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Distinct regulators of *Shh* transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node

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SUMMARY

The establishment of the floor plate at the ventral midline of the CNS is dependent on an inductive signaling process mediated by the secreted protein Sonic hedgehog (Shh). To understand molecularly how floor plate induction proceeds we identified a Shh-responsive regulatory element that directs transgene reporter expression to the ventral midline of the CNS and notochord in a *Shh*-like manner and characterized critical cis-acting sequences regulating this element. Cross-species comparisons narrowed the activity of the *Shh* floor plate enhancer to an 88-bp sequence within intron 2 of *Shh* that included highly conserved binding sites matching the consensus for homeodomain, Tbx and Foxa transcription factors. Mutational analysis revealed that the homeodomain and Foxa binding sites are each required for activation of the *Shh* floor plate enhancer, whereas the Tbx site was required for repression in regions of the CNS where *Shh* is not normally expressed. We further show that Shh enhancer activity was detected in the mouse node from where the floor plate and notochord precursors derive. Shh reporter expression was restricted to the ventral (mesodermal) layer of the node in a pattern similar to endogenous *Shh*. X-gal-positive cells emerging from the node were only detected in the notochord lineage, suggesting that the floor plate and notochord arise from distinct precursors in the mouse node.

Key words: *Shh*, Floor plate, Notochord, Node, Gene regulation, Central nervous system

INTRODUCTION

The floor plate comprises a highly specialized group of glial cells that occupy the ventral midline of the neural tube in vertebrate embryos. Although the size and shape of the floor plate differs from species to species, several of its important functions appear to be conserved. The floor plate has been deemed an organizing center given that it serves as a source of signals required to pattern the neural tube along the dorsal/ventral axis, attract and repel axons, as well as provide trophic support to axons en route to their final target destination (Placzek, 1995; Burstyn-Cohen et al., 1999; Wang and Tessier-Lavigne, 1999; Giger and Kolodkin, 2001; Charron et al., 2003). In the absence of a functional floor plate the ventral midline becomes highly disorganized because of a failure in the specification of some ventral neuronal progenitors and the lack of guidance cues for other neurons (Matise et al., 1998; Matise et al., 1999; Ding et al., 1998; Charron et al., 2003).

The origin of the floor plate has been the subject of ongoing controversy (Placzek et al., 2000; Le Douarin and Halpern, 2000). Clarification of where and when the floor plate derives bears significantly on understanding the molecular mechanisms underlying its development. The prevailing hypothesis stipulates that an instructive signaling event between the axial mesoderm and the medial cells of the overlying neural plate results in floor plate induction (Jessell, 2000). A challenge to this model has been proposed which attests that in chick, zebrafish and frog embryos, the ventral midline of the neural tube is derived from a population of precursors shared with the notochord that are situated in Henson's node (chick), the organiser (frog) and the embryonic shield (zebrafish) (Le Douarin and Halpern, 2000; Lopez et al., 2003). Segregation of these precursors into either a floor plate or notochord lineage is thought to depend, at least in zebrafish and frogs, on delta-notch signaling (Appel et al., 1999; Lopez et al., 2003). In the chick, floor plate precursors come to occupy their ultimate position in the CNS by intercalating into the medial aspect of the neural plate (Catala et al., 1996; Teillet et al., 1998). Although these two models of floor plate development are not mutually exclusive their compatibility in mammals remains to be determined.

Much of our knowledge of floor plate development has emerged from the study of the Sonic hedgehog (Shh) signaling pathway. With the observation that Shh, a secreted protein, is expressed in the node and notochord prior to floor plate differentiation it was deemed an ideal candidate to mediate the induction of the floor plate and other ventral neuronal cell types (Echelard et al., 1993; Roelink et al., 1995; Marti et al., 1995; Ericson et al., 1996). Several studies have determined that Shh is indeed the notochord-derived signal that promotes patterns of cellular growth and differentiation within the ventral neural tube including the homeogenetic (like-by-like) induction of its

own expression in the floor plate (see Jessell, 2000). Misexpression of *Shh* or downstream effectors in its signal transduction pathway is sufficient to induce the formation of an ectopic floor plate (Echelard et al., 1993; Sasaki and Hogan, 1994; Roelink et al., 1995; Ruiz i Altaba et al., 1995; Epstein et al., 1996; Hynes et al., 1995; Hynes et al., 1997; Lee et al., 1997). Moreover, loss of Shh function results in mouse embryos lacking a floor plate (Chiang et al., 1996).

The Shh-dependent pathway resulting in floor plate formation relies on triggering a transcription factor cascade culminating in the stable expression of *Shh* in the ventral midline of the neural tube. Shh signaling from the notochord activates Gli2, a zinc-finger transcriptional regulator, in the overlying neural plate (Matise et al., 1998; Ding et al., 1998). Gli2, which is required for floor plate development, is responsible for initiating the transcription of Foxa2 (formerly Hnf3b) (Sasaki et al., 1997; Matise et al., 1998; Ding et al., 1998). Although, the misexpression of *Foxa2* in the CNS can under certain conditions result in the ectopic activation of *Shh*, it remains unclear whether Foxa2 is required to regulate *Shh* transcription within sites of endogenous expression including the floor plate (Ruiz i Altaba et al., 1995; Hynes et al., 1997). Attempts at addressing this question through conventional loss-of-function studies is confounded by the requirement for Foxa2 in node formation, resulting in *Foxa2*$^{-/-}$ embryos that lack both the notochord and floor plate (Ang and Rossant, 1994; Weinstein et al., 1994).

Efforts to determine whether Foxa2 and/or other genes regulate *Shh* transcription in the floor plate have thus turned to the analysis of cis-acting sequences controlling *Shh* expression (Epstein et al., 1999; Müller et al., 1999; Müller et al., 2000; Goode et al., 2003). Multiple enhancers were shown to regulate the expression of *Shh* at discrete positions along the anteroposterior axis of the mouse neural tube (Epstein et al., 1999). Regulatory sequences mediating the activity of two of these enhancers, *Shh* brain enhancer 1 (Sbe1) and *Shh* floor plate enhancer 2 (Sfpe2), were found to reside on contiguous DNA fragments within the second intron of the *Shh* gene. Sbe1 activity alone was sufficient to direct reporter expression to rostral regions of the CNS including the ventral midline of the midbrain and portions of the diencephalon. Importantly, the combination of Sbe1 and Sfpe2 activities were required to direct reporter expression to more caudal regions of the neural tube including the floor plate of the hindbrain and spinal cord, as neither enhancer on its own was sufficient for this expression (Epstein et al., 1999). Further indication of the cooperative nature of these two enhancers came from mutational studies showing that the deletion of two Foxa2 binding sites in sequences mediating Sbe1 activity compromised the ability of Sfpe2 to consistently drive reporter expression to the floor plate (Epstein et al., 1999). These data suggest that Foxa2 must be acting with additional transcription factors to regulate *Shh* expression in the floor plate. To ascertain the identity of the cooperating transcription factors we sought to uncover the critical regulatory sequences mediating Sfpe2 activity.

We report here that cross-species comparison of intron 2 sequences from mouse, human, chicken and zebrafish have narrowed Sfpe2 activity to an 88-bp interval. A key feature of this floor plate element is its responsiveness to Shh signaling, probably reflecting that the transcription factors promoting *Shh* expression in the floor plate are activated by notochord-derived Shh. Further inspection of the 88-bp sequence identified three highly conserved binding sites matching the consensus for homeodomain, T-box related (Tbx) and Foxa transcription factors. Our studies reveal that the combined action of homeodomain and Foxa2 proteins is required to positively regulate transcription from the *Shh* floor plate enhancer, whereas the Tbx factor has a role in repressing transcription from areas of the CNS where *Shh* is not normally expressed. Because Sfpe2 activity was also observed in the node of early somite-stage embryos it provided the opportunity to trace the lineage of *Shh*-expressing cells from this structure. Interestingly, both Sfpe2 reporter activity and *Shh* expression were restricted to the ventral (mesodermal) layer of the node and notochord plate with no apparent mixing of X-gal-positive ventral cells with X-gal-negative dorsomedial cells (prospective floor plate). Thus, in contrast to the chick, floor plate cells in the mouse embryo do not derive from precursors shared with the notochord that subsequently insert into the neural plate, but are generated by inductive Shh signaling that initiates in the node.

MATERIALS AND METHODS

Generation of reporter constructs

All regulatory sequences assayed were cloned into the NotI restriction site of a reporter vector comprising the *Shh* promoter (1.1 kb *Eco*RI-*Sac*II), *lacZ* cDNA, SV40 large T antigen poly(A) site, and sequences mediating Shh brain enhancer-1 (Sbe1) activity (0.5 kb *Eco*RI-*Xho*I) in an arrangement that keeps with the native orientation of the *Shh* gene. The generation of reporter constructs 1 and 2 (Rc1 and Rc2) was reported previously (Epstein et al., 1999). DNA sequences tested in Rc3-8 were generated by PCR amplification, the primer sequences for which are available upon request.

To generate the mouse HR-c trimer (Rc9), excess amounts of the 88 bp HR-c fragment, derived from Rc7 upon NotI digestion, were used for ligation into the NotI site of the same reporter construct yielding multi-copy clones. Rc9 comprises a total of 3 copies of the HR-c fragment in the reverse orientation with respect to the *lacZ* cDNA. HR-c sequences from chicken and zebrafish were generated by PCR amplification of their respective genomic DNA using the following primers: Rc10, (E133) 5′-ATTAGCGGCCGCGG-ATTTTAATTAGAGAACCCAAA-3′ and (E134) 5′-ATTAGCGG-CCGCTAGTAGTGTAGCAGCTCTACAAA-3′; Rc11, (E138) 5′-ATTAGCGGCCGCGGTTTTTAATTAGAGCAGTCCAGG-3′ and (E139) 5′-ATTAGCGGCCGCTAGGCTGTAAAGCTTCTCTAAA-AAAC-3′. The orientation of the trimeric HR-c fragments cloned in Rc10 and Rc11 is the same as that in Rc9. The E number in parentheses refers to the lab stock code for each primer.

To test the requirement of the homeodomain binding-site in HR-c (Rc12), the following pair of PCR primers were used to create a small deletion overlapping the TAATTA core motif at the 5′ end of HR-c: (E59) 5′-ATTAGCGGCCGCCCACACAAGTCTCGGGCTTTCA-CACC-3′ and (E34) 5′-ATTAGCGGCCGCTAGTCGTTGTAGCA-ACTGGACAAACATTCCAGAGGTTTGC-3′. Point mutations designed to disrupt DNA binding at the recognition sequences for FoxH1, T-box protein and Foxa2 were constructed by PCR with the following primer pairs. The mutated residues are underlined. FoxH1, (E33) 5′-ATTAGCGGCCGCGGATTTTAATTAGAAAATCCGAG-CAAGTCTCGGGCTTTCA-3′, and (E34); T-box (Rc14), (E147) 5′-ATTAGCGGCCGCGGATTTTAATTAGAAAATCCACACAAGTC-TCGGGCTTTGGGGGCTTGGGCAAACCTCTGG-3′ and (E34); T-box (Rc14.1), (E243) 5′-ATTAGCGGCCGCGGATTTTAATTAG-AAAATCCACACAAGTCTCGGGCTTTCATTTCTTGGGCAAAC-

CTCTGG-3′ and (E34); Foxa2, (E32) and (E86) 5′-ATTAGCGGCCGCTAGTCGTTGTAGCAACTGGCGGGGGCTTC-CAGAGGTTTGCCCAAGGTG-3′. Each of the PCR products was cloned into the *Shh* reporter cassette as a trimer to generate Rc13-15. The integrity and orientation of all constructs containing PCR-generated fragments was confirmed by DNA sequencing.

Production and genotyping of transgenic mice

Transient transgenic embryos or mouse lines were generated by pronuclear injection into fertilized eggs derived from either (BL6xSJL)F_1 (Jackson Labs) or FVBN (Charles River) strains essentially as described (Hogan et al., 1994). Transgenes were prepared for microinjection as described (Epstein et al., 1996). The genotyping of embryos or adult mice carrying reporter constructs was performed by PCR using Proteinase K-digested yolk sacs or tail biopsies as DNA templates. An upstream primer directed against the *Shh* promoter (E56) 5′-GACAGCGCGGGGACAGCTCAC-3′ and a downstream primer directed against *lacZ* (E68) 5′-AAGGGCGATCGGTGCGGGCC-3′ were used as described (Epstein et al., 1999). The $Shh^{+/-}$ animals were kindly provided by H. Westphal (NIH) (Chiang et al., 1996) and maintained on a CD-1 background (Charles River). $Ptc^{lacz/+}$ animals were procured from the Jackson Labs (Bar Harbor, ME, USA).

Whole-mount β-galactosidase and in situ hybridization

The assessment of β-galactosidase activity was performed by histochemical staining using either X-gal (GibcoBRL) or Salmon-gal (Biosynth) as substrates (Epstein et al., 2000). Transgenic embryos were stained from 30 minutes to overnight depending on the strength of transgene expression. Whole-mount RNA in situ hybridization was performed essentially as described (Matise et al., 1998) using digoxygenin-UTP-labeled *Shh*, *Foxa2* (B. Hogan), and *lacZ* riboprobes. After whole-mount staining some embryos were fixed in 4% paraformaldehyde, embedded in 4% agarose and sectioned on a vibratome at 50-75 μm.

Neural explant cultures

Embryos generated from matings between $Rc9^{+};Shh^{+/-}$ males and $Shh^{+/-}$ females were dissected in ice-cold L15 medium (GibcoBRL) between 8.0 and 8.5 dpc (3-6 somites), and dissociated in 1 mg/ml dispase (Boehringer Mannheim) essentially as described (Alder et al., 1999). Neural tissue isolated from the presumptive anterior spinal cord was bissected along the midline and cultured on Transwell filters (0.4 μm, Costar) floating on 47.5% Dulbecco's minimum essential medium (Specialty Media), 47.5% F-12 Ham's nutrient mixture (GibcoBRL), 2 mM glutamine (GibcoBRL), 100 U/ml penicillin-streptomycin (GibcoBRL), and 5% rat serum (Harlan) in the presence or absence of 1 μM recombinant Shh-N protein. After 30 hours of culture in a CO_2 incubator at 37°C, explants were fixed and stained with X-gal. The genotype of each neural explant was established by PCR using DNA from Proteinase K digested yolk sacs as template with the following primers directed against neo (to detect the mutant *Shh* allele) (E131) 5′-GAACAAGATGGATTGCACGCAG-3′ and (E132) 5′-TTCAGTGACAACGTCGAGCACA-3′); *Shh* intron2 (to detect the wild-type allele) (E133) 5′-TGAGCAGCGGTAATC-CAGCC-3′ and (E134) 5′-CTCCAGGATCATGCTTTTGGC-3′; and the Rc9 transgene (E56 and E68).

RESULTS

Comparative sequence analysis identifies a conserved *Shh* regulatory element

Given that vertebrate species show similar patterns of *Shh* expression in the CNS and that regulatory sequences directing floor plate expression have been localized to intron 2 in mouse, chicken and zebrafish (Epstein et al., 1999; Müller et al., 1999), we explored the premise that conservation of sequence underscores conservation of function. Comparative sequence analysis of the 746-bp fragment of mouse DNA previously attributed with Sfpe2 activity was undertaken with comparable regions from human, chicken and zebrafish using ClustalW algorithms (MacVector, Oxford Molecular) (Fig. 1A). As expected, alignment of mouse and human sequence showed the highest degree of overall homology at 67%. Conservation of chicken and zebrafish sequences was found on average to be lower when compared to that of mouse, with homologies of 44% and 36%, respectively. On closer inspection however, the 4-way alignment revealed higher homology scores over short stretches of sequence compared to the overall average. Of these short stretches of sequence, three homologous regions corresponding to HR-a (nucleotide position: 60-139), HR-b (184-233) and HR-c (221-308) were remarkable given that all of the 2-by-2 comparisons between mouse and the individual species in question displayed higher homologies than the overall average for that species (Fig. 1A).

To test the functional relevance of the conserved sequences, each of the homologous regions was assayed for its ability to direct transgene expression to the floor plate of the hindbrain and spinal cord either independently or in combination. A series of reporter constructs (Rc1-8) were generated that contained a *lacZ* reporter cassette cloned upstream of fragments of the 746-bp region mediating Sfpe2 activity (Fig. 1B). The Sbe1 region, which is also required for floor plate expression, served as an internal control for transgene expression (gray oval in Fig. 1A,B). Transgenic embryos were generated with each of the reporter constructs and assayed for Sfpe2 activity at 9.5 dpc by X-gal staining. Whereas transgenic embryos carrying Rc3 or Rc4 showed strong X-gal staining in cranial regions similar to Rc1 they failed to express *lacZ* in the floor plate, suggesting that neither HR-a nor HR-b (in conjunction with Sbe1) was sufficient for this expression (Fig. 1B). When tested in combination (Rc5), the two regions still failed to direct transgene expression to the floor plate ruling out the possibility that they operate in tandem. Transgenic embryos expressing Rc7, however, showed X-gal staining in the ventral midline throughout the length of the spinal cord, supporting a role for HR-c in mediating Sfpe2 activity (Fig. 1B,C). In comparison to embryos carrying the full 746-bp element, those transgenic for Rc7 showed consistently weaker staining in the floor plate, suggesting that HR-c probably operates in conjunction with additional sequences (Fig. 1C: compare Rc2 with Rc7). To determine whether HR-c was required for Sfpe2 activity we deleted the 88-bp fragment from the full-length 746-bp regulatory region (Rc8). Of the 10 transgenic embryos carrying Rc8, 6 displayed very weak and patchy X-gal staining in the floor plate whereas the other 4 showed a complete absence of spinal cord expression despite the presence of strong staining in the midbrain (Fig. 1C). These results suggest that an 88-bp fragment encompassing HR-c constitutes an essential component of Sfpe2 and that in cooperation with Sbe1, HR-c is sufficient to direct *lacZ* expression to the floor plate in a *Shh*-like manner.

HR-c constitutes a functionally conserved floor plate and notochord enhancer

If one copy of HR-c is sufficient to interact with Sbe1 for floor

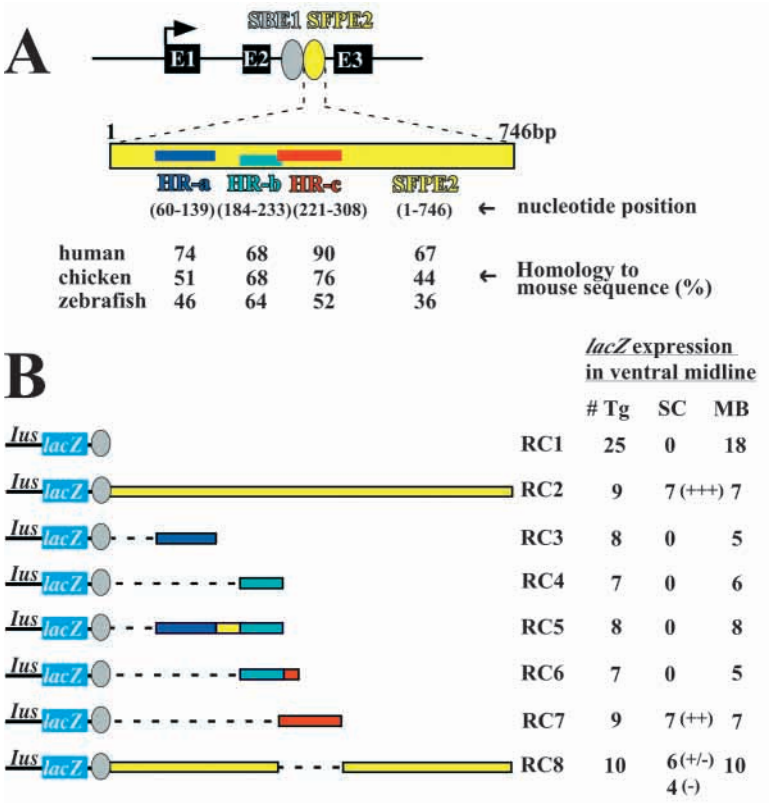


Fig. 1. Conservation of *Shh* regulatory sequences across phyla. (A) A schematic of the *Shh* locus showing the location of coding exons (black boxes), noncoding sequences (solid line) and intronic enhancers (gray oval, Sbe1; yellow oval, Sfpe2). The sequence of the 746 bp region of intron 2 overlapping Sfpe2 activity in the mouse was compared with human, chicken and zebrafish, identifying three regions of high sequence homology corresponding to homology region-a (HR-a, blue), HR-b (green) and HR-c (red). (B) Reporter constructs (RC) designed to assay Sfpe2 activity. DNA segments from the 746 bp region were cloned downstream of a reporter cassette containing a minimal *Shh* promoter (Ius), *lacZ* gene (light-blue box) and Sbe1 sequences (gray oval). To the right of the constructs are the results of the transgenic expression analysis indicating the total number of transgenic embryos generated for each construct (#Tg) and the number of embryos that stained in the ventral midline of the spinal cord (SC) and midbrain (MB). The consistency of staining intensity and minimal time to initiate staining were used to subjectively classify the spinal cord expression as strong (+++), moderate (++), weak and patchy (+/–), or absent (–). The *lacZ* expression generated in the midbrain is regulated by Sbe1 and serves as a positive control for transgene expression. Rc1 and Rc2 were reported previously (Epstein et al., 1999). (C) X-gal staining of transgenic embryos carrying reporter constructs at 9.5 dpc. The cranial staining depicted by the embryo carrying Rc1 is also representative of the staining pattern seen in embryos expressing Rc3-6.

plate expression, albeit at weaker levels compared to the full-length fragment, we reasoned that a construct containing multimers of HR-c might recapitulate the full extent of Sfpe2 activity. Three copies of HR-c were cloned into the reporter cassette in head to tail configuration and tested in transgenic embryos (Rc9). In all 12 embryos carrying Rc9, X-gal staining was detected in the floor plate in a pattern indistinguishable from those carrying the entire 746-bp fragment (Rc2) as assessed by the intensity of X-gal staining as well as the minimal time required to initiate staining (30 minutes) (Fig. 2A). Upon sectioning the embryos, strong staining was detected in both the floor plate and notochord (Fig. 2D), indicating that the multimerized HR-c construct faithfully recapitulates these domains of endogenous *Shh* expression (Epstein et al., 1999).

The comparison of *Shh* regulatory sequences between mouse, human, chicken and zebrafish proved successful in identifying a highly conserved 88-bp fragment sufficient to cooperate with Sbe1 in targeting transgene expression to the floor plate and notochord in a *Shh*-like manner. To determine whether this enhancer was functionally conserved in non-mammalian species, we cloned trimers of HR-c from chicken

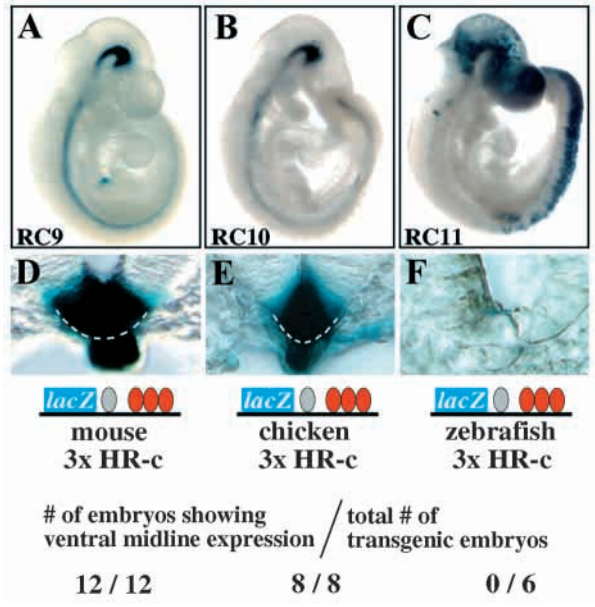


Fig. 2. Functional conservation of the 88 bp homology region-c (HR-c) element. X-gal staining of transgenic embryos at 9.5 dpc carrying three copies of HR-c from mouse (A,D), chicken (B,E) and zebrafish (C,F). Transverse sections through the spinal cord at the level of the forelimb of representative embryos revealed strong floor plate and notochord expression from mouse (D) and chicken (E) but not zebrafish (F) DNA. The ectopic staining from Rc11 was seen in all six transgenic embryos. The broken line in D,E mark the boundary between the floor plate and notochord.

and zebrafish DNA into the reporter cassette in the same orientation as the mouse HR-c trimer (Rc9). All eight transgenic embryos carrying chicken HR-c (Rc10) revealed strong X-gal staining in the floor plate and notochord in a manner similar to those expressing mouse HR-c (Rc9) (Fig. 2B,E). In contrast, none of the embryos carrying zebrafish HR-c showed ventral midline staining in the spinal cord, despite consistent expression in the ventral midbrain and other sites of ectopic expression (Fig. 2C,F). Given that floor plate enhancer activity has been localized to intron 2 sequences of the zebrafish *Shh* gene (Müller et al., 1999), it is probable that critical regulatory sequences have been displaced from zebrafish HR-c.

The *Shh* floor plate enhancer is responsive to Shh signaling

The induction of *Shh* expression in the floor plate is dependent on Shh signaling from the underlying axial mesoderm (reviewed by Jessell, 2000). If the sequences mediating Sfpe2 activity contain the requisite complement of transcription factor-binding sites we reasoned that they should be responsive to Shh signaling. However, if sequences outside of these sequences are also required, then the reporter should not be activated by Shh. Sfpe2 responsiveness to Shh signaling was assayed in neural explants derived from wild-type and $Shh^{-/-}$ embryos. The use of explants from $Shh^{-/-}$ embryos offers the advantage of performing the experiments in an environment free of prior exposure to Shh and extraneous floor plate signals. Stable transgenic mouse lines carrying three copies of HR-c (Rc9) were crossed onto a $Shh^{+/-}$ background and subsequently intercrossed to generate wild-type and $Shh^{-/-}$; $Rc9^{+}$ embryos. Spinal cord explants were isolated from 3-6 somite stage embryos, which precede the onset of *Shh* expression in the CNS, and cultured in the presence or absence of recombinant N-Shh for 30 hours. X-gal staining could not be detected in the majority of wild-type; $Rc9^{+}$ or any of the $Shh^{-/-}$; $Rc9^{+}$ neural explants cultured in the absence of N-Shh (Fig. 3A,B). The few cells that stained positive in the wild-type culture probably reflect their exposure to Shh signaling from the notochord prior to being explanted. In the presence of N-Shh, a significant portion of each explant stained positive for *lacZ* expression in both wild-type; $Rc9^{+}$ (n=16) and $Shh^{-/-}$; $Rc9^{+}$ (n=3) genotypes (Fig. 3C,D). These data show that Shh signaling is both necessary and sufficient to activate the Sfpe2 enhancer, confirming that the combination of Sbe1 and HR-c possess the appropriate array of transcription factor-binding sites needed to respond to Shh signaling.

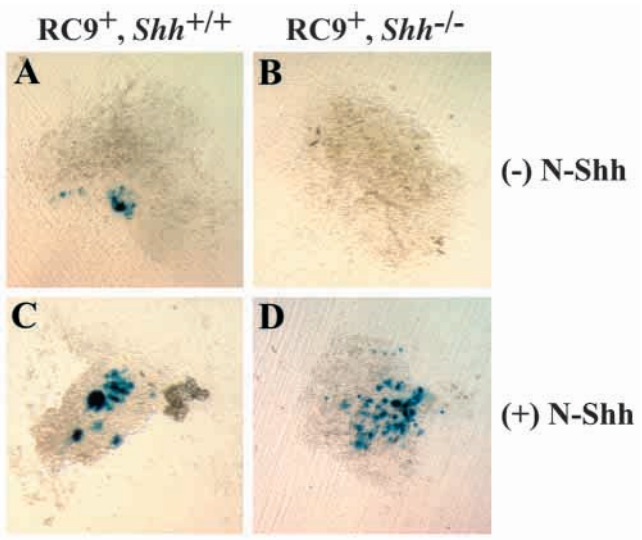


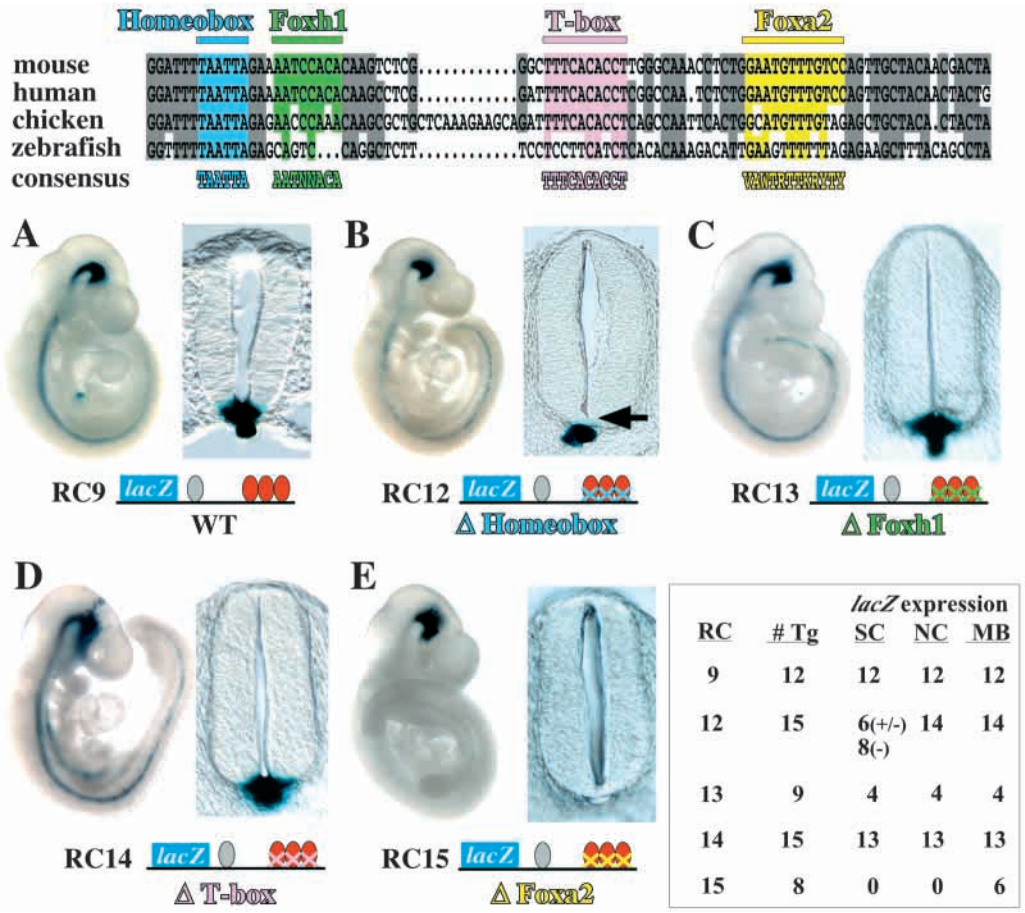
Fig. 3. Homology region-c (HR-c) is responsive to Shh signaling. Neural explants isolated from wild-type (A,C) or $Shh^{-/-}$ (B,D) embryos carrying Rc9 were cultured in the presence (+) or absence (–) of N-Shh for 30 hours. A few wild-type cells showed X-gal staining when cultured in the absence of N-Shh (possibly reflecting their prior exposure to Shh in vivo), however this number was significantly increased when cultured in the presence of N-Shh (compare A with C, n=16). No cells showed X-gal staining in explants derived from $Shh^{-/-}$, $Rc9^{+}$ embryos grown in the absence of N-Shh (B), in marked contrast to the significant number of positive stained cells from the corresponding explant grown in the presence of N-Shh (D, n=3).

Conserved binding sites in HR-c are required for floor plate and notochord expression

To identify the cis-acting elements required for Sfpe2 activity, we surveyed the HR-c nucleotide sequence for consensus binding sites recognized by known transcription factors using the Transcription Element Search System (TESS, University of Pennsylvania), as well as by comparing some candidate consensus sites by eye. This analysis recognized many transcription factor-binding sites in the 88-bp segment, but only those sites showing significant homology across phyla or some prior indication for a role in floor plate development were considered. Four binding sites matching the consensus for homeodomain, T-box and two distinct winged-helix-containing proteins, Foxh1 (formerly Fast1) and Foxa (Hnf3), were identified. To address the in vivo significance of the candidate binding sites on floor plate and notochord transcription, reporter constructs were generated harboring small deletions or point mutations in each of the core recognition sequences. The mutations were chosen based on their ability to prohibit DNA binding by the corresponding transcription factor (Overdier et al., 1994; Awatramani et al., 1997; Labbé et al., 1998; Smith, 1998). Mutated sequences were cloned in the same context as the wild-type trimeric HR-c reporter construct (Rc9) and assayed in transgenic embryos.

Two homeodomain binding-sites were identified within an AT-rich segment of DNA spanning approximately 20 bp including sequence proximal to HR-c (Fig. 4 and data not shown). Remarkably, this 20-bp stretch was virtually 100% conserved across phyla with only a single nucleotide substitution identified in zebrafish. The ATTA cores of the two homeodomain recognition sequences were found in opposite orientation separated by a two base-pair overlap. Sequences outside the core ATTA motif did not match recognition sequences for any of the transcription factors known to partner with Hox proteins, including Pbx and Meis.

The effect of deleting the homeodomain binding-sites in HR-c was dramatic as each of the 14 transgenic embryos expressing Rc12 showed either a complete absence (8/14) or greatly reduced (6/14) expression of *lacZ* in the floor plate (compare Fig. 4A with 4B). Patchy floor plate expression was revealed in a few of the embryos similar to that shown in Fig. 6H. In contrast to the loss of reporter expression in the floor



RC	# Tg	*lacZ* expression SC	NC	MB
9	12	12	12	12
12	15	6(+/-) 8(-)	14	14
13	9	4	4	4
14	15	13	13	13
15	8	0	0	6

Fig. 4. Requirement of conserved DNA binding sites in homology region-c (HR-c) on floor plate and notochord expression. Alignment of HR-c sequences from mouse, human, chicken and zebrafish is shown at the top. Conserved sequences are shaded in gray. DNA recognition sequences matching the consensus for homeobox (blue), Foxh1 (green), T-box (pink) and Foxa (yellow) transcription factors are shown. (A-E) X-gal staining of transgenic embryos at 9.5 dpc carrying reporter constructs with (A) wild-type Rc9, or (B-E) mutant DNA binding sites. Adjacent to each embryo is a transverse section through lumbar regions of the spinal cord. Underneath each figure is a schematic of the reporter construct. The table to the right indicates the total number of transgenic embryos generated for each construct and their patterns of expression. The arrow in B points to the floor plate, which is devoid of X-gal staining. NC, notochord.

plate, the deletion of the homeodomain binding-sites had no consequence on X-gal staining in the notochord. To determine whether the highly homologous stretch of DNA overlapping the homeodomain recognition sequences was sufficient for floor plate expression, a construct containing these sequences was tested in transgenic embryos (Fig. 1B, Rc6). No X-gal staining was detected in the floor plate of embryos expressing Rc6 (Fig. 1B). Taken together, these results suggest that highly conserved homeodomain binding-sites are required but not sufficient for Sfpe2-directed floor plate transcription, alluding to the probability that additional sites within HR-c are also necessary.

An 8 bp sequence matching the consensus for the Foxh1 protein is situated three base pairs distal to the homeodomain binding site in HR-c (Labbé et al., 1998). Although this sequence appeared conserved between mouse and human it diverged significantly from chicken and zebrafish. Despite the lack of sequence conservation across phyla we chose to assess the requirement of the Foxh1 site for Sfpe2 activity because Foxh1 can mediate aspects of nodal signaling and at least in zebrafish, nodal signaling is sufficient to induce *Shh* transcription (Weisberg et al., 1998; Müller et al., 2000). Point mutations in the Foxh1 recognition sequence that abrogate Foxh1 binding (Labbé et al., 1998) had no consequence on floor plate or notochord expression, suggesting that Foxh1 does not participate directly in Sfpe2-mediated transcription (Fig. 4C, Rc13).

A highly conserved Tbx binding site showing a perfect match with the consensus was also identified in HR-c (Smith, 1998) (Fig. 4). Given that the founding member of the Tbx family, *brachyury*, is expressed in the axial mesoderm and that other members of this large transcription factor family have been described in the ventral midline, the Tbx site seemed to be a good candidate for regulating Sfpe2 activity. Nevertheless, no embryos expressing the Rc14 transgene, which contains mutations in the Tbx binding-site, showed any alteration to the pattern of X-gal staining in the floor plate or notochord as compared to embryos carrying the wild-type transgene (compare Fig. 4A with 4D). Unexpectedly, loss of the Tbx binding-site did result in alterations to the pattern of X-gal staining in the diencephalon. Normally, the rostral boundary of X-gal staining derived from Sbe1 coincides with prosomere 3 (Fig. 5A). However, in all 13 transgenic embryos expressing the mutated Tbx binding-site construct (Rc14), the rostral boundary of X-gal staining extended into prosomere 5 (Fig. 5B). Moreover, X-gal staining was detected in the ventral midline in contrast to where *Shh* is normally expressed in the rostral diencephalon – within two stripes adjacent to the midline (Fig. 5C,D). To confirm that the ectopic *Shh* reporter activity indeed resulted from the loss of the Tbx binding site rather than the inadvertent creation of a new binding site supporting *Shh* reporter activation, a second construct was generated containing a different set of point mutations (Rc14.1; see Materials and Methods). Transgenic embryos carrying this construct showed precisely the same ectopic expression of *Shh* reporter activity in the ventral midline of the rostral

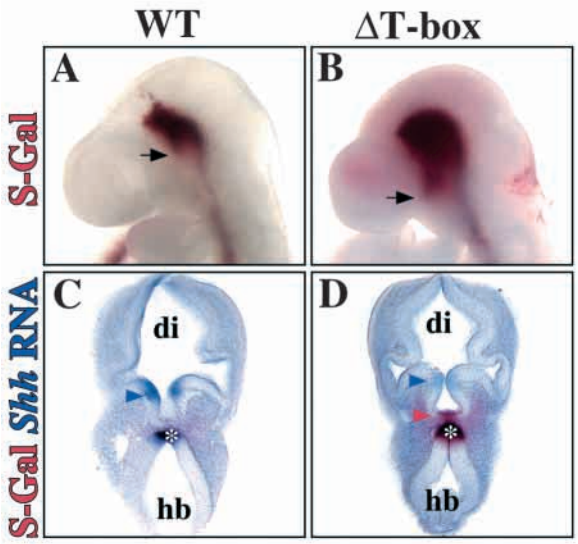


Fig. 5. Loss of a T-box binding site in homology region-c (HR-c) results in ectopic expression in the diencephalon. (A,B) Whole-mount salmon-gal staining of transgenic embryos at 9.5 dpc expressing either (A) wild-type (Rc9) or (B) mutated T-box (Rc14) constructs. The arrows in A and B point to the rostral limit of salmon-gal staining detected at the level of prosomeres 3 and 5, respectively. (C-D) Transverse sections through the rostral diencephalon of transgenic embryos expressing either (C) wild-type Rc9, or (D) mutated T-box (Rc14), double-stained for *lacZ* reporter activity (salmon-gal) and *Shh* mRNA (BM-purple). The blue arrowhead points to the stripe of lateral expression of endogenous *Shh* mRNA adjacent to the ventral midline in this region of the diencephalon (di). The salmon-colored arrowhead in D points to the ectopic reporter activity in the ventral midline of the diencephalon. The asterisk marks the floor plate of the hindbrain (hb).

diencephalon (*n*=4, data not shown). These results suggest that sequences encoding a consensus binding site for Tbx proteins are required to recruit a transcription factor, probably a member of the Tbx family, which functions to repress Sfpe2 activity and by extension, *Shh* transcription, from the ventral midline of the diencephalon.

The remaining transcription factor binding-site to be analyzed in HR-c was one recognized by Foxa proteins (Overdier et al., 1993). As the sequence of the Foxa site in mouse HR-c differed slightly from the consensus, electromobility shift assays were performed to verify that Foxa proteins could bind efficiently to the proposed site. All three Foxa family members were found capable of binding to the recognition sequence (data not shown). Previous studies indicated that Foxa binding sites in sequences mediating Sbe1 activity are required for consistent reporter expression in the floor plate when assayed in the presence of Sfpe2 (Epstein et al., 1999). Given this finding, we reasoned that the Foxa site contained in HR-c might be redundant. In contrast to this prediction however, embryos expressing a transgene with point mutations in the Foxa binding site of HR-c failed to activate *lacZ* in any cells of the floor plate or notochord (Fig. 4E). This data indicates that although the Foxa site in HR-c is required for Sfpe2 activity, it is not sufficient to mediate floor plate expression on its own, given our earlier finding that Rc12-expressing embryos – which carry a small deletion overlapping the homeodomain binding sites – fail to show significant levels of X-gal staining in the floor plate despite the presence of an intact Foxa binding site (Fig. 4B). We thus conclude that Sfpe2 activity in the floor plate is governed by the combined positive actions of Foxa2 and homeodomain-containing transcriptional regulators.

Homeodomain and Foxa2 binding sites are required at the initial stages of *Shh* transcription in the floor plate

In the CNS of the developing mouse embryo, the expression of *Shh* initiates at the 7-8 somite stage within the ventral midline of the prospective midbrain and then extends rostrally to the forebrain and caudally to the hindbrain and spinal cord (Echelard et al., 1993). By the 13-somite stage, the ventral midline expression intensifies and is continuous along the extent of the anteroposterior neuraxis. We verified that transgenic lines carrying a wild-type enhancer construct (Rc9) recapitulated this early progression of *Shh* transcription (Fig. 6). With exception to the domain of *Shh* expression in the rostral forebrain, embryos expressing the wild-type enhancer construct (Rc9) simulated the ontogeny of *Shh* transcription in the ventral midline of the CNS. With this information in hand, we next sought to determine whether the consequences of mutating the homeodomain and Foxa recognition sequences on X-gal staining in the floor plate and notochord reflected alterations in the initiation and/or maintenance of *lacZ* expression.

Stable mouse lines expressing the mutated homeodomain binding-site transgene (Rc12) were generated to test the role of the homeodomain site on ventral midline expression at early stages of CNS development. At the 6-somite stage, X-gal staining was detected in the notochord of embryos from 4 independent lines in a pattern similar to embryos carrying the wild-type transgene (Fig. 6A,B). By the 9-somite stage, X-gal staining was detected in the region of the presumptive midbrain in embryos from all 4 lines expressing Rc12. However, unlike embryos expressing the wild-type enhancer construct, staining was excluded from the ventral hindbrain, thus indicating that the caudal progression of Sfpe2 activity was impaired by the mutation in the homeodomain binding-site (Fig. 6C,D). By the 13-15 somite stage, X-gal staining was completely absent from the floor plate in 3 of 4 transgenic lines carrying the homeodomain binding-site mutation (Rc12), with the fourth showing patchy expression in the rostral portion of the spinal cord (Fig. 6F,H). The patchy X-gal staining could result from limited activity of the enhancer in the absence of the homeodomain binding site or perdurance of β-gal from an earlier progenitor cell. To distinguish between these two possibilities whole-mount in situ hybridization was performed to detect *lacZ* mRNA transcripts. Transgenic embryos carrying the wild-type enhancer construct (Rc9) showed high levels of *lacZ* mRNA in the floor plate and notochord at the 13-somite stage (Fig. 6I). Embryos derived from the reporter line showing patchy X-gal staining in the floor plate showed weak and patchy expression of *lacZ* mRNA consistent with the pattern of X-gal staining (Fig. 6J,K). Because the *lacZ* expression is only detected at the time that *Shh* is normally transcribed in the floor plate (after the 8-somite stage) it argues against the probability that the X-gal-positive cells were derived from a precursor that expressed *lacZ* at earlier stages. These studies confirm that the homeodomain binding-sites in HR-c are

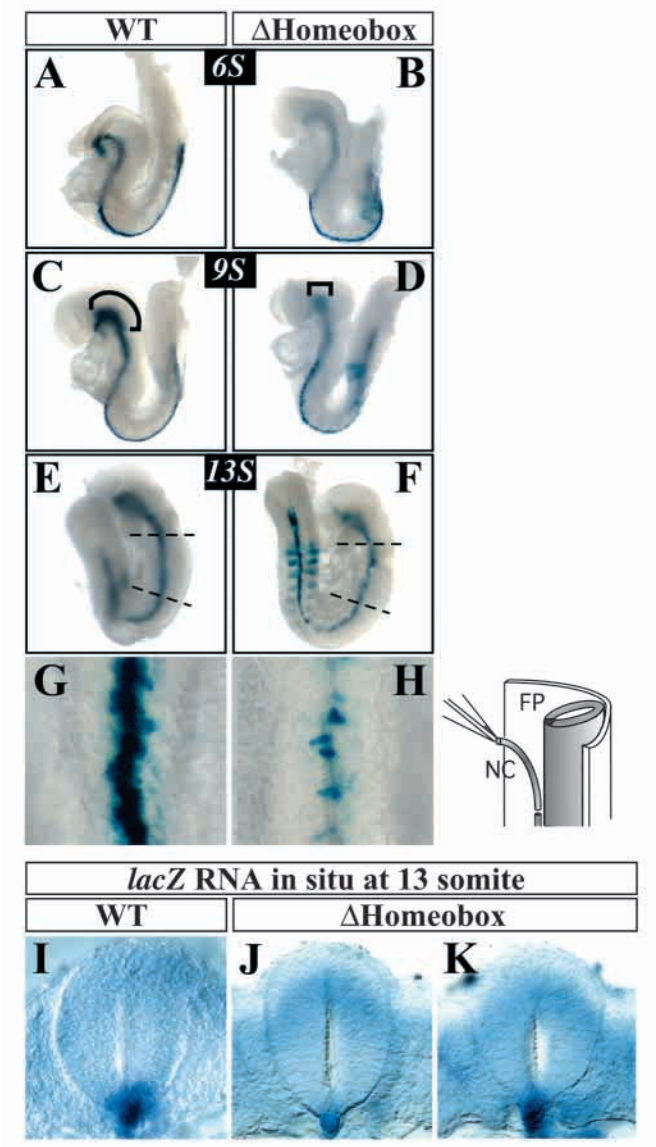


Fig. 6. The homeodomain binding-sites in homology region-c (HR-c) are required at the onset of floor plate expression. Whole-mount X-gal staining of embryos between 6 and 13 somites from reporter lines of mice expressing wild-type Rc9 (A,C,E,G), or mutated homeobox Rc12 (B,D,F,H) transgenes. Ventral midline expression from Sfpe2 is delayed as early as the 9-somite stage in embryos expressing Rc12 (compare C with D). Ectopic *lacZ* expression was detected in the somites of one of the lines carrying the homeobox mutation. G and H show ventral views of the floor plate after the notochord has been removed as shown in the diagram. Transverse sections of spinal cords from Rc9 (I) and Rc12 (J and K) expressing embryos stained for *lacZ* mRNA by whole-mount in situ hybridization. The floor plate expression of *lacZ* in Rc12 transgenics was patchy and thus appeared on sections as either absent (J) or weak (K). Brackets in C,D indicate extent of cranial reporter activity. Broken lines in E,F indicate the thoracic regions of the spinal cord that are magnified in G,H.

required for the initiation of Sfpe2 activity in the floor plate between 9 and 13 somites.

With respect to the Foxa binding site, at no stage of development analyzed did embryos expressing a reporter construct containing a mutated Foxa binding-site (Rc15) display X-gal staining in the floor plate or notochord, suggesting that Foxa2 is also required at the initiation of Sfpe2 activity. Because of the failure to activate reporter expression at early stages in embryos carrying binding-site mutations, we cannot exclude the possibility that homeodomain and Foxa proteins also function to maintain Sfpe2 activity at later stages.

Tracing the lineage of *Shh*-expressing cells in the node

In addition to the floor plate and notochord we observed Sfpe2 activity in the node of the mouse embryo as early as the one-somite stage (Fig. 7A and data not shown). In comparing the distribution of X-gal staining in the node of 5-6 somite stage embryos to that of endogenous *Shh* we observed that both are restricted to the ventral (mesodermal) layer (Fig. 7A-D). Examination of thin sections (6 μm) generated through X-gal-stained embryos at the 5-6 somite stage (n=7) from the level of the posterior node to the hindbrain confirmed the absence of any X-gal-positive cells in the dorsal layer of the node or ventral midline of the neural plate (Fig. 7A). Only the ventral layer of the node and its derivatives – the notochord progenitors – showed X-gal staining at the 5-somite or any other stage examined prior to the onset of *Shh* transcription in the CNS (n=37 embryos examined between 0 and 7 somite stages, data not shown). This contrasts with the expression of *Shh* in the chick, which shows a uniform distribution at low levels in both the ventral and dorsal (ectodermal) layers of Henson's node (Teillet et al., 1998). Lineage tracing studies of Henson's node using chick-quail chimeras suggested that the floor plate and notochord are derived from a common precursor (Catala et al., 1996; Teillet et al., 1998). The same is unlikely to be true in the mouse because X-gal staining is not detected in the dorsal layer of the node, from where the floor plate is derived. Furthermore, the clear demarcation between mesoderm and ectoderm as evidenced by the segregation of X-gal-positive and X-gal-negative cells in and around the node (Fig. 7A), argues against the probability that the mouse floor plate arises from a common precursor with the notochord that subsequently inserts into the medial portion of the neural plate as was previously reported for the chick (Catala et al., 1996; Teillet et al., 1998). The expression of *Ptc* and *Foxa2* in the dorsal layer of the node and medial neural plate at more rostral axial levels in response to Shh signaling is consistent with the model that floor plate development initiates in the node via inductive signaling (Fig. 7F,G).

DISCUSSION

The establishment of the notochord and floor plate as organizing centers within the vertebrate embryo relies on the precise temporal and spatial control of *Shh* transcription within these tissues. Failure to properly initiate *Shh* expression in the notochord and floor plate has profound consequences on the development of a variety of cell types in the ventral neural tube and paraxial tissues (Chiang et al., 1996; Gustaffson et al., 2002; Riccomagno et al., 2002). The notochord and floor plate serve not only as conduits for Shh signaling but are themselves dependent on Shh function given that the notochord is not maintained and the floor plate is not induced in $Shh^{-/-}$ embryos (Chiang et al., 1996). Elucidation of the genes regulating *Shh*

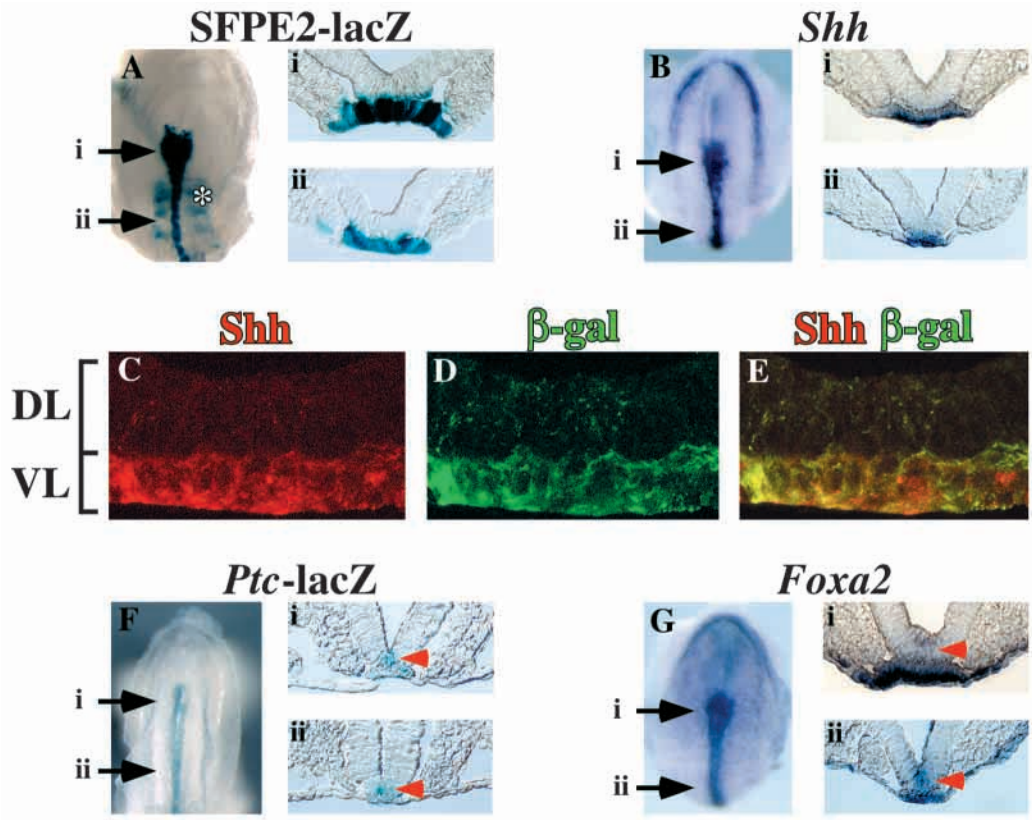


Fig. 7. Lineage tracing of *Shh*-expressing cells in the mouse node. (A,B) Posterior-ventral views of 5-6 somite stage mouse embryos showing (A) Sfpe2 reporter activity and (B) *Shh* expression in the (i) node and (ii) ventral midline. Asterisk in A marks ectopic X-gal staining in the somites. Sections generated through the embryos in A and B at the level of the (i) node and (ii) second most newly formed somite show that X-gal staining and *Shh* expression are restricted to the ventral (mesodermal) layer of the node and notochordal plate, respectively. (C-E) Shh and β-gal immunostaining colocalize to the ventral layer of the node in a 5-6 somite stage embryo carrying the Sfpe2 reporter. (F,G) Ventral views of 5-6 somite stage mouse embryos showing (F) *Ptc-lacZ* and (G) *Foxa2* expression in the (i) node and (ii) ventral midline. Sections taken through the embryos in F and G are at the level of the (i) node and (ii) second most newly formed somite. Both *Ptc-lacZ* staining and *Foxa2* expression localize to the dorsal and ventral layers of the node and to their derivatives, the floor plate and notochord, respectively. Red arrowheads point to the expression of *Ptc-lacZ* and *Foxa2* in the presumptive floor plate. DL, dorsal layer; VL, ventral layer.

transcription will therefore benefit our understanding of ventral midline development.

Functional conservation of *Shh* regulatory sequences

Our approach towards identifying regulators of *Shh* expression in the notochord and floor plate relied on comparing candidate regulatory sequences from multiple organisms to narrow down the critical elements based on homology. The assumption that conservation of sequence underscores conservation of function was validated with the identification of a highly conserved 88-bp fragment from intron 2 of the *Shh* gene (HR-c) that cooperates with Sbe1 to direct *lacZ* reporter expression to the floor plate and notochord in transgenic mice. Further confirmation of this principle was obtained when the corresponding chicken DNA was found sufficient to direct transgene expression to the notochord and floor plate in mouse embryos.

A survey of the 88-bp fragment for transcription factor binding sites identified those matching the consensus for homeodomain, Tbx and winged-helix family members. In determining the requirement of each binding site on transgene expression we uncovered a novel interaction between homeodomain and Foxa2 proteins as binding sites for each were required for the initiation of *Shh* floor plate enhancer activity. In addition to the positive elements mediating Sfpe2 function, we found that a well-conserved Tbx binding site is required for the repression of transgene expression in a region of the ventral diencephalon where *Shh* is not normally detected. The identity of the Tbx protein that represses Sfpe2 activity is presently unknown.

Interestingly, the more divergent zebrafish HR-c sequence which was incapable of directing ventral midline expression in transgenic embryos showed significant sequence homology in and around the area of the homeodomain recognition sequences but not in the vicinity of the Foxa binding site. *Shh* floor plate enhancer activity has been mapped in zebrafish to sequences inclusive of the conserved homeodomain binding-site and extending 160 bp upstream of HR-c (Müller et al., 1999). Upon surveying these sequences we identified a Foxa binding site located in the reverse orientation 25 bp downstream of a previously reported Tbx site. This suggests that the transcriptional control mechanisms responsible for regulating *Shh* expression in the floor plate of zebrafish, although slightly displaced in position, may remain conserved in function.

Cooperative interactions regulate *Shh* floor plate enhancer activity

Our studies suggest that Foxa2 is not sufficient to mediate Sfpe2 function and that cooperative interactions with a homeodomain transcription factor are required to direct reporter expression to the floor plate in a *Shh*-like manner. These results are seemingly inconsistent with previous reports documenting that forced expression of Foxa2 is sufficient to activate *Shh* transcription (Hynes et al., 1995; Ruiz i Altaba et

al., 1995). However, additional observations are supportive of our conclusion. First, Foxa2 is expressed along the length of the floor plate yet the activities of the enhancers regulating *Shh* are regionalized along the anteroposterior axis of the neural tube (Epstein et al., 1999). Moreover, the sequences mediating Sbe1 and Sfpe2 activity, although both possessing Foxa binding sites, cannot independently direct reporter expression to the floor plate even when multimerized. Therefore, additional transcription factors must be acting in concert with Foxa2 to regulate *Shh* expression in the floor plate. To reconcile differences between our results and the Foxa2 gain-of-function studies, we speculate that: (1) Foxa2 may be inducing the expression of the cooperating transcription factor(s) (see Fig. 8B); (2) Foxa2 may only be capable of activating *Shh* transcription where the cooperating transcription factor(s) is/are expressed. Restrictions in where Foxa2 can activate *Shh* within the neural tube have been described (Ruiz i Altaba et al., 1995); and 3) ectopic expression of Foxa2 may be activating *Shh* transcription through enhancers other than Sfpe2.

Our data implicating homeodomain binding-sites in the regulation of Shh floor plate enhancer activity leaves open the identity of the trans-acting factor binding to these sites. A survey of genes encoding homeodomain proteins expressed in the ventral midline of the mouse CNS identified members of the Arx, Hox, Lmx and Nkx families (Miura et al., 1997; Li and Lufkin, 2000; Riddle et al., 1995; Pabst et al., 1998; Vallstedt et al., 2001). Despite this large number of factors, members of the Nkx family stand out as candidate regulators of Sfpe2 based on their temporal and spatial overlap with *Shh*. At least four *Nkx* genes including *Nkx2.2*, *2.9*, *6.1* and *6.2* are expressed in the ventral midline of the CNS prior to the onset of *Shh* transcription (Pabst et al., 1998; Qiu et al., 1998; Briscoe et al., 1999; Vallstedt et al., 2001) (and data not shown). The list of candidates can be further narrowed based on the divergent DNA-binding properties exhibited by Nkx2 [T(T/C)AAGT(A/G)(C/G)TT] and Nkx6 (TTAATTAC) class family members (Watada et al., 2000; Mirmira et al., 2000). Because the homeodomain binding-site in HR-c better matches the consensus for Nkx6 versus Nkx2 family members – confirmed by our own DNA binding studies showing that Nkx6.1 but not Nkx2.2 could bind to the homeodomain site in HR-c (unpublished data) – we favor Nkx6.1 and Nkx6.2 as candidate regulators of Sfpe2 activity.

Notwithstanding the agreement of our data with a role for Nkx6 family members in regulating *Shh* expression in the floor plate, genetic studies supporting the requirement of *Nkx6* genes in this process have not been forthcoming (Sander et al., 2000; Vallstedt et al., 2001; Cai et al., 2001). Thus, we cannot rule out the possibility that homeodomain proteins other than Nkx6.1 or Nkx6.2 regulate Sfpe2. Furthermore, detecting a down-regulation in *Shh* transcription in *Nkx6* mutants may be confounded by the presence of another floor plate enhancer (Sfpe1), located upstream of the *Shh* gene which may compensate in the absence of Sfpe2. It is interesting to note that the regulation of *Shh* expression in more rostral regions of the CNS is also dependent on an *Nkx* gene. In embryos lacking Nkx2.1, *Shh* expression in the ventral telencephalon is completely absent. Not surprisingly *Nkx2.1* is the only *Nkx* family member expressed in this region of the CNS (Sussel et al., 1999).

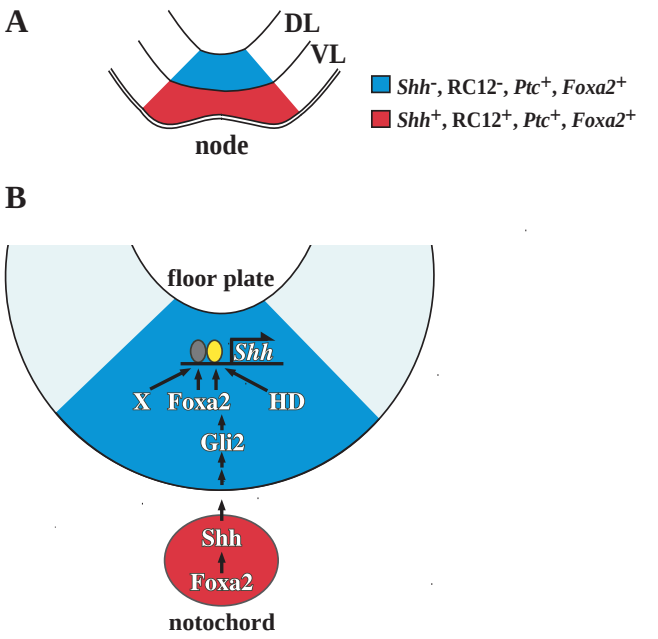


Fig. 8. Homeogenetic induction of *Shh* expression in the floor plate. (A) Schematic of the mouse node depicting the segregation of gene expression patterns in the dorsal (blue) and ventral (red) layers (DL and VL, respectively). Whereas Shh and Sfpe2 reporter activity are restricted to the ventral layer, the Shh target genes *Ptc* and *Foxa2* are expressed in both the DL and VL. (B) Schematic of the notochord and floor plate showing the transcriptional cascade resulting in *Shh* expression. In the notochord, Foxa2 activates Shh, which then signals to the overlying neural plate. For simplicity, only transcriptional components of the Shh pathway resulting in its expression in the floor plate is shown. In response to Shh signaling, Gli2 activates *Foxa2* expression in the neural plate. Foxa2, in cooperation with a homeodomain (HD) protein and at least one other transcriptional activator (X) bind *Shh* regulatory elements (Sbe1, gray oval and Sfpe2, yellow oval) to stimulate *Shh* transcription in the floor plate.

To explain how Foxa2 and the cooperating homeodomain factor may be interacting to regulate *Shh* transcription we draw from previous studies which have shown that the binding of Foxa proteins to their recognition sites on active enhancers can result in the stabilization of nucleosome position, thus facilitating the binding of additional transcription factors to the enhancer complex (Cirillo et al., 1999; Chaya et al., 2001). Foxa2 may be functioning in a similar capacity on Sfpe2 by promoting the stable binding of homeodomain proteins such as Nkx6 family members. This model is particularly attractive because it can also explain why Foxa2 sites in Sbe1 are unable to compensate when a similar site in Sfpe2 is mutated. Foxa2 may only be able to act locally in generating an environment permissive for transcription, concordant with the close proximity of Foxa2 binding sites to those for factor X in Sbe1 and the homeodomain site in Sfpe2 (Fig. 8).

The floor plate and notochord do not share a common precursor in the node

That the mutation in the homeodomain binding-sites in HR-c only affected reporter expression in the floor plate stipulates that the cooperating homeodomain factor is not involved in

regulating Sfpe2 activity in the notochord. This result bears significance on the timing of floor plate specification and argues that although initial steps may be occurring in the node, the final step in the process happens well after floor plate progenitors have emerged from the node.

Using Sfpe2 reporter activity to trace the lineage of *Shh*-expressing cells in the node we showed that X-gal staining was restricted to the ventral layer, as was endogenous *Shh*. Consequently, only the notochordal plate, a mesodermal derivative emerging from the ventral layer of the node was positive for X-gal staining. Because the floor plate precursors residing in the dorsal layer of the node showed no X-gal staining, we conclude that floor plate and notochord progenitors in the mouse node do not derive from a common origin (Fig. 8A). These results are consistent with previous dye I labeling studies of the mouse node (Beddington, 1994; Sulik et al., 1994) but are in disagreement with data from the chick which supports a common origin for floor plate and notochord precursors (Catala et al., 1996; Teillet et al., 1998). In the chick, floor plate precursors in the node segregate from a common population of progenitors and subsequently insert into the medial position of the overlying neural plate (Catala et al., 1996; Teillet et al., 1998). Because mixing between X-gal-positive ventral cells and X-gal-negative dorsal cells in or around the mouse node was not observed, we conclude that floor plate precursors in the mouse are not generated by the same mechanism as in chick. Instead, we concur with the prevailing model that the mouse floor plate forms by inductive Shh signaling.

Our observation that the Shh target genes *Ptc* and *Foxa2* are expressed in the dorsal layer of the node (Fig. 8A) offers further support that the process of floor plate induction begins in the mouse node at early somite stages and doesn't terminate until *Shh* transcription is activated in the ventral midline of the CNS between 8 to 12 somite stages. In this homeogenetic model of floor plate induction, Shh secreted from the axial mesoderm signals to the overlying neural plate to activate effectors of the Shh signal transduction cascade (Fig. 8B). A consequence of this vertical signaling step is the initiation of *Shh* transcription, through the direct binding of Foxa2 and a homeodomain protein to sequences in HR-c. Given that sequences mediating Sbe1 activity are also required for floor plate expression, we speculate that additional transcriptional activators are participating in the regulation of *Shh* expression (Fig. 8B). Identifying the critical sequences mediating Sbe1 activity and the factors binding to these sites should further elucidate how *Shh* expression is activated in the floor plate of the mouse spinal cord.

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REFERENCES

Alder, J., Lee, K. J., Jessell, T. M. and Hatten, M. E. (1999). Generation of cerebellar granule neurons in vivo by transplantation of BMP-treated neural progenitor cells. *Nat. Neurosci.* **2**, 535-540.

Ang, S.-L. and Rossant, J. (1994). HNF-3β is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

Appel, B., Fritz, A., Westerfield, M., Grunwald, D. J., Eisen, J. S. and Riley, B. B. (1999). Delta-mediated specification of midline cell fates in zebrafish embryos. *Curr. Biol.* **9**, 247-256.

Awatramani, R., Scherer, S., Grinspan, J., Collarini, E., Skoff, R., O'Hagan, D., Garbern, J. and Kamholz, J. (1997). Evidence that the homeodomain protein Gtx is involved in the regulation of oligodendrocyte myelination. *J. Neurosci.* **17**, 6657-6668.

Beddington, R. S. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Burstyn-Cohen, T., Tzarfaty, V., Frumkin, A., Feinstein, Y., Stoeckli, E. and Klar, A. (1999). F-Spondin is required for accurate pathfinding of commissural axons at the floor plate. *Neuron* **23**, 233-246.

Cai, J., Qi, Y., Wu, R., Modderman, G., Fu, H., Liu, R. and Qiu, M. (2001). Mice lacking the Nkx6.2 (Gtx) homeodomain transcription factor develop and reproduce normally. *Mol. Cell. Biol.* **21**, 4399-4403.

Catala, M., Teillet, M. A., De Robertis, E. M. and Le Douarin, M. L. (1996). A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development* **122**, 2599-2610.

Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003) The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with Netrin-1 in midline axon guidance. *Cell* **113**, 11-23.

Chaya, D., Hayamizu, T., Bustin, M. and Zaret, K. S. (2001). Transcription factor FoxA (HNF3) on a nucleosome at an enhancer complex in liver chromatin. *J. Biol. Chem.* **276**, 44385-44389.

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.

Cirillo, L. A. and Zaret, K. S. (1999). An early developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA. *Mol. Cell* **4**, 961-969.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C.-C. (1998). Diminished Sonic Hedgehog signaling and lack of floor plate differentiation in *Gli2* mutant mice. *Development* **125**, 2533-2543.

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 and limb polarity. *Cell* **75**, 1417-1430.

Epstein, D. J., Marti, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122**, 2885-2894.

Epstein, D. J., Martinu, L., Michaud, J. L., Losos, K. M., Fan, C.-M. and Joyner, A. L. (2000). Members of the bHLH-PAS family regulate *Shh* transcription in forebrain regions of the mouse CNS. *Development* **127**, 4701-4709.

Epstein, D. J., McMahon, A. P. and Joyner, A. L. (1999). Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. *Development* **126**, 281-292.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Giger, R. J. and Kolodkin, A. L. (2001). Silencing the siren: guidance cue hierarchies at the CNS midline. *Cell* **10**, 1-4.

Goode, D. K., Snell, P. K. and Elgar, G. K. (2003). Comparative analysis of vertebrate Shh genes identifies novel conserved non-coding sequence. *Mamm. Genome* **14**, 192-201.

Gustafsson, M. K., Pan, H., Pinney, D., Liu, Y., Lewandowski, A., Epstein, D. J. and Emerson, C. P., Jr (2002). *Myf5* is a direct target of long range Shh signaling and Gli regulation for muscle specification. *Genes Dev.* **16**, 114-126.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hynes, M., Poulsen, K., Tessier-Lavigne, M. and Rosenthal, A. (1995).

Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.

Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli1. *Neuron* **19**, 15-26.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Labbé, E., Silvestri, C., Hoodless, P. A., Wrana, J. L. and Attisano, L. (1998). Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol. Cell* **2**, 109-120.

Le Douarin, N. M. and Halpern, M. E. (2000). Discussion point. Origin and specification of the neural tube floor plate: insights from the chick and zebrafish. *Curr. Opin. Neurobiol.* **10**, 23-30.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). *Gli1* is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.

Li, X. and Lufkin, T. (2000). Cre recombinase expression in the floorplate, notochord and gut epithelium in transgenic embryos driven by the Hoxa-1 enhancer III. *Genesis* **26**, 121-122.

Lopez, S. L., Paganelli, A. R., Siri, M. V., Ocana, O. H., Franco, P. G. and Carrasco, A. E. (2003). Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in *Xenopus*. *Development* **130**, 2225-2238.

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

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. P. and Joyner, A. L. (1998). *Gli2* is required for induction of floor plate but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759-2770.

Matise, M. P., Lustig, M., Sakurai, T., Grumet, M. and Joyner, A. L. (1999). Ventral midline cells are required for the local control of commissural axon guidance in the mouse spinal cord. *Development* **126**, 3649-3659.

Mirmira, R. G., Watada, H. and German, M. S. (2000). Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. *J. Biol. Chem.* **275**, 14743-14751.

Miura, H., Yanazawa, M., Kato, K. and Kitamura, K. (1997). Expression of a novel aristaless related homeobox gene 'Arx' in the vertebrate telencephalon, diencephalon and floor plate. *Mech. Dev.* **65**, 99-109.

Müller, F., Albert, S., Blader, P., Fischer, N., Hallonet, M. and Strahle, U. (2000). Direct action of the nodal-related signal cyclops in induction of sonic hedgehog in the ventral midline of the CNS. *Development* **127**, 3889-3897.

Müller, F., Chang, B., Albert, S., Fischer, N., Tora, L. and Strahle, U. (1999). Intronic enhancers control expression of zebrafish sonic hedgehog in floor plate and notochord. *Development* **126**, 2103-2116.

Overdier, D. G., Porcella, A. and Costa, R. (1994). The DNA-binding specificity of the Hepatocyte Nuclear Factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. *Mol. Cell. Biol.* **14**, 2755-2766.

Pabst, O., Herbrand, H. and Arnold, H. H. (1998). Nkx2-9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS. *Mech. Dev.* **73**, 85-93.

Placzek, M. (1995). The role of the notochord and floor plate in inductive interactions. *Curr. Opin. Genet. Dev.* **4**, 499-506.

Placzek, M., Dodd, J. and Jessell, T. M. (2000). Discussion point. The case for floor plate induction by the notochord. *Curr. Opin. Neurobiol.* **10**, 15-22.

Qiu, M., Shimamura, K., Sussel, L., Chen, S. and Rubenstein, J. L. (1998). Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. *Mech. Dev.* **72**, 77-88.

Riccomagno, M. M., Martinu, L., Mulheisen, M., Wu, D. K. and Epstein, D. J. (2002). Specification of the mammalian cochlea is dependent on Sonic hedgehog. *Genes Dev.* **16**, 2365-2378.

Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M. and Tabin, C. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* **83**, 631-640.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Ruiz i Altaba, A., Jessell, T. M. and Roelink, H. (1995). Restrictions to floor plate induction by hedgehog and winged helix genes in the neural tube of frog embryos. *Mol. Cell. Neurosci.* **6**, 106-121.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.

Sasaki, H. and Hogan, B. L. M. (1994). HNF-3β as a regulator of floor plate development. *Cell* **76**, 103-115.

Sasaki, H., Hui, C.-C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for *HNF-3β* floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.

Smith, J. (1998). T-box genes: what they do and how they do it. *Trends Genet.* **15**, 154-158.

Sulik, K., Dehart, D. B., Iangaki, T., Carson, J. L., Vrablic, T., Gesteland, K. and Schoenwolf, G. C. (1994). Morphogenesis of the murine node and notochordal plate. *Dev. Dyn.* **201**, 260-278.

Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Teillet, M. A., Lapointe, F. and Le Douarin, N. M. (1998). The relationships between notochord and floor plate in vertebrate development revisited. *Proc. Natl. Acad. Sci. USA* **95**, 11733-11738.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.

Wang, H. and Tessier-Lavigne, M. (1999). En passant neurotrophic action of an intermediate axonal target in the developing mammalian CNS. *Nature* **401**, 765-769.

Watada, H., Mirmira, R. G., Kalamaras, J. and German, M. S. (2000). Intramolecular control of transcriptional activity by the NK2-specific domain in NK-2 homeodomain proteins. *Proc. Natl. Acad. Sci. USA* **97**, 9443-9448.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E. (1994). The winged-helix transcription factor HNF-3β is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.

Weisberg, E., Winnier, G. E., Chen, X., Farnsworth, C. L., Hogan, B. L. and Whitman, M. (1998). A mouse homologue of FAST-1 transduces TGF beta superfamily signals and is expressed during early embryogenesis. *Mech. Dev.* **79**, 17-27.