the establishment of an anterior-posterior polarity in the midbrain (reviewed by Joyner, 1996). *shh* or other members of the *hh* family have not been identified as an essential signaling activity in this dorsal brain region. However, the *sal* stripe at the MHB clearly expands in response to ectopic *shh* expression (Fig. 5C). It expands from only a few cell diameters width in wild-type embryos (Fig. 5B) up to five fold in the AP direction in response to ectopic HH activity (Fig. 5C). Conversely, inhibition of *hh* signaling in PKA* injected embryos, which efficiently suppresses *sal* transcription in all *hh* signaling centers, also prevents the *sal* stripe expression at the MHB (Fig. 6B).

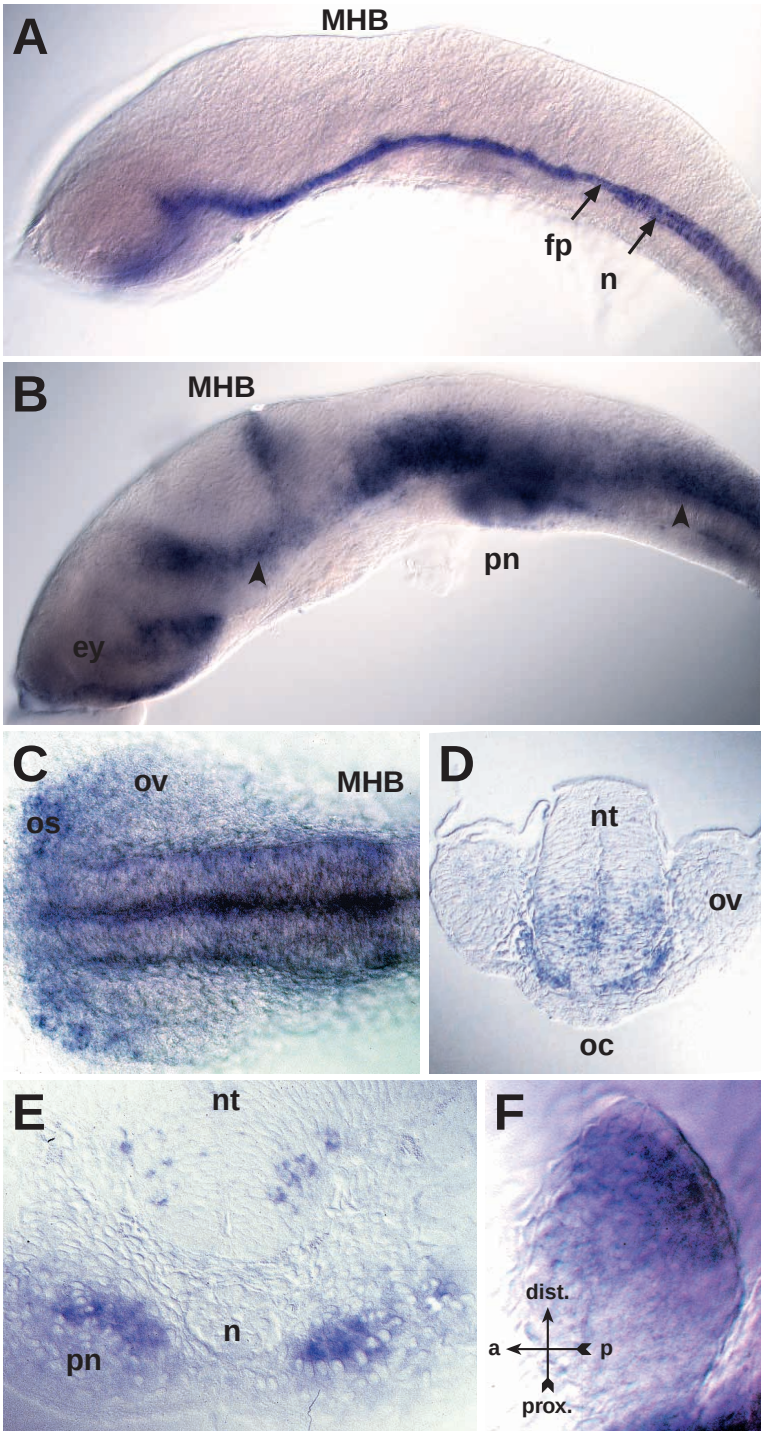


**Fig. 4.** Medaka *sal* expression is confined to *hh* signaling centers. (A) Lateral view of a stage 24 embryo (early somitogenesis) stained for medaka *shh*. *shh* is detected in prechordal mesoderm and along the AP-axis in the ventralmost part of the CNS (floorplate). More caudally expression is detected in the notochord. (B-F) *sal* expression. (B,C) *sal* is detected in proximal part of optic vesicle, in optic stalk and in optic chiasm, revealed by transverse sections at the level of the eye in stage 19 embryos (D). *sal* transcripts are detected in forming neural tube (arrowheads in B) restricted to its ventral part (D) and subsequently in differentiating neurons (E; see also Fig. 3). (B,E) *sal* expression is found in the mesodermally derived pronephros. (F) In outgrowing pectoral fins *sal* expression is confined to the posterior part. In addition to known *hh* signaling centers *sal* is also expressed in the MHB (B,C) where no *shh* expression is detected (A). ey, eye; fp, floorplate; MHB, mid-hindbrain boundary; n, notochord; nt, neural tube; oc, optic chiasm; os, optic stalk; ot, otic vesicle; ov, otic vesicle; pn, pronephros.

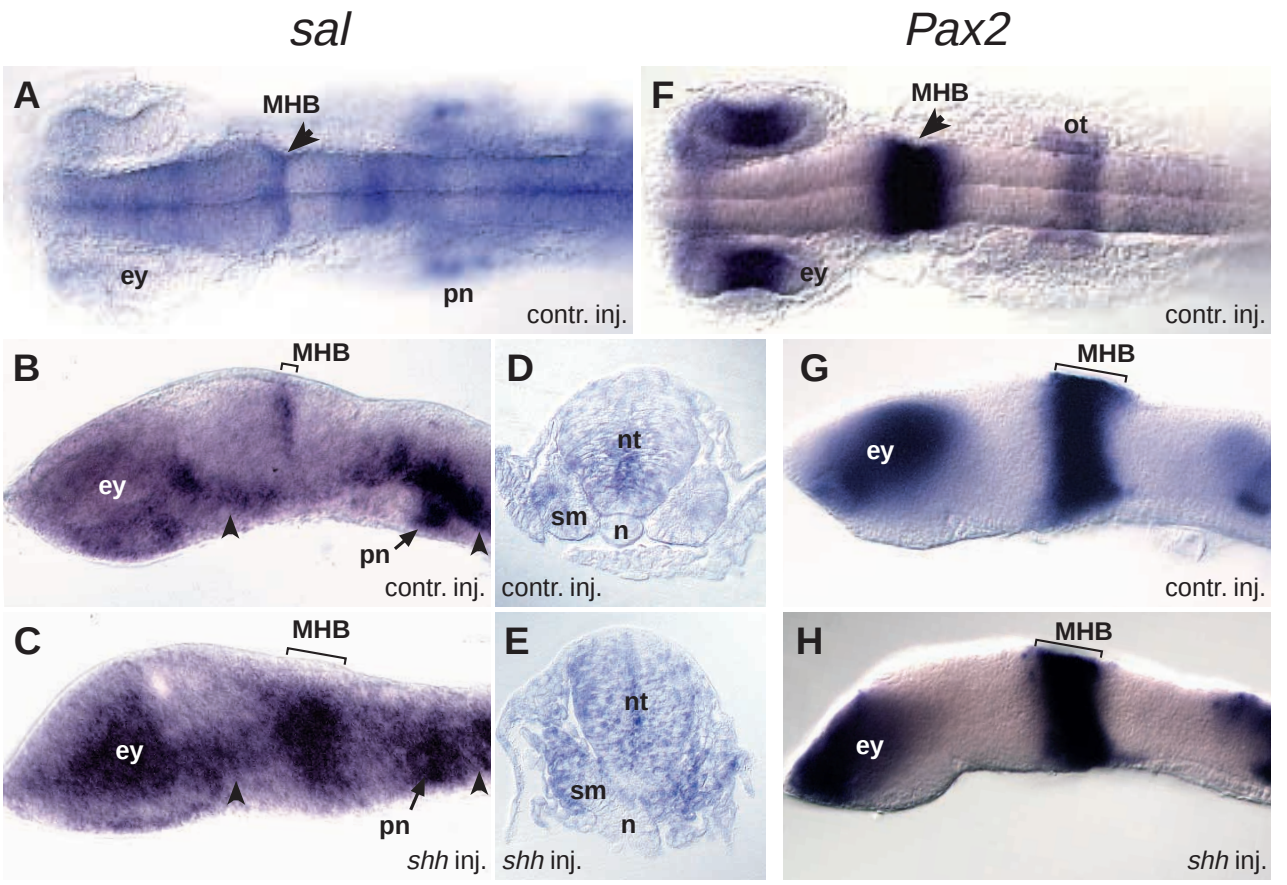


**Fig. 5.** *sal* expression domains adjacent to *shh* expression are expanded by ectopic *shh*. Detection of *sal* (A-E) and *Pax2* transcripts (F-H) at stage 22, anterior is left. Compare expression patterns in control (A,B,D,F,G; XEF1α, ambion) and *shh* injected (C,E,H) embryos. (A) *sal* stripe (arrowhead) in the MHB is within *Pax2* expression domain (F). Faint expression in the eye, restricted to proximal structures (dorsal view). (B) Lateral view of same embryo. (C) Upon *shh* injection *sal* expression expands distally in eye, dorsally in neural tube (arrowhead) and in pronephros (arrow). *sal* stripe is expanded in AP-direction. Compare dimensions of *sal* stripe (bar) with the *Pax2* domain in H. Cross sections of (D) control and (E) *shh*-injected embryos at the level of the spinal cord. *sal* expression in somites and neural tube is expanded. (F) Dorsal view shows *Pax2* expression in entire MHB and in proximal parts of the eye. (G) *Pax2* expression in control and (H) *shh* injected embryos. *Pax2* is detected in entire eye. Dimensions of MHB expression domain are unaffected. Abbreviations: ey, eye; MHB, mid-hindbrain boundary; n, notochord; nt, neural tube; ot, otic vesicle; pn, pronephros; sm, somite.

As *hh* signaling has not been found to be required for AP-patterning in the dorsal midbrain, we asked whether the experimentally induced HH effect on *sal* stripe expression is an indirect one, such as an HH-induced overproliferation of the *sal*-expressing cells. To clarify this, we examined the expression domain of *Pax2*, a cellular marker for the size of the MHB area (Krauss et al., 1992; Brand et al., 1996). Figs 5H, 6D show that *Pax2* expression in the eye depends on HH activity, as reported previously. But neither ectopic *shh* nor PKA* significantly altered the dimension of the MHB *Pax2* expression domain, indicating that the expansion of the *sal* stripe is not caused by cell proliferation events in response to ectopic HH activity. This suggests that *sal* expression in the MHB also depends directly on *hh* signaling.

## DISCUSSION

### *sal* is a general target of *hh* signaling

Signaling by *hh* and the vertebrate *hedgehog* gene family is a patterning mechanism involved in many different developmental processes in *Drosophila* and vertebrates. The secreted HH protein diffuses and binds to its receptor complex consisting of at least two multipass membrane spanning proteins, SMOOTHENED and PATCHED (Stone et al., 1996; Marigo et al., 1996a). The receptors *patched* in chicken and *ptc1* in zebrafish, which are the vertebrate homologues of *Drosophila patched*, are expressed in an *hh*-dependent manner in *hh* signaling centers adjacent to known sites of *hh* expression (Marigo and Tabin, 1996; Concordet et al., 1996). In vertebrates, target genes that can mediate *hh* patterning on the transcriptional level have been described only for a few signaling centers. Here we report the identification of a novel hedgehog target gene, the medaka gene *sal*. It encodes all features diagnostic for a DNA-binding zinc finger protein and thus, like its *Drosophila* homologue, it is likely to act as a transcriptional regulator. In contrast to other transcription factors which act as *hh* targets in a subset of the *hh* signaling centers, *sal* expression is found in all known *hh* signaling centers. *sal* expression expands in response to increased HH activity and is repressed in response to PKA* and thus, it

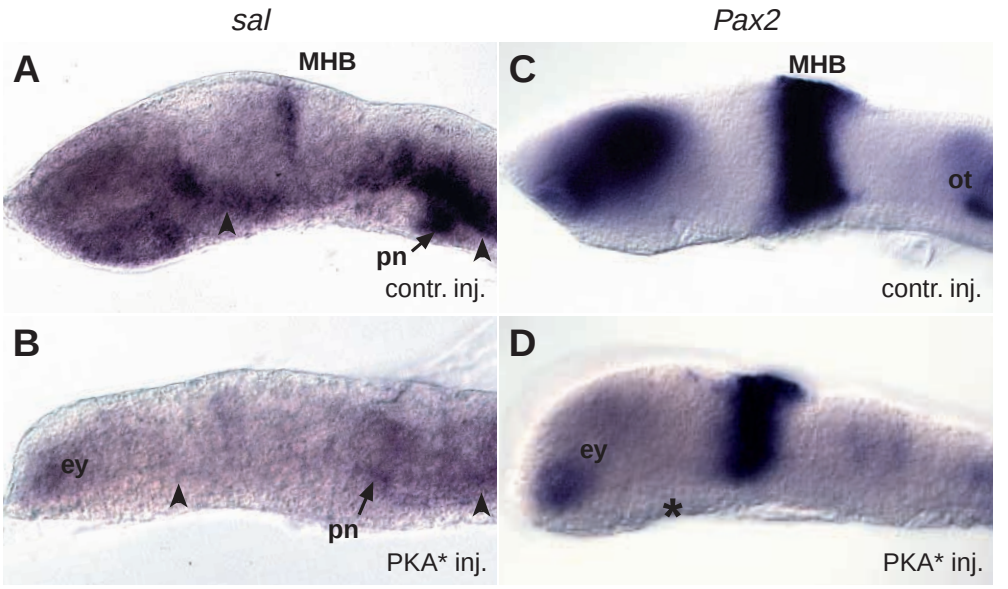


**Fig. 6.** Suppression of *hh* signaling prevents *sal* expression. Detection of *sal* (A,B) and *Pax2* transcripts (C,D) in lateral views of stage 22 embryos. Compare expression patterns of control (XEF1α, ambion; A,C) and PKA* injected (B,D) embryos. (A) Wt *sal* expression in the eye and ventral neural tube (arrowheads) as well as in the pronephros (arrow) is strongly reduced or completely absent in PKA* injected embryos (B). No *sal* stripe expression is detectable in the MHB. (C) *Pax2* expression in control and (D) PKA* injected embryos. Expression in the eye is suppressed by PKA* whereas expression in the MHB is unaffected. Axial mesoderm (asterisk) underlying the neural tube is extended towards anterior upon PKA* injection. Abbreviations as in Fig. 5

appears to be a general target of *hh* in the medaka fish. The finding of a competence area surrounding the wild-type *sal* expression domains provides some evidence for an immediate control of *sal* by *hh* although, at the moment, we cannot decide whether this control by *hh* signaling is direct or mediated.

*sal* expression is observed in all known *hh* signaling centers including the optic stalk and anterior optic vesicle where *shh* acts in proximodistal pattering. This domain of *sal* expression is expanded upon ectopic HH activity whereas blocking of *hh* signaling by PKA* prevents its expression. In the proximal part of the eye, *nk 2.2* (Barth and Wilson, 1995) and *Pax2* have been reported as *hh* target genes (Ekker et al., 1995b; Macdonald et al., 1995). Unlike *sal*, which is *hh* dependent in all *hh* signaling centers, the expression of *nk2.2* is limited to the proximal parts of the developing eye and the ventral part of the neural tube. *Pax2* shows a responsiveness to *hh* signaling only in the eye expression domain (Ekker et al., 1995b; Macdonald et al., 1995 and our results), while its expression in the MHB and at other locations does not depend on *hh* signaling.

*sal* expression in the neural tube, somites and limb buds is reminiscent of the expression pattern of the zinc-finger transcription factor GLI in chick (Marigo et al., 1996b) which was shown to be controlled by *shh* in the limb. In the neural tube SHH exerts its patterning activity in two phases. First, naive neural plate cells are converted to ventralized progenitors (positive for *sal* and GLI). In a second step SHH emanating from the floor plate directly induces motoneurons. Peak concentrations of SHH, which acts as a morphogen in the neural tube, induce exclusively the expression of floor plate markers (e.g. HNF3β/*axial*), while slightly lower concentrations induce expression of motoneuron specific markers and intermediate concentrations induce the expression of interneuron markers (Ericson et al., 1996). This patterning activity is reflected by the expression of *sal* which is initially present over the entire ventral part of the neural tube (excluded from the floorplate), but is subsequently refined to motoneurons and is later also detected in interneurons. In contrast, GLI is expressed in the ventral neural tube without further refinement (Marigo et al., 1996b). Late dorsal *sal* expression in sensory neurons could likely represent a long range induction through SHH secreted from the floorplate diffusing up through the lumen of the neural tube, as suggested for *patched* in chicken (Marigo and Tabin, 1996). This induction might also be due to the activity of other members of the *hh* family expressed in dorsal regions of the spinal chord like *banded hh* in *Xenopus* (Ekker et al., 1995a). Alternatively, dorsally expressed BMP4 (Liem et al., 1995), the vertebrate homologue of *Drosophila dpp*, which activates *spalt* in the *Drosophila* wing imaginal disc (Lecuit et al., 1996; Nellen et al., 1996; de Celis et al., 1996), may be required for late *sal* transcription in sensory neurons.

Ectopic expression of *shh* leads to an expansion of the wild-type *sal* expression domains and does not cause de novo expression of *sal*. In contrast the *hh* target genes zp50 (Hauptmann and Gerster, 1996) and *axial* (Strähle et al., 1993) are expressed at ectopic locations upon *shh* injection, indicating different competences in the *hh* response. In *shh* injected embryos *zp50* is expressed in ectopic domains with a drastic loss of wt expression domains (Hauptmann and Gerster, 1996). *axial*, like *sal*, upon *shh* injection exhibits a widening of its ventral neural tube expression. In addition ectopic de novo expression was detected in the MHB region (Krauss et al., 1993) indicating a competence for *hh* signaling. The widening of the *sal* expression domains upon *shh* injection is best explained by assuming that the ectopic *shh* adds to the endogenous concentration of HH and thereby increases the critical concentration thresholds of HH in the signaling centers. The finding that no de novo *sal* expression domains are observed indicates that additional competence factors determine the response to ectopic HH activity, which allow *sal* activation only in the competent tissue. Taken together, these results indicate that *sal* is indeed a specific and general target of *hh* signaling.

### Does the *sal* stripe define a new *hh* signaling center?

In addition to the known *hh* signaling centers we found that the *sal* expression domain at the MHB expands in response to *hh* signaling. Ectopic transplantation studies in chick and quail have demonstrated that the MHB, the junction of mid- and hindbrain, acts as an organizer that has a strong polarizing

activity on midbrain and hindbrain (Bally Cuif et al., 1992; Otero et al., 1993). So far the expression pattern and functional analysis of known *hedgehog* family members is consistent with a role in dorsoventral patterning of the CNS. However, no member of the *hh* family has been shown to be expressed in an area around the MHB nor has it been shown that HH activity is involved in AP-patterning at the MHB.

Our results, however, indicate that the *sal* stripe expression domain responds directly to ectopic *hh* signaling. This finding and the notion that *sal* is expressed in all known *hh* signaling centers, suggest that the *sal* stripe expression depends on non-detectable amounts of known members of the HH family of secreted molecules or on an unknown member of the family. This argument is consistent with the finding that ectopic PKA* represses *sal* expression in all these domains, including the MHB.

A distinct HH competence region could be established by cell migration events in the neural keel at the level of the MHB. In this view, a cluster of cells which is initially in contact with HH emanating from the ventral midline could migrate dorsally in a highly coordinated manner and give rise to the MHB region expressing *sal* in response to the initial SHH activity in the ventral midline. We have not observed dorsal migration of *sal*-expressing cells within the corresponding region and thus, such a mechanism underlying HH-dependent *sal* expression at the wild-type MHB appears unlikely.

A second possibility that may account for HH-dependent *sal* expression at the MHB is a localized dorsal competence responding to long range *shh* signaling after its ventral expression in the neural tube. The expression pattern of *patched*, one of the receptor components of the *hh* signaling cascade in the midbrain-hindbrain boundary of chicken (Marigo and Tabin, 1996), is consistent with this proposal. Additional evidence in support of this model is provided by the observation of ectopic MHB expression of another *hh* target gene, *axial* (Strähle et al., 1993) in response to ectopic *shh* (Krauss et al., 1993). *axial* is normally expressed at the ventral midline of the neural tube and in the diencephalic-mesencephalic boundary (Strähle et al., 1993). Upon injection of *shh* mRNA, *axial* is ectopically expressed in the region of the MHB (Krauss et al., 1993) indicating a localized competence to *hh* signaling in the MHB. In contrast to *sal*, which is detected by the early neurula stage in the dorsal part of the MHB area, the dorsal *patched* expression seems to evolve from a more ventrally localized expression domain. Marigo and Tabin (1996) suggest that *patched* expression in the MHB is likely to represent a long range action of the ventral SHH signal, facilitated through diffusion of SHH in the lumen of the neural tube. At the time when the *sal* stripe is first detected in the prospective MHB, a neural tube has not yet formed because of fish secondary neurulation (Papan and Campos-Ortega, 1994). This property of fish development makes it unlikely that long range diffusion of ventral SHH through the lumen of the neural tube would be the cause for the *sal* stripe expression, but we cannot rule out long range signaling through the neural keel itself.

A third scenario, based on the proposal of a local HH competence in the MHB, involves an additional novel source of *hh*. This proposes a novel member of the family, as none of the *hh* family members described so far has been shown to be expressed dorsally in the MHB. This could be induced by FGF8, a diffusible molecule prominently involved in the patterning at the MHB (Crossley et al., 1996a) and a member of the fibroblast growth factor family of secreted factors. FGF8 is required for AP patterning of the midbrain and it also mediates AP patterning in the developing limb where it induces *shh* expression in the ZPA (Crossley et al., 1996b). This link between the two signaling pathways in the limb and the dependence of the MHB *sal* stripe on *hh* signaling consistently argues for an unknown member of the *hh* family to be involved in the *sal* induction and pattern organizing activity at the MHB.

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## REFERENCES

**Bally Cuif, L., Alvarado Mallart, R. M., Darnell, D. K. and Wassef, M.** (1992). Relationship between Wnt-1 and En-2 expression domains during early development of normal and ectopic met-mesencephalon. *Development* **115**, 999-1009.

**Barrio, R., Shea, M. J., Carulli, J., Lipkow, K., Gaul, U., Frommer, G., Schuh, R., Jackle, H. and Kafatos, F. C.** (1996). The spalt-related gene of Drosophila melanogaster is a member of an ancient gene family, defined by the adjacent, region-specific homeotic gene spalt. *Dev. Genes Evolution* **206**, 315-325.

**Barth, K. A. and Wilson, S. W.** (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

**Bitgood, M. J. and McMahon, A. P.** (1995). Hedgehog and BMP genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

**Brand, M., Heisenberg, C.-P., Jiang, Y.-J., Beuchle, D., Lun, K., vanEeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nüsslein-Volhard, C.** (1996). Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. *Development* **123**, 179-190.

**Chang, D. T.** (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* **120**, 3339-3353.

**Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A.** (1996). Cyclopia and defective axial patterning in mice lacking Sonic Hedgehog gene function. *Nature* **383**, 407-413.

**Chitnis, A. B. and Kuwada, J. Y.** (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10**, 1892-1905.

**Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W.** (1996). Spatial regulation of a zebrafish patched homologue reflects roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.

**Crossley, P. H., Martinez, S. and Martin, G. R.** (1996a). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.

**Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R.** (1996b). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-136.

**de Celis, J. F., Barrio, R. and Kafatos, F. C.** (1996). A gene complex acting downstream of dpp in Drosophila wing morphogenesis. *Nature* **381**, 421-424.

**Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P.** (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-30.

**Ekker, S. C., McGrew, L. L., Lai, C.-J., Lee, J. J., v. Kessler, D. P., Moon, R. T. and Beachy, P. A.** (1995a). Distinct expression and shared activities of members of the *hegehog* gene family of *Xenopus laevis*. *Development* **121**, 2337-2347.

**Ekker, S. C., Ungar, A. R., Greenstein, P., v. Kessler, D., Porter, J. A., Moon, R. T. and Beachy, P. A.** (1995b). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

**Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessel, T. M.** (1996). Two critical periods of Sonic Hedgehog signalling required for the specification of notor neuron identity. *Cell* **87**, 661-673.

**Fan, C.-M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M.** (1995). Long-range sclerotome induction by sonic hedgehog: Direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signalling pathway. *Cell* **81**, 457-465.

**Fan, C.-M. and Tessier-Lavigne, M.** (1994). Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

**Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P.** (1996). Protein kinase A is a common negative regulator of Hedgehog signalling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.

**Hammerschmidt, M., Brook, A. and McMahon, A. P.** (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.

**Hauptmann, G. and Gerster, T.** (1996). Complex expression of the *zp-50 pou* gene in the embryonic zebrafish brain is altered by overexpression of *sonic hedgehog*. *Development* **122**, 1769-1780.

**Hollemann, T., Schuh, R., Pieler, T. and Stick, R.** (1996). Xenopus Xsal-1, a vertebrate homolog of the region specific homeotic gene spalt of Drosophila. *Mech. Dev.* **55**, 19-32.

**Iwamatsu, T.** (1994). Stages of normal development in the medaka *Oryzias latipes*. *Zoo. Sci.* **11**, 825-839.

**Jiang, J. and Struhl, G.** (1995). Protein kinase A and hedgehog signaling in Drosophila limb development. *Cell* **80**, 563-72.

**Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C.** (1994). Ectopic expression of sonic hedgehog alters dorsal-ventral patterning of somites. *Cell* **79**, 1165-1173.

**Joore, J., Fasciana, C., Speksnijder, J. E., Kruijer, W., Destrée, O. H. J., v. d. Eijnden-v. Raaij, A. J. M., de Laat, S. W. and Zirkovic, D.** (1996). Effects of retinoic acid on the expression of retinoic acid receptors during zebrafish embryogenesis. *Mech. Dev.* **55**, 3-18.

**Joyner, L. J.** (1996). Engrailed, Wnt and Pax genes regulate midbrain-hindbrain development. *Trends Genet.* **12**, 15-19.

**Kohlhase, J., Schuh, R., Dowe, G., Kühnlein, R. P., Jäckle, H., Schroeder, B., Schulz-Schaeffer, W., Kretzschmar, H. A., Köhler, A., Müller, U., Raab-Vetter, M., Burkhardt, E., Engel, W. and Stick, R.** (1996). Isolation, characterization and organ-specific expression of two novel human zinc finger genes related to spalt of Drosophila. *Genomics* **38**, 291-298.

**Krauss, S., Concordet, J.-P. and Ingham, P. W.** (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

**Krauss, S., Maden, M., Holder, N. and Wilson, S. W.** (1992). Zebrafish pax[b] is involved in the formation of the midbrain-hindbrain boundary. *Nature* **360**, 87-89.

**Krieg, P. A. and Melton, D. A.** (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucl. Acids Res.* **12**, 7057-7070.

**Kühnlein, R. P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J. F., Gehring, W. J., Jäckle, H. and Schuh, R.** (1994). Spalt encodes an evolutionary conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo. *EMBO J.* **13**, 168-179.

**Lawrence, P. A. and Struhl, G.** (1996). Morphogens, compartments, and pattern: lessons from Drosophila? *Cell* **85**, 951-961.

**Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M.** (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

**Lee, J. J., von Kessler, D. P., Parks, S. and Beachy, P. A.** (1992). Secretion and localized transcription suggests a role in positional signalling for products of the segmentation gene hedgehog. *Cell* **70**, 777-789.

**Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J. and Parkhurst, S. M.** (1995). Signal transduction by cAMP-dependent protein kinase A in Drosophila limb patterning. *Nature* **373**, 711-5.

**Li, W., Ohlmeyer, J. T., Lane, M. E. and Kalderon, D.** (1995). Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. *Cell* **80**, 553-62.

**Liem, K. F., Jr., Tremml, G., Roelink, H. and Jessell, T. M.** (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-79.

**López-Martinez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A. and Fallon, J. F.** (1995). Limb-patterning activity and restricted posterior localization of the amino-terinal product of Sonic hedgehog cleavage. *Curr. Biol.* **5**, 791-796.

**Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W.** (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

**Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J.** (1996a). Biochemical evidence that Patched is the Hedgehog receptor. *Nature* **384**, 176-179.

**Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J.** (1996b). Sonic hedgehog differentially regulates expression of Gli and Gli3 during limb development. *Dev. Biol.* **180**, 273-283.

**Marigo, V. and Tabin, C. J.** (1996). Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93**, 9346-9351.

**Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P.** (1995). Requirement of 19K form of sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

**Nellen, D., Burke, R., Struhl, G. and Basler, K.** (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

**Oliver, G., Loosli, F., Köster, R., Wittbrodt, J. and Gruss, P.** (1996). Ectopic lens induction in fish in response to the murine homeobox gene Six3. *Mech. Dev.* **60**, 233-239.

**Orellana, S. A. and McKnight, S.** (1992). Mutations in the catalytic subunit of cAMP-dependent proein kinase results in unregulated biological activity. *Proc. Natl. Acad. Sci. USA* **89**, 4726-4730.

**Otero, R. A., Sotelo, C. and Alvarado Mallart, R. M.** (1993). Chick-quail chimeras with partial cerebellar grafts an analysis of the origin and migration of cerebellar cells. *J. Comp. Neurol.* **333**, 597-615.

**Ott, T., Kaestner, K. H., Monaghan, A. P. and Schütz, G.** (1996). The mouse homolog of the region specific homeotic gene spalt of Drosophila is expressed in the developing nervous system and in mesoderm-derived structures. *Mech. Dev.* **56**, 117-128.

**Pan, D. and Rubin, G. M.** (1995). cAMP-dependent protein kinase and hedgehog act antagonistically in regulating decapentaplegic transcription in Drosophila imaginal discs. *Cell* **80**, 543-552.

**Papan, C. and Campos-Ortega, J. A.** (1994). On the formation of the neural keel and neural tube in the zebrafish Danio (Brachydanio) rerio. *Rouxs Archiv. Dev. Biol.* **203**, 178-186.

**Piperno, G. and Fuller, M. T.** (1985). Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J.Cell Biol.* **101**, 2085-2094.

**Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C.** (1993). Sonic Hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

**Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and et al.** (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-75.

**Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F. and Rosenthal, A.** (1996). The tumour-suppressor gene patched encodes a candidate receptor for sonic hedgehog. *Nature* **384**, 129-134.

**Strähle, U., Blader, P., Henrique, D. and Ingham, P. W.** (1993). *Axial*, a zebrafish gene expressed along the developing body axis, shows altered expression in *cyclops* mutant embryos. *Genes Dev.t* **7**, 1436-1446.

**Strutt, D. I. and Mlodzik, M.** (1995). Ommatidial polarity in the Drosophila eye is determined by the direction of furrow progression and local interactions. *Development* **121**, 4247-4256.

**Sturtevant, M. A., Biehs, B., Marin, E. and Bier, E.** (1997). The spalt gene links the A/P compartment boundary to a linear adult structure in the Drosophila wing. *Development* **124**, 21-32.

**Tabata, T. and Kornberg, T. B.** (1994). Hedgehog is a signalling protein with a key role in patterning Drosophila imaginal discs. *Cell* **76**, 89-102.

**Tanabe, Y., Roelink, H. and Jessell, T. M.** (1995). Induction of motor neurons by sonic hedgehog is independent of floor plate differentiation. *Curr. Biol.* **5**, 651-658.

**Wittbrodt, J. and Rosa, F. M.** (1994). Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin. *Genes Dev.* **8**, 1448-1462.

**Zardoya, R., Abouheif, E. and Meyer, A.** (1996). Evolutionary analyses of hedgehog and Hoxd-10 genes in fish species closely related to the zebrafish. *Proc. Natl. Acad. Sci. USA* **93**, 13036-13041.

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