The activity of Neurogenin1 is controlled by local cues in the zebrafish embryo

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SUMMARY

Zebrafish neurogenin1 encodes a basic helix-loop-helix protein which shares structural and functional characteristics with proneural genes of Drosophila melanogaster. neurogenin1 is expressed in the early neural plate in domains comprising more cells than the primary neurons known to develop from these regions and its expression is modulated by Delta/Notch signalling, suggesting that it is a target of lateral inhibition. Misexpression of neurogenin1 in the embryo results in development of ectopic neurons. Markers for different neuronal subtypes are not ectopically expressed in the same patterns in neurogenin1-injected embryos suggesting that the final identity of the ectopically induced neurons is modulated by local cues. Induction of ectopic motor neurons by neurogenin1 requires coexpression of a dominant negative regulatory subunit of protein kinase A, an intracellular transducer of hedgehog signals. Moreover, the pattern of endogenous neurogenin1 expression in the neural plate is expanded in response to elevated levels of Hedgehog (Hh) signalling or abolished as a result of inhibition of Hh signalling. Together these data suggest that Hh signals regulate neurogenin1 expression and subsequently modulate the type of neurons produced by Neurogenin1 activity.

Key words: Neurogenin, vertebrate neurogenesis, proneural genes, Sonic hedgehog, zebrafish

INTRODUCTION

Tissues flanking the neural plate, such as the notochord and the surface ectoderm, emit signals that result in the region-specific diversification of the neuroectoderm. Subsequently, signals generated within the neuroectoderm are involved in the control of region-specific differentiation of the neural tube (Tanabe and Jessell, 1996).

Neurogenesis is best understood in the fruit fly (Campos-Ortega, 1995). In Drosophila the neuroectoderm contains groups of cells, so-called proneural clusters, from which individual precursors are singled out to develop as neurons through a process known as lateral inhibition (Artavanis-Tsakonas and Simpson, 1991). Proneural clusters are characterised by expression of proneural genes such as achaete-scute and atonal which belong to the basic helix-loop-helix (bHLH) family of transcriptional regulators (Campuzano and Modolell, 1992; Jarman et al., 1993). These transcription factors control the expression of the neurogenic gene Delta (Kunisch et al., 1994). Evidence suggests that high levels of Delta expression in one cell of the proneural cluster leads to the activation of the Notch signalling pathway in neighbouring cells. Activation of Notch results in repression of the proneural genes, a concomitant decrease in Delta expression and thus drives cells into a differentiation program that will ultimately give rise to epidermis (Heitzler and Simpson, 1991).

The mechanisms leading to the establishment of neuronal fate are less well understood in vertebrates. The presence of genes homologous to Notch and Delta as well as to achaete-scute and atonal suggests, however, that similar mechanisms of neurogenesis may exist in vertebrates and flies (Lewis, 1996; Salzberg and Bellen, 1996; Lee, 1997). Overexpression of neuroD or neurogenin, vertebrate homologues of the Drosophila gene atonal, leads to the formation of ectopic neurons in Xenopus embryos suggesting that these genes share some properties with their Drosophila counterpart (Lee et al., 1995; Ma et al., 1996). Furthermore, the expression pattern of vertebrate Delta homologues within the neural plate suggests that a process similar to lateral inhibition acts within the neuroectoderm of the vertebrate embryo to single out particular cells to develop into neurons (Chitnis et al., 1995; Henrique et al., 1995). In accordance, misexpression of Delta results in inhibition of neurogenesis in Xenopus embryos (Chitnis et al., 1995).

Despite our growing understanding of the molecular mechanisms by which cells are singled out from proneural clusters to become neurons, much less is known about how the position of the proneural clusters is established or how distinct neuronal identities are subsequently specified. In vertebrates, Sonic hedgehog (Shh) expressed in the notochord underlying the neural plate and subsequently in the floorplate of the neural plate/tube induces ventral cell types such as motor neurons (Roelink et al., 1994; Marti et al., 1995a; Tanabe et al., 1995). Bone morphogenetic proteins (BMPs) expressed in the non-neural ectoderm flanking the neural plate, have been implicated in the specification of dorsal cell fates such as sensory neurons.
and neural crest cells (Liem et al., 1995). It is less clear, however, at which level the signalling centres controlling D/V pattern in the neural tube act on neurogenesis. It is possible that these centres directly regulate neurogenesis by inducing clusters of neuronal precursor cells at distinct dorsoventral coordinates of the neural tube that are homologous to proneural clusters of *Drosophila*. Alternatively, they may act downstream of neurogenesis by affecting the regionally distinct differentiation of neurons.

We report here the cloning and functional analysis of the proneural gene neurogenin1 (*ngn1*) from zebrafish. *ngn1* is expressed at early neurula stages in most, if not all, neurogenic regions of the neural plate. Misexpression of *ngn1* results in the formation of ectopic neurons in the ectoderm. Remarkably, distinct subtypes of neurons are ectopically induced in a region restricted manner suggesting that local cues modulate the activity of Ngn1. The activity of Ngn1 is modulated by lowering the activity of protein kinase A. Furthermore, we provide evidence which indicates that the expression of *ngn1* is directly regulated in the neural plate in response to ventralising signals. These data support the model that Hedgehog signalling acts on both the formation of proneural clusters immediately flanking the midline of the neural plate and the subsequent region-specific differentiation of neurons within this region.

**MATERIALS AND METHODS**

**Fish stocks, embryo production**
The wild-type zebrafish line wtOX is derived from fish purchased from the Goldfish Bowl, Oxford, UK, and has been bred for several years in the laboratory. Fish were bred and raised as described by Westerfield (1993).

**Cloning of neurogenin1 and neuroD**
PCR cloning from genomic DNA was accomplished using fully degenerate primers designed to the conserved peptide sequences NNRRNRMH and NYI Walt within the bHLH domain of MATH4 family proteins or PCY CKTQ and PPY GTMD within the loop of the bHLH domain and the conserved C-terminal region of human and *Xenopus* NeuroD; the resulting amplified fragments were subcloned into the EcoRI/BamHI sites of pBS II KS (Stratagene) and sequenced. Subsequent screening of a genomic library (Stachel et al., 1993) was carried out using one of the PCR fragments as probe; DNA was purified from positive phage using Nucleobond columns following the manufacturer’s instructions (Macherey-Nagel) and sequenced. The sequences have been submitted to GenBank (Accession numbers: *ngn1*, AF017301; *nrd*, AF017302). To confirm the identity of the predicted initiation methionine, 5’ RACE was performed on RNA purified from 18-hour old embryos as previously described (Frohman, 1990); the RACE products were subcloned and sequenced. To generate plasmid based clones for *ngn1* and *nrd* for use as templates for probes for in situ hybridisation, the predicted coding regions of the two genes were amplified using primers spanning the translation initiation and termination codons and subcloned. All other manipulations were performed as described by Sambrook et al. (1989).

**Plasmid construction and RNA injection**
The coding region of *ngn1* was amplified using Pfu polymerase (Stratagene) and subcloned into the expression vectors pCS2 and pCS2MT (Turner and Weintraub, 1994); to exclude the introduction of errors during amplification, all PCR generated constructs were sequenced. pCS2MT: *ngn1* contains the *ngn1* coding regions fused in-frame downstream of six copies of an epitope from the cMYC protein that is recognised by the monoclonal antibody 9E10 (Evan et al., 1985).

All expression constructs described above were linearised using *Not* I prior to transcription as were the *Xenopus* Delta-1 and Delta-1*Sm* constructs (Chitnis et al., 1995), pCS2:Shh (Strähle et al., 1997), pCS2MT:dnReg (Strähle et al., 1997) and the pCS2-mutPKACat (Concordet et al., 1996); pSP64Tnuc/gal (Vise et al., 1991) was linearised with *Xho* I. Capped RNAs were made using an SP6 transcription kit (Ambion) following the manufacturer’s instructions. The integrity of synthetic RNA was assessed on agarose gels and the RNAs were diluted to a final concentration of 200 μg/ml RNA and 0.2% phenol red immediately prior to injection. Injections into newly fertilised embryos were carried out using a gas driven microinjector (Eppendorf).

To normalise for variation between different injection experiments, large batches of one- to two-cell stage embryos were injected and split for the analysis with the various neuronal markers; the expression of the MYC epitope was also assayed as an indicator of the success of the injection. We have found that injection into one blastomere at the two-cell stage leads to a reasonable frequency of embryos which express the injected RNA unilaterally in neural plate stage embryos.

**In situ hybridisation and immunohistochemistry**
In situ hybridisation was performed essentially as described by Oxtoby and Jowett (1993). Immunohistochemistry using the mouse monoclonal antibodies zn12 (anti L2/HNK1; Trevarrow et al., 1990) and 9E10 (Evan et al., 1985) was carried out as previously described (Strähle et al., 1993, 1996).

β-gal RNA-injected embryos were fixed in BT-Fix (Westerfield, 1993) for 1-2 hours at 4°C, washed 3 times in 1x PBS, 0.1% NP40, washed once in β-gal staining buffer (1x PBS, 4 mM MgCl2, 3 mM K[Fe(CN)6], 3 mM K3[Fe(CN)6]) and incubated at 37°C in 1 ml β-gal staining buffer with 0.2% Xgal. After staining was complete, embryos were refixed in BT-fix overnight prior to analysis by immunohistochemistry with the zn12 antibody or in situ hybridisation with the *ngn1* DIG probe.

**RESULTS**

*ngn1*/Math4C genes form a subgroup distinct from *Xenopus* neurogenin-related and *ngn2*/Math4A genes
Previously, the mammalian *aortal* homologue 4A (*ngn2*/Math4A) gene was isolated from mouse in a two-hybrid screen for proteins that interact with the bHLH domain of MASH1 (Gradwohl et al., 1996). Subsequent low stringency screening of a murine genomic library led to the isolation of two additional members of the murine Math4 family (*ngn3*/Math4B and *ngn1*/Math4C; Cau et al., 1997). In order to isolate zebrafish homologues of this family, PCR against zebrafish genomic DNA was performed using degenerate oligonucleotide primers designed to conserved regions within the bHLH domain of the three murine members of the NGN/MATH4 family. Fragments isolated in this manner encoded a bHLH domain with high amino acid identity to NGN1/MATH4C. The complete coding region of zebrafish *ngn1* was determined by sequencing directly from genomic phage that were isolated using the zebrafish PCR fragment; like the three murine proteins, the putative zebrafish protein is encoded within a single exon. 5’ RACE indicated that the 5’ untranslated region of the mature transcript corresponding to the zebrafish gene begins 232 bps upstream of the initiation methionine predicted from the genomic sequence and contains no additional methionine residues (data not shown).
Alignment of the predicted amino acid sequence of the zebrafish Ngn1 protein with the three murine NGN/MATH4 proteins and two recently identified Xenopus family members (Xenopus neurogenin-related 1a and 1b, X-NGNR-1a and 1b; Ma et al., 1996) indicate that the putative zebrafish protein is most closely related to murine Ngn1/MATH4C both within (85% identity) and outside (31.3% identity) the bHLH domain (Fig. 1A; data not shown). Zebrafish Ngn1 is more distinctly related to murine NGN2/MATH4A and X-NGNR-1a and 1b, both within (83.3% and 78.3% identity, respectively) and outside the bHLH (15.4% and 14% identity, respectively). Thus, the ngn1/Math4C and zebrafish ngn1 genes form a branch of the family distinct from that of ngn2/Math4A, X-ngnr-1a and X-ngnr-1b (Fig. 1B).

**ngn1 is expressed early in the neural plate**

The spatial and temporal pattern of expression of zebrafish ngn1 was analysed by in situ hybridisation (Fig. 2). ngn1 is strongly expressed in the neural plate of embryos at the 3-somite stage. Anteriorly, expression is restricted to distinct clusters of cells including the primordia of the trigeminal ganglion (Fig. 2A,E). In more posterior regions of the neural plate, cells expressing ngn1 appear to be arranged in three rostralcaudal rows; while the medial stripe of ngn1 expression immediately overlying the notochord presumably represents precursors of primary motor neurons, the lateral domains of expression represent precursors of interneurons and sensory neurons. Interestingly, ngn1 expression is not uniform in these regions of the neural plate as solid clusters of highly expressing cells are separated by cells expressing lower levels of ngn1 both within specific rows and in the regions of the neural plate separating the rows (Fig. 2B-D,F). The most posterior neural plate surrounding the tail bud shows large regions devoid of ngn1 expression with the exception of stripes at the lateral margins of the neural plate and a group of cells overlying the notochord anlage in the tailbud (Fig. 2D). Examination of sections revealed that the expression of ngn1 is restricted to the neural plate and is not present in the underlying mesoderm (Fig. 2G). At 24 hours (h), ngn1 expression is found in many distinct regions of the brain; throughout the spinal cord and in the trigeminal and other cranial ganglia (data not shown).

The timing and pattern of expression of ngn1 in the neural plate suggests that it might be expressed in neural progenitor cells. If this were the case, one would expect that ngn1 expression should precede that of other neural markers. Zebrafish neuroD (nrd; for cloning of nrd see Materials and Methods) expression is first detected at the 1 somite stage in cells of the trigeminal ganglion, cells in the anterior forebrain and weakly in cells in the lateral neural plate, presumably corresponding to the Rohon-Beard sensory neurons (Fig. 3A,B). Like nrd, islet-1 (isl-1) expression in the neural plate is first detected in the trigeminal ganglion and Rohon-Beard cells but also in a subset

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**Fig. 1.** Sequence comparison of atonal related genes. (A) Alignment of the bHLH domains of zebrafish Neurogenin1 (Z Ngn1) and zebrafish NeuroD (Z Nrd) with those of related genes. NGN1/MATH4C (Cau et al., 1997), NGN2/MATH4A (Ma et al., 1996; Gradwohl et al. 1996), Xenopus Neurogenin Related 1a and 1b (X-NGNR-1a, X-NGNR-1b; Ma et al., 1996), NGN3/MATH4B (Sommer et al., 1996; Cau et al., 1997), murine NEUROD/BETA2/BHF1 (Lee et al., 1995; Naya et al., 1995), murine MATH3 (Takebayashi et al., 1997), murine MATH2/NEX-1 (Bartholoma and Nave, 1994; Shimizu et al., 1995), murine KW8/NEUROD2/NDRF (McCormick et al., 1996), murine MATH1 (Akazawa et al., 1995), *Drosophila* Atonal (Jarman et al., 1993). The consensus residues among NGN genes are indicated. (B) Radial phylogenetic tree of NGN and NEUROD family members distinct from that of Ngn1/MATH4C, X-NGNR-1a and X-NGNR-1b.
of motor neurons (Fig. 3C,D) (Korzh et al., 1993; Inoue et al., 1994). While ngn1 expression can already be detected at late gastrula stages (9h; Fig. 3F), the neural expression of nrd and isl-1 are not detected before the 1-somite stage (10.5h; data not shown). Furthermore, expression of ngn1 is stronger, is present in many more cells and extends more posteriorly in the neural plate at the 1-somite stage than either that of nrd or isl-1 (Fig. 3E and data not shown).

**ngn1 expression is modulated by lateral inhibition**

Expression levels of ngn1 are not uniform within the neural plate. Even within rows of expressing cells, significant variation in the level of ngn1 RNA is observed (e.g. Fig. 2B,F). In *Drosophila*, expression of the proneural bHLH genes within the proneural clusters is under the control of the Delta/Notch signalling cascade through a process known as lateral inhibition; it has recently been shown that lateral inhibition also operates during vertebrate neurogenesis (Chitnis et al., 1995). To test whether the levels of expression of ngn1 could be modulated by lateral inhibition, RNAs encoding wild-type *Xenopus* Delta-1 or Delta-1^Stu, an antimorphic variant of X-Delta-1, were injected into one blastomeres of a two cell stage embryo. To mark injected cells, embryos were co-injected with β-gal RNA and the location of the enzymatic activity was revealed prior to in situ hybridisation with ngn1 antisense probe.

While misexpression of X-Delta-1 abolishes or strongly reduces expression of ngn1 in 3 somite stage embryos (Fig. 4A,B; n=35), regions misexpressing the antimorphic X-Delta-1^Stu show an increase of ngn1 expression at this stage (Fig. 4C,D; n=48). Interestingly, the increase of ngn1 expression in X-Delta-1^Stu injected embryos is confined to the rows of cells normally expressing ngn1, where expression appears to become more uniform (Fig. 4C,D). In summary, these data suggest that ngn1 is a target of lateral inhibition.

**Misexpression of ngn1 induces the formation of ectopic neurons**

The early onset and the pattern of ngn1 expression in the neural plate and its sequence homology with *Drosophila* proneural genes suggested that it might act as a positive regulator of neural differentiation. To test this hypothesis, synthetic ngn1 RNA was injected into one- to two-cell stage zebrafish embryos and the expression of isl-1 (Korzh et al., 1993; Inoue et al., 1994) was analysed. Strong ectopic expression of isl-1 was detected in the non-neural ectoderm on the ventral side of injected embryos at the 3-somite and 18-somite stages (Fig. 5A-C,E,F); uninjected embryos did not display ectopic isl-1 expression (Fig. 5D). The effects of the injections are summarised in Table 1. At the 18-somite stage, a high proportion of injected embryos displayed ectopic isl-1 expression in the surface ectoderm overlaying the body axis (Fig. 5F). Interestingly, the number of isl-1-expressing cells within the neural tube of injected embryos was not significantly altered in comparison to uninjected controls (compare Fig. 5G with Fig. 5D). While cells expressing isl-1 ectopically were usually isolated in the yolk sac ectoderm at the 3-somite stage (Fig. 5A), isl-1+ cells in the yolk sac ectoderm of 18-somite stage embryos frequently formed clusters (Fig. 5C). isl-1+ cells in the ectoderm over the body axis remained isolated or formed smaller clusters of two to three cells (Fig. 5F). Consistently, injected embryos showed loss of one or both eyes and expansion of the isl-1 expression domain in the area of the trigrimal ganglion (Fig. 5E,H).

As isl-1 is also expressed in non-neural tissues such as the
pancreas and the prechordal plate mesoderm (Korzh et al., 1993; Inoue et al., 1994), we wished to confirm that induction of ectopic isl-1 expression corresponded to the induction of ectopic neurons. Injected embryos were immunohistochemically stained with the monoclonal antibody zn12 which is specific for the L2/HNK1 epitope expressed on Rohon-Beard sensory neurons, the trigeminal ganglion and a number of neuronal processes in the nervous system of 24h zebrafish embryos (Metcalfe et al., 1990; Trevarrow et al., 1990). Loss of the eye in injected embryos was correlated with the development of ectopic ganglion-like structures expressing the L2/HNK1 epitope (Fig. 5H). Moreover, injected embryos had ectopic neuronal processes in the surface ectoderm of the body axis and the yolk sac (Fig 5I and data not shown) indicating that misexpression of ngn1 induces ectopic neurons. As with isl-1, no change in the quantity and distribution of the L2/HNK1 epitope was detected in the neural ectoderm itself (data not shown). Injection of βgal RNA as control did not induce the ectopic expression of the L2/HNK1 epitope (Table 1; Fig. 5J,K).

The neuroectoderm itself appeared refractory to the induction of ectopic expression of neural markers in response to misexpression of ngn1 (compare Fig. 5F with G, and L with M). One explanation for this might be differential stability of the injected RNA/translated protein in the neuroectoderm. To address this possibility, a tagged version of Ngn1 (Ngn1-MYC) was constructed by fusing the ngn1 coding region in-frame downstream of 6 copies of a MYC-epitope, which is recognised by the monoclonal antibody 9E10 (Evan et al., 1985); the tagged Ngn1-MYC construct was functionally equivalent to the untagged version (Table 1). When one blastomere of the two-cell stage embryo was injected, embryos were frequently observed that had MYC epitope immunoreactivity predominantly on one side of the embryo only (Fig. 5N). The mosaic distribution of the Ngn1-MYC may explain the unilateral induction effects that we observed (e.g. Fig. 5H), but it cannot account for the inability of ectopic Ngn1 to induce isl-1 within the neural plate, as the neural plate of injected embryos expressed Ngn1-MYC at levels indistinguishable from levels

Fig. 3. ngn1 is expressed earlier and more widely than zebrafish nrd and isl-1. (A,B) nrd expression in 1-somite stage embryos. nrd is expressed in a row of cells in the anterior neural plate (arrowhead in A), the trigeminal ganglion (tg) and in two lateral rows of cells in the trunk neural plate (arrow in B). Dorsal views onto anterior neural plate (A) and at mid trunk level (B). (C,D) isl-1 expression in 1s embryos. isl-1 is expressed in the pillow (arrow head in C); the trigeminal ganglion (tg) and in two medial and two lateral rows of cells in the posterior neural plate (arrows in D). Dorsal views onto anterior neural plate (C) and at mid trunk level (D). (E) ngn1 expression in 1-somite stage embryo. Dorsal view onto neural plate at posterior hindbrain/anterior trunk level. (F) ngn1 expression at the 90% epiboly stage; view is dorsal onto the hindbrain anlage, anterior up. ngn1 is already expressed whereas isl-1 and nrd are not expressed in the late gastrula. The lateral extent of the neural plate is marked by arrowheads; arrows indicate the trigeminal ganglia, which are widely spaced at this stage. Abbreviations: tg, trigeminal ganglion.

Fig. 4. ngn1 expression is regulated by Delta. (A,B) Embryos at the 3-somite stage co-injected with X-Delta-1 and β-gal RNA into one blastomere at the 2-cell stage. Embryos were stained for β-gal activity (turquoise) before in situ hybridisation with ngn1 antisense probe (purple). ngn1 expression (arrows) is strongly reduced or completely abolished in the injected side (turquoise). (C,D) Embryos at the 3-somite stage co-injected with the antimorphic X-Delta-1Stu and βgal RNA into one blastomere at the 2-cell stage. Expression of X-Delta-1Stu results in up-regulation of ngn1 expression (purple, arrows) in the injected side (turquoise) in comparison to the uninjected control side. (A,C) Whole mounts in dorsal views onto the neural plate at the level of posterior hindbrain/anterior trunk; anterior up. B and D show flat preparation of different embryos from those in A and C, respectively; anterior up. Abbreviations: in, injected side; co, uninjected control side. Scale bar, 60 μm in A,C and 25 μm in B, D.
Fig. 5. Misexpression of ngn1 induces ectopic neurons. (A) Ventral view of 3-somite stage, ngn1 RNA-injected embryo showing ectopic isl-1 expression in the non-neural ectoderm of the yolk sac (arrowheads). (B) Transverse section through yolk sac of ngn1 RNA-injected embryo showing ectopic isl-1-expressing cell in the yolk sac ectoderm. (C,D) ngn1 RNA-injected embryo (C), and uninjected control (D), at the 18-somite stage hybridised to isl-1 probe. Ventral view of injected embryo (C) shows the ectopic isl-1-expressing cells in the yolk sac (arrowhead) forming cell clusters at this stage. Control embryo (D) is shown in lateral view. (E-G) ngn1-injected embryos analysed for isl-1 expression at the 18-somite stage. (E) Lateral view of the head with an expanded trigeminal ganglion (arrows, tg; compare to D); (F,G; lateral views, dorsal up). Optical sections at the level of the surface ectoderm (F) and the midline of the neural tube of the same injected embryo. Position of frames along the anteroposterior axis is indicated in D. While injected embryos develop numerous isl-1-expressing cells in the surface ectoderm (arrowheads in F) the number of isl-1-expressing Rohon-Beard (rb in G) and motor neurons (arrow in G) in the neural tube is unaltered in injected embryos (compare with uninjected control in D). (H) Head of ngn1 RNA-injected 24h embryo immunohistochemically stained with antibody zn12 which recognises the L2/HNK1 epitope. Embryo (dorsal view onto head, anterior left) shows loss of an eye and concomitant unilateral ectopic formation of a ganglion-like structure (arrowhead) and unilateral expansion of the trigeminal ganglion (arrows). (I) Optical section through surface ectoderm at the level of the hindgut of ngn1 RNA-injected 24h embryo immunohistochemically stained with antibody zn12. Neurons (arrowheads) with processes which contact each other develop ectopically in the epidermis of injected embryos. (J, K) View onto yolk sac (J) and optical section through body axis (K) of embryos injected with β-gal RNA. Embryos were stained for β-gal activity (turquoise) and immunohistochemically with antibody zn12 (brown). Expression of β-gal did not cause the formation of ectopic neurons. Only the normal expression of the L2/HNK1 epitope could be detected in injected embryos (K, arrowheads). (L,M) ngn1-myc RNA-injected embryo (L) and uninjected control (M) at the 3-somite stage hybridised to isl-1 probe. The embryo shown in L was injected in both blastomeres at the two-cell stage. Ectopic isl-1-expressing cells are found lateral to, but not within the neural plate. Views onto the neural plate at the level of the posterior hind brain/anterior trunk, anterior up. The optical section through the embryo shown in L is taken from a deeper position than the section in M to show ectopic neurons (arrowheads) on both sides of the neural plate. The normal pattern of isl-1 expression in the neural plate is therefore out of focus in the injected embryo. (N,O) Embryos injected with ngn1-myc RNA in one blastomere at the two-cell stage and immunohistochemically stained with anti-myc antibody 9E10 at the 3-somite stage to visualise ectopic expression of Ngn1-MYC. View is dorsal with anterior up (N) and optical transverse section through neural plate with dorsal up (O). Ngn1-MYC is strongly expressed in the neural plate (arrowhead in O) in the injected side (in). Lack of immunostaining on the uninjected side (co) shows that the MYC immunoreactivity is specific. Abbreviations: e, eye; y, yolk; tg, trigeminal ganglion; rb, Rohon-Beard sensory neurons; in, injected side of embryo; co, control side of embryo. Scale bar represents 50 μm in A,C,D,L,M,N,O; 25 μm in E,F,G,J,K; and 12 μm in B,I.
Table 1. Ectopic development of neurons in ngn1 injected embryos

<table>
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<th>Probe</th>
<th>% ectopic expression (n)</th>
<th>Stage</th>
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<tr>
<td>ngn1</td>
<td>isl-1</td>
<td>50% (173)</td>
<td>3s†</td>
</tr>
<tr>
<td>ngn1-myc</td>
<td>isl-1</td>
<td>56% (52)</td>
<td>18s†</td>
</tr>
<tr>
<td>ngn1</td>
<td>zn12</td>
<td>62.5% (32)</td>
<td>24h</td>
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†Somites.

Table 2. Ectopic activation of neural markers in the non-neural ectoderm in ngn1 injected embryos at the 3-somite and 24h stage

<table>
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<th>Injected RNA</th>
<th>Probe</th>
<th>% ectopic expression</th>
</tr>
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<tbody>
<tr>
<td>ngn1-myc</td>
<td>isl-1</td>
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<td>27% (49)*</td>
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<tr>
<td>ngn1-myc</td>
<td>hlx-1</td>
<td>67% (49)</td>
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The strong expression of the L2/HNK1 epitope on the surface of ectopically induced neurons in ngn1-injected embryos as well as their bipolar morphology and frequent aggregation into ganglion-like structures in the yolk sac ectoderm suggested that the ectopically induced neurons follow the specific differentiation pathway characteristic of Rohon-Beard or trigeminal ganglion neurons. To address this question more directly, the expression of markers of general neuronal character as well as markers for specific subtypes of neurons that differentiate at defined positions within the neural tube was analysed in ngn1-myc-injected embryos at the 3-somite and the 24h stages. For this experiment, a large batch of embryos was injected with ngn1-myc and split prior to analysis with the various markers in order to reduce potential differences between separate injection experiments.

Ectopic activation of isl-1 was detected in 66% of 3-somite stage embryos and 73% of 24h embryos, percentages that compare well with the presence of the MYC-epitope immunoreactivity in injected embryos (66%; Table 2). Similar to isl-1, which marks neurons of both dorsal and ventral character in the neural tube, the wild-type expression of islet-2 (isl-2) is detected in dorsal sensory neurons and motor neurons beginning at the 7-somite stage (Appel et al., 1995); isl-2+ motor neuron are distinct from isl-1+ motor neurons. Ectopic expression of isl-2 was not detected at the 3-somite stage in ngn1-injected embryos. By 24h, however, strong ectopic expression was detected in the ectoderm of both the body axis and the yolk sac, in a manner indistinguishable from ectopic isl-1 expression (Fig. 6C,D). In unmanipulated 24h embryos, nrd is strongly expressed in cranial ganglia and several other neuronal clusters in the head but only weakly and transiently in the Rohon-Beard cells of the spinal cord. We were unable to detect the expression of nrd in ventral motor neurons at any stage up to 28h (Fig. 3A,B; data not shown). Ectopic nrd expression was strongly induced by misexpression of ngn1 at both the 3-somite and 24h stages (Table 2; Fig. 6E-I). Cells expressing ectopic nrd were not, however, uniformly distributed in the non-neural ectoderm at the 3-somite stage as were those expressing isl-1. Rather, the induction of ectopic nrd appeared to be restricted to cells in the anterior half of the embryo.

Zebrafish achaete-scute homologue 1b (zash1b) is expressed in cells at an intermediate position along the dorsoventral axis of the spinal cord at 24h, presumably in cells of interneuronal character, and in a number of distinct regions in the brain (Allende and Weinberg, 1994). Misexpression of ngn1-myc resulted in an expansion of a dorsally located cluster of zash1b-expressing cells in the forebrain at the 3-somite stage (data not shown; Table 2). By 24h, additional ectopic zash1b+ cells were evident in the non-neural ectoderm (Fig. 6J-M; Table 2). Unlike ectopic isl-1+, isl-2+ and nrd+ cells, however, ectopic zash1b+ expressing cells were not widely distributed in the yolk sac ectoderm or in the ectoderm covering the body axis.

In contrast to the widespread pattern of expression of zash1b in putative interneurons in unmanipulated embryos, pax6b and hlx-1 are expressed only in subsets of interneurons (Fjose et al., 1994; Mikkola et al., 1992). We were unable to induce ectopic expression of pax6b at any stage (data not shown; Table 2) and ectopic hlx-1 expression was only detected in cells close to the hindgut (Fig. 6M).

Expression of lim-3 is detected in primary motor neurons beginning at the 5-somite stage, but it is not expressed in cells...
at dorsal positions in the spinal cord of the zebrafish embryo (Appel et al., 1995). We were unable to detect ectopic activation of \textit{lim-3} at either the 3-somite or the 24h stage in \textit{ngn1}-myc-injected embryos, suggesting that motor neurons are not induced by misexpression of \textit{ngn1} (data not shown). In summary, these data indicate that \textit{ngn1} misexpression induces ectopic neurons of dorsal and to a lesser extent lateral character but not of ventral character. The spatially restricted ectopic activation of \textit{nrd} and \textit{zash1b} may reflect the influence of local modulators of the fate of the ectopic neurons induced by misexpression of \textit{ngn1}.

### The activity of \textit{ngn1} is modulated by inhibition of protein kinase A

The Shh signalling pathway has been implicated in the induction of motor neurons (Marti et al., 1995; Roelink et al., 1995). Thus, Shh is a candidate for a local cue that regulates neuronal phenotype by modulating the activity of proneural genes such as \textit{ngn1} in the ventral neural plate. Our inability to induce the ectopic formation of motor neurons may reflect the lack of exposure of the non-neural ectoderm to the ventralising Shh signal. We, therefore, wondered whether it might be possible to induce motor neurons ectopically in \textit{ngn1}-injected embryos by concomitantly activating the Shh signalling pathway. Shh signalling leads to a down-regulation of protein kinase A (PKA) activity in responding tissues. To mimic signalling we co-injected a dominant negative regulatory subunit of mouse PKA (\textit{dnReg}; Clegg et al., 1992; Hammerschmidt et al., 1996; Strähle et al., 1997) with \textit{ngn1}; injected embryos were subsequently analysed using \textit{lim-3} as a motor neuron marker.

Co-injection of \textit{ngn1} with 100 ng/\textmu{l} \textit{dnReg} RNA resulted in the induction of ectopic \textit{lim-3}-expressing cells in non-neural ectoderm (Table 2; Fig. 7A). Lowering the concentration of co-injected \textit{dnReg} RNA led to a concomitant reduction in the frequency of ectopic \textit{lim-3} expression (Table 2). Ectopic expression of \textit{lim-3} in non-neural ectoderm was not induced by misexpressing of \textit{dnReg} RNA alone or increasing the concentration of \textit{dnReg} RNA co-injected with \textit{ngn1} to 400 ng/\textmu{l} (Table 2, see also Hammerschmidt et al., 1996). These data indicate that the identity of the neurons induced ectopically by misexpression of \textit{ngn1} can be changed by lowering PKA activity and suggest that Hh signals may act as local cues that modulate Ngn1 activity.

![Fig. 6. Misexpression of \textit{ngn1} causes ectopic development of distinct types of neurons (arrowheads) in a region specific manner. (A,B) \textit{ngn1}-myc-injected (A) and uninjected control (B) embryo hybridised to \textit{isl-1} probe at the 3-somite stage. Lateral views; anterior up. (C,D) \textit{ngn1}-myc-injected (C) and uninjected control (D) showing \textit{isl-2} expression at the 24h stage. Ectopic \textit{isl-2} expression is detectable in the non-neural ectoderm. Optical sections through surface ectoderm at the level of the hindgut extension. Dorsal up, anterior left. (E,F) Ectopic \textit{nrd} expression in \textit{ngn1}-myc-injected (E) and uninjected (F) control embryo at the 3-somite stage. \textit{nrd}-expressing cells are detectable in the anterior half of the yolk sac ectoderm. Lateral views, anterior up. (G-I) Ectopic \textit{nrd} expression is detectable over the yolk (G) and over the surface ectoderm covering the body axis in 24h \textit{ngn1}-myc-injected (I) but not in uninjected controls (H). \textit{nrd} expression in the nasal placodes is expanded in \textit{ngn1}-myc-injected embryos (arrow). Ventral views, anterior up (G,H). Lateral view, anterior left, dorsal up (I). (J-L) Ectopic \textit{zash1b} expression is found in cells lateral to the body axis in \textit{ngn1}-myc-injected (J,L) but not in uninjected controls (K). Dorsal view, anterior up (J,K). Lateral view, dorsal up, anterior left (L). (M) \textit{ngn1}-myc-injected embryos express \textit{hix-1} ectopically in cells in the endoderm. Scale bar: 50 \textmu{m} (A,B,E-H,J,K), 25 \textmu{m} (C,D,I,L) and 12 \textmu{m} (M).](image)
ngn1 expression is regulated by the Hh signalling pathway

A dorsal expansion of lim-3 expression was detected within the neural tube in embryos injected with dnReg RNA, irrespective of whether ngn1 RNA had been co-injected (compare Fig. 7B with C; see also Hammerschmidt et al., 1996). islet-1-expressing motor neurons at ventrolateral positions of the neural tube were also expanded dorsally (data not shown; also see Hammerschmidt et al., 1996). To investigate whether this dorsal expansion of motor neurons is paralleled by a change in ngn1 expression in the neural plate, we injected dnReg RNA and analysed ngn1 expression at the 5-somite stage.

Injection of dnReg RNA into both blastomeres at the two-cell stage led to a lateral expansion of ngn1 expression flanking the midline of the neural plate at the 5-somite stage (Fig. 8A). In 53% of injected embryos (n=51), the medial rows of ngn1 expression appear as two broad stripes flanking the midline rather than the fine rostrocaudal rows seen in wild-type embryos (compare Fig. 8A with 8C). The lateral rows of ngn1-expressing cells appear to be unaffected by misexpression of dnReg. The same effect on ngn1 expression was observed when shh RNA was injected (Fig. 8B). Thus, the dorsal expansion of lim-3 expression in the neural tube of one-day old dnReg-injected embryos is preceded by a lateral expansion of ngn1 expression at the neural plate stage.

To address this point further, RNA coding for a constitutively active form of protein kinase A (PKA*) was injected. Misexpression of PKA* blocks Shh signalling (Concordet et al., 1996; Hammerschmidt et al., 1996) and thereby would be expected to down-regulate ngn1 expression. Injection of PKA* into one blastomere of the two-cell stage embryo resulted in unilateral loss of ngn1 expression (Fig. 8D; n=45); injection of β-gal RNA alone did not cause loss of ngn1 expression (Fig. 8H). Expression of the zebrafish Delta homologue, delta B, which is expressed in a pattern similar to isl-1 in the 5 somite stage embryo (C. Haddon, J. Lewis, pers. comm.) is also abolished in PKA*-injected embryos, indicating that loss of ngn1 expression is accompanied by loss of primary neurons in both medial and lateral aspects of the neural plate (data not shown). Loss of ngn1 expression does not appear to be a consequence of overactivation of lateral inhibition in PKA*-injected embryos. We also regard it as unlikely that modulation of PKA activity decreases cell proliferation; the width of the neural plate is not significantly altered by small clones of PKA*-expressing cells in which ngn1 expression has been partially abolished (for example see Fig. 8D). We noted, however, a moderate expansion of the neural plate in embryos with large regions expressing PKA* (Fig. 8E,G). Taken together, these data are consistent with regulation of ngn1 expression by PKA and the notion that ngn1 expression is responsive to Hh signalling.

DISCUSSION

We report here the cloning and functional analysis of the zebrafish bHLH gene ngn1 which shares significant sequence homology with the murine ngn1/Math4C gene. The region-specific activity of misexpressed ngn1 in the embryo, as well as the effects of co-expression of the dominant negative regulatory subunit of PKA, suggests that ngn1 activity is regulated at the functional level by local cues; one such local cue being Shh. In addition, we provide evidence that induction of ngn1 expression provides one interface at which dorsoventral positional information is fed into the region specific development of neurons in the neural tube. Our data suggest that Hh signals are involved in both the establishment of ngn1 expression at the midline of the neural plate and its function once its expression has been established.

zebrafish ngn1 is a vertebrate proneural gene

Expression of proneural genes in Drosophila define equivalence groups of cells, the so-called proneural clusters, from which single cells are selected by lateral inhibition to become a neural precursor. Drosophila proneural genes positively control Delta expression in the proneural cluster. Delta protein in turn activates the Notch signalling pathway in neighbouring cells which leads to the cessation of proneural gene expression in these cells and their entry into an epidermal differentiation program. In contrast, the Delta-expressing cell within the proneural cluster maintains proneural gene expression and enters a neuronal differentiation program (Artavanis-Tsakonas and Simpson, 1991; Campos-Ortega, 1995).

ngn1 is initially expressed in broad regions of the neural plate.
including the medial and lateral expression domains which give rise to primary motor neurons and Rohon-Beard sensory neurons, respectively. Like its *Drosophila* counterparts, *ngn1* is expressed in more cells than the scattered primary neurons that eventually differentiate from these regions. As predicted from the maturation of a proneural cluster in *Drosophila*, the levels of *ngn1* expression vary from cell to cell within these regions of the neural plate. While misexpression of wild-type Delta inhibits *ngn1* expression, blocking lateral inhibition with an antimorphic Delta leads to an up-regulation of *ngn1* expression resulting in a more uniform level of expression within the endogenous domains of *ngn1* expression. Furthermore, misexpression of *ngn1* induced delta B expression in the zebrafish embryo (P. B., C. Haddon, J. Lewis and U. S., unpublished). Thus, *ngn1* appears to control zebrafish *delta* expression and at the same time is down-regulated by lateral inhibition, like proneural genes in *Drosophila*. A further characteristic of proneural genes is their ability to convey neural potential to ectodermal cells. In accordance, *ngn1* transforms ectodermal cells into neurons.

Zebrafish *ngn1* shares structural and functional properties with the related but distinct *Xenopus ngnr-1a* and *1b* (Ma et al., 1996) suggesting redundancy in gene function in vertebrate neurogenesis. Expression of mouse *ngn1/Math4C, ngn2/Math4A* or *ngn3/Math4B* (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996; Cau et al., 1997) induces ectopic activation of *isl-1* expression in the non-neural ectoderm of zebrafish embryos in a manner indistinguishable from zebrafish *ngn1* (P. B. and U. S., unpublished). Presently, we are addressing whether the zebrafish genome encodes other *ngn1* related genes.

Misexpression of *X-ngnr-1a* in *Xenopus* induces coherent patches of non-neuroectodermal cells to express neural markers at the neural plate stage. We never detected coherent regions of cells expressing neural markers ectopically in the zebrafish non-neural ectoderm at the neural plate stage in response to misexpression of *ngn1* or any of the mouse *ngn* genes. As *ngn2/Math4A*, the closest mouse homologue of *X-ngnr-1A*, induces the same pattern of scattered cells in the zebrafish yolk sac ectoderm, it is likely that the more widespread effects seen in *Xenopus* reflect differences in the responsiveness of the non-neural ectoderm of the two organisms rather than a difference in the inducing properties of the two genes.

**Function of *ngn1* is controlled by local cues**

*ngn1* appears to be expressed in most if not all neurogenic regions in the neural plate at the 3-somite stage when compared with the expression pattern of zebrafish *nrp* (this report), *isl-1* (Korzh et al., 1993; Inoue et al., 1994) or elav/HuC (Kim et al., 1996). Misexpression of *ngn1* induces a variety of neuronal subtypes, the expression of which only marginally precedes their endogenous expression in the neural plate (P. B. and U. S., unpublished). In addition, the temporal order of expression of different markers at ectopic positions appears to be the same as for the endogenous genes in the neural plate. These data suggest that the non-neural ectoderm has to acquire competence to respond to *ngn1* misexpression and subsequently undergoes the same temporal sequence of differentiation as cells in the neuroectoderm.

![Fig. 8. Expression of *ngn1* is modulated by Shh.](image)
Ectopic expression of neuronal markers was spatially restricted in embryos misexpressing \( \textit{ngn1} \); the restricted patterns of ectopic expression differed depending on the specific marker being used. While \( \textit{isl-1} \)- and \( \textit{isl-2} \)-expressing cells were found over the whole yolk sac, ectopic \( \textit{nd} \) expression was restricted to the anterior half of the yolk sac and ectopic \( \textit{zash1b} \) expression was restricted to the region immediately lateral to the body axis. These data strongly suggest that local cues modify the response of a cell to \( \textit{ngn1} \) expression.

Hh signalling appears to provide one local cue that modulates \( \textit{ngn1} \) function. Ectopic development of motor neurons was only observed when a dominant negative regulatory subunit of PKA was co-expressed with \( \textit{ngn1} \). Inhibition of PKA activity is a general mechanism by which Hh signals are transduced within target cells (Hammerschmidt et al., 1996). Shh induces floor plate and motor neurons in neural plate explants at a distinct concentration; high concentrations induce floor plate, while 2- to 3-fold lower concentrations are required for the specification of motor neurons (Roelink et al., 1995; Ericson et al., 1996). In agreement with this dose-dependent activity of Shh, we found that the concentration of co-injected \( \textit{dnReg} \) RNA was critical for the development of ectopic \( \textit{lim-3} \) expressing cells as concentrations both too high or too low were unable to induce \( \textit{lim-3} \) expression when co-injected with \( \textit{ngn1} \).

It is intriguing that the majority of the ectopic neurons found in the non-neural ectoderm of \( \textit{ngn1} \)-injected embryos were dorsal neural tube type neurons. In chicken embryos, the two members of the TGF\( \beta \) family, BMP4 and BMP7, have been implicated in the induction of dorsal neurons in the neural tube (Liem et al., 1995). In zebrafish, as in \textit{Xenopus}, the ventral regions of gastrulating embryos, from which the non-neural ectoderm is derived, is a source of BMP4 (Fainsod et al., 1994; Papan and Campos-Ortega, 1994; Schmidt et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Nikaido et al., 1997). It is tempting to speculate that \( \textit{ngn1} \) function may, therefore, also be modulated by BMP signals to yield neurons of dorsal character. Furthermore, low levels of BMP, perhaps in combination with low levels of Hh signals, may cause differentiation of interneurons. The restricted distribution of \( \textit{zash1b}^{+} \) ectopic interneurons in \( \textit{ngn1} \)-injected embryos may reflect just such a balance between dorsalising and ventralising signals.

In striking contrast to the ectopic development of neurons in the ectoderm, the neural plate appeared largely refractory to misexpression of \( \textit{ngn1} \). Development of additional neurons in regions of the neural plate which normally express \( \textit{ngn1} \) may be prevented by lateral inhibition. In regions which normally do not express \( \textit{ngn1} \), it is less likely, however, that a Delta/Notch mediated mechanism prevents activation of additional neurons. Misexpression of the dominant-negative form of Delta did not cause activation of \( \textit{ngn1} \) expression in regions normally not expressing the gene. Also, Delta genes are expressed in a pattern very similar to \( \textit{ngn1} \) in the neural plate of the zebrafish embryo (C. Haddon and J. Lewis; personal communication). These regions of the neural plate may be refractory to \( \textit{Ng} \) activity because co-activators are absent. Alternatively, during development of the peripheral nervous system in \textit{Drosophila}, regions outside the proneural clusters are characterised by the presence of inhibitors of proneural gene function such as \textit{hairy} and \textit{extramacrochaetae} (Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991, 1992; Ohsako et al., 1994; Van Doren et al., 1994). It is therefore also possible that the presence of repressors independent of the Delta/Notch signalling pathway block differentiation of ectopic neurons in non-neurogenic regions of the early neural plate.

\( \textit{ngn1} \) expression is regulated by hedgehog signals

While misexpression of \( \textit{ngn1} \) was not sufficient to induce additional neurons in the neural tube, misexpression of \( \textit{shh} \) or \( \textit{dnReg} \) led to ectopic induction of motor neurons at lateral aspects of the neural tube (Hammerschmidt et al., 1996; this report). The dorsal expansion of motor neurons at 24h was anticipated by a lateral expansion of the medial rows of \( \textit{ngn1} \) expression in the neural plate in \( \textit{shh} \) and \( \textit{dnReg} \)-injected embryos. Our data suggest that the medial rows of \( \textit{ngn1} \) expression are dependent on Hh signals secreted from the notochord and the floor plate, the endogenous sources of several Hh protein in the zebrafish (Krauss et al., 1993; Ekker et al., 1995; Currie and Ingham, 1996). As misexpression of \( \textit{ngn1} \) alone did not lead to ectopic muscle neuron induction in the neural tube, however, other factors induced by Hhs must be required in addition to \( \textit{ngn1} \) for development of motor neurons.

Misexpression of \( \textit{shh} \) or \( \textit{dnReg} \) leads to an expansion of ventral character that is reflected in both lateral expansion of \( \textit{ngn1} \) expression as well as ectopic development of motor neurons in lateral aspects of the neural tube. The neural tube is, however, not completely ventralised in injected embryos; Rohon-Beard cells appear to develop normally in dorsal regions of the neural tube. Thus dorsal specification cannot be inhibited by misexpression of \( \textit{dnReg} \) or \( \textit{shh} \), in agreement with previous findings (Hammerschmidt et al., 1996; Strähle et al., 1997). This might be a simple reflection of the fact that Patched, the Shh receptor, is not expressed in the dorsal-most regions of the neural tube (Concordet et al., 1996). In view of the complex pattern of expression of \( \textit{ngn1} \) in the neural plate it is clear that Hh signals cannot be the only determinants of \( \textit{ngn1} \) expression. \( \textit{ngn1} \) is likely to be the target of a number of signals acting along the AP and DV axis. It is possible that \( \textit{ngn1} \) expression in the lateral neural plate is controlled by BMP4/7 and that the interplay of the two signalling centres causes the striped pattern of \( \textit{ngn1} \) in the posterior neural plate. During development of the autonomous nervous system of the mouse, expression of another vertebrate proneural gene homologue, \textit{Mash1}, has been proposed to depend on BMP2 signalling (Shah et al., 1996). Inhibition of \( \textit{ngn1} \) expression in the lateral neural plate in PKA*-injected embryos may reflect the inability of endogenous signals other than Hhs to overcome artificially high levels of PKA activity; PKA* may thus act as a repressor of \( \textit{ngn1} \) expression in a dominant fashion.

In conclusion, Hh signals appear to be one class of regulators of \( \textit{ngn1} \) expression as well as modulators of the identity of the neurons induced by \( \textit{ngn1} \) activity. This dual function is in accordance with the postulated two step mechanism of motor neuron induction by Shh in chicken neural plate explants (Ericson et al., 1996). From antibody inhibition experiments it has been concluded that Shh first ventralises neural precursors which then are given a neuronal identity in a second Shh-dependent step. Our data suggest that, during neurogenesis in
the zebrafish embryo, the initial step of the two step mechanism of Shh action may involve the induction of ngn1 expression.

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