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Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in *Xenopus*

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SUMMARY

We analysed the role of Notch signalling during the specification of the dorsal midline in *Xenopus* embryos. By activating or blocking the pathway we found that Notch expands the floor plate domain of *sonic hedgehog* and *pintallavis* and represses the notochordal markers *chordin* and *brachyury,* with a concomitant reduction of the notochord size. We propose that within a population of the early organiser with equivalent potential to develop either as notochord or floor plate, Notch activation favours floor plate development at the expense of the notochord, preferentially before mid gastrula. We present evidence that *sonic hedgehog* down-regulates *chordin,* suggesting that secreted Sonic hedgehog may be involved or reinforcing the cell-fate switch executed by Notch. We also show that Notch signalling requires Presenilin to modulate this switch.

Key words: Notch, Sonic hedgehog, Chordin, Floor plate, Notochord, Presenilin, *Xenopus laevis*

INTRODUCTION

The floor plate is an epithelial structure located on the ventral midline of the vertebrate neural tube and constitutes an important source of signals involved in the induction of motor neurons and axonal pathfinding (Tanabe and Jessell, 1996; Colamarino and Tessier-Lavigne, 1995; Stoeckli and Landmesser, 1998). Two cell populations have been described as contributing to this structure: one in the midline, the medial floor plate (MFP), which expresses *sonic hedgehog (shh)* and *hnf3β*, and the other one, the lateral floor plate (LFP), which expresses *hnf3β* and flanks the medial cells (Odenthal et al., 2000). Studies on the avian embryo showed that the floor plate does not develop after removal of the caudal notochord but appears ectopically after grafting notochordal tissue onto the lateral or dorsal regions of the neural tube. Therefore, it was concluded that the floor plate is induced by the notochord (Placzek et al., 2000). This scenario, reinforced by work with other vertebrate models, considers Shh as the inducer (Tanabe and Jessell, 1996) and this is supported by the finding that mice lacking *shh* function fail to develop the floor plate (Chiang et al., 1996). It was proposed from over-expression experiments in frog embryos that secreted Shh from the notochord promotes Gli1 expression in the midline of the developing neural plate. Gli1, in turn, activates the winged-helix transcription factor *hnf3β,* or the related gene *pintallavis (plvs)* in *Xenopus,* which then activates *shh* in floor plate precursors (Lee et al., 1997). Finally, Shh induces motor neurons in the neighbouring ventral neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Roelink et al., 1995; Marti et al., 1995; Ericson et al., 1996; Lee et al., 1997).

Careful experiments revisited those studies performed on birds and found that the reason why floor plate did not develop after the removal of the notochord was that the floor plate precursors were removed also, since Hensen's node (equivalent to the amphibian's Spemann's organiser) generates both midline structures (Catala et al., 1996; Teillet et al., 1998; Le Douarin and Halpern, 2000). This agrees with several studies in vertebrates including chicken, mouse, fish and amphibians, which show that precursors of the floor plate, notochord and dorsal endoderm originate within the organiser (Spemann and Mangold, 1924; Selleck and Stern, 1991; Catala et al., 1995; Catala et al., 1996; Wilson and Beddington, 1996; Shih and Fraser, 1995; Melby et al., 1996; Amacher et al., 2002; Latimer et al., 2002). Therefore, if the notochord and the floor plate share the same embryonic origin, it will be necessary to reconcile this scenario with the role and the hierarchy of the molecules described above. This is complicated by the findings that in zebrafish embryos, only the development of the LFP seems to be Shh dependent, while the medial cells appear to be dependent on Nodal activity (Rebagliati et al., 1998; Sampath et al., 1998; Schauerte et al., 1998; Odenthal et al., 2000).

Notch signalling is best known from its central role in lateral inhibition during neurogenesis. Within a proneural cluster, the future neuron is the source of the membrane-bound ligand

Delta, which interacts with the receptor Notch on the surface of the neighbouring cells. The receptor is cleaved to render the intracellular domain ($Notch^{ICD}$) that enters the nucleus and, in association with *CSL* intracellular transducers such as Su(H), activates the transcription of target genes that lead to the suppression of the neuronal fate in the cells surrounding the neuronal precursor (Schroeter et al., 1998; Bray, 1998; Wolfe and Haas, 2001; Kopan and Goate, 2002).

Different models provided evidence that Presenilins facilitate the Notch signalling pathway. Most authors favour the idea that their γ-secretase activity, which mediates the proteolytic cleavage of the amyloid precursor protein (APP), is also involved in the final proteolytic step that cleaves the Notch receptor to produce $Notch^{ICD}$ (De Strooper et al., 1999; Struhl and Greenwald, 1999, Struhl and Greenwald, 2001; Chan and Jan., 1999; Taniguchi et al., 2002). It was also shown that a γ-secretase-independent mechanism may play a partial role in Notch signal transduction (Berezovska et al., 2000; Berechid et al., 2002) and other research suggested that Presenilin is required before the generation of $Notch^{ICD}$ (Ye et al., 1999; Ray et al., 1999).

We have previously shown that both *shh* and *presenilin* repress primary neurogenesis in *Xenopus laevis* embryos (Franco et al., 1999; Paganelli et al., 2001). Since *presenilin* stimulates *shh* expression in the floor plate, we suggested that the effects of *presenilin* could be exerted through *shh*. We also proposed that in the primary neurogenesis cascade, *shh* acts very upstream of the lateral inhibition step mediated by Notch, modulating the activity of prepattern genes. Bearing in mind the relationship between Notch and Presenilin, and because lateral inhibition does not account for the repression of primary neuron development after *presenilin* overexpression, we wondered whether Notch signalling could modulate *shh* expression.

To answer this question, we either activated or prevented Notch signalling in *Xenopus* embryos and found that Notch stimulates *shh* and *plvs* expression in the floor plate and represses the notochordal markers *chordin (chd)* and *brachyury (bra)*. These changes are accompanied by an expansion of the floor plate and a reduction of the notochord size. We propose that Notch may execute a binary decision, favouring floor plate development at the expense of the notochord, and this preferentially occurs before mid gastrula. We also show that *shh* down-regulates *chd* and suggest that *shh* itself may be involved in reinforcing the binary decision executed by Notch. We present evidence that Presenilin can also modulate this switch in a Notch-dependent way.

MATERIALS AND METHODS

Embryological manipulations, RNA synthesis, morpholinos and injections

Albino *Xenopus laevis* embryos were obtained using standard methods (Ruiz i Altaba, 1993), staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) and fixed with MEMFA (Harland, 1991).

Synthetic capped mRNAs for microinjection were obtained as described previously (Franco et al., 1999). To direct the material to the future dorsal midline populations, injections were delivered into the animal hemisphere of one blastomere at the 2-cell stage at approximately 30-40° from the equator and close to the cleavage plane, following the observations of Vodicka and Gerhart (Vodicka and Gerhart, 1995). The *Xotch* antisense oligodeoxynucleotide *(Xotch Mo)* used was a 25-mer morpholino oligo (Gene Tools, LLC) with the base composition 5′-GCACAGCCAGCCCTATCCGATCCAT-3′. The *X-ps-α* antisense morpholino oligonucleotide *(X-ps-α Mo)*, the standard control morpholino oligo *(Control Mo)* and the *X-ps-α* construction used for in-vitro transcription were the same as those employed by Paganelli et al. (Paganelli et al., 2001). The templates for mRNA synthesis has been described previously: *X-shh* (Franco et al., 1999); $notch^{ICD}$, *hGR/ICD22* and $X\text{-}su(H)^{DBM}$ (Wettstein et al., 1997). Nuclear translocation of *hGR/ICD22* was induced with dexamethasone (Sigma, D4902).

For RNA interference of *shh* function, a deletion was made in the construct used for making *X-shh* antisense probes described by Franco et al. (Franco et al., 1999) by digestion with *Eco*0109 I and *Bst*EII, followed by fill-in and ligation. The resulting construct contains a 935 bp insert encoding the N-terminal region of X-Shh, from bp 51 to bp 986 of the cDNA sequence (Ekker et al., 1995). Sense and antisense RNA were obtained after linearisation with *Eco*RI or *Kpn*I and transcription with T7 or T3 mRNA polymerase, respectively. 1 μg of each template was used for in vitro transcription, which was carried out with the Megascript kit (Ambion). Synthetic RNAs were purified with the Qiagen RNeasy mini kit. Equimolar amounts of sense and antisense RNAs were annealed in 1 mM $MgSO_4$, 30 mM NaCl at 70°C for 15 minutes and then at 37°C for 45 minutes. The quality of double strand RNA (ds-RNA) was tested by native agarose gel electrophoresis in TBE in the presence of ethidium bromide. Gel mobility was shifted according to ds-RNA of the expected length.

The amounts of synthetic mRNAs, ds-RNA and morpholinos injected are indicated in the table and figures. All injections included 0.5 ng of *nuc-lacZ* mRNA as tracer. For comparison, equal amounts of *nuc-lacZ* mRNA were delivered in each set of experiments.

Semi-quantitative RT-PCR, X-gal staining, in situ hybridisation, C-myc immunohistochemistry and histology

Semi-quantitative RT-PCR analysis was performed essentially as described by Paganelli et al. (Paganelli et al., 2001). The number of cycles and the template input for PCR were determined empirically in each case, within the linear range of amplification. The forward (F) and reverse (R) primer sequences, the product sizes, and the number of cycles were as follows: *ef1α:* F 5′-CAGATTGGTCCTGG-ATATGC-3′, R 5′-ACTGCCTTGATGACTCCTAG-3′, 268 bp., 26 cycles for stage 12, 25 cycles for stage 15; *X-shh:* F 5′-ATGCT-GGTTGCGACTC-3′; R 5′-CCCGCCAGACTTGG-3′, 581 bp., 36 cycles for stage 12, 32 cycles for stage 15.

X-gal staining, preparation of digoxigenin-labeled antisense RNA probes and whole-mount in situ hybridisation were performed as described previously (Franco et al., 1999), except that the proteinase K step was omitted in in situ hybridisation when embryos would be further processed for immunohistochemistry.

50 μm sections were cut using an Oxford Vibratome and mounted onto gelatine coated slides as described by Hollemann et al. (Hollemann et al., 1996), except that 4% formaldehyde was used instead of glutaraldehyde during the embedding, in order to reduce background fluorescence. For immunolocalization of the C-myc epitope, slides were washed three times in PBS, for 10 minutes each, incubated for 1 hour at room temperature in blocking buffer containing 5% nonfat milk (Molico, Nestlé) in PBS, then overnight at 4°C with mouse 9E10 anti-Myc monoclonal antibody (Santa Cruz) diluted 1/500 in blocking buffer, in a wet chamber. The following day, slides were washed three times at room temperature with PBS containing 0.1% Tween 20 (10 minutes each), twice with PBS (10 minutes each), and incubated for 2 hours at room temperature in a wet chamber in the dark with anti-mouse immunoglobulins-FITC (Dako F0232) diluted 1/200 in blocking buffer. After washing with TBS

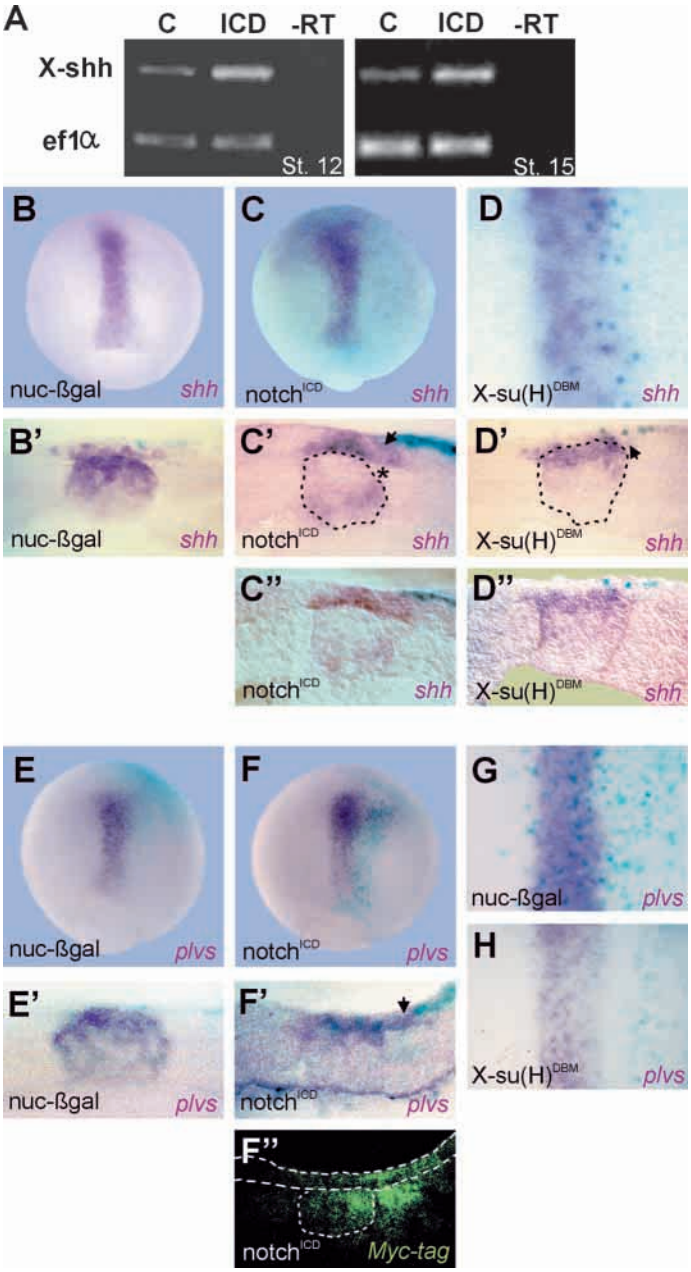

Fig. 1. Notch signalling up-regulates *shh* and *plvs* in the floor plate domain. (A) Semiquantitative RT-PCR analysis of *X-shh* transcripts at stage 12 (left) and 15 (right). C, control embryos; ICD, embryos bilaterally injected with 1 ng of *notch*^{ICD} mRNA per blastomere at the 2-cell stage; –RT, PCR amplification without the addition of reverse transcriptase. The constitutively ef1α transcript was used as internal standard. (B-D″) In situ hybridisation of *shh*. (E-F′,G,H) In situ hybridisation of *plvs*. (F″) Immunofluorescence revealing the Myc-tag epitope. Whole embryos in B,C,D,E,F,G,H are shown in dorsal views, anterior up. The *nuc-lacZ* (nuc-βgal) tracer is visualised by the turquoise X-gal staining. (B) Stage 13 embryo injected with 2.5 ng of *nuc-lacZ*. (C) Stage 13 embryo injected with 1 ng of *notch*^{ICD}. (D) Stage 13 embryo injected with 2 ng of *X-su(H)*^{DBM} shown at high magnification to appreciate the down-regulation of *shh* on the injected side (right). (B′) Transverse section of a stage 14 embryo injected with 2.5 ng of *nuc-lacZ*. (C′,C″) Transverse section of a stage 14 embryo injected with 1 ng of *notch*^{ICD}. Arrow points to the expanded floor plate domain of *shh* on the injected side, while loss of notochordal tissue is indicated by an asterisk. (D′,D″) Transverse section of a stage 14 embryo injected with 2 ng of *X-su(H)*^{DBM}. Arrow points to the depletion of *shh* transcripts from the floor plate domain on the injected side. The broken line in C′,D′ indicates the notochord contour determined by the Nomarski interference contrast view shown in C″,D″, respectively. (E) Stage 12 embryo injected with 1.5 ng of *nuc-lacZ*. (F) Stage 12 embryo injected with 1 ng of *notch*^{ICD}. (E′) Transverse section of a stage 14 embryo injected with 1.5 ng of *nuc-lacZ*. (F′,F″) Transverse section of a stage 14 embryo injected with 1 ng of *notch*^{ICD}. The arrow in F′ indicates the expanded floor plate domain of *plvs*. The broken lines in F″ outlines the notochord and neuroectodermal contour of the section shown in F. Ectopic floor plate cells that inherited the *notch*^{ICD} mRNA, as revealed by the Myc-tag epitope, express *plvs*. (G) Stage 13 embryo injected with 2 ng of *nuc-lacZ* shown at high magnification. (H) Stage 13 embryo injected with 2 ng of *X-su(H)*^{DBM} shown at high magnification to appreciate that *plvs* expression is strongly reduced on the superficial layer of the injected side (right) corresponding to the floor plate domain, while a deeper staining, corresponding to the notochord, remains.

(pH 7.5) containing 0.1% Tween 20, slides were mounted in PBS:glycerol (1:2).

RESULTS

Notch expands the floor plate domain of shh and plvs

To investigate whether Notch signalling can modulate *shh* expression, we first examined the effects of bilateral injections of *notch*^{ICD} mRNA, which encodes a constitutively active form of the receptor independent of ligand binding, and analysed the expression of *shh* by semiquantitative RT-PCR. Active Notch significantly increased the levels of *shh* transcripts, and this was evident both at late gastrula and at neurula stages (Fig. 1A). Next, we wanted to know whether changes in the spatial distribution of *shh* transcripts could account for this up-regulation. For this purpose, we examined the effects of unilateral injections of *notch*^{ICD} by in situ hybridisation.

Early neurulae showed that *notch*^{ICD} enhances *shh* expression on the injected side (83% of injected embryos, *n*=24; Fig. 1C). This was evident as an increase in the density of *shh*-positive cells and/or a lateral expansion of the domain. As with *presenilin* injections, we did not observe ectopic expression of *shh* in other regions of the embryo.

To corroborate whether endogenous Notch activity was indeed involved in this modulation, we prevented Notch signalling by injecting *X-su(H)*^{DBM} mRNA, which encodes a dominant-negative variant of the Notch transducer X-Su(H). Whole embryos at the neural plate stage showed that *shh* transcripts were down-regulated (82%, *n*=90; Fig. 1D).

Since *shh* mRNA is present both in the prospective floor plate and notochord at this stage, we wanted to know which structures were affected. For this purpose, we analysed transverse sections of more advanced neurulae, when the notochord and floor plate are more clearly distinguished from each other. Control embryos show *shh* transcripts in the floor

plate and a dorsal-ventral gradient in the notochord, with highest levels close to the floor plate (Fig. 1B′). Embryos injected with *notch*^{ICD} revealed an expansion of the *shh* domain corresponding to the floor plate and a concomitant reduction of the notochord size on the injected side (Fig. 1C′,C″). Conversely, *X-su(H)*^{DBM}-injected embryos showed a decrease in *shh* transcripts in the floor plate domain, while the notochord size was simultaneously augmented on the injected side (Fig. 1D′,D″).

The Notch-induced up-regulation of *shh* in the floor plate domain may happen by a direct or indirect regulation and/or by favouring the development of the floor plate. Therefore, we studied the consequences of activating the Notch pathway on the expression of another gene that was described to participate in floor plate development. *Plvs* mRNA precedes and overlaps *shh* expression and is first detected in the dorsal marginal zone of late blastulae. During gastrulation it is expressed at the dorsal midline by cells that undergo convergent-extension movements. At the early neurula stage, transcripts are distributed throughout the dorsal midline in the three germ layers, i.e. the prospective floor plate, the notochord and the dorsal endodermal cells lining the archenteron (Ruiz i Altaba and Jessell, 1992) (Fig. 1E,E′).

Whole embryos at the neural plate stage show that *plvs* expression also increases on the injected side when the Notch pathway is stimulated (75%, *n*=16; Fig. 1F) and transverse sections confirm that this occurs in the floor plate domain, which appears expanded (Fig. 1F′,F″). Conversely, *X-su(H)*^{DBM}-injected embryos showed a decrease in *plvs* expression (95%, *n*=19; Fig. 1H). Thus, Notch signalling positively regulates the expression of two genes that were shown to be able of promoting floor plate development.

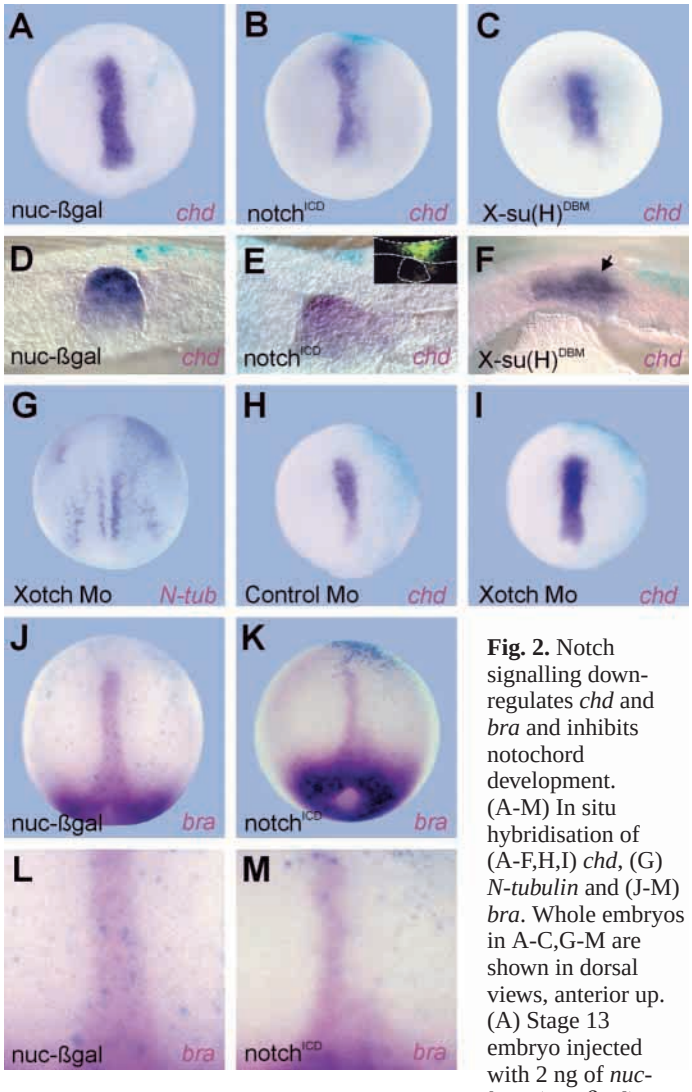

Fig. 2. Notch signalling down-regulates *chd* and *bra* and inhibits notochord development. (A-M) In situ hybridisation of (A-F,H,I) *chd*, (G) *N-tubulin* and (J-M) *bra*. Whole embryos in A-C,G-M are shown in dorsal views, anterior up. (A) Stage 13 embryo injected with 2 ng of *nuc-lacZ* (nuc-βgal). (B) Stage 13 embryo injected with 1 ng of *notch*^{ICD}. (C) Stage 12.5 embryo injected with 2 ng of *X-su(H)*^{DBM}. (D) Transverse section of a stage 14 embryo injected with 2.5 ng of *nuc-lacZ* . (E) Transverse section of a stage 14 embryo injected with 2 ng of *notch*^{ICD}. The inset shows the immunolocalization of the Myc-tag epitope to reveal inheritance of *notch*^{ICD} mRNA. (F) Transverse section of the same embryo shown in C. The arrow points to *chd*-positive cells that adopted more dorsal positions. (G) Stage 15 embryo injected with 5 ng of *Xotch Mo* in the animal hemisphere of one blastomere at the 2-cell stage, to assay for the activity of the antisense morpholino. Notice the increase in the number of *N-tubulin*-positive primary neurons on the injected side (right), as expected after impairing the Notch pathway. (H) Stage 12.5 embryo injected with 5 ng of *Control Mo*. (I) Stage 12.5 embryo injected with 5 ng of *Xotch Mo*. (J) Stage 13 embryo injected with 1.5 ng of *nuc-lacZ*. (K) Stage 13 embryo injected with 1 ng of *notch*^{ICD}. (L,M) Higher magnification of the same embryos shown in J,K, respectively.

Notch decreases *chd* and *bra* expression and restricts notochord development

To further study the effect of Notch signalling on notochord development, we analysed whether activating or blocking the pathway could change the expression of the mRNA encoding the secreted polypeptide Chd. In early neurulae, *chd* transcripts are normally present in the notochord and the prechordal mesoderm (Sasai et al., 1994) (Fig. 2A,D). At this stage we found that *notch*^{ICD} strongly reduced *chd* expression on the injected side (66%, *n*=35; Fig. 2B). Transverse sections of slightly more advanced embryos again showed that the notochord size, now visualised by the expression of *chd*, was reduced on the injected side, while the overlying layer containing the prospective floor plate was concomitantly thickened (Fig. 2E). In contrast, when Notch signalling was interfered with by injecting *X-su(H)*^{DBM} or an antisense morpholino oligonucleotide against *Xenopus notch-1 (Xotch Mo)*, *chd* expression was increased, appearing as a more dense and superficial staining in whole embryos (63%, *n*=49 for *X-su(H)*^{DBM}; 75%, *n*=16 for *Xotch Mo*; Fig. 2C,I). Transverse sections revealed that this effect was the result of an increase of *chd*-positive cells, which adopted more dorsal positions, as if they were occupying the normal place of floor plate cells (arrow, Fig. 2F).

Therefore, Notch signalling inhibits *chd* expression and this may occur through a direct or indirect regulation and/or by inhibiting the development of the dorsal axial mesoderm. Thus, we studied the effects of activating the Notch pathway on the expression of *bra*, which is necessary for notochord development (Chesley, 1935; Halpern et al., 1993; Conlon et al., 1996; Smith, 1997).

In *Xenopus*, transcription of *bra* begins at mid-blastula transition, but strongest levels are achieved when gastrulation starts. At this stage, transcripts are distributed

throughout the entire marginal zone (presumptive mesodermal cells). During gastrulation, mesodermal cells that migrate anteriorly turn-off *bra* expression with the exception of the notochord. During neurula stages, transcripts persist only in the notochord and in a circumblastoporal ring (Smith et al., 1991) (Fig. 2J,L).

Neurula stage embryos showed that *notch^{ICD}* strongly reduced the notochordal expression of *bra* (86%, *n*=42; Fig. 2K,M). We conclude that activation of the Notch pathway down-regulates the expression of two notochordal markers, the transcription factor Bra and the secreted polypeptide Chd, and concomitantly decreases the notochord size; at the same time, Notch up-regulates two molecules expressed by the floor plate, the transcription factor Plvs and the secreted factor Shh, and increases the floor plate size. All this evidence suggests that a binary choice determines between floor plate and notochordal fates and when Notch signalling is active, favours floor plate development at the expense of the notochord.

The floor plate versus notochord switch executed by Notch mainly occurs before mid gastrula

To determine the time of highest competence for the proposed binary decision in response to Notch signalling, we performed time-course experiments by injecting *hGR/ICD22* mRNA, a construction encoding the ligand-binding domain of the human glucocorticoid receptor fused to the amino termini of Notch^{ICD}. Thus, nuclear translocation of Notch^{ICD} can be induced upon dexamethasone administration (Wettstein et al., 1997). Control embryos were injected with the construction but left untreated. Two windows of induction with 10 μM dexamethasone were assayed: one that included the first half of gastrulation (from stage 9+ to stage 11) and the other included the second half (from stage 11 to stage 13). In situ hybridisation revealed that in response to Notch signalling, nearly three-fold more embryos were able to up-regulate *shh* before stage 11 than after this stage. Meanwhile, *chd* down-regulation occurred in nearly two-fold more embryos before stage 11 than after this stage (Table 1). These results suggest that the Notch-mediated binary decision that chooses floor plate fate in preference to the notochord for the trunk region mainly takes place around early gastrulation. To further test this hypothesis, we analysed whether the effect of activating or blocking Notch signalling on *chd* expression could also be detected at early gastrula. We chose *chd* instead of *shh* because of its stronger and broader expression in the early organiser (see below), so that unambiguous differences in transcripts levels between the injected and non-injected side could be distinguished. We found that *notch^{ICD}* decreased *chd* transcripts in the organiser on the injected side (90%, *n*=21; Fig. 3B; see also Fig. 5B,C), while *X-su(H)^{DBM}* produced the opposite effect (63%, *n*=49; Fig. 3C).

Thus, the down-regulation of *chd* by Notch signalling is already initiated at early gastrula, when the competence of *chd* and *shh* for responding to active Notch is highest.

Table 1. *Shh* and *chd* are more susceptible to regulation by Notch during the first half of gastrulation

Dexamethasone	Marker analysed	Increase	Decrease	No change	*n*
Untreated	*shh*	0 (0%)	0 (0%)	17 (100%)	17
	chd	0 (0%)	0 (0%)	12 (100%)	12
From st. 9 to st. 11	*shh*	8 (80%)	0 (0%)	2 (20%)	10
	chd	0 (0%)	5 (56%)	4 (44%)	9
From st. 11 to st. 13	*shh*	3 (43%)	0 (0%)	4 (57%)	7
	chd	0 (0%)	3 (20%)	12 (80%)	15

Embryos were injected with 1 ng of *hGR/ICD22* mRNA and left untreated or were treated with 10 μM dexamethasone during the periods indicated.

Xenopus notch-1 transcripts are present in the early organiser and later in floor plate precursors

It was reported that *Xenopus notch-1 (Xotch)* transcripts are

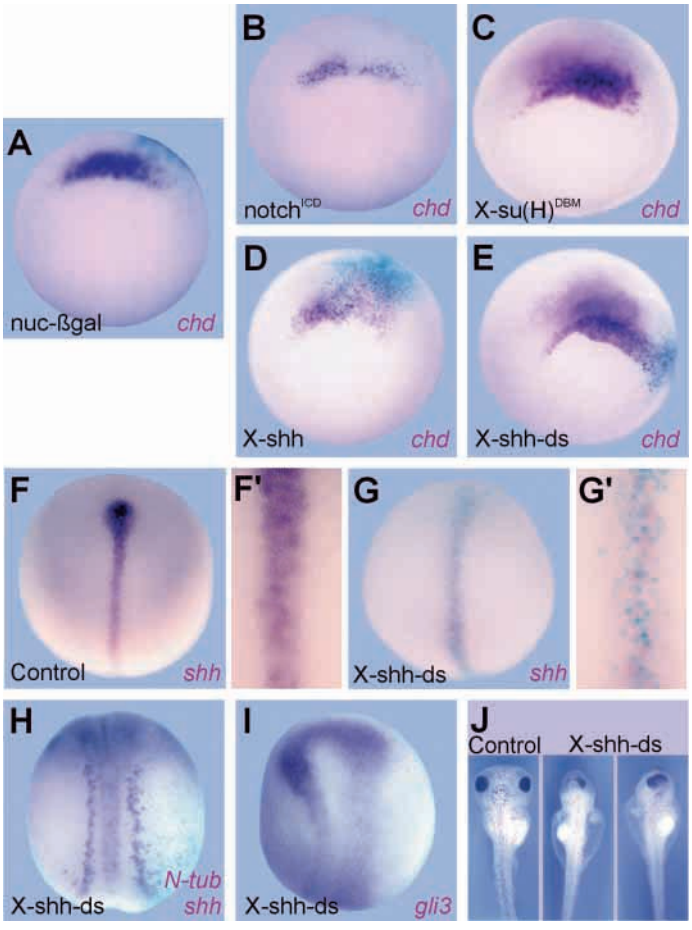

Fig. 3. Shh signalling down-regulates *chd*, resembling the effect of Notch. (A-E) In situ hybridisation of *chd* in early gastrulae, vegetal views, dorsal up, injected side on the right. (F-G′) In situ hybridisation of *shh* in neurulae, dorsal views, anterior up. (H) In situ hybridisation of *N-tubulin* and *shh* in neurulae. (I) In situ hybridisation of *gli3* in neurulae. (J) External morphology at tadpole stages. (A-E) Embryo injected with (A) 2 ng of *nuc-lacZ*, (B) 1 ng of *notch^{ICD}*, (C) 2 ng of *X-su(H)^{DBM}*, (D) 1 ng of *X-shh* mRNA, (E) 2 ng of *X-shh-ds* RNA. (F) Control embryo. (F′) Higher magnification of the embryo shown in F. (G) Embryo injected with 2 ng of *X-shh-ds* RNA to show the degradation of endogenous *shh* transcripts (77% of injected embryos, *n*=22). (G′) Higher magnification of the embryo shown in G. (H,I) Embryos injected with 2 ng of *X-shh-ds* RNA on the right side. (J) Control embryo (left) and two embryos injected with 1 ng of *X-shh-ds* RNA per blastomere at the 2-cell stage showing different grades of cyclopia (right).

present in domains of primary neurogenesis from late gastrula stages (Chitnis et al., 1995). RNAse protection assays revealed maternal and zygotic *Xotch* mRNA and its presence in the three germ layers, especially in the dorsal mesoderm, when gastrulation starts (Coffman et al., 1990). A more precise study of the early distribution would help to understand whether *Xotch* transcripts are present at the right time and place to elicit the effects suggested above. Therefore, we performed in situ hybridisation of early and late gastrula/early neurula stages and attempted to correlate *Xotch* mRNA distribution with the expression patterns of *chd* and *shh*.

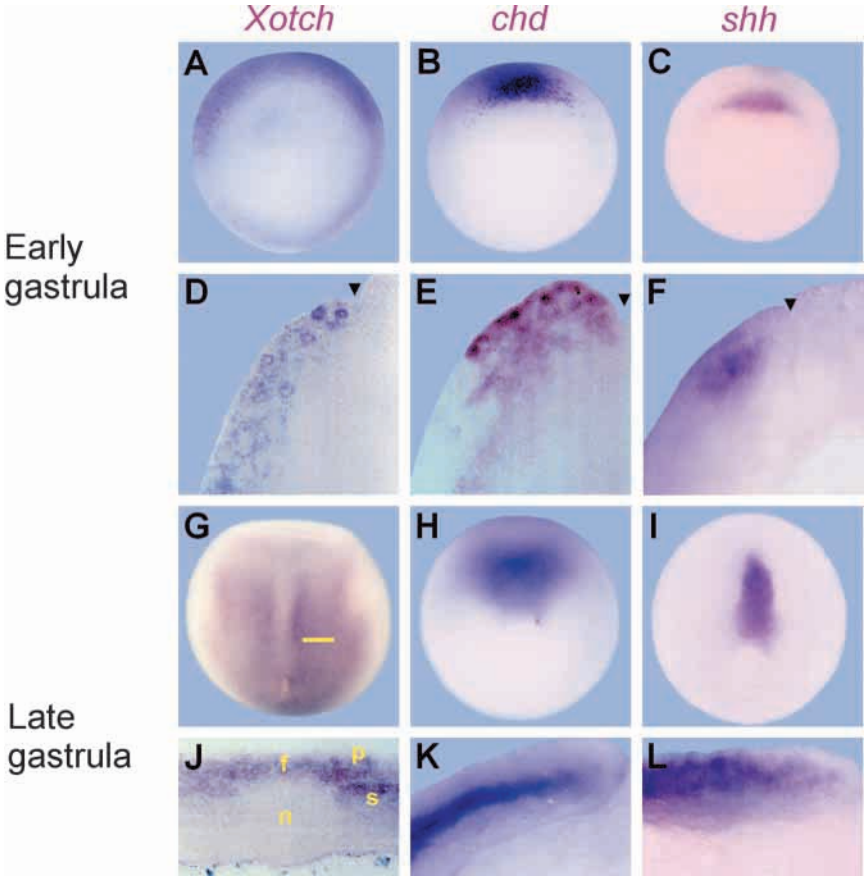

Fig. 4. Comparison of the expression patterns of *Xotch*, *chd* and *shh*. In situ hybridisation of (A,D,G,J) *Xotch*, (B,E,H,K) *chd* and (C,F,I,L) *shh*. (A-C) Vegetal view of an early gastrula, stage 10.25, dorsal side up, showing (A) the distribution of *Xotch* mRNA in the dorsal marginal zone, (B) the expression of *chd* in the organiser and (C) *shh* transcripts, first seen at this stage in the organiser and found in a subdomain of the *chd* territory. (D-F) Vibratome section in the sagittal plane of an early gastrula showing (D) the expression of *Xotch* in the epithelial and subepithelial layers of the organiser, (E) the expression of *chd* in the epithelial and subepithelial layers and in the deep zone of the organiser and (F) a patch of cells expressing *shh* in the subepithelial layer of the organiser, with some faint staining in the epithelial layer. The arrowheads in D,E,F point to the dorsal blastoporal groove. Distinctions in the cellular composition of the gastrula organiser followed the criteria of Hausen and Riebesell (Hausen and Riebesell, 1991). (G) Late gastrula/early neurula embryo, dorsal view, anterior up, showing the distribution of *Xotch* transcripts. Interestingly, we observed asymmetries in several embryos, with higher levels of *Xotch* mRNA on the right side. The yellow line indicates the plane of section shown in J. (H) Dorsal view of a late gastrula, anterior up, showing the expression of *chd* in the involuted cells. (I) Dorsal view of a late gastrula, anterior up, showing the expression of *shh* in the dorsal midline. (J) Transverse vibratome section at the level of the posterior trunk of the same embryo as in G. *Xotch* transcripts are found in proneural domains (p) of the neural plate, presomitic mesoderm (s) and in the developing floor plate (f) but are not found in the notochord (n). Notice higher levels of *Xotch* mRNA in the proneural and presomitic domains on the right side. More anterior sections also showed the presence of *Xotch* transcripts in prospective floor plate cells, although at lower levels, and their absence from the notochord (not shown). (K) Sagittal section of the same embryo shown in H, anterior to the left; the blastopore is at the right margin. *Chd* transcripts are distributed along the notochord. (L) Sagittal section of the same embryo shown in I, anterior to the left; the blastopore is at the right margin. *Shh* transcripts are found in the three dorsal midline layers, in a dorsal to ventral gradient.

At early gastrula, *Xotch* transcripts are present both in the involuting and non-involuting dorsal marginal zone (Fig. 4A) and extend towards the animal pole but are absent from the ventral marginal zone and the vegetal yolk mass (not shown). Sagittal sections reveal *Xotch* transcripts in the epithelial and subepithelial layers of the dorsal blastopore lip (Fig. 4D), where *chd* is strongly expressed (Fig. 4B,E). *Shh* transcripts are first detected at this time (Fig. 4C), but are delayed in relation to *chd*, which began to be expressed in the dorsal marginal zone shortly before gastrulation started (Sasai et al., 1994). It is noticeable that *shh* expression is more confined than *chd* and *Xotch*: transcripts are mainly found in several cells in the subepithelial layer of the organiser, with some faint distribution in the epithelial layer (Fig. 4F). Neither *Xotch* nor *shh* mRNA are detected in the deep zone of the organiser, which mostly contains the involuted precursors of the prechordal mesoderm and expresses *chd*. Therefore, when gastrulation starts, a population of cells containing notochord precursors expresses the three markers analysed.

When gastrulation ends, apart from the known expression in the presomitic mesoderm and neural ectoderm, *Xotch* transcripts are detected in floor plate precursors, with higher levels in the posterior region, and are absent from the notochord (Fig. 4G,J). Around this time, *chd* mRNA is restricted to the developing notochord (Fig. 4H,K) and *shh* expression is evident in the three dorsal midline layers, i.e. the prospective floor plate, the notochord and the lining of the archenteron (Fig. 4I,L). Across these populations, a dorsal to ventral gradient of *shh* expression is evident, which later correlates with the higher levels of expression found in the floor plate and in the dorsal region of the notochord (Fig. 1B′).

Thus, at early gastrula, *Xotch* transcripts are present in a population of cells in the organiser which is known to contain notochord precursors and co-express *chd* and *shh*, giving support to the idea that Notch signalling may be involved in the regulation of these genes and in cell-fate decisions

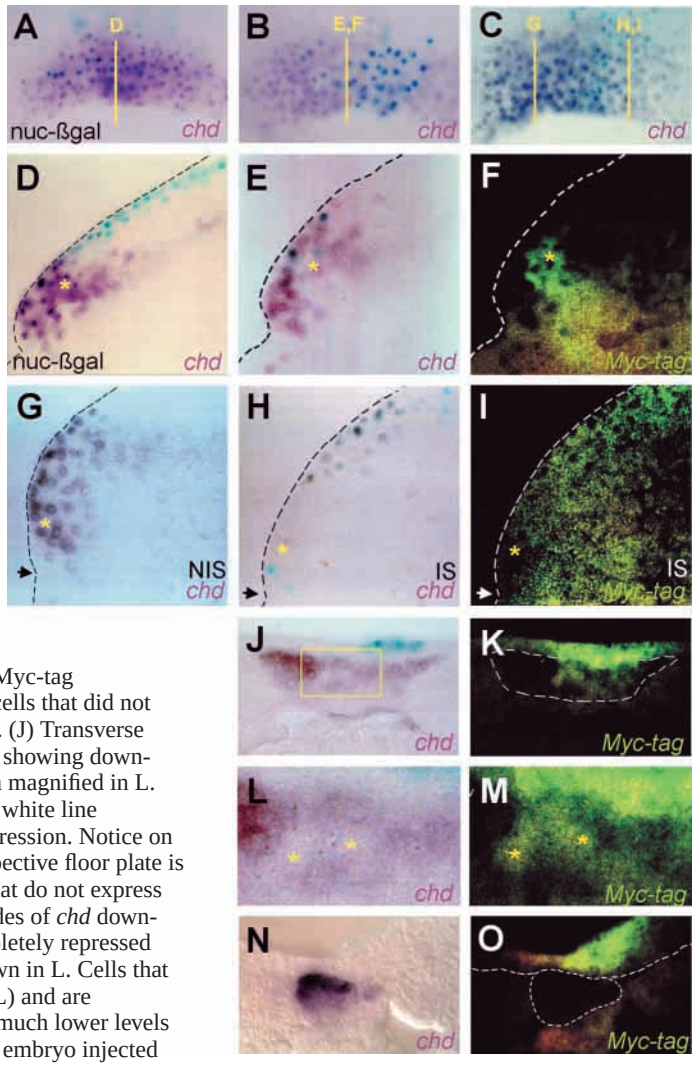

Fig. 5. Notch down-regulates *chd* cell-autonomously and non cell-autonomously. (A-E,G,H,J,L,N) In situ hybridisation of *chd*. (F,I,K,M,O) Immunofluorescence revealing the Myc-tag epitope. In A-C the yellow lines indicate the plane of section of subsequent panels, as indicated. (A) Early gastrula injected with 2 ng of *nuc-lacZ* (nuc-βgal). (B) Early gastrula injected with 1 ng of *notch*^{ICD}. Notice the repression of *chd* coincident with the X-gal-positive patch. (C) Another early gastrula injected with 1 ng of *notch*^{ICD} showing on the injected side (right) repression of *chd* at a distance from the X-gal stained cells, which also show down-regulation of *chd*. (D) Sagittal section of the *nuc-lacZ*-injected embryo shown in A. The asterisk marks cells with strong expression of *chd* in the organiser and serves as reference for comparing similar cell locations in the sections in E,F. (E) Sagittal section of the *notch*^{ICD}-injected embryo shown in B. Notice the strong repression of *chd* in some of the organiser cells (asterisk; compare with D). (F) Myc-tag immunolocalization of the same section shown in E. Notice that the patch of cells that inherited the *notch*^{ICD} mRNA (asterisk) coincides with the patch of cells that down-regulated *chd* expression. (G) Parasagittal section at 250 μm from the sagittal plane, non-injected side, of the embryo shown in C. The asterisk marks cells with strong expression of *chd* in the organiser and serves as a reference for comparing with similar cell locations in the contralateral section shown in H,I. (H) Parasagittal section at 250 μm from the sagittal plane, injected side, of the embryo shown in C. Notice the strong down regulation of *chd* on the injected side. (I) Myc-tag immunolocalization of the same section shown in H. Notice that some cells that did not inherited the *notch*^{ICD} mRNA (asterisk) down-regulated *chd* expression. (J) Transverse section of a stage 13 embryo injected with 1 ng of *notch*^{ICD} mRNA and showing down-regulation of *chd* on the injected side. The yellow box indicates the area magnified in L. (K) Myc-tag immunolocalization of the section shown in J. The broken white line indicates the limits of the developing notochord, as revealed by *chd* expression. Notice on the injected side that the boundary between the notochord and the prospective floor plate is not clearly defined and is mostly populated by Myc-tag-positive cells that do not express *chd*. (L) Higher magnification of the section in J, showing different grades of *chd* down-regulation on the injected side. Asterisks mark two cells that have completely repressed *chd* expression. (M) Myc-tag immunolocalization of the same area shown in L. Cells that inherited *notch*^{ICD} mRNA do not express *chd* (asterisks, compare with L) and are intermingled with Myc-tag-negative cells that express *chd*, although in much lower levels than cells on the non-injected side. (N) Transverse section of a stage 15 embryo injected with 1 ng of *notch*^{ICD} mRNA showing the down-regulation of *chd* and the reduction of the notochord on the injected side, viewed with Nomarski interference contrast optics. (O) Myc-tag immunolocalization of the same section shown in N. Notice that all cells that inherited the *notch*^{ICD} mRNA are *chd* negative and do not contribute to the notochord but populate the overlying layer containing the prospective floor plate, which appears thickened on the injected side. The broken white line delineates the contour of the notochord and the neural epithelium as identified in N.

during the specification of the dorsal midline structures. Later on, among the dorsal midline layers, *Xotch* transcripts are only present in the developing floor plate, suggesting that notochord cells in the trunk can no longer respond to Notch ligands to divert towards floor plate fate.

Notch activity down-regulates *chd* expression both in cell-autonomous and non cell-autonomous ways

Early gastrulae injected with *notch*^{ICD} showed that the patches of X-gal staining coincided with the territories of *chd* repression but in some embryos, repression of *chd* was also seen towards more lateral regions at a distance from the X-gal patch (Fig. 5B,C). Because X-gal staining could be underestimating the cells harbouring the injected *notch*^{ICD} mRNA, we made use of the c-Myc epitope fused to the Notch^{ICD} fragment encoded by the synthetic mRNA that we injected in our experiments. Thus, we were able to identify more precisely those cells that inherited and translated the *notch*^{ICD} mRNA by immunofluorescence for the Myc-tag and compared their localisation with the expression of *chd*.

Sagittal sections of early gastrulae showed down-regulation of *chd* expression in patches of Myc-tag-positive cells (compare Fig. 5E,F with 5D). In addition, *chd* down-regulation was also observed in Myc-tag-negative cells that were surrounded by Myc-tag-positive cells (compare Fig. 5H,I with 5G). Transverse sections of early neurulae also revealed Myc-tag-positive cells displaying a strong down-regulation of *chd* intermingled with Myc-tag-negative cells that did express *chd*, although in much lower levels than cells on the non-injected side (Fig. 5J-M). Therefore, since *chd* expression was repressed in the same cells that inherited the *notch*^{ICD} mRNA and also in neighbouring cells, our results suggest that active Notch has the ability to down-regulate *chd* expression in both cell-autonomous and non-cell-autonomous ways. Transverse sections of more advanced neurulae showed that Myc-tag-positive cells were *chd* negative and rarely contributed to the

notochord; instead, they were located in the overlying layer containing the floor plate (Fig. 5N,O; see also Fig. 2E), which appeared thickened, while the notochord was concomitantly reduced in size and *chd*-positive cells were decreased on the injected side as shown before, indicating that cells that inherited *notch*^{ICD} mRNA diverted their notochord fate and became incorporated into the floor plate. This developmental series suggests that cells within the organiser are able to repress *chd* expression in response to active Notch and later segregate to the prospective floor plate.

Shh down-regulates *chd*

Bearing in mind the preceding results and the proposed role of Shh as inducer of floor plate development, we wanted to analyse whether an enhancement of Shh signalling could contribute to reduce the number of *chd*-positive cells. Gastrulae unilaterally injected with *X-shh* mRNA showed a decrease in *chd* expression in the early organiser (83%, *n*=36; Fig. 3D), in a manner reminiscent to that obtained after Notch activation (Fig. 3B). Next, we wanted to know what happened if we interfered with *shh* function. Double-stranded RNA has been successfully used as a potent and specific reagent for silencing different genes in *Xenopus* embryos (Oelgeschläger et al., 2000; Nakano et al., 2000; Lau et al., 2001; Zhou et al., 2002). When we specifically degraded the endogenous transcripts with *X-shh* double-stranded RNA *(X-shh-ds)* (compare Fig. 3G,G′ with F,F′), we observed a significant increase of *chd*-positive cells on the injected side (44%, *n*=43; Fig. 3E), resembling the effect of blocking Notch signalling with *X-su(H)*^{DBM} (Fig. 3C). Because the X-gal staining was not reduced in *X-shh-ds*-injected embryos when compared with those injected with *nuc-lacZ* mRNA alone (compare Fig. 3E with 3A), we conclude that the phenotype that we observe is not due to non-specific mRNA degradation or to non-specific interference of protein translation. To verify the specificity of the effects produced by *X-shh-ds*, we analysed the expression of *N-tubulin* and *gli3*, which were shown to be down-regulated by *X-shh* overexpression (Franco et al., 1999). We also examined the distance between the optic vesicles in tadpoles, since targeted disruption of the mouse *shh* gene leads to cyclopia (Chiang et al., 1996). As expected, *X-shh-ds* increased the number of primary neurons (55%, *n*=38; Fig. 3H), expanded the *gli3* domain (55%, *n*=38; Fig. 3I) and produced several grades of cyclopia (80%, *n*=35; Fig. 3J). Moreover, another hint that the action of *X-shh-ds* RNA is specific is that it does not deplete other unrelated endogenous transcripts, for example *N-tubulin* or even *chd* (notice in Fig. 3H that the X-gal staining is extensively distributed on the injected side of the embryo, indicating that *X-shh-ds* RNA, although present in the domains of *N-tubulin* expression, does not promote the degradation of *N-tubulin* mRNA; for *chd* see Fig. 3E). Thus, Shh signalling specifically down-regulates *chd* and this effect is already evident when the proposed switch controlling notochordal and floor plate fates modulated by Notch mainly takes place. This correlation suggests that Shh signalling may be in part mediating the effect of Notch in the specification of the different cell populations that configure the dorsal midline.

Presenilin up-regulates *shh* and down-regulates *chd* in a Notch-dependent way

Because Presenilins have been implicated in Notch signalling, we wanted to know whether Notch mediates the up-regulation of *shh* promoted by *presenilin*. Therefore, we unilaterally co-injected *Xenopus* embryos with *presenilin-α (X-ps-α)* and *X-su(H)*^{DBM} mRNAs and analysed the expression of *shh* at neurula stages. When injected alone, *X-ps-α* expands *shh* expression on the injected side (84%, *n*=19; Fig. 6B), as we have previously described (Paganelli et al., 2001). Transverse sections show a lateral expansion of the floor plate domain and a concomitant reduction of the notochord domain on the injected side (Fig. 6E), resembling the consequences of Notch activation. When Notch signalling was prevented by co-injection of *X-su(H)*^{DBM} (i.e. down-stream of the processing step of the receptor where Presenilin is thought to intervene), *X-ps-α* was unable to up-regulate *shh*. Moreover, the effect of blocking the Notch pathway prevailed, and we observed a reduction of *shh* in the floor plate domain (82%, *n*=34; Fig. 6C,F).

If Presenilin affects *shh* expression and the development of the floor plate and the notochord by facilitating Notch signalling, then, *chd* expression should be impaired by *presenilin* in the same way as after Notch activation. A set of experiments was performed to test this hypothesis. Over-expression of *X-ps-α* resulted in a down-regulation of *chd* (41%, *n*=39; Fig. 6H,K). Conversely, blocking *X-ps-α* translation with a specific antisense morpholino oligonucleotide had the opposite effect, and in these X-Ps-α-depleted embryos *chd* expression appeared as a more dense and superficial staining than in embryos injected with a control morpholino (80%, *n*=5; compare Fig. 6N,P with M,O), similar to the effect observed after blocking Notch signalling with *X-su(H)*^{DBM}. When Notch signalling was prevented by co-injection of *X-su(H)*^{DBM}, *X-ps-α* was unable to down-regulate *chd*, and the effect of blocking Notch signalling prevailed again: *chd* was up-regulated and transverse sections showed a higher density of *chd*-positive cells on the injected side (57%, *n*=23; Fig. 6I,L).

Together, these results suggest that Notch signalling requires Presenilin activity to modulate the binary switch that decides between notochord and floor plate fates.

DISCUSSION

Although substantial evidence that Presenilins facilitate Notch signalling has been collected, we found that Notch-mediated lateral inhibition was probably not responsible for the repression of primary neurogenesis after *presenilin* overexpression in *Xenopus* (Paganelli et al., 2001). However, since *presenilin* was able to up-regulate *shh* expression, which also represses primary neurogenesis (Franco et al., 1999), we considered the possibility that Notch could be participating earlier in the cascade, modulating *shh* expression. We confirm this hypothesis and also present evidence that the Notch pathway is involved in cell fate decisions during the specification of the dorsal midline.

Our data show that Notch signalling promotes the expression of *shh* and *plvs*, two markers of floor plate specification, and together expands the floor plate. At the same time it represses the expression of both notochordal markers examined, *chd* and *bra*, while concomitantly reduces the notochord size.

Grafting and ablation experiments in birds have shown that

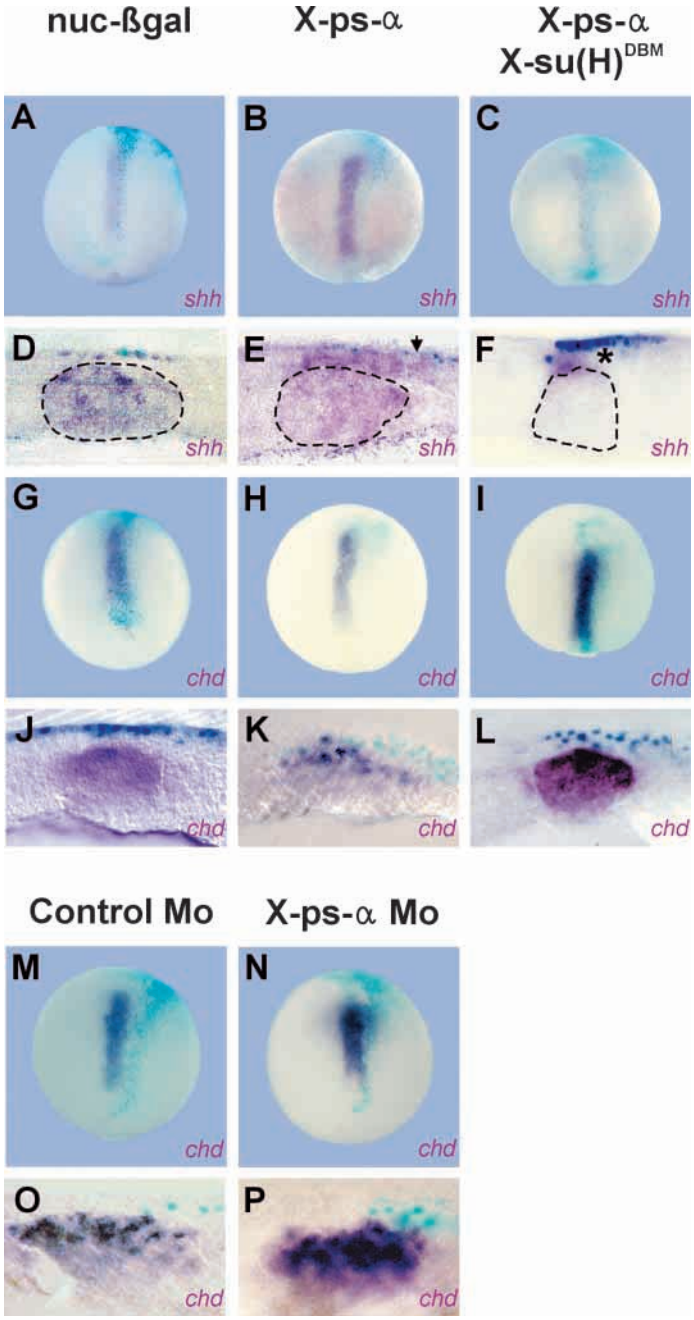

Fig. 6. Presenilin up-regulates *shh* and down-regulates *chd* in a Notch-dependent way. In situ hybridisation of (A-F) *shh* and (G-P) *chd*. Whole embryos in A-C,G-I,M,N are shown in dorsal views, anterior up. (A) Stage 14 embryo injected with 4.5 ng of *nuc-lacZ* mRNA (nucβgal). (B) Stage 14 embryo injected with 2 ng of *X-ps-α*. (C) Stage 14 embryo injected with 2 ng of *X-ps-α* plus 2 ng of *X-su(H)*^{DBM}. (D) Transverse section of the same embryo shown in A. (E) Transverse section of the same embryo shown in B. Notice the expansion of the floor plate domain of *shh* on the injected side (arrow). (F) Transverse cut of a stage 16 embryo injected with 2 ng of *X-ps-α* plus 2 ng of *X-su(H)*^{DBM}. Owing to the strong inhibition, the visualisation of *shh* expression in 50 μm vibratome sections from these embryos was difficult. In order to appreciate differences between the injected and non-injected side, we show here a transverse cut obtained with a scalpel. The asterisk indicates the repression of *shh* expression in the floor plate domain on the right side, where X-gal stained cells are preferentially located. Notice that the notochord is complementarily augmented on this side. Overall, the notochord is larger than in embryos of the same stage injected with equal amounts of *nuc-lacZ* mRNA (not shown). The broken line in D-F outlines the notochord. (G) Stage 13 embryo injected with 4 ng of *nuc-lacZ* mRNA. (H) Stage 13 embryo injected with 2 ng of *X-ps-α*. (I) Stage 13 embryo injected with 2 ng of *X-ps-α* plus 2 ng of *X-su(H)*^{DBM}. (J,K,L) Transverse sections of the embryos shown in G, H and I respectively. (M) Stage 12.5 embryo injected with 5 ng of a control morpholino. (N) Stage 12.5 embryo injected with 5 ng of an antisense morpholino oligonucleotide against *X-ps-α*. (M,P) Transverse sections of the embryos shown in M and N respectively. All sections, except F, are Nomarski interference contrast views.

the notochord and the floor plate derive from the Hensen's node during primary neurulation, and from the cordoneural hinge (the remains of Hensen's node) during secondary neurulation [(Catala et al., 1995; Catala et al., 1996; Teillet et al., 1998) for revision see Le Douarin and Halpern (Le Douarin and Halpern, 2000)]. Lineage tracing revealed that the *Xenopus* late organiser, which is the equivalent of the avian cordoneural hinge, also originates both structures during tail formation (Gont et al., 1993) and the pioneer work of Spemann and Mangold in amphibians clearly demonstrated that the implanted dorsal lip differentiates into notochord and floor plate in the trunk [see figure 25 in Spemann and Mangold (Spemann and Mangold, 1924)]. When the cellular origin of the *Xenopus* organiser was traced in the 32-cell embryo, the progeny of the B1 blastomere gave rise to 70% of the organiser, and descendants were found both in the notochord and the floor plate [see figure 6 in Vodicka and Gerhart (Vodicka and Gerhart, 1995)]. Fate maps of the embryonic shield, the teleost equivalent of the Spemann's organiser, also established that this region contributes to both structures (Shih et al., 1995; Melby et al., 1996; Amacher et al., 2002; Latimer et al., 2002). Therefore, if the floor plate and the notochord derive from a common cell population, our results indicate that Notch may be executing a binary cell-fate decision: when active, it promotes floor plate specification at the expense of the notochord. This is consistent with other findings in zebrafish embryos: missense mutants of the Notch ligand *delta A (dlA)* develop excess notochord and reduced numbers of floor plate and hypochord cells, while overexpression of *dlA* leads to the opposite effect (Appel et al., 1999).

For the trunk region, the proposed binary decision appears to take place mainly around the beginning of gastrulation, since the effect of activating or blocking Notch signalling on *chd* expression is already evident at early gastrula and the competence of *chd* and *shh* to respond to active Notch is highest from stage 9+ to stage 11. Therefore, at least two parallel mechanisms seem to contribute to stop this Notch-mediated cell-fate switch in the dorsal midline precursors while gastrulation proceeds: first, *Xotch* transcripts ultimately disappear from the developing notochord, suggesting that these cells become refractory to divert to floor plate in response to Notch ligands; second, the competence of the binary switch for responding to active Notch decreases throughout gastrulation. Interestingly, *X-delta-1* transcripts are present in the dorsal blastopore lip at stage 10.5 (Ma et al., 1996) and then disappear

from the involuted cells (Wittenberger et al., 1999). Functional experiments should elucidate whether X-Delta-1 is the ligand that operates the proposed binary switch, but its down-regulation in the involuted cells may underlie another key component in the mechanisms that contribute to stop Notch signalling in the dorsal midline.

Besides the previously reported up-regulation of *shh*, we show here that *presenilin* also down-regulates *chd*. Because both activities were prevented when the transduction of the Notch pathway was impeded, we conclude that Presenilin is required by Notch signalling during the binary switch that specifies dorsal midline fates. *X-ps-α* expression appears to be ubiquitous, but transcripts are present at the right time to modulate Notch signalling during dorsal midline development (Tsujimura et al., 1997).

Notch signalling up-regulates the expression of molecules involved in floor plate specification

Evidence from several sources suggest that Notch activation may trigger a cascade linking *shh* and *plvs*, which ultimately results in favouring floor plate development. First, *notch*^{ICD} injection increases both transcripts in floor plate precursors (this paper). Second, their expression domains overlap, but *plvs* precedes *shh*, beginning at late blastula (Ruiz i Altaba and Jessell, 1992) (this work). After neural tube closure *plvs* mRNA is replaced by transcripts from the closely related gene *hnf3β*, and it was proposed that the combined expression of both transcription factors in *Xenopus* is equivalent to that of *hnf3β* in rats and mice (Ruiz i Altaba et al., 1993a). Third, functional correlation from overexpression experiments in frog embryos associates *plvs/hnf3β* and *shh* with the specification of floor plate fate: *hnf3β* promotes the ectopic expression of floor plate markers including *shh* and *hnf3β* itself, in the neural tube; *plvs* promotes the ectopic expression *of F-spondin*, a marker of differentiated floor plate, in the dorsal neural tube, and *shh* induces the ectopic expression of *F-spondin*, *hnf3β* and *shh* itself (Ruiz i Altaba et al., 1993b; Ruiz i Altaba et al., 1995; Roelink et al., 1994). It could be argued that in these studies, the ectopic expression of floor plate markers within the neural ectoderm was obtained at tadpole stages and could not be detected earlier, when floor plate is normally specified. Furthermore, in *Xenopus* embryos the regulatory relationship between *plvs* and *shh* was not directly tested and the ability of *hnf3β/plvs* and *shh* to promote floor plate development was not analysed in the context of their normal functional domain. Therefore, functional experiments in this context should be carried out to test the hierarchy of the relationship between *shh* and *plvs* upon Notch activation.

Evidence from other vertebrate models also indicate that *shh* and *hnf3β* are functionally linked. Mice lacking *shh* activity did not develop a distinct floor plate despite the presence of a differentiated notochord during early stages, and *hnf3β* expression in the ventral neural tube was never initiated (Chiang et al., 1996). However, mice homozygous for targeted mutations of the *hnf3β* gene failed to develop the notochord and the floor plate and lacked *shh* expression, but the severe impairment in the development of the node and its derivatives did not allow us to determine whether *hnf3β* is directly required for *shh* expression (Ang and Rossant, 1994; Weinstein et al., 1994). However, HNF3-binding sites have been found in the promoter and other regulatory regions of the mouse and zebrafish *shh* gene, including intronic enhancers that direct the expression in floor plate and notochord, and it was suggested that *shh* expression in both structures is regulated by HNF3-dependent and independent mechanisms (Chang et al., 1997; Müller et al., 1999; Epstein et al., 1999). However, the enhancer that directs *hnf3β* expression in floor plate cells in mice contains a Gli binding site, which was proposed to respond to Shh signalling (Sasaki et al., 1997). Thus, evidence collected from mice suggest that Shh protein from the notochord induces HNF3β during specification of the floor plate and HNF3β in turn activates *shh* expression in floor plate cells. Analysis in zebrafish support the idea that *shh* is a target of HNF3β, nevertheless, it was proposed that, unlike floor plate development in the mouse, zebrafish embryos employ two distinct mechanisms for floor plate specification, one dependent on Nodal activity, which induces MFP, and the other dependent on Shh, which induces LFP (Schauerte et al., 1998; Odenthal et al., 2000).

Notch down-regulates the expression of molecules involved in dorsal axial mesoderm development

In *Xenopus*, *chd* is able to promote notochord development in mesodermalised animal caps and in u.v.-ventralised embryos, both in cell-autonomous and non cell-autonomous ways (Sasai et al., 1994). Chd is a potent antagonist of ventralising BMPs (Sasai et al., 1995; Piccolo et al., 1996), and it was suggested that notochord formation requires co-repression of both BMP and Wnt signalling (Yasuo and Lemaire, 2001). Inactivation of Chd protein by the metalloprotease Xolloid leads to strong ventralised phenotypes up to neurula stages, and later the notochord is frequently absent (Piccolo et al., 1997). Lack of Chd activity in zebrafish embryos disrupts posterior notochord development, and Ntl (Bra) protein is absent from the posterior notochord (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). Thus, Chd has been mainly regarded as an inhibitor of ventralising signals during mesodermal patterning and its requirement for notochord development may reflect this fact.

Experiments conducted in *Xenopus* embryos showed that *bra* is one of the direct targets of mesoderm-inducing factors and promotes development of posterior mesoderm in ectodermal explants (Smith et al., 1991). The dorsal-ventral character of this mesoderm depends on *bra* concentration, the highest dose being able to promote somitic muscle but never notochord formation (Cunliffe and Smith, 1992; Cunliffe and Smith, 1994), and hence, it is unable to induce *chd* in this kind of explants (Taira et al., 1997). However, when co-expressed with either *plvs* or the BMP antagonist *noggin* (which is also present in dorsal mesoderm), *bra* can promote notochord development (Cunliffe and Smith, 1994; O'Reilly et al., 1995). Besides, lack of *bra* function in mouse and zebrafish or changing its behaviour from transcriptional activator to repressor in *Xenopus* results in the absence of posterior mesoderm and failure of notochord differentiation (Chesley, 1935; Halpern et al., 1993; Conlon et al., 1996). It appears therefore that the transcription factor Bra is necessary but not sufficient in the pathway that leads to notochord differentiation, and other molecules such as Plvs and BMP antagonists may cooperate in this process. In this context, it will be interesting to test whether the combined action of *bra* and the BMP antagonist *chd* is sufficient to promote notochord

formation. In conclusion, our results show that Notch signalling down-regulates the expression of two molecules required for notochord development.

Notably, it was demonstrated that the floor plate, revealed by *shh* expression, is widened in zebrafish *ntl* mutant embryos (Halpern et al., 1997), suggesting that Bra activity antagonises floor plate development while promoting notochord formation. Moreover, in line with our findings, it was recently described that Notch represses *ntl* cell-autonomously (Latimer et al., 2002). Therefore, *bra* may constitute a key target in the binary switch that decides between notochord and floor plate fates under the control of Notch signalling. Further experimentation will be needed to elucidate whether a hierarchical relationship links *chd* and *bra* in this switch.

Notch signalling may be required for medial floor plate specification

The enhancement of *shh* and *plvs* transcripts that we observe could be due to a Notch-dependent up-regulation of both genes or it may be a consequence of favouring floor plate development. Analogously, *chd* and *bra* down-regulation may be the result of their repression by active Notch or a consequence of disfavouring notochord development. However, as discussed above, *shh* and *plvs* have been implicated in the specification of floor plate fate, and *bra* and *chd* are necessary for proper notochord formation. Therefore, more than being regarded as mere markers, changes in the expression of these genes appear to be inherent in the fate decisions that are taking place. Thus, it seems more likely that Notch signalling triggers floor plate specification at the expense of the notochord through the opposite regulation of *plvs/shh* and *chd/bra*. Although further experimentation should elucidate if *shh* is required for Notch effects, and whether the activation of *shh* is direct or indirect, we have shown that *shh* down-regulates *chd*, and this can already be seen at early gastrula, suggesting that an enhancement of Shh signalling may be necessary for limiting the number of notochordal precursors. This mechanism may underlie some aspects of the role of *shh* in promoting floor plate development.

Classically, Notch activation has been considered as a mechanism by which a cell remains in a progenitor state to be available for subsequent waves of differentiation or as a way to repress the specification of certain cell types in favour of others. However, recent evidence suggests that Notch may have an instructive role in specifying glial fate (Gaiano et al., 2000; Morrison et al., 2000; Furukawa et al., 2000; Scheer et al., 2001) (for a review, see Gaiano and Fishell, 2002; Lundkvist and Lendahl, 2001). In this scenario, there could be at least two explanations for the role of Notch signalling in the development of the dorsal midline. (1) A permissive role for floor plate development, which implies that within the population of dorsal midline precursors, Notch activation may repress the notochordal fate and allow the development of floor plate identity through some default mechanism. In this context, it is intriguing that some markers of floor plate specification are also expressed by the notochord (e.g. *shh*, *plvs*) whereas notochordal markers seem to be exclusively present in the notochord (e.g. *bra*, *chd*), and depleting embryos of Bra activity, as in the zebrafish mutant *ntl* (Halpern et al., 1997), favours floor plate development at the expense of the notochord. Active Notch may thus deplete the dorsal midline precursors of molecules required for the specification of notochord, allowing floor plate to develop. (2) An instructive role, implying that, apart from the repression of genes required for notochord development, Notch signalling may actively promote floor plate specification by increasing the expression of genes that specify floor plate fate (e.g. *shh*, *plvs*).

In conclusion, we propose that the early organiser contains a population of cells with the potential to develop either as floor plate or notochord. Activation of Notch (which is present in the dorsal blastopore lip) in response to a ligand, which may be X-Delta-1 but this remains to be elucidated, may switch-off the genetic program for notochord specification in a subset of cells (evident by repression of *bra* and the cell-autonomous down-regulation of *chd*) and switch-on the program for floor plate development (instructive hypothesis), including the enhancement of *plvs* and *shh* expression, or allow this program to proceed (permissive hypothesis). In turn, secreted Shh could refine the segregation of both populations by limiting the number of notochordal (*chd*-positive) cells by a non cell-autonomous mechanism. This is consistent with the developmental profile of *chd* and *shh* expression. Their spatial patterns partially overlap in the early gastrula organiser but *chd* precedes *shh* and displays a broader domain. Later, when gastrulation finishes, this spatial relationship is reversed: while *shh* is expressed throughout the dorsal midline cell populations, *chd* expression is excluded from the prospective floor plate and the dorsal lining of the archenteron, and thus covers a subset of the *shh* expression domain. This dynamic profile and the results of our functional experiments may thus underlie a negative feed back of *shh* over *chd* (Fig. 7). Whether the cell-autonomous down-regulation of *chd* elicited by Notch is due to the activation of a transcriptional repressor or is mediated by secreted Shh acting on the same cell, where it is activated by Notch (i.e. in an autocrine way), remains to be elucidated.

Finally, we have observed that at the early neural plate stage *shh* and *plvs* expression completely overlap in the floor plate

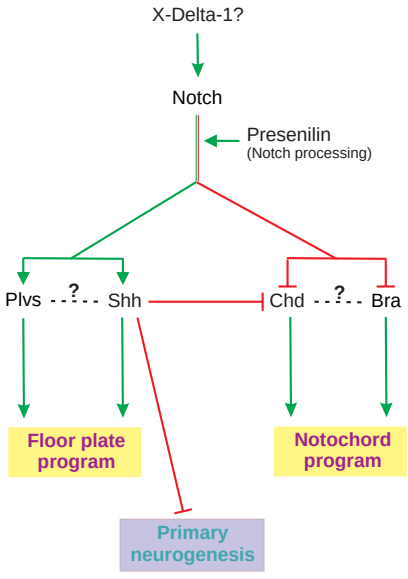

Fig. 7. Proposed model for the genetic interactions involved in dorsal midline specification.

domain (data not shown). If *shh* expression is restricted to the MFP and *hnf3β/plvs* is expressed in both floor plate populations in *Xenopus*, as has been described for zebrafish, mouse and rat embryos (Odenthal et al., 2000), our observation would suggest that LFP has not been specified yet. Hence, at this stage, these cells co-expressing *shh* and *plvs*, which we have shown to be susceptible to Notch signalling, may correspond to the MFP. This raises the possibility that the MFP derives from the same organiser population as the notochord, and thus Notch may either permit or instruct some cells to adopt MFP fate, while repressing the notochordal fate. Indeed, recent findings from the avian embryo demonstrate that the MFP derives from Hensen's node while the LFP is formed by the neuralised ectoderm (Charrier et al., 2002). Whether Shh signalling from the MFP further induces LFP on the neural ectoderm in *Xenopus*, as suggested for zebrafish and avian embryos (Odenthal et al., 2000; Charrier et al., 2002), remains to be tested.

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