Development 127, 3889-3897 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2578

Direct action of the Nodal-related signal Cyclops in induction of *sonic hedgehog* in the ventral midline of the CNS

Ferenc Müller, Stephanie Albert, Patrick Blader, Nadine Fischer, Marc Hallonet and Uwe Strähle*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France
*Author for correspondence (e-mail: uwe@titus.u-strasbg.fr)

Accepted 15 June; published on WWW 22 August 2000

SUMMARY

The secreted molecule Sonic hedgehog (Shh) is crucial for floor plate and ventral brain development in amniote embryos. In zebrafish, mutations in *cyclops* (*cyc*), a gene that encodes a distinct signal related to the TGFβ family member Nodal, result in neural tube defects similar to those of *shh* null mice. *cyc* mutant embryos display cyclopia and lack floor plate and ventral brain regions, suggesting a role for Cyc in specification of these structures. *cyc* mutants express *shh* in the notochord but lack expression of *shh* in the ventral brain. Here we show that Cyc signalling can act directly on *shh* expression in neural tissue. Modulation of the Cyc signalling pathway by constitutive activation or inhibition of Smad2 leads to altered *shh* expression in zebrafish embryos. Ectopic activation of the *shh* promoter occurs in response to expression of Cyc signal transducers in the chick neural tube. Furthermore an enhancer of the *shh* gene, which controls ventral neural tube expression, is responsive to Cyc signal transducers. Our data imply that the Nodal related signal Cyc induces *shh* expression in the ventral neural tube. Based on the differential responsiveness of *shh* and other neural tube specific genes to Hedgehog and Cyc signalling, a two-step model for the establishment of the ventral midline of the CNS is proposed.

Key words: TGFβ, Smad2, FAST-1, FoxH1, Nodal, HNF3β, Floor plate, Chick electroporation, *shh* promoter

INTRODUCTION

The secreted signalling molecule sonic hedgehog (Shh) plays a crucial role in the specification of the floor plate and ventral brain identity. Shh is expressed in the organizer and subsequently in the axial mesoderm and ventral neural tube, regions shown by tissue recombination experiments in amniote embryos to secrete ventral neural tube inducing signals (Placzek et al., 1990; Yamada et al., 1991). Mouse embryos that carry null alleles of *shh* fail to form ventral neural tube structures and display cyclopia (Chiang et al., 1996). Similarly, floor plate differentiation is blocked in mice deficient in Gli2, a mediator of Shh signalling (Ding et al., 1998; Matise et al., 1998). Furthermore, recombinant Shh induces floor plate and ventral brain gene expression when administered to neural plate explants of amniote embryos in vitro (Roelink et al., 1994; Marti et al., 1995). Taken together, this body of evidence strongly suggests that *shh* is both necessary and sufficient for specification of the ventral neural tube. It has been suggested that Shh may control its own expression in the ventral neural tube. This autoregulatory loop was proposed to involve the winged helix transcription factor HNF3β, which is both an immediate target of Shh signalling (Ruiz i Altaba et al., 1995a; Sasaki et al., 1997) and a regulator of the *shh* gene (Ruiz i Altaba et al., 1995b; Chang et al., 1997). There is, however, little direct evidence supporting a role for Shh in regulating its own expression. Moreover, based on the analysis of enhancers of the mouse and zebrafish *shh* gene it is apparent that HNF3β-independent mechanisms also contribute to controlling *shh* expression in the ventral neural tube (Epstein et al., 1999; Müller et al., 1999a).

Unlike $shh^{-/-}$ mouse embryos, zebrafish embryos lacking *shh* activity (*sonic-you* mutants), show only moderate defects in the ventral neural tube. *sonic-you* mutants lack lateral floor plate cells but form the ventralmost part of the neural tube, which is known as the floor plate proper or medial floor plate (Odenthal et al., 2000; Schauerte et al., 1998). Thus, *shh* appears to be less critically required for floor plate induction in zebrafish, although the activity of the related genes, *tiggy-winkle hedgehog* (Ekker et al., 1995) and *echidna hedgehog* (Currie and Ingham, 1996) are also expressed in the developing body axis and may partially compensate for the lack of *shh* activity in the mutant.

In contrast to mutations in *shh*, mutations in *cyclops* (*cyc*) and *squint* (*sqt*) result in a loss of floor plate, defects in the ventral forebrain and cyclopia (Hatta et al., 1991; Feldman et al., 1998), very similar to the phenotype of $shh^{-/-}$ mouse embryos (Chiang et al., 1996). The *cyc* and *sqt* genes encode TGFβ-like molecules, which are very closely related to mouse Nodal (Feldman et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998). Although $cyc^{-/-}$ mutant embryos form a notochord that expresses *shh* mRNA, mutant embryos fail to

form a correctly differentiated ventral neural tube and 1-day-old embryos lack expression of genes normally expressed in the medial floor plate, including *shh, twhh, netrin1* and *axial/HNF3β* (Krauss et al., 1993; Strähle et al., 1993, 1996, 1997a; Ekker et al., 1995). In addition, *cyc/sqt* double mutants fail to form mesendoderm (Feldman et al., 1998), a phenotype also observed in Nodal-deficient mouse embryos (Zhou et al., 1993; Conlon et al., 1994).

Although it has been demonstrated that Cyc activity is required for development of the ventral neural tube, it remains unclear whether Cyc regulates this process by acting directly on neuroectodermal cells or whether it controls the expression and/or processing of ventral neural tube inducing signals in the midline mesoderm. In particular, it is unknown how Cyc signalling interacts with or impinges upon the known function of Hedgehog proteins in specifying ventral neural tube identity. We therefore investigated the role of Cyc in neural tube patterning in the zebrafish by employing expression of dominant negative and constitutively active signal transducers of Cyc signalling in wild-type and mutant zebrafish embryos. We show that a constitutively active form of the Cyc signal transducer Smad2 can activate *shh* expression in the neural tube in a cell-autonomous fashion. In addition, an enhancer that was previously shown to mediate ventral neural tube expression of *shh* in zebrafish and mouse embryos was demonstrated to be responsive to Cyc signalling by electroporation in the chick neural tube. In contrast, the *shh* gene has been found to be much less responsive to Hh signals than other floor plate markers such as *netrin1* or *axial/HNF3β,* suggesting that autoinducing mechanisms play a less important role in the establishment of *shh* expression in the zebrafish neural tube. Taken together our experiments provide evidence for a crucial role for Cyc signalling in control of *shh* expression in the ventral neural tube.

MATERIALS AND METHODS

Constructs and microinjection of fish embryos

Cloning, sequence and expression of zebrafish *smad1, smad2* and the production of expression vectors have been published elsewhere (Müller et al., 1999b). *CMV::Smad2^{CA}* and *smad2^{CA}* mRNA were produced from the pCS2(+) based construct containing the N-terminally truncated *Smad2A1-239* (Müller et al., 1999b). The plasmids *–563shh::lacZ, –2.2shh::lacZ* and *I1+I2/–2.2shh::lacZ* were described previously (Müller et al., 1999a). *pCSdnReg* was used to synthetise *PKI* mRNA (Strähle et al., 1997) and *pCS2(+)Fast-1^{SID}* (Chen et al., 1997) containing the deletion variant of *Xenopus* FAST-1 for *FAST-1^{SID}* mRNA. Synthetic RNA was synthesized and injected into 1-, 2- or 16-cell stage zebrafish embryos as described (Müller et al., 1999b). RNA was injected at a concentration of 50 ng/μl (*Smad2^{CA}*) 500 ng/μl (*Smad1*), 50 ng/μl (*FAST-1^{SID}*) and 100 ng/μl (*PKI*). The expression plasmid *CMV::Smad2^{CA}* was injected into 1- and 2-cell-stage fish embryos at a concentration of 20 ng/μl.

In situ analysis

In situ hybridisation and immunohistochemistry on fish embryos was performed essentially as described (Hauptman and Gerster, 1994; Strähle et al., 1996).

Chick electroporation and β-gal expression analysis

Plasmids were injected into chick embryos into the folding neural tube at the unsegmented somite level at 2 μg/μl. *I1+I2/–2.2shh::lacZ* or other *lacZ*-containing constructs were injected together with or without the expression vectors described above and/or *pCS:2(+)GFP*, the construct *pCS2(+)FAST-1* (Chen et al., 1997), *pCS2(+)NDR2* (Rebagliati et al., 1998a,b). Electroporation of stage 11-12 chick embryos (Hamburger and Hamilton, 1951) was carried out by placing platinum electrodes lateral to the neural tube at the unsegmented mesoderm level onto the vitelline membrane and application of electric pulses (3 times, 30 μseconds, 100 V/cm, with 1 second intervals) generated by a TBX square pulse electroporator (Muramatsu et al., 1997). Embryos were fixed at stage 18-19 and stained for β-gal activity as described (Müller et al., 1999a). Transverse sections of the X-gal stained embryos were cut manually.

RESULTS

Smad2 and FAST-1 act in the Cyc signalling pathway

To investigate the possible role of Cyc in patterning of ventral neural tube, we first tested whether putative downstream mediators of Cyc/Nodal signalling could be used as tools to mimic or interfere with Cyc signalling. TGFβ signals are transduced intracellularly by Smad proteins (reviewed in Attisano and Wrana, 1998; Kretzschmar and Massague, 1998; Whitman, 1998). Indirect evidence in mouse and zebrafish indicated that Smad2 is a downstream, intracellular transducer of the Nodal subclass of TGFβ signals (Gritsman et al., 1999; Nomura and Li, 1998). Consistent with this idea, injection of synthetic mRNA for an amino-terminal truncated, constitutively active form of *smad2* (*smad2^{CA}*) into early cleavage-stage zebrafish embryos resulted in malformations in 1-day-old embryos (94.0%, *n*=310) in a manner reminiscent of those injected with *cyc* mRNA (Fig. 1A-C; Erter et al., 1998; Rebagliati et al., 1998b; Müller, 1999b). Most strikingly, *smad2^{CA}*-injected embryos lacked posterior structures. Furthermore, those embryos expressed *shh* ectopically (90.5%, *n*=74, Fig. 1E,F) similarly to Cyc-injected embryos (Kiecker et al., 2000).

Biochemical evidence suggests that Smad2-dependent gene regulation involves the DNA binding partner FAST-1, a forkhead/winged helix domain protein (Chen et al., 1997). Deletion of the DNA binding domain of FAST-1 generates a dominant inhibitor of Smad2 activity, (FAST-1^{SID}; Chen et al., 1997; Watanabe and Whitman, 1999). As predicted, the phenotype of *FAST-1^{SID}* mRNA-injected embryos was very similar to that of compound mutants for *cyc* and *sqt* (85.0%, *n*=56; Fig. 1D), displaying deficiencies in mesendoderm formation, ventral neural tube patterning and profound cyclopia (Figs 1D, 2C). *FAST-1^{SID}* mRNA-injected embryos lacked *shh* expression (94.7%, *n*=38; Fig. 1G). Moreover, *FAST-1^{SID}* efficiently blocked the activity of coinjected *Smad2^{CA}* (Fig. 1H; 96.2%, *n*=56) or Cyc (88.0%, *n*=75; data not shown). Together, these data show that Smad2^{CA} and FAST-1^{SID} are efficient tools to manipulate the Cyc/Sqt signalling pathway, consistent with important roles for Smad2 and FAST-1 downstream of Cyc/Sqt signals in the zebrafish embryo.

Modulation of the Cyc signal transduction pathway affects *shh* expression in the neural tube

In addition to posterior truncations, *Smad2^{CA}*-injected embryos displayed malformations of the eyes characterised by an expansion of the *pax-2.1* expression domain into the distal

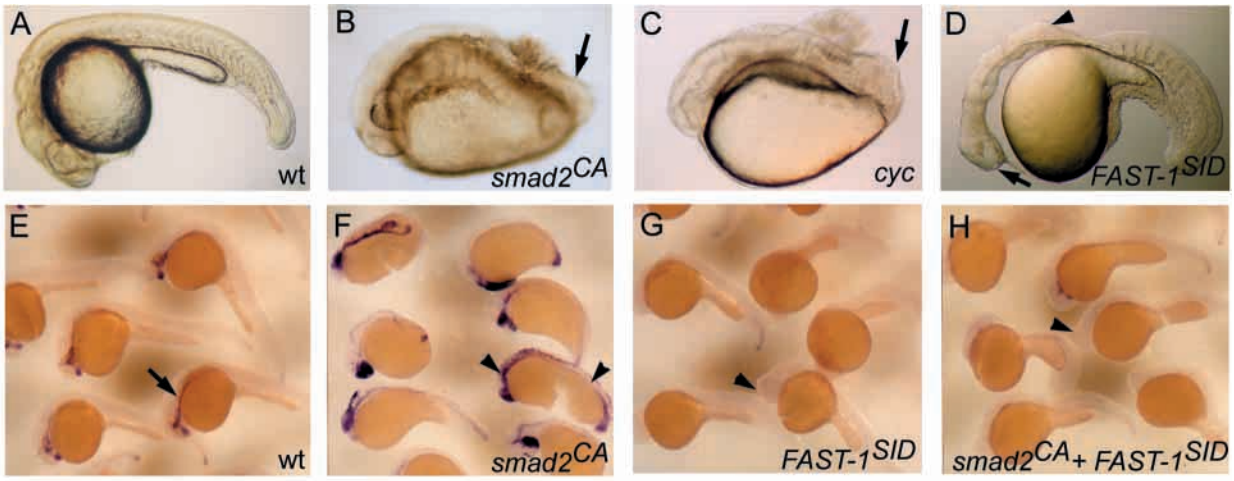

Fig. 1. Smad2^{CA} and FAST-1^{SID} mimic gain- and loss-of-function phenotypes of the Cyc/Sqt signals, respectively. (A) Uninjected control embryo. B,C: expression of constitutively active *Smad2^{CA}* (B) causes malformations similar to the effects of *cyc* misexpression (C). Injected embryos lack posterior trunk and tail (arrows in B,C). (D) Injection of dominant negative *FAST-1^{SID}* mimics the *cyc/sqt* double mutant phenotype (Feldman et al., 1998), characterised by severely disrupted dorsoventral patterning of the neural tube, lack of mesendoderm and a cyclopic eye (arrow). Arrowhead indicates the position of the otic vesicle. (A-D) Lateral views anterior to the left. (E-H) FAST-1^{SID} blocks the activity of constitutively active Smad2^{CA}. (E) Uninjected control embryos; arrow indicates *shh* expression in the ventral midline. (F) Embryos injected with *smad2^{CA}*. Arrowheads indicate ectopic *shh* expression. (G) Embryos injected with *FAST-1$^{SID.}$* Arrowhead points at an embryo with no expression of *shh* in the midline. (H) Embryos coinjected with *Smad2^{CA}* and *FAST-1^{SID}*. Arrowhead points at an embryo with complete loss of *shh* expression.

(dorsal) parts of the eye (53.0%, *n*=45; Fig. 2A,B). This expansion of *pax2.1* expression is reminiscent of the effects of misexpression of *shh* (Ekker et al., 1995; MacDonald et al., 1995). Moreover, a loss of the brain ventricles was also observed, again similar to the effects caused by ectopic expression of *shh* mRNA, suggesting that Smad2^{CA} may affect eye development indirectly through activation of *shh* expression. Consistent with this interpretation, *shh* expression in the brain was increased in *Smad2^{CA}*-injected embryos (Fig. 1F; compare Fig. 2A with B and F with G). In contrast, injection of mRNA encoding the structurally related but functionally distinct *smad1* (Müller et al., 1999b) affected neither *shh* nor *pax2.1* expression (data not shown and Kiecker et al., 2000). Finally, when Smad2 activity was blocked by expression of *Fast-1^{SID}*, *shh* and *pax-2.1* expression were abolished in the ventral brain and eye, respectively, (Fig. 2C; 70.0%, *n*=30), reminiscent of the *cyc* mutant phenotype (Fig. 2D).

We next tested whether misexpression of *smad2^{CA}* can rescue the *cyc$^{-/-}$* phenotype in *cyc^{b16}* embryos. Injection of *smad2^{CA}* mRNA at the 16- to 32-cell stage resulted in a mosaic distribution of the expressed protein, and hence allowed the unambiguous identification of mutant embryos due to mosaic rescue. *Smad2^{CA}* partially rescued both *shh* and *pax2.1* expression in *cyc^{b16}* mutant embryos (53.8%, *n*=26 embryos with a *cyc* mutant phenotype; Fig. 2E,H). Moreover, injection of *Smad2^{CA}* into early embryos resulted in rescue of the mutant eye phenotype (only 14.4% injected embryos showed cyclopia (*n*=188), versus 25.9% in the uninjected control batch (*n*=266).

To address whether expression of Smad2^{CA} affects *shh* in the neurectoderm directly, we investigated whether Smad2^{CA} acts on *shh* expression in a cell-autonomous manner. For this purpose, a Myc-epitope tagged version of *smad2^{CA}* was expressed from a plasmid (*CMV::smad2^{CA}*), resulting in a highly mosaic distribution of expression in order to allow analysis at a single cell resolution (Müller et al., 1999a). In the neurectoderm of early somite-stage *cyc* mutant and wild-type embryos injected with *CMV::smad2^{CA}*, ectopic expression of *shh* was only detected in neural cells, which also expressed the myc epitope (Fig. 2I, 79.5% ectopic *shh*+ cells were also myc+; *n*=39). A similar result was obtained in the neural tube of 24 hour wild-type injected embryos (Fig. 2J). Such a high frequency of colocalisation suggests that *shh* can be activated cell-autonomously by Smad2^{CA}, and is inconsistent with an indirect action of Smad2^{CA} via induction of a second signal. Taken together, these results imply that the Cyc pathway can regulate expression of *shh* in neural epithelial cells.

We have also addressed whether *smad2^{CA}* is able to induce other ventral neural marker genes. Both *axial/HNF3β* (82.2% *n*=37) and *netrin1* (78.9%, *n*=38) are expressed ectopically in the neural tube of *smad2^{CA}-injected* embryos (Fig. 2; compare K with L and M with N, respectively). Expression of both *netrin1* (64.8%, *n*=37; Fig. 6B) and *axial/HNF3β* (data not shown) was abolished or significantly reduced in the neural tube of FAST-1^{SID} expressing embryos, similar to Shh expression.

The zebrafish *shh* promoter is activated by Smad2^{CA} in the chick neural tube

We next asked whether previously identified *shh* promoter/enhancer regions, which drive expression in the ventral neural tube, could be activated by Smad2 and FAST-1. We utilised the *I1+I2/–2.2shh::lacZ* construct that harbours the intronic enhancer region in front of the –2.2 kb *shh* promoter. This construct drives *lacZ* expression in the midline of fish and mouse embryos (Müller et al., 1999a), suggesting conservation of the underlying regulatory mechanisms. We employed electroporation into the chick neural tube as this technique

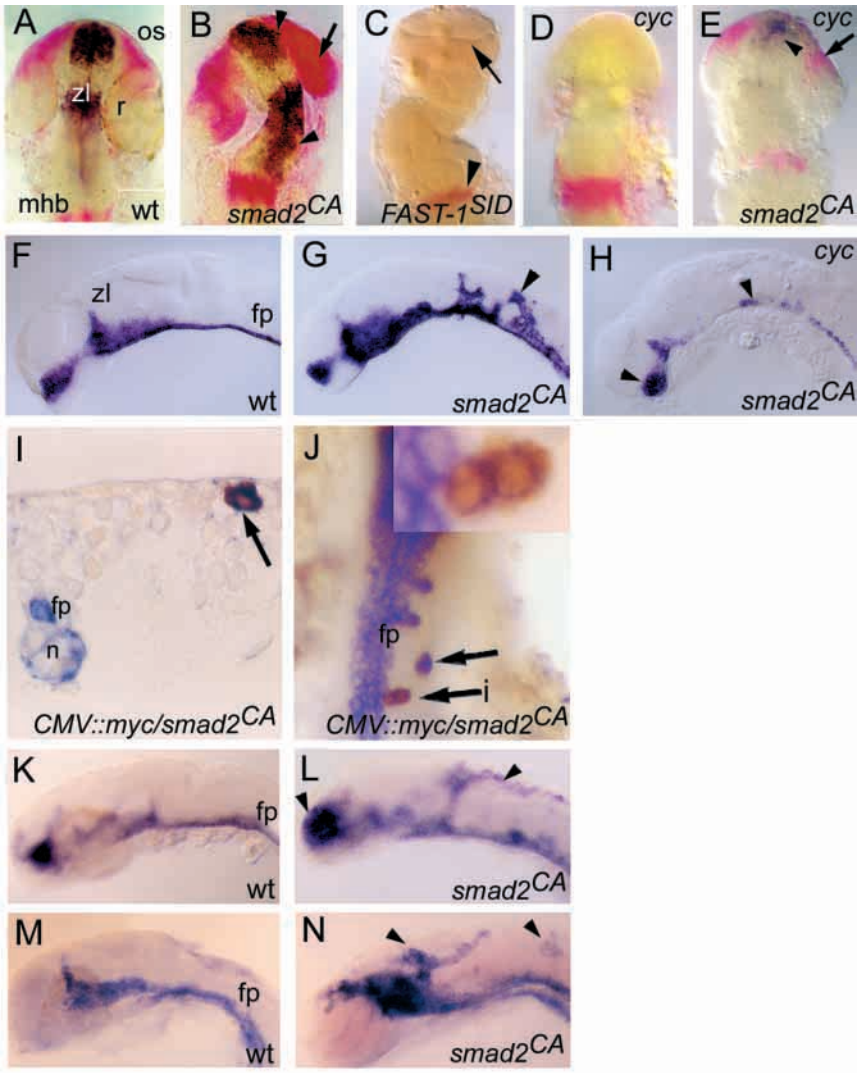

Fig. 2. Smad2^{CA} induces ectopic expression of ventral neural markers while FAST-1^{SID} abolishes ventral neural tube specific gene expression. (A-H) Smad2^{CA} induces *pax2.1* expression in the distal eye (red) and *shh* expression in the dorsal neural tube (blue/black). (A,F) Uninjected control embryos showing normal *shh* expression in the ventral neural tube and *pax2.1* expression (A) in the proximal part of the eye, optic stalk and the midbrain/hindbrain boundary. (B,G) *Smad2^{CA}* causes expansion of *shh* expression (arrowheads) paralleled by an expansion of *pax-2.1* expression into distal parts of the eye (red, arrow in B). (C,D) Injection of *FAST-1^{SID}* (C) causes effects similar to the phenotype of the *cyc* mutant (D). *FAST-1^{SID}* expressing embryos have cyclopia (arrow in C), elicit loss of *shh* expression in the ventral brain and *pax-2.1* expression in the remaining single eye while *pax2.1* expression in the midbrain/hindbrain boundary is present (arrowhead in C). (E,H) Mosaic expression of *Smad2^{CA}* rescues *shh* (arrowheads in E,H) and *pax-2.1* expression (arrow in E) in *cyc* mutant embryos. (A-E) Dorsal views anterior to the top; (F-H) lateral views, anterior left. (I,J) *Smad2^{CA}* activates *shh* in a cell-autonomous manner in the neuroectoderm. Expression of Myc-tagged *Smad2^{CA}* driven by the CMV promoter (brown) results in cell-autonomous activation of *shh* expression (blue) in the neural plate of early somite-stage wild-type embryo (arrow in I). Similarly in (J), arrows and the magnified insert of cells noted by the arrow labelled i, indicate colocalisation of *shh* expression and *Smad2^{CA}* in lateral cells of the hindbrain of a 24 hour embryo. (I) Transverse section through the anterior neural plate. (J) Dorsal view of the hindbrain, anterior up. (K-N) Activation of ventral neural markers by expression of *Smad2^{CA}*. Uninjected control embryos with expression of *netrin1* (K) and *axial/HNF3β* (M). Injection of *Smad2^{CA}* results in ectopic activation of *netrin1* (arrowheads in L) and *axial/HNF3β* (arrowheads in N) in the neural tube of 24 hour embryos. (K-N) Lateral views, anterior to the left. fp, floor plate; mhb, midbrain hindbrain boundary; n, notochord; os, optic stalk; r, retina; zl, zona limitans.

directly targets expression into neuroepithelial cells in situ (reviewed in Itasaki et al., 1999) and leaves other tissues such as notochord unaffected, as demonstrated by the pattern of expression of Green Fluorescent Protein (GFP) from coelectroporated *CMV::GFP* in stage-19 embryos (Fig. 3A,B). The *I1+I2/–2.2shh::lacZ* construct was electroporated (Muramatsu et al., 1997) into the folding neural plate in stage 10-12 chicken embryos, either alone or together with plasmids encoding regulators or empty plasmid vector as controls. Floor plate-restricted expression of *lacZ* was observed in the chick embryos electroporated with *I1+I2/–2.2shh::lacZ* (10/10 embryos, Fig. 3C,D). Coelectroporation of *CMV::cyc* and *I1+I2/–2.2shh::lacZ* resulted in the ectopic activation of *lacZ* in cells of the lateral neural tube (8/10 embryos; Fig. 3E). Similarly, ectopic activation of *lacZ* was noted when *CMV::smad2^{CA}* and *I1+I2/–2.2shh::lacZ* were coelectroporated (8/10 embryos, Fig. 3F). Ectopic activation of endogenous *shh* expression was also detected by in situ hybridisation of Smad2^{CA} expressing embryos, albeit at a lower frequency (3/15 embryos, data not shown). As a control, no ectopic *lacZ* expressing cells were detected when *CMV::smad1* was electroporated together with the *I1+I2/–2.2shh::lacZ* reporter construct, although floor plate-specific expression was maintained (*n*=7 embryos; data not shown). Dorsal expansion and an increase in the number of *lacZ* expressing cells were observed in the lateral neural tube when embryos were electroporated with *I1+I2/–2.2shh::lacZ*, *CMV::smad2^{CA}* and *CMV::FAST-1* (8/13 embryos, Fig. 3G,H). Taken together these results suggest that Cyc, and its downstream transducers Smad2 and FAST-1, are regulators of *shh* expression in neuroepithelial cells.

The *shh* gene harbours at least one Cyc responsive enhancer

The regulatory architecture of the *shh* gene is complex. As previously shown, multiple enhancer regions contribute to the

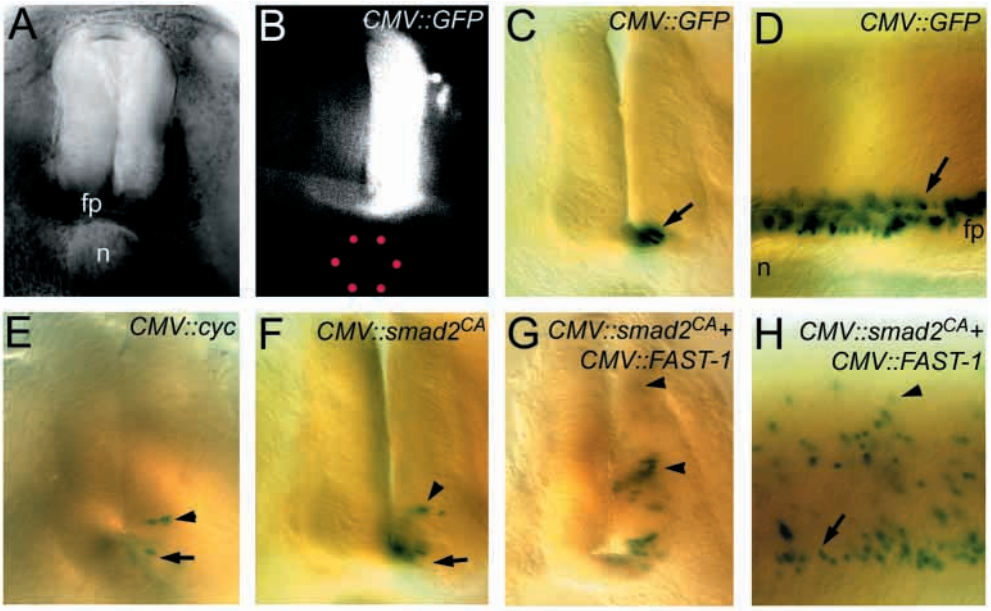

Fig. 3. Regulatory regions in the *I1+I2/–2.2shh::lacZ* reporter construct drive expression in the floor plate and respond to Cyc signals upon electroporation into the chicken neural tube. (A,B) Bright field (A) and fluorescent image (B) of a cross section of a chick neural tube anterior to the hindlimb bud at stage 19. The neural tube was transfected by injection with a *CMV::GFP* construct into the groove of the folding posterior neural plate at stage 10-12 and electroporation in situ. GFP expression is evident throughout the right half of the neural tube, but never detected in the notochord (depicted with red dots). (C,D) β-gal activity is restricted to the floor plate (arrows) in neural tubes coelectroporated with *I1+I2–2.2shh::lacZ* and *CMV::GFP*. (E-H) Electroporation of *CMV::cyc* (E), *CMV::smad2*^{CA} (F), or *CMV::smad2*^{CA} with *CMV::FAST-1* (G,H) causes ectopic activation of *I1+I2/–2.2shh::lacZ* (arrowheads in E-H). Arrows in E,F and H indicate *lacZ* expression in the floor plate. A-C and E-G are cross sections, dorsal up. (D,H) Lateral views of the spinal cord between the limb buds. (C-H) Expression vectors coelectroporated with *I1+I2–2.2shh::lacZ* are indicated in the top right corner of panels. fp, floor plate; n, notochord.

control of *shh* expression in the body axis. To locate the region responsible for activation by FAST-1/Smad2^{CA}, deletion constructs were tested by electroporation into the chick neural tube. The *–2.2shh::lacZ* reporter, which contains 2430 bp upstream of the transcription start site but lacks the intronic enhancer regions, was shown to drive expression in the ventral neural tube of zebrafish embryos (Müller et al., 1999a). When the *–2.2shh::lacZ* construct was electroporated alone into the chick neural tube (Fig. 4C) *lacZ* expression was confined to the floor plate (5/6 embryos), as in the case of the full-length *I1+I2/–2.2shh::lacZ* construct (Fig. 4A). Also, expression from the *–2.2shh::lacZ* construct could be activated ectopically by coexpression of FAST-1/Smad2^{CA} (6/6 embryos, Fig. 4D). In contrast, expression from construct *–563shh::lacZ*, which contains 563 bp upstream of the transcription start site of the *shh* promoter (Chang et al., 1997), failed to drive expression

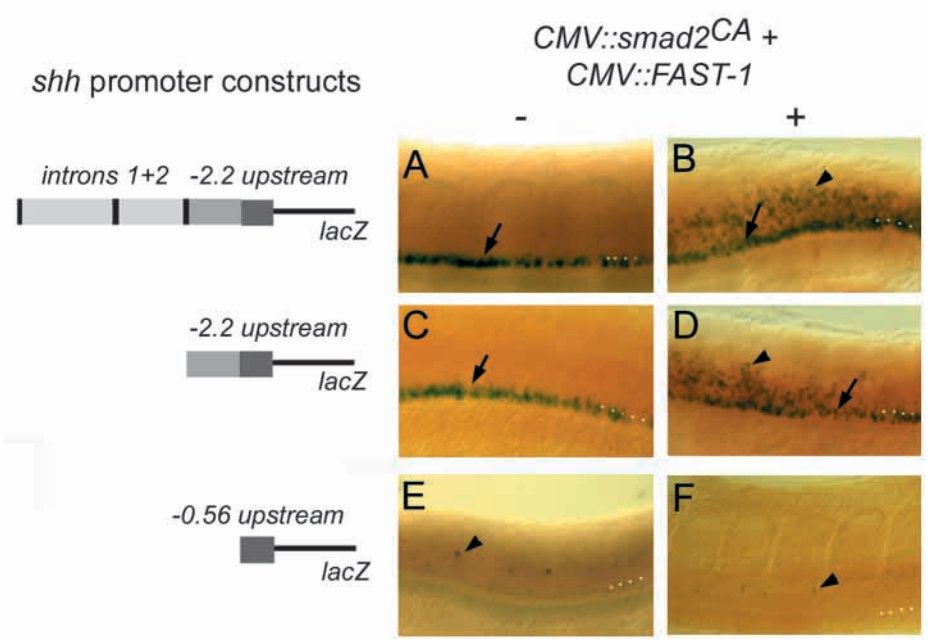

Fig. 4. The upstream enhancer region of the *shh* gene is responsive to Cyc signalling. (A,B) Neural tubes electroporated with *I1+I2/–2.2shh::lacZ* alone (A) or together with *CMV::smad2*^{CA} and *CMV::FAST-1* (B). (C,D) Neural tubes electroporated with *–2.2shh::lacZ* alone (C) or together with *CMV::smad2*^{CA} and *CMV::FAST-1* (D). The 2430 bp *shh* upstream sequence in *–2.2shh::lacZ* drives floor plate-specific expression (C, arrow) and can be activated ectopically by coexpression of *CMV::smad2*^{CA} and *CMV::FAST-1* (D, arrowhead) similarly to *I1+I2/–2.2shh::lacZ* (B, arrowhead). (E,F) The *–563shh::lacZ construct* containing the 563 bp proximal promoter region does not mediate floor plate specific expression (E), and can not be activated by coexpressed *CMV::smad2*^{CA} and *CMV::FAST-1*. Weak expression of *lacZ* is seen in few scattered cells in the electroporated neural tube (arrowheads in E,F). DNA constructs containing different genomic fragments of the *shh* locus are indicated schematically on the left. (A-H) Lateral views onto the trunk between the limb buds of stage 18-19 chick embryos. Anterior to the left. White dots indicate the dorsal border of the floor plate.

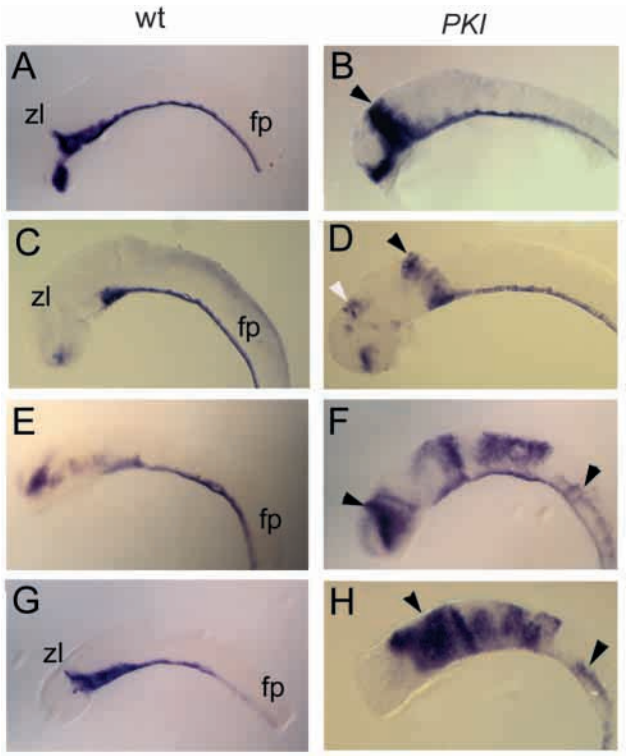

Fig. 5. PKI activates ventral neural markers with different efficiency. Embryos were injected with the PKI mRNA. Groups of control embryos (A,C,E,G) and injected embryos (B,D,F,H) were split and hybridised either to *shh* (A,B), *twhh* (C,D), *netrin1* (E,F) or *axial/HNF3_* (G,H) antisense probes. Ectopic expression of the paralogous genes *shh* and *twhh* was observed in the anterior brain. Activation of *shh* is limited to the region of the zona limitans in the diencephalon (B, arrowhead). Ectopic patches of *twhh* expression were observed in the midbrain (black arrowhead in D) and around the zona limitans (white arrowhead in D), but never posterior to the midbrain. In contrast, strong ectopic activation of *netrin1* (F) and *axial/HNF3_* (H) was observed in the midbrain and posterior to the midbrain (arrowheads). fp, floor plate; zl, zona limitans.

of *lacZ* in the floor plate (Fig. 4E). Instead, weak expression was detected in very few scattered cells throughout the neural tube (7/6 embryos), consistent with previous findings that the *–563shh* promoter region is not sufficient to drive floor plate specific expression (Müller et al., 1999a). Moreover, the *–563shh::lacZ* construct was not responsive to coexpressed FAST-1/Smad2^{CA} (7/8 embryos, compare Fig. 4F with E). It was upregulated in response to coexpressed *axial/HNF3β* (data not shown), consistent with the presence of multiple HNF3β binding sites in the promoter (Chang et al., 1997). The finding that *–2.2shh:lacZ* but not *–563shhlacZ* was responsive to Smad2/FAST-1 suggests that the upstream region between –2430 and –563 contains elements that are required for activation by FAST-1/Smad2.

shh expression cannot be activated by Hh signalling as efficiently as other floor plate marker genes

The direct effect of Cyc signalling on *shh* expression in the neural tube prompted us to re-examine the role of Hedgehogs in this process. We utilised expression of a dominant negative regulatory subunit of protein kinase A (PKI), which mimics Hh signalling (Concordet et al., 1996; Hammerschmidt et al., 1996) and compared the responsiveness of *shh* expression with that of other floor plate markers such as *netrin1* and *axial/HNF3β*. To minimise variation, *PKI* mRNA was injected into a large batch of zebrafish embryos, which was then split for staining with the different probes. Ectopic activation of *shh* by *PKI* was restricted to an expansion of *shh* expression in the zona limitans of the diencephalon (56.3%, *n*=126; Fig. 5A,B). Similarly, a restricted activation was observed for the *shh* homolog *twhh* in the diencephalon and the posterior mesencephalon (67.9%, *n*=112; Fig. 5C,D). In contrast, expression of *PKI* elicited strong ectopic activation of the floor plate marker genes *netrin1* (88.7%, *n*=71) and *axial/HNF3β* (83.9%, *n*=93; Fig. 5E,F and G,H, respectively), beginning from the diencephalon and extending into the rest of the brain overlying the floor plate. Thus, *shh* and *twhh* appear to be less responsive to Hedgehog signalling than *netrin1* and *axial/HNF3β*. This suggests that the regulatory mechanisms underlying the expression of *shh/twhh* in the floor plate differ from those controlling *netrin1* and *axial/HNF3β*.

cyc mutants may lack expression of ventral neural tube markers such as *netrin1* because they fail to establish *shh* (and *twhh*) expression in the neurectoderm. If this epistatic relationship were true, one would predict that *netrin1* or the ventral eye marker *pax2.1* can be induced by forced activation of Hh signalling when Cyc/Sqt signalling is blocked. To this end Cyc/Sqt signalling was inhibited by expression of *FAST-1^{SID}*, while the Hh pathway was simultaneously activated by coexpression of *PKI*. In the resulting double-injected zebrafish embryos, *netrin1* (Fig. 6A-C) was broadly activated ectopically (79.6% *n*=37) while no *netrin1* expression or only traces in the midbrain were observed in *FAST-1^{SID}*-injected embryos (80.6% *n*=31). *axial/HNF3β* was activated by *PKI* in a similar manner in embryos with blocked Cyc/Sqt signalling (data not shown). *pax2.1* expression in the eye was strongly induced (85.7%, *n*=49; Fig. 6D-F) while *shh* expression was only rescued in the area of the zona limitans of *FAST-1^{SID}* expressing embryos (77.1% of embryos showed complete lack of *shh* expression or were restricted to the zona limitans, *n*=35). These results show that *netrin1*, *axial/HNF3β* and *pax2.1* can be activated by Hh signalling in the absence of Cyc signalling. These results are, furthermore, consistent with the notion that *cyc* mutants lack expression of ventral neural tube marker genes because they failed to establish *shh (twhh)* expression in the CNS.

DISCUSSION

We provide evidence that Smad2 and FAST-1 are regulators of the *shh* gene. Our data furthermore suggest that Smad2 and FAST-1 or closely related molecules are transducers of the Nodal-like signals Cyc and Sqt, consistent with previous findings in both mouse and zebrafish embryos (Gritsman et al., 1999; Nomura and Li, 1998). It has been shown that *cyc* mutants lack *shh* expression in the neural tube but express *shh* mRNA in the notochord (Krauss et al., 1993). Our results support a direct role for Cyc signalling in the regulation of *shh* expression in the neural tube. In particular, we have shown that Smad2^{CA} activates *shh* in a cell-autonomous fashion in the zebrafish embryo. In addition, the upstream *shh* promoter/

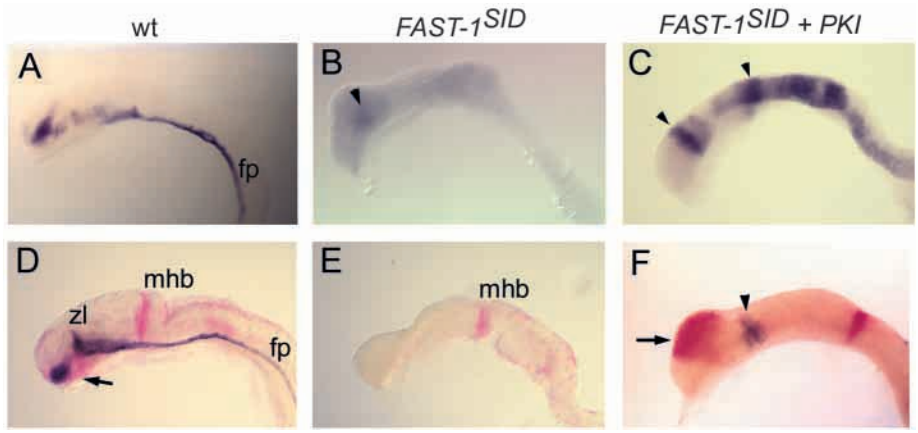

Fig. 6. PKI can activate *netrin1* and *pax2.1* in the absence of Cyc signalling. (A,D) Control uninjected embryos. (B,E) Embryos injected with *FAST-1*^{SID} mRNA. (C,F) Embryos double-injected with *FAST-1*^{SID} and *PKI* mRNA. Embryos were stained with either *netrin1* probe (A-C) or with a combination of *shh* (blue/black) and *pax2.1* (red) probes (D-F). Expression of *netrin1* and *shh* in the ventral neural tube and *pax2.1* in the eye is lost in embryos injected with *FAST-1*^{SID} (B,E). Traces of *netrin1* expression and unaffected *pax2.1* activity are detected in the more dorsal domain in the midbrain (arrowhead in B) and in the midbrain-hindbrain boundary (mhb) respectively. (C,F) Activation of *netrin1* is evident throughout the brain of embryos coinjected with *FAST-1*^{SID} and *PKI* (arrowheads in C) while *shh* expression was restricted to the zona limitans region (arrowhead in F). *pax2.1* expression in the anterior brain is strongly activated (arrow in F). All panels are lateral views of the head of 24 hour embryos. fp, floor plate; mhb, midbrain/hindbrain boundary; zl, zona limitans.

enhancer is activated in response to coexpression of Cyc and Smad2^{CA}/FAST-1 in the chick neural tube. Furthermore, the transactivation by Smad2^{CA}/FAST-1 depends on an enhancer that was previously shown to mediate ventral neural tube expression.

Our data best fit a model in which Cyc signalling is required for the establishment of *shh* expression in the neuroectoderm (Fig. 7). This mode of regulation also operates presumably on the paralogous gene *twhh* (Ekker et al., 1995; Krauss et al., 1993). Hh signalling, as assessed by ectopic induction of floor plate marker genes such as *netrin1* and *axial (HNF3β)*, is not impaired in *cyc* mutant embryos (Hammerschmidt et al., 1996; Strähle et al., 1997a) or in embryos injected with *FAST-1*^{SID} (this study). We propose a two-step model to explain the lack of floor plate and ventral brain identity in *cyc* mutant embryos (Fig. 7). The first step requires the action of Cyc signalling to establish *shh* (*twhh*) expression in the ventral neural tube. Once turned on in the neural tube, local Hh signals lead to activation of the downstream Hh target genes *netrin1*, *pax2.1* or *axial (HNF3β)*, a step that can occur in the absence of Cyc signalling. Our data, however, do not rule out that these genes can also be activated directly by Cyc signalling in an Hh-independent manner (Fig. 7, stippled arrow).

Our data imply indirectly an involvement of FAST-1 in the regulation of *shh*. In agreement with this, it was recently shown that *schmalspur*, which has a ventral neural phenotype very similar to *cyc* mutants, encodes Fast-1/FoxH1, a zebrafish homologue of FAST-1 (D. Meyer, personal communication). It remains to be established whether Smad2/FAST-1 interacts directly with the *shh* enhancer. Several homologies to the binding sites of FAST-1 (FAST binding elements, FBE (Chen et al., 1996; Labbe et al., 1998; Zhou et al., 1998) are present in the −2430/−563 region. It is likely that other enhancer regions in addition to the −2430/−563 upstream region are also responsive to Cyc signalling. For example, the intronic ar-C enhancer, which mediates notochord and ventral neural tube expression (Müller et al., 1999a), also contains an FBE. The FBEs, however, are not the only regions in these enhancers that are able to drive ventral neural tube expression. It remains to be determined whether these other regions are also responsive to Cyc signalling, as Smad2 may also act indirectly by activation of other transcription factors. An example of such a factor may be HNF3β.

HNF3β is crucial for notochord and floor plate development in mouse embryos (Ang and Rossant, 1994; Weinstein et al., 1994) and has been proposed to act both upstream and downstream of Shh signalling during midline development (Ruiz i Altaba et al., 1995b; Chang et al., 1997; Sasaki et al., 1997). In this model, Shh was envisaged to activate HNF3β in the neural tube, which would then turn on *shh* expression. HNF3β was also shown to be a target of activins (Strähle et al., 1993) and may be responsive to Cyc/Nodal signals. Several lines of evidence suggest, however, that HNF3β, even though necessary, is not sufficient to control *shh* expression in the ventral neural tube. First, enhancers in the mouse and zebrafish *shh* gene that drive expression in the ventral neural tube lack HNF3β binding sites, indicating that there are HNF3β-independent mechanisms of *shh* regulation (Epstein et al., 1999; Müller et al., 1999a). Moreover, although strongly ectopically activated by *PKI* in the CNS, HNF3β is not able to induce *shh* and *twhh* to the same extent in the neural tube of the zebrafish embryo. Smad2^{CA}/FAST-1 do not activate the

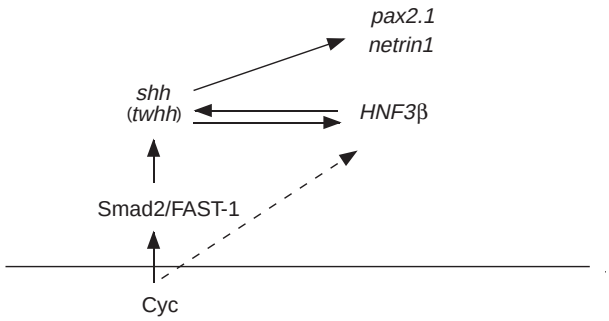

Fig. 7. A model of the function of Nodal and Shh signalling for activation of ventral neurectoderm markers during floor plate induction. Note that the model depicts developmental pathways; arrows may therefore not reflect single steps or direct molecular interactions.

–563shh promoter, which contains functional HNF3β sites. Furthermore, the *–563shh* promoter is not able to drive floor plate expression (Müller et al., 1999a; this study), suggesting that the HNF3β binding sites in the promoter region are not sufficient.

Bone morphogenetic proteins (BMP) inhibit development of ventral neural character in explant cultures (Liem et al., 1995). Furthermore, mouse embryos that lack the BMP inhibitor Noggin fail to develop a floor plate, suggesting that the activity of BMPs has to be blocked for floor plate, differentiation to occur (McMahon et al., 1998). It was previously noted that Smad2 and the BMP transducer Smad1 can compete for the common partner Smad4 in *Xenopus* embryos (Candia et al., 1997). Although we regard this competition as unlikely to be the main mechanism, Cyc signalling could nevertheless contribute to inhibition of BMP signalling via activation of Smad2 and subsequent sequestering of Smad4.

Transplantation and ablation experiments showed that the notochord has floor plate inducing activity, leading to the proposal that signals from the notochord induce floor plate (Placzek et al., 1990). Results of cell labelling experiments carried out in chick embryos, together with the expression patterns of floor plate and notochord-specific genes, suggested, however, that floor plate differentiation may be well under way before cells have taken up their final position in notochord and floor plate (Catala et al., 1996). Arguments based on marker gene expression in the zebrafish led to similar conclusions (Le Dourain and Halpern, 2000). Our results, which support a direct role for Cyc signalling in inducing expression of *shh* in the CNS and the early expression of *cyc* in the zebrafish shield and tail bud (Rebagliati et al., 1998a,b), argue in favour of an early specification of floor plate identity before the notochord has formed.

Shh expressed from the mature notochord is apparently not sufficient to induce its own expression in the zebrafish embryo as *cyc* mutants strongly express *shh* in the notochord (Krauss et al., 1993; Strähle et al., 1997b; Odenthal et al., 2000). This raises the question of the function of Hh signalling in this process in the zebrafish. When we overexpressed the Hh signal transducer PKI in the zebrafish embryo, we observed an expansion of *shh* expression, although this was limited to the zona limitans. This suggests that *shh* can induce its own expression, to some extent. Other ventral genes such as *netrin1* or *pax2.1* were, however, much more strongly activated by the same concentration of the mimic of Hh activity, *PKI*. One possibility is that only very high concentrations of Shh signalling may be able to efficiently auto-induce *shh* expression, a situation that we may not be able to achieve by injection of *PKI* mRNA. The presence of multiple *hh* related genes has so far precluded an analysis of Hh function in medial floor plate induction in the zebrafish (Currie and Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993; Schauerte et al., 1998). Irrespective of the role of Hh signals, the *cyc* mutant phenotype, together with our results, points to a prominent role for the Nodal related signal Cyc in the induction of *shh* expression in the zebrafish neural tube.

The mouse knock-out of *shh* is, due to its design, not informative as to whether *shh* is required to induce its own expression (Chiang et al., 1996). This raises the question whether Nodal or Nodal-related factors may also play a role in regulating *shh* expression in amniotes. Mouse embryos lacking Nodal activity are so severely malformed (Zhou et al., 1993; Conlon et al., 1994) that they do not allow conclusions to be made regarding whether Nodal has a function in the ventral neural tube. Several lines of indirect evidence argue, however, in favour of an involvement of Nodal signalling. Firstly, transgenes harbouring the control regions of the zebrafish *shh* gene drive reporter gene expression in the ventral neural tube of mouse embryos (Müller et al., 1999a). Secondly, mutations that affect the Nodal signal pathway in the mouse also cause defects in the ventral CNS. *smad2/+*, *nodal/+* transheterozygote mouse embryos show defects similar to *shh* mutant mice (Nomura and Li, 1998) Moreover, loss of *shh* expression occurs in the anterior brain in mouse embryos lacking *smad2* gene function, suggesting that a similar regulatory relationship exists between Smad2 and *shh* in the mouse (Heyer et al., 1999). We thus propose that the induction of *shh* expression by Nodal signals is fundamental to ventral neural tube development in higher vertebrates.

We thank I. David, S. Ekker, C. Kimmel, S. Krauss, M. Kühn and M. Whitman for plasmids. We are indebted to Siew L Ang and Carol Fode for critical reading of this manuscript and to S. Wilson for comments and discussion. We are grateful to D. Biellmann A. Karmin and O. Nkundwa, for care of the fish, D. Biellmann for art work, and to T. Ding for sectioning. This work was supported by an EMBO fellowship and AFM (F.M.), FRM (F.M., P.B.), ARC (S.A.) and the EC (P.B.). We are also grateful to the Institute National de la Sante et de la Recherche Medicale, the Centre National de la Recherche Scientifique, the Hopital Universitaire de Strasbourg, ARC, CNRS Projet Genome, AFM and La Ligue contre le Cancer.

REFERENCES

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

Attisano, L. and Wrana, J. L. (1998). Mads and Smads in TGF beta signalling. *Curr. Opin. Cell Biol.* **10**, 188-194.

Candia, A. F., Watabe, T., Hawley, S. H., Onichtchouk, D., Zhang, Y., Derynck, R., Niehrs, C. and Cho, K. W. (1997). Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124**, 4467-4480.

Catala, M., Teillet, M.-A., De Robertis, E. M. and Le Douarin, N. M. (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.

Chang, B.-E., Fischer, N., Blader, P., Ingham, P. and Strähle, U. (1997). Axial (HNF3β) and retinoic acid receptors are regulators of the zebrafish sonic hedgehog promoter. *EMBO J.* **16**, 3955-3964.

Chen, X., Rubock, M. J. and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-β signalling. *Nature* **383**, 691-696.

Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G. and Whitman, M. (1997). Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* **389**, 85-89.

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.

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

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-1928.

Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452-455.

Ding, Q., J, M., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C.

(1998). Diminished Sonic hedgehog signalling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2759-2770.

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

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

Erter, C. E., Solnica-Krezel, L. and Wright, C. V. E. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer. *Dev. Biol.* **204**, 361-372.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal- related signals. *Nature* **395**, 181-185.

Gritsman, K. Z.hang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.

Hamburger, V. and Hamilton, L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

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

Hauptman, G. and Gerster, T. (1994). Two-colour hybridisation whole mount in situ hybridisation to vertebrate and Drosophila embryos. *Trends. Genet.* **10**, 266.

Hatta, K., Kimmel, B. C., Ho, R. K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Heyer, J., Escalante-Alcade, D., Lia, M., Boettinger, E., Edelmann, W., Stewart, C.L., Kucherlapati, R. (1999). Postgastrulation Smad2-deficient embryos show defects in embryo turning and anterior morphogenesis. *Proc. Natl. Acad. Sci. USA* **96**, 12595-12600.

Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999). Shocking developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.* **1**, 203-207.

Kiecker, C., Müller, F., Wei, W., Glinka, A., Strähle, U. and Niehrs, C. (2000). Phenotypic effects in Xenopus and zebrafish suggest that one eyed pinhead functions in an active fashion. *Mech. Dev.* **94**, 37-46.

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

Kretzschmar, M. and Massague, J. (1998). SMADs: mediators and regulators of TGF-beta signaling. *Curr. Opin. Genet. Dev.* **8**, 103-111.

Labbe, E., Silvestri, C., Hoodless, P., Wrana, J. and Attisano, A. (1998). Smad2 and smad3 positively and negatively regulate TGFb dependent transcription through the forkhead DNA-binding protein FAST-2. *Mol. Cell* **2**, 109-120.

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

Liem, K. F., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

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

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

Matise, M., Epstein, D., Park, H., Platt, K. and Joyner, A. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2795-2770.

McMahon, J., Takada, S., Zimmerman, L., Fan, C., Harland, R. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **10**, 1438-1452.

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

Müller, F., Blader, P., Rastegar, S., Fischer, N., Knochel, W. and Strähle, U. (1999b). Characterisation of zebrafish smad1, smad2 and smad5: the amino-terminus of Smad1 and 5 is required for specific function in the embryo. *Mech. Dev.* **88**, 73-88.

Muramatsu, T., Mizutani, Y., Yasushige, O. and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Comm.* **230**, 376-380.

Nomura, M. and Li, E. (1998). Smad2 role in mesoderm formnation, left-right patterning and cranifacial development. *Nature* **393**, 786-790.

Odenthal, J., van Eeden, F., Haffter, P., Ingham, P. and Nüsslein-Volhard, C. (2000). Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev. Biol.* **219**, 350-363.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. M. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-988.

Rebagliati, M. R., Toyama, R., Haffter, P. and Dawid, I. B. (1998a). Cyclops encodes a nodal-related factor involved in midline signalling. *Proc. Natl. Acad. Sci. USA* **95**, 9932-9937.

Rebagliati, M. R., Toyama, R., Fricke, C., Haffter, P. and Dawid, I. B. (1998b). Zebrafish nodal related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev. Biol.* **199**, 261-272.

Roelink, H., Augsburger, A., Heemskerk, J., Kortzh, V., Norlin, S., Altaba, R. i., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T. M. (1995a). Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3 beta. *Dev. Biol.* **170**, 299-313.

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

Sampath, K., Rubinstein, A. L., Cheng, A. M. S., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. E. (1998). Induction of the zebrafish ventral brain and floor plate requires Cyclops/Nodal signalling. *Nature* **395**, 185-189.

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

Schauerte, H., van Eeden, F., Fricke, C., Odenthal, J., Strähle, U. and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983-2993.

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

Strähle, U., Blader, P. and Ingham, P. W. (1996). Expression of axial and sonic hedgehog in wildtype and midline defective zebrafish embryos. *Int. J. Dev. Biol.* **40**, 929-940.

Strähle, U., Fischer, N. and Blader, P. (1997a). Expression and regulation of a *netrin* homologue in the zebrafish embryo. *Mech. Dev.* **62**, 147-160.

Strähle, U., Jesuthasan, S., Blader, P., Garcia-Vilalba, P., Hatta, K. and Ingham, P. (1997b). One-eyed pinhead is required for floor plate development in the zebrafish embryo. *Genes Function* **1**, 131-148.

Watanabe, M. and Whitman M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early *Xenopus* embryo. *Development* **126**, 5621-5634.

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

Whitman, M. (1998). Smads and early developmental signalling by the TGFβ superfamily. *Genes Dev.* **12**, 2445-2462.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous systen: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.

Zhou, S., Zawel, L., Lengauer, C., Kinzler, K. and Vogelstein, B. (1998). Characterization of human FAST-1 a TGFβ and Activin signal transducer. *Mol. Cell* **2**, 121-127.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. and Kühn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* **361**, 543-547.