

The Notch-target gene *hairy2a* impedes the involution of notochordal cells by promoting floor plate fates in *Xenopus* embryos

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Summary

We have previously shown that the early *Xenopus* organiser contains cells equally potent to give rise to notochord or floor plate, and that Notch signalling triggers a binary decision, favouring the floor plate fate at the expense of the notochord. Now, we present evidence that Delta1 is the ligand that triggers the binary switch, which is executed through the Notch-mediated activation of *hairy2a* in the surrounding cells within the organiser, impeding their

involution through the blastopore and promoting their incorporation into the *hairy2a*+ notoplate precursors (future floor-plate cells) in the dorsal non-involuting marginal zone.

Key words: Delta, Notch, Hairy2a, Floor plate, Notochord, *Xenopus laevis*

Introduction

The floor plate (FP) is an epithelial structure located on the ventral midline of the vertebrate neural tube, extending from the midbrain to the tail region, and constituting an important source of signals involved in the induction of motor neurons and guidance for axonal pathfinding (Tanabe and Jessell, 1996; Colamarino and Tessier-Lavigne, 1995; Stoeckli and Landmesser, 1998). Two cell populations contribute to this structure: the medial FP (MFP) in the midline and the flanking lateral FP (LFP). In zebrafish and mouse embryos, *foxa2* [formerly known as *hnf3b*; for unified nomenclature of the winged helix/forkhead transcription factors see Kaestner et al. (Kaestner et al., 2000)] is found in both populations, while sonic hedgehog (*shh*) is only expressed by the MFP. In chicken, medial and lateral cells initially express both markers, but *foxa2* becomes later restricted to the MFP, while some *shh* expression remains in the LFP (reviewed by Strähle et al., 2004).

The origin of the FP has been subject of a recent controversy, which can be synthesized in three models, based on those described by Strähle and colleagues (Strähle et al., 2004). (1) The 'induction' model postulates that the FP derives from the neural ectoderm and is induced by secreted Shh from the notochord (Placzek et al., 2000; Tanabe and Jessell, 1996; Chiang et al., 1996). (2) The 'allocation' model postulates a common origin for notochord and MFP in the vertebrate's organiser, with MFP precursors invading the midline of the overlying neural plate (Spemann and Mangold, 1924; Selleck and Stern, 1991; Gont et al., 1993; Wilson and Beddington, 1996; Catala et al., 1995; Catala et al., 1996; Shih and Fraser,

1995; Melby et al., 1996; Teillet et al., 1998; Amacher et al., 2002; Latimer et al., 2002) (see also Le Douarin and Halpern, 2000). In this model, Shh is regarded as a factor necessary for survival of FP cells and for maintenance of their phenotype, and as an inducer of anterior and lateral FP (Le Douarin and Halpern, 2000; Thibert et al., 2003; Charrier et al., 2002; Patten et al., 2003; Rebagliati et al., 1998; Sampath et al., 1998; Schauerte et al., 1998; Odenthal et al., 2000). (3) The 'induction and allocation' model tends to reconcile the experimental evidence from the first two models by proposing that the inductive step for the MFP [involving Shh and/or Nodal signalling, which varies among vertebrate species (see Strähle et al., 2004)] takes place before the segregation of MFP and notochord precursors emerging from the organiser. Then, specified MFP precursors populate the midline of the neural plate.

In *Xenopus* embryos the term notoplate designates the midline neural plate cells that later become the FP of the neural tube. It was reported that the notoplate arises from the dorsal non-involuting marginal zone (DNIMZ), a region of ectoderm located just above the dorsal involuting marginal zone (DIMZ) or dorsal lip. The latter contains mesodermal cells that enter through the blastopore during gastrulation and extend along the anterior-posterior (AP) axis, giving rise to the prechordal plate and notochord. The notoplate precursors remain in the ectodermal layer but, together with the notochord precursors, undergo convergent-extension movements during gastrulation, resulting in the midline extension of the future FP along the AP axis (Jacobson, 1981; Keller et al., 1985; Keller and Danilchik, 1988).

We have recently described that before mid-gastrula Notch

executes a binary cell-fate switch that favours FP development at the expense of the notochord, leading to the specification of the different cell populations that contribute to the dorsal midline (DML) in *Xenopus* (López et al., 2003). As a corollary, we proposed that the early organiser indeed contains cells that have the potential to develop either as notochord or FP, but the question of whether they constitute a mixed population or occupy different compartments within the organiser remained unanswered. In addition, we described that Notch signalling activates *shh* expression, and secreted Shh would amplify the effects of the binary decision by inhibiting notochord specification. By this means, Shh would refine the segregation of both cell-populations initially started by Notch. This mechanism could in part underlie the role of *shh* as a FP inducer during the early event proposed by the third model of FP formation.

The main goal of the present work is to understand the molecular and cellular mechanisms that govern the development of the DML structures, beginning from their precursors in the Spemann's organiser. In particular, we wanted to answer the following questions: (1) Which is the ligand(s) for the Notch receptor that triggers the cell-fate switch FP vs. notochord? (2) Which is the Notch-target gene(s) that executes this switch? (3) How do cells from the Spemann's organiser give rise to the FP?

Materials and methods

Embryological manipulations, RNA synthesis, morpholinos and injections

Albino *Xenopus laevis* embryos were obtained using standard methods (Ruiz i Altaba, 1993) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Synthetic capped mRNAs for microinjection were obtained as described previously (Franco et al., 1999). The *hairy2a* antisense oligodeoxynucleotide (AMOh) used was a 3'-carboxyfluorescein-tagged 25-mer morpholino oligonucleotide (Gene Tools, LLC) with the base composition 5'-ATGGTATCTGCGGGCATGTTTCAGTT-3', complementing the *hairy2a* sequence from -8 to +17 relative to the initiation codon. The templates for *X-notch^{ICD}* and *X-su(H)^{DBM}* mRNA synthesis were described by Wettstein et al. (Wettstein et al., 1997), and those for *X-delta1* and *X-delta1^{STU}*, by Chitnis et al. (Chitnis et al., 1995). The full-length *X-hairy2a* cDNA construct in pCS2+, kindly provided by Dave Turner, was digested with *NotI* and transcribed with SP6 RNA polymerase.

Samples were injected as previously described (López et al., 2003). The amounts of synthetic mRNAs and morpholino injected are indicated in the figures. Some injections included 0.5 ng of *nuc-lacZ* mRNA as tracer.

X-gal staining, in situ hybridisation, immunohistochemistry and histology

X-gal staining, preparation of digoxigenin-labelled antisense RNA probes and whole-mount in situ hybridisation were performed as described previously (Franco et al., 1999; Pizard et al., 2004), except that the proteinase K step was omitted in in situ hybridization. The *hairy2a* template (Turner and Weintraub, 1994) was digested with *BamHI* and transcribed with T7 RNA polymerase. For double in situ hybridization, fluorescein-labelled antisense RNA probes were prepared with fluorescein-12-UTP (Amersham). Embryos were hybridised with digoxigenin and fluorescein-labelled probes simultaneously, washed and blocked according to the standard protocol and incubated first with one of the antibodies conjugated with alkaline phosphatase (AP) (1/2000 of anti-digoxigenin-AP or

1/5000 of anti-fluorescein-AP, Fab fragments; Amersham). The corresponding probe was revealed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or Magenta Phos (Sigma). Inactivation of the AP was carried out (65°C 30 minutes in methanol, two 5-minute washes in methanol at room temperature), followed by two 5-minute washes in MAB buffer and 15 minutes incubation in blocking reagent before adding the second AP-conjugated antibody, which was revealed with NBT+BCIP or BCIP alone (Sigma).

The c-myc epitope harboured by the *notch^{ICD}* or *su(H)^{DBM}* constructs used for mRNAs injections and the fluorescein tag of AMOh were detected by immunohistochemistry. For this purpose, after in situ hybridization we performed the AP-inactivation, washing and blocking steps as described above. Then, embryos were incubated for 4 hours at room temperature with mouse 9E10 anti-Myc monoclonal antibody (Santa Cruz) diluted 1/500 or with anti-fluorescein-AP (Fab fragments, Amersham) diluted 1/5000, both in blocking reagent (the same as for in situ hybridization). The unbound antibodies were washed three times with MAB, 10 minutes each, at room temperature, and overnight at 4°C. The following day, embryos injected with AMOh were revealed with BCIP or Magenta Phos, as in the in situ hybridization protocol, and embryos injected with *notch^{ICD}* or *su(H)^{DBM}* were incubated for 4 hours at room temperature with anti-mouse IgG-AP (Santa Cruz) diluted 1/500 or with anti-mouse IgG-HRP (Dako) diluted 1/100 in blocking reagent. After washing the excess of antibody as before, the anti-mouse IgG-AP was revealed with BCIP or Magenta Phos, as in the in situ hybridization protocol. Embryos incubated with the HRP-conjugated antibody were washed twice with TBS (10 mM Tris-HCl pH 6.5, 150 mM NaCl), 5 minutes each, equilibrated 30 minutes at room temperature with DAB solution (0.5 mg/ml of 3,3'-diaminobenzidine (Sigma) in 10 mM Tris-HCl pH 6.5) and revealed with 0.009% of H₂O₂ in DAB solution.

For histology, 50 µm sections were taken in an Oxford Vibratome and mounted onto gelatine coated slides, as described by Hollemann et al. (Hollemann et al., 1996).

Results

The expression patterns of *delta1* and *hairy2a* suggest that both may be involved in the DML binary switch

We searched for Notch ligands and direct target genes whose expression patterns in *Xenopus* could suggest their participation in the DML binary switch. The presence of *delta1* transcripts in the dorsal blastopore lip at stage 10.5 has been reported (Ma et al., 1996). Among the bHLH-O transcriptional repressors that mediate Notch signalling isolated from *Xenopus* (Davis and Turner, 2001), *hairy2a* was very interesting because, apart from bordering the neural ectoderm, it is also present along the DML in open neural plate-stage embryos (Turner and Weintraub, 1994). A more precise study of the early distribution of both transcripts would help to determine whether they are present at the right time and place to be involved in the proposed cell-fate switch. Therefore, we analysed the distribution of *delta1* and *hairy2a* transcripts in more detail by in situ hybridization and compared their expression patterns.

In the dorsal marginal zone, evident transcription of both genes appears in the organiser region around stage 10.5 (Fig. 1). *delta1*-positive (*delta1+*) cells are scattered in the dorsal lip (asterisks, Fig. 1B,B'), and strongest expression is found in the rest of the marginal zone (arrow, Fig. 1B,B'). Sagittal sections of these embryos show that, in the organiser, *delta1* transcripts are only found in the dorsal mesoderm that has not yet involuted (arrow, Fig. 1B''), whereas involuted axial mesoderm

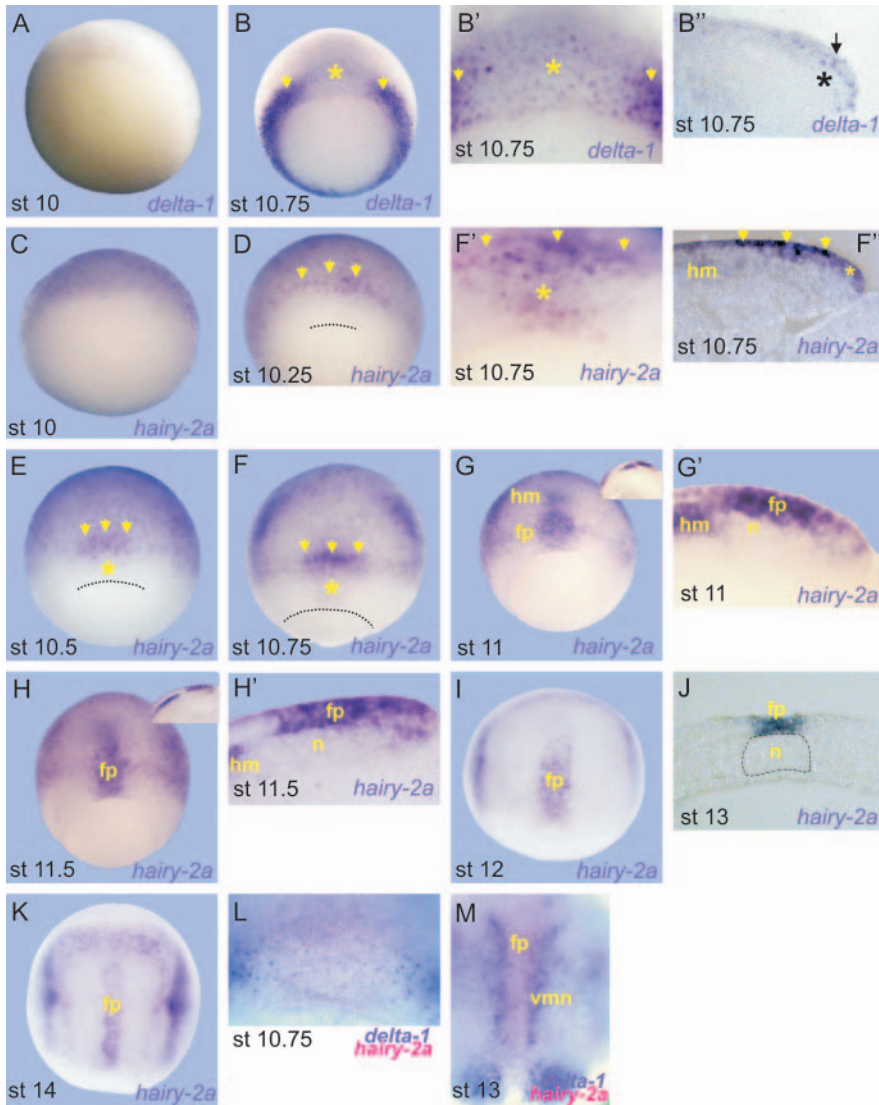


Fig. 1. Comparative expression patterns of *delta1* and *hairy2a*. In situ hybridization of (A-B'') *delta1* and (C-K) *hairy2a*, (L,M) *delta1* (purple) and *hairy2a* (magenta) in control embryos. Stages (st) are indicated at the bottom of each figure; fp, prospective floor plate; n, notochord; hm, head mesoderm; vmn, *delta1* stripe corresponding to the domain of developing primary ventral motor neurons; dotted line, dorsal blastoporal groove. The insets in G and H show low magnifications of the corresponding sagittal sections in G' and H'. See text for details.

pintallavis) was reported to participate in FP development (Lee et al., 1997). *FoxA4a* mRNA is first detected in the dorsal marginal zone of late blastulae and persists during gastrulation in the DML cells that undergo convergent-extension movements. At the early neurula stage, transcripts are distributed throughout the DML in the three germ layers, i.e. the prospective FP, the notochord and the dorsal endodermal cells lining the archenteron (Ruiz i Altaba and Jessell, 1992). At gastrula stages (around st. 11), *foxa4a* and *hairy2a* have partially overlapping domains (Fig. 2B,B'). In the DNIMZ, the outer limit of *foxa4a* coincides with the *hairy2a* arc that marks the prospective notoplate (asterisks). While the entire DIMZ in the organiser expresses *foxa4a*, only some scattered cells are *hairy2a*+ (arrow). At this stage, single in situ hybridization of *foxa4a* distinguishes a superficial population of cells with strong expression of *foxa4a*, which overlaps the *hairy2a* arc that demarcates the prospective notoplate (asterisks, Fig. 2C,C'). Double in

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does not express this gene (asterisk, Fig. 1B''). At the same time, *hairy2a*+ cells are distributed in an arc on the DNIMZ (arrowheads, Fig. 1D-F''). This arc gradually accumulates more *hairy2a*+ cells and ultimately converges and extends along the AP axis, forming the notoplate (prospective FP) (G-K,M). Interestingly, at early and mid-gastrula stages several *hairy2a*-expressing cells are scattered on the DIMZ (asterisks, Fig. 1F-F''), where scattered *delta1* cells are also found (Fig. 1L). Later, at neural plate stage, *hairy2a* is strongly expressed in the prospective FP, flanked by bilateral stripes of *delta1* corresponding to the proneural domains of ventral motor neurons. Among the involuted DML cells, *hairy2a* is only found in the head mesoderm, while the notochord is devoid of transcripts (Fig. 1G-M).

Next, we analysed the expression of *hairy2a* in the context of other markers of notochord and FP fates. *Chordin* (*chd*) transcripts are normally present in the notochord and the prechordal mesoderm (Sasai et al., 1994). Double in situ hybridization of *chd* and *hairy2a* revealed that their territories are mutually exclusive. While *chd* is expressed in notochordal cells, *hairy2a* is only found in FP precursors (Fig. 2A,A'). The winged-helix transcription factor *foxa4a* (formerly known as

situ hybridization of *chd* and *foxa4a* reveals that these genes have partially overlapping domains (Fig. 2D-E''). In early gastrulae, while both are expressed in the DIMZ and in the involuted notochordal cells, only *foxa4a* is found in the DNIMZ, in an area corresponding to the *hairy2a*+ arc that demarcates the future notoplate (asterisk, Fig. 2D,D'). In late gastrulae *foxa4a* transcripts are found in the prospective FP, which is devoid of *chd* transcripts, as *chd* is only expressed by the notochord (Fig. 2E''). Expression of *shh* in the dorsal marginal zone starts later than that of *chd* and *foxa4a*, and *shh* transcripts are ultimately found in FP and notochord cells (Ekkert et al., 1995; López et al., 2003). Double in situ hybridization of *shh* and *hairy2a* show that both genes are co-expressed in the prospective FP, but the latter is excluded from the notochord, where *shh* transcripts are also found (Fig. 2F-G').

In conclusion, *hairy2a* is an interesting marker for the FP precursors and, as a Notch-target gene, it is a good candidate for mediating some of the molecular changes involved in DML cell-fate decisions. The scattered *delta1*+ cells in the organiser may be the source of the ligand that triggers the Notch pathway on the surrounding cells, leading them to activate *hairy2a*

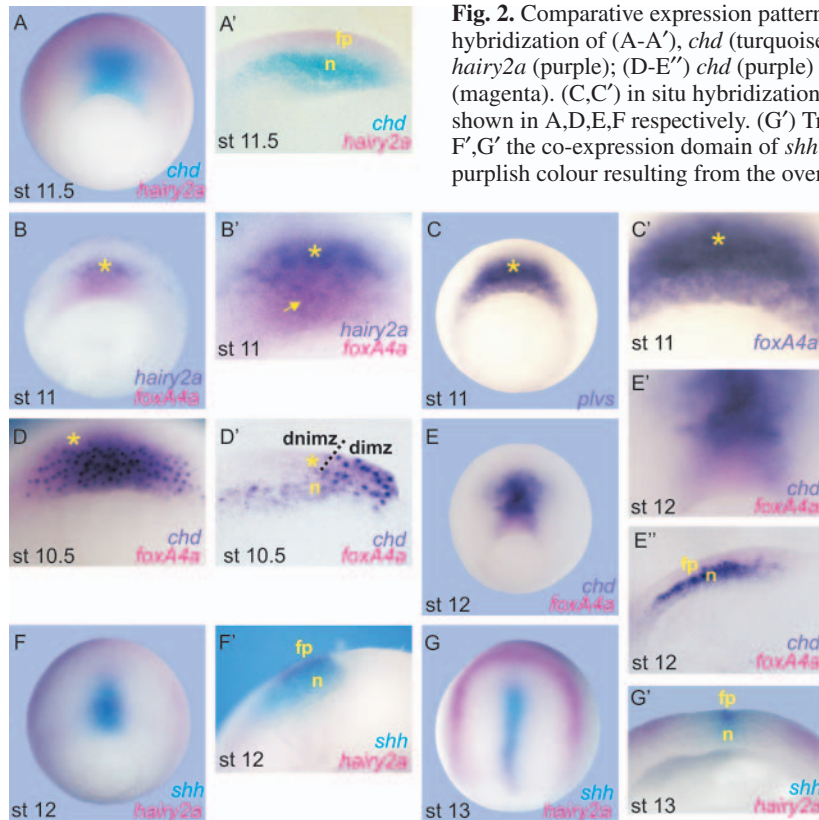


Fig. 2. Comparative expression patterns of *hairy2a*, *chd*, *foxa4a* and *shh*. Double in situ hybridization of (A-A'), *chd* (turquoise) and *hairy2a* (magenta); (B,B') *foxa4a* (magenta) and *hairy2a* (purple); (D-E'') *chd* (purple) and *foxa4a* (magenta); (F-G') *shh* (turquoise) and *hairy2a* (magenta). (C,C') in situ hybridization of *foxa4a*. (A',D',E'',F') Sagittal sections of the embryos shown in A,D,E,F respectively. (G') Transverse section of the embryo shown in G. Notice that in F',G' the co-expression domain of *shh* and *hairy2a* in the FP can be distinguished because of the purplish colour resulting from the overlapping of magenta and turquoise staining. Stages (st) are indicated at the bottom of each figure; fp, prospective floor plate; n, notochord; dnimz, dorsal non-involuting marginal zone; dimz, dorsal involuting marginal zone; dotted line indicates the presumptive limit of involution. See text for details.

increases the FP size, as previously shown with other markers that are co-expressed by FP and notochord (López et al., 2003).

To test whether Delta1 could be the ligand that triggers the binary switch executed by Notch in the DML precursors, we overexpressed *delta1* or blocked the ligand function with the antimorph *delta1^{STU}* (Chitnis et al., 1995) and looked for changes in the expression patterns of *chd* and *hairy2a* (as notochord and FP markers, respectively). Overexpression of *delta1* reduced the number of *chd*+ cells in the organiser (77% of injected embryos, $n=13$, Fig. 3E,E', red asterisk) and increased the number of the *hairy2a*+ ones (49%, $n=49$, Fig. 3G,G', green asterisk). Injection of *delta1^{STU}* mRNA resulted in the opposite effects (increase of *chd*+ cells: 76%, $n=38$, Fig. 3F,F', green asterisk; decrease of *hairy2a*+ cells: 63%, $n=16$, Fig. 3H,H', red asterisk).

From these results, we conclude that *hairy2a* behaves as a Notch target in the DML precursors and that Delta1 is the ligand capable of switching on the binary decision executed by Notch in the DML precursors.

hairy-2a represses the notochordal fate

To assess whether *hairy2a* is able to execute the cell-fate switch triggered by Notch in the DML, we firstly analysed the effects of overexpressing or blocking *hairy2a* on notochord development by looking at the expression of two notochordal markers: *chd* and *brachyury* (*bra*). When we injected 1 ng of *hairy2a* mRNA, *chd* was drastically repressed in the Spemann's organiser after dorsal injections (94%, $n=33$, Fig. 4A, red asterisk). Paradoxically, ventral or lateral injections resulted in ectopic *chd* transcription in the rest of the marginal zone (100%, $n=27$; arrowhead, Fig. 4B). When sibling control embryos reached the neural plate stage, all dorsally injected embryos were arrested at gastrulation. *Chd*+ cells were reduced in number, they could not migrate anteriorly and remained mostly in the outer layer encircling the blastopore, which was unable to complete its closing (100%, $n=23$; Fig. 4C, red asterisk). Interestingly, at the neurula stage, ventrally injected embryos developed a normal dorsal axis (white arrow, Fig. 4D), but the ectopic *chd*+ cells remained close to the ventral blastopore lip and were unable to migrate and extend anteriorly (100%, $n=20$, arrowhead, Fig. 4D). Lower doses of *hairy2a* mRNA also repressed dorsal *chd* expression and resulted in ectopic *chd*+ cells on ventral or lateral mesodermal locations in a similar proportion of injected embryos (e.g. for *chd* repression in dorsally injected embryos: 86% with 0.5 ng of

expression. In early gastrulae, an arc of *hairy2a*+, *foxa4a*+, *chd*-negative (*chd*-) cells located in the DNIMZ demarcates the prospective notoplate. During the course of the gastrulation, this group of cells also becomes *shh*+, re-accommodates along the AP axis by convergent-extension movements and will form the FP of the neural tube.

Delta1 down-regulates *chd* and activates *hairy2a*, and the latter behaves as a Notch target in the DML precursors

In order to determine whether *hairy2a* is a target of Notch in the DML precursors, we activated or prevented Notch signalling with *notch^{ICD}* or *su(H)^{DBM}* mRNA, respectively, and analysed the expression of *hairy2a*. To identify those cells that inherited and translated the injected mRNAs, we revealed by immunohistochemistry the c-myc epitope fused as a tag to the *notch^{ICD}* and *su(H)^{DBM}* fragments. Injection of *notch^{ICD}* mRNA, which encodes a constitutively active form of the receptor independent of ligand binding, produced an enlargement of the *hairy2a* domain on the injected side, both in gastrulae and neurulae (85%, $n=122$, Fig. 3A,A',C-C', green asterisks). To corroborate whether endogenous Notch activity was indeed involved in this modulation, we injected *su(H)^{DBM}* mRNA, which encodes a dominant negative variant of the Notch transducer Su(H). We observed a decrease of *hairy2a*+ cells both in gastrulae and neurulae (70%, $n=114$, Fig. 3B,B',D-D', red asterisks). Transverse sections of neural-plate stage embryos confirmed that these changes occurred in the FP precursors (Fig. 3C'', green asterisk; D'', red asterisk). Therefore, *hairy2a*, whose midline expression at the trunk level exclusively marks FP cells, corroborates that Notch signalling

hairy2a, $n=14$; 95% with 0.25 ng of *hairy2a*, $n=20$) but qualitatively, the effects were gradually weaker (not shown). However, with 0.25 ng injections, gastrulation could better proceed, allowing us to examine other markers at neural plate stages (see below). To block *hairy2a* function we injected an antisense morpholino oligonucleotide complementary to a sequence of *hairy2a* comprising the initiation codon (AMOh). 10 ng of AMOh increased the number of *chd*+ cells on the injected side, both in gastrulae (Fig. 4E-E''', green asterisks) and neurulae (not shown) (82%, $n=45$). When co-injected with 0.5 ng of *hairy2a* mRNA, 10 ng of AMOh reversed the down-regulation of *chd* that produces *hairy2a* mRNA alone, demonstrating that this antisense morpholino specifically interferes with the translation of *hairy2a* transcripts [*chd*+ cells decreased in only 10% of the dorsally injected embryos, the remaining ones evidenced an increase of *chd*+ cells (67%) or were unaffected (23%), $n=30$; Fig. 4F,F', green asterisk].

In early gastrula, *bra* expression is normally observed in a ring demarcating the entire marginal zone, broader at the ventral side when the dorsal cells begin to involute at the blastopore (Fig. 4G,G'). Injection of *hairy2a* mRNA represses *bra* expression in all locations, including the organiser (91% with 0.25 ng of *hairy2a* mRNA, $n=44$, Fig. 4H-I, red asterisks; 100% with 1 ng, $n=18$, not shown; the effects are qualitatively stronger with higher doses of mRNA). However, dorsal injection of 10 ng of AMOh increases *bra*+ cells, both in gastrulae and neurulae (74%, $n=43$, Fig. 4J-K', green asterisks).

Together, these results show that *hairy2a*, like Notch signalling, represses *bra* and *chd* in the Spemann's organiser. Therefore, *hairy2a* may be the mediator of Notch in the repression of the notochordal fate that takes place during the DML cell-fate switch. In addition, an excess of *hairy2a* activity interferes with the normal movement of *chd*+ (notochordal) cells, blocking their involution.

hairy 2a favours the FP fate

We next addressed the question of whether *hairy2a* was able to promote the FP fate as does Notch signalling. Therefore, we overexpressed or blocked *hairy2a* and looked at the expression of two FP markers: *foxa4a* and *shh*. However, these genes are also expressed in the notochord progenitors. Although at stage 11 it is possible to discern the contribution of *foxa4a* to the arc of the notoplate, the low expression of *shh* at this time does not allow the determination of the FP and notochord components

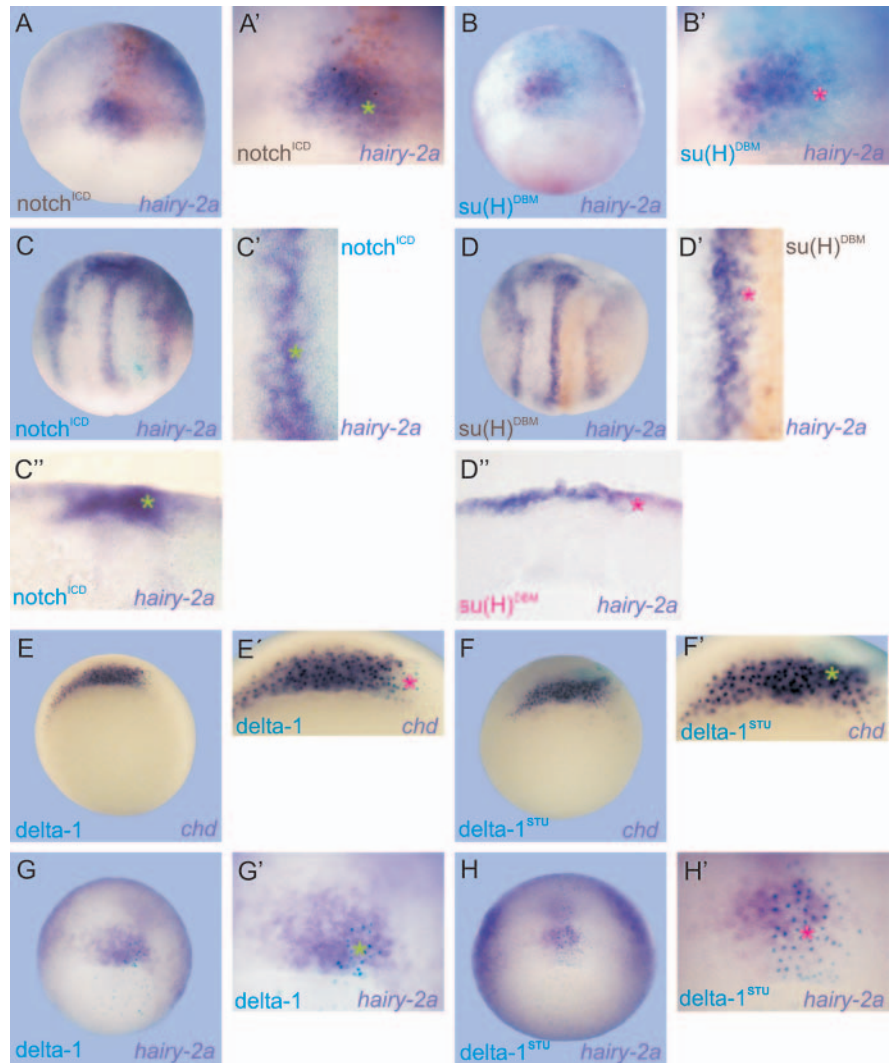


Fig. 3. Notch signalling up-regulates *hairy2a* in the DML precursors. *delta1* represses the notochordal marker *chd* and activates the FP marker *hairy2a*. (A,C) In situ hybridization of *hairy2a* (purple) in embryos injected with 1 ng of *notch*^{ICD} mRNA fixed at (A) gastrula stage, brown c-myc staining, or (C) at neural plate stage, turquoise c-myc staining. (A',C') Higher magnifications of A and C, respectively. (C'') Transverse section of the embryo shown in C. (B,D) In situ hybridization of *hairy2a* (purple) in embryos injected with 2 ng of *su(H)*^{DBM} mRNA fixed at (B) gastrula (turquoise c-myc staining) or (D) at neural plate stages (c-myc brown staining). (B',D') Higher magnifications of B and D, respectively. (D'') Transverse section of another neurula injected with 2 ng of *su(H)*^{DBM} (magenta c-myc staining), in situ hybridization of *hairy2a* (purple). (E,F) In situ hybridization of *chd* (purple) in gastrula stage embryos injected with (E) 1 ng of *delta1* mRNA (turquoise X-gal staining) or (F) with 0.5 ng of *delta1*^{STU} mRNA (turquoise X-gal staining). (E',F') Higher magnifications of E and F, respectively. (G,H) In situ hybridization of *hairy2a* (purple) in gastrula stage embryos injected with (G) 1 ng of *delta1* mRNA (turquoise X-gal staining) or (H) with 0.5 ng of *delta1*^{STU} mRNA (turquoise X-gal staining). (G',H') Higher magnifications of G and H, respectively. Green asterisks indicate increase effects and red asterisks, decreased effects. In all embryos the injected side is oriented to the right.

that contribute to the *shh* domain. Thus, we were interested in analysing the effects of *hairy2a* at neural plate stages, when both components can be clearly distinguished. Although 1 ng of *hairy2a* mRNA had the strongest effects on mesodermal markers at gastrula stages and also promoted ectopic expression of *foxa4a* on ventral locations (arrowhead, Fig.

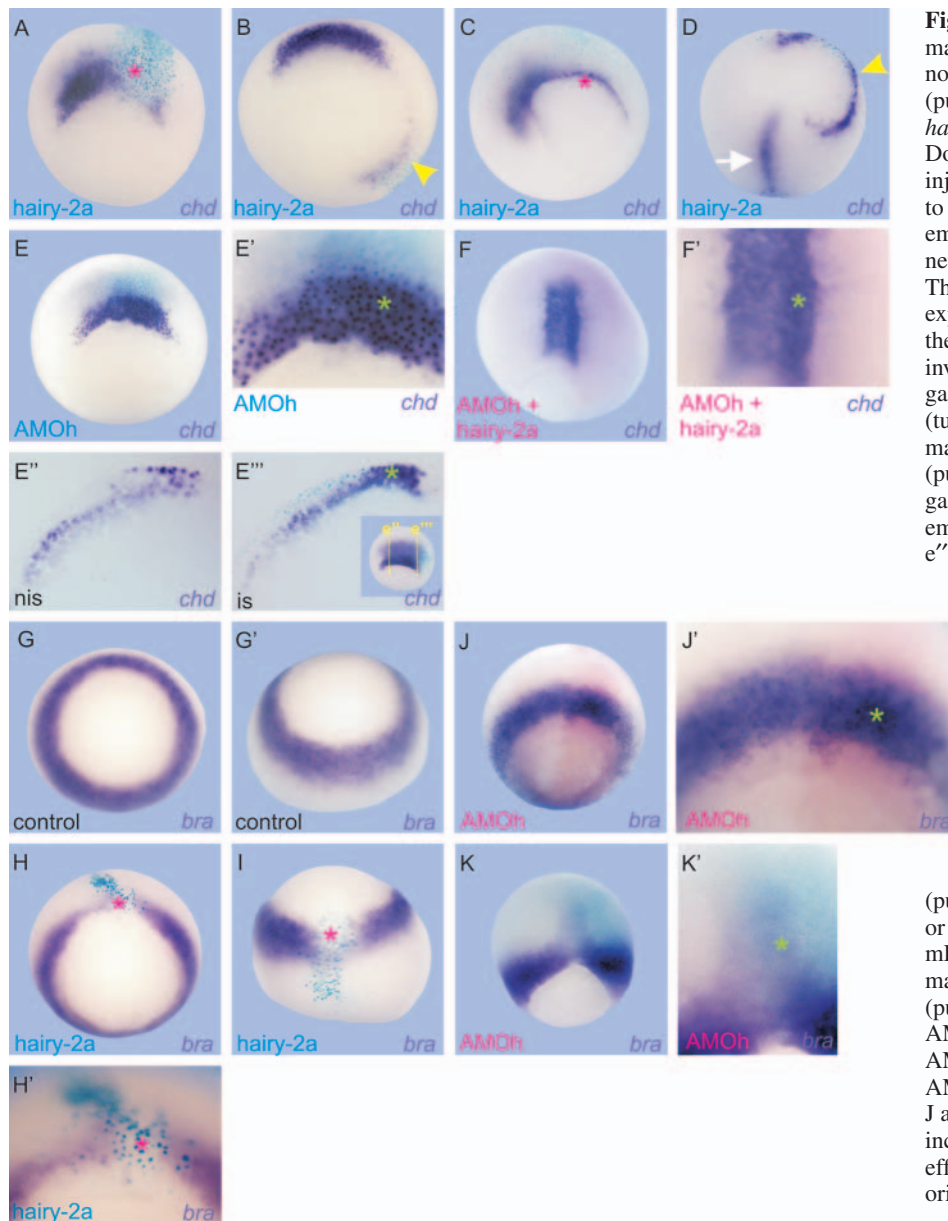


Fig. 4. *hairy2a* down-regulates the notochordal markers *chd* and *bra* and impairs involution of notochordal cells. (A-D) *chd* expression (purple) in embryos injected with 1 ng of *hairy2a* mRNA (turquoise X-gal staining). (A) Dorsally injected gastrula. (B) Ventrally injected gastrula. The yellow arrowhead points to the ectopic *chd*⁺ cells. (C) Dorsally injected embryo, fixed when sibling controls reached the neurula stage. (D) Ventrally injected neurula. The white arrow points to the normal axis expressing *chd*. The yellow arrowhead points to the ectopic *chd*⁺ cells, which were unable to involute. (E) *chd* expression (purple) in a gastrula injected with 10 ng of AMO (turquoise X-gal staining). (E') Higher magnification of E. (E'', E''') *chd* expression (purple) in parasagittal sections of another gastrula injected with 10 ng of AMO (whole embryo shown in the inset in E'''; yellow lines e'', e''' indicate the levels of the sections shown in E'', E''', respectively). (E'') Non-injected side. (E''') Injected side. The turquoise dots reveal the *lacZ* tracer. (F) *chd* expression (purple) in a late gastrula injected with 0.5 ng of *hairy2a* mRNA plus 10 ng of AMO. The magenta staining reveals the distribution of the injected AMO. (F') Higher magnification of F. (G) *bra* expression in a control gastrula, vegetal view. (G') Ventrally tilted view of the same embryo shown in G. (H, I) *bra* expression (purple) in gastrula stages embryos (H) dorsally or (I) ventrally injected with 0.25 ng of *hairy2a* mRNA (turquoise X-gal staining). (H') Higher magnification of H. (J, K) *bra* expression (purple) in embryos injected with 10 ng of AMO fixed at (J) early gastrula, magenta AMO staining, or (K) late gastrula, turquoise AMO staining. (J', K') Higher magnifications of J and K, respectively. Green asterisks indicate increase effects and red asterisks, decrease effects. In all embryos the injected side is oriented to the right.

Table 1. Notch signalling changes DML cell fates through the activation of *hairy-2a*

Expression of *chd* in the gastrula organiser

Injection	<i>chd</i> increase	<i>chd</i> decrease	<i>chd</i> without changes	<i>n</i>
Notch ^{ICD} 1 ng	4 (8%)	37 (79%)	6 (13%)	47
AMO 10 ng	33 (81%)	5 (12%)	3 (7%)	41
Notch ^{ICD} 1 ng + AMO 10 ng	20 (83%)	2 (8%)	2 (8%)	24

Expression of *foxa4a* in the notoplate of gastrula stage embryos and prospective FP of neurulae

Injection	<i>foxa4a</i> increase	<i>foxa4a</i> decrease	<i>foxa4a</i> without changes	<i>n</i>
Notch ^{ICD} 1 ng	16 (64%)	7 (28%)	2 (8%)	25
AMO 10 ng	6 (14%)	24 (54%)	14 (32%)	44
Notch ^{ICD} 1 ng + AMO 10 ng	2 (6%)	27 (82%)	4 (12%)	33

Expression of *shh* in the prospective FP of neural plate stage embryos

Injection	<i>shh</i> increase	<i>shh</i> decrease	<i>shh</i> without changes	<i>n</i>
Notch ^{ICD} 1 ng	5 (83%)	0	1 (17%)	6
AMO 10 ng	1 (5%)	11 (58%)	7 (37%)	19
Notch ^{ICD} 1 ng + AMO 10 ng	2 (15%)	10 (77%)	1 (8%)	13

Changes for each marker were scored in individual embryos by comparison between the injected and non-injected side after three different injections (Notch^{ICD} alone, AMO alone or Notch^{ICD} plus AMO). Embryos were classified into three phenotypes, according to the variation of each marker on the injected side (increase, decrease, without changes). Absolute values indicate the number of embryos that show the phenotype indicated at the top of each column. The corresponding percentages are shown between brackets. *n* indicates the total number of embryos analysed for each injection.

5A''), this dose severely interfered with gastrulation movements, and we were unable to distinguish between the FP and the notochordal components of *shh* expression in these extremely affected embryos. Thus, we decided to lower the dose until we were able to analyse *shh* in neurulae, but preserving the effects, although milder, on notochordal markers. This compromise could be reached with 0.25 ng of *hairy2a* mRNA, as described above. In these conditions, overexpression of *hairy2a* increased *foxa4a*+ cells in the notoplate precursors (80%, *n*=15, Fig. 5A,A', green asterisk) and increased *shh*+ cells in the prospective FP (67%, *n*=27, Fig. 5D-D'', green asterisks). In contrast, blocking *hairy2a* with 10 ng of AMOh decreased *foxa4a*+ cells in the notoplate in gastrulae and in the prospective FP in neurulae (54%, *n*=44, Fig. 5B-C'', red asterisks) and *shh*+ cells in the prospective FP in neural plate stage embryos (58%, *n*=19, Fig. 5E-E'', red asterisks). In conclusion, *hairy2a*, like Notch signalling, increases *foxa4a*+ and *shh*+ cells within the prospective FP domain. These results suggest that *hairy2a* is a mediator of Notch in the promotion of FP specification that takes place during the DML cell-fate switch. Overall, *hairy2a* is able to promote the FP fate at the expense of the notochord, and this was confirmed by double in situ hybridization of *chd* and *foxa4a* in injected embryos: 0.25 ng of *hairy2a* mRNA increase the domain of *chd*-*foxa4a*+ cells (FP precursors, green asterisk) and concomitantly decrease the domain of *chd*+*foxa4a*+ cells (notochord precursors, red asterisk) (79% and 92%, respectively; *n*=14) (Fig. 5F).

***hairy2a* mediates the cell-fate switch executed by Notch in the DML precursors**

If *hairy2a* is required for the cell-fate switch induced by Notch, then, blocking *hairy2a* activity would impede the effects of *notch^{ICD}* on DML development. Therefore, we performed co-injections of 10 ng of AMOh plus 1 ng of *notch^{ICD}* mRNA and compared the effects on *chd*, *foxa4a* and *shh* expression with those obtained after injecting embryos with 1 ng of *notch^{ICD}* mRNA alone or 10 ng of AMOh alone (Table 1, Fig. 6). As previously shown (López et al., 2003), *notch^{ICD}* alone decreased the number of notochordal precursors (*chd*+ cells) in the organiser (Fig. 6A, red asterisk) and increased the number of *foxa4a*+ and *shh*+ cells in the FP domain (Fig.

6D,D',G,G', green asterisks). The opposite results were observed in embryos injected with AMOh alone (Fig. 6B,

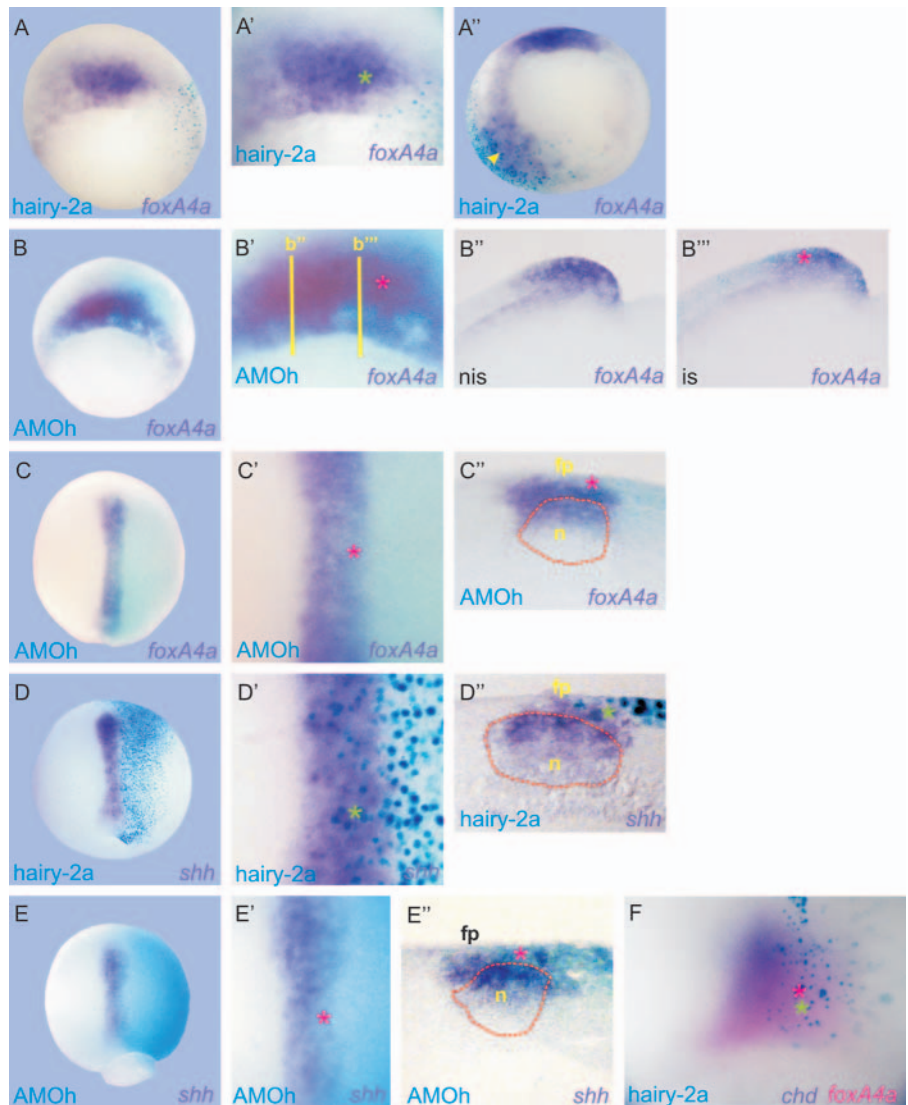


Fig. 5. *hairy2a* up-regulates *foxa4a* and *shh* in the FP precursors. (A) *foxa4a* expression (purple) in a gastrula dorsally injected with 0.25 ng of *hairy2a* mRNA (turquoise X-gal staining). (A') Higher magnification of A, revealing the increase of *foxa4a*+ cells in the notoplate precursors (green asterisk). (A'') *foxa4a* expression (purple) in a gastrula ventrally injected with 1 ng of *hairy2a* mRNA (turquoise X-gal staining). The arrowhead points to the ectopic *foxa4a*+ cells in the ventral mesoderm region. (B,C) *foxa4a* expression (purple) in (B) gastrula or (C) neural plate stage embryos injected with 10 ng of AMOh, turquoise staining reveals the distribution of AMOh. (B',C') Higher magnification of B and C, respectively. In B' the yellow lines indicate the plane of the parasagittal sections shown in B'' and B''', respectively. (C'') Transverse section of the embryo shown in C. (D) *shh* expression (purple) in a neurula injected with 0.25 ng of *hairy2a* mRNA (turquoise X-gal staining). (D') Higher magnification of D, revealing the increase of *shh*+ superficial cells corresponding to the prospective FP. (D'') Transverse section of the same embryo shown in D. Notice the increase of *shh*+ cells in the FP domain on the injected side (green asterisk). (E) *shh* expression (purple) in a neurula injected with 10 ng of AMOh, turquoise staining reveals the distribution of AMOh. (E') Higher magnification of E, revealing the decrease of *shh*+ superficial cells corresponding to the prospective FP. (E'') Transverse section of the embryo shown in E. Notice the decrease of *shh*+ cells in the FP domain on the injected side (red asterisk). (F) Expression of *chd* (purple) and *foxa4a* (magenta) in a late gastrula embryo injected with 0.25 ng of *hairy2a* mRNA (turquoise X-gal staining). nis, non-injected side; is, injected side; fp, prospective floor plate; n, notochord. The dotted red line demarcates the notochord. See text for details.

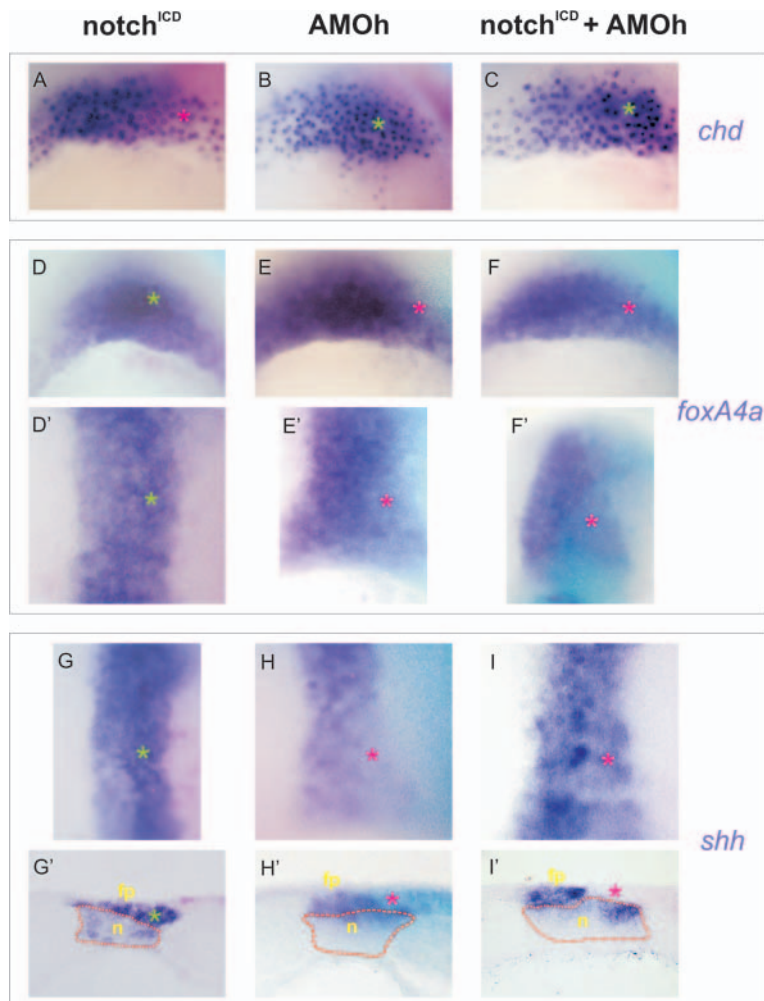


Fig. 6. *hairy2a* mediates the cell-fate switch executed by Notch in the DML precursors. (A-C) *chd* expression (purple) in gastrulae injected with (A) 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining), (B) 10 ng of AMOH (magenta staining) or (C) 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining) plus 10 ng of AMOH. (D-F) *foxa4a* expression (purple) in gastrulae injected with (D) 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining), (E) 10 ng of AMOH (turquoise staining) or (F) 1 ng of *notch*^{ICD} mRNA (turquoise, c-myc staining) plus 10 ng of AMOH. (D'-F') *foxa4a* expression (purple) in late gastrula/early neurula embryos injected with (D') 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining), (E') 10 ng of AMOH (turquoise staining) or (F') 1 ng of *notch*^{ICD} mRNA (turquoise c-myc staining) plus 10 ng of AMOH. (G-I) *shh* expression (purple) in neurula embryos injected with (G) 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining), (H) 10 ng of AMOH (turquoise staining) or (I) 1 ng of *notch*^{ICD} mRNA (magenta c-myc staining) plus 10 ng of AMOH. (G'-I') Transverse sections of the embryos shown in G-I. fp, prospective floor plate; n, notochord. The dotted red line demarcates the notochord. Green asterisks indicate increase effects and red asterisks, decrease effects. In all embryos the injected side is oriented to the right.

green asterisk, *chd*+ cells increase; E, E', H, H', red asterisks, *foxa4a* and *shh* repression) or co-injected with *notch*^{ICD} plus AMOH (Fig. 6C, green asterisk, *chd* increase; F, F', I, I', red asterisks, *foxa4a* and *shh* repression). Thus, AMOH could reverse the effects of *notch*^{ICD} on DML markers, as expected. We conclude that Notch signalling represses the notochordal fate and promotes FP specification through the activation of *hairy2a*.

Discussion

We have previously proposed that Notch signalling may be executing a binary cell-fate decision in the *Xenopus* organiser within a bipotential cell population: when active, it promotes FP specification at the expense of the notochord (López et al., 2003). However, the question of whether these precursors are mixed or if they occupy different compartments within the organiser remained unanswered.

We present evidence that Delta1 is the ligand that triggers this cell-fate switch, and that *hairy2a* is the Notch target that mediates the repression of the notochordal fate and the promotion of FP development. We found that *hairy2a* is able to repress genes that are involved in dorsal axial mesoderm development, such as *chd* and *bra* (Chesley, 1935; Smith et al., 1991; Cunliffe and Smith, 1992; Cunliffe and Smith, 1994; Halpern et al., 1993; Sasai et al., 1994; Sasai et al., 1995; O'Reilly et al., 1995; Piccolo et al., 1996; Piccolo et al., 1997; Hammerschmidt et al., 1996; Conlon et al., 1996; Schulte-Merker et al., 1997), and to promote the expression of genes related to FP specification, such as *shh* and *foxa4a* (Ruiz i Altaba et al., 1993; Ruiz i Altaba et al., 1995; Roelink et al., 1994; Chiang et al., 1996; Chang et al., 1997; Sasaki et al., 1997; Epstein et al., 1999; Müller et al., 1999). Since *hairy2a* is a transcriptional repressor, at least two alternative explanations for its role in DML development arise. (1) A permissive role for FP development, which implies that *hairy2a* represses genes that specify the notochordal fate and allows the development of FP identity through some default mechanism. In this context, it is intriguing that markers of FP specification are also expressed by the notochord (e.g. *shh*, *foxa4a*), the only exception being *hairy2a* itself, whereas notochordal markers seem to be exclusively present in the notochord (e.g. *bra*, *chd*). *hairy2a* may thus deplete the DML precursors of molecules required for the specification of notochord, allowing FP to develop. (2) An instructive role, which implies that, apart from the repression of genes required for notochord development, *hairy2a* may indirectly promote FP specification by repressing a negative regulator of genes that specify FP fate (e.g. *shh*, *foxa4a*).

Bra behaves as a transcriptional activator (Conlon et al., 1996), and the zebrafish homologue is *no tail* (*ntl*). Notably, while the notochord does not differentiate in *ntl* mutant embryos, the FP is widened (Halpern et al., 1997). This suggests that Bra activity antagonises FP development while promoting notochord formation. Therefore, *bra* may constitute a key target in the Notch-dependent binary switch, and it may be directly repressed by *hairy2a*. Further experimentation will be needed to elucidate whether a hierarchical relationship links *hairy2a* and *bra* in this switch in *Xenopus*. Interestingly, although *hairy2a* is able to activate *chd* ectopically in ventral or lateral mesoderm, it represses *bra* in all locations. Since *chd*+ *bra*-cells are unable to involute and migrate properly and *bra* is involved in convergent-extension movements (Conlon and Smith, 1999; Kwan and Kirschner, 2003), it is conceivable that

hairy2a interferes with them by repressing *bra*. We suggest that the specification of FP or notochord fates is intimately linked to cell movements during gastrulation and that *hairy2a* constitutes a master gene operating on both events.

It was recently described that the zinc finger transcriptional activator *Churchill* (*ChCh*) stops the ingression of cells through the primitive streak in chicken embryos (Sheng et al., 2003). The authors postulated that during normal embryogenesis a decision between paraxial mesodermal and neural fates is made by establishing the boundary that restricts cell ingression during gastrulation. We propose that an analogous mechanism may take place during DML development, with *hairy2a* stopping the ingression of notochord cells that, otherwise, would have been incorporated into the notochord. This specialised neural vs. mesodermal switch for DML precursors is envisaged through the observation that the *Xenopus* FP retains the potential to differentiate into neurons, since the ectopic expression of the proneural gene *X-ngnr1* in the FP precursors turns on *N-tubulin* expression, and the bHLH-O transcriptional repressor *XHRT1*, which is expressed in the prospective FP, represses neurogenesis (Taelman et al., 2004). Interestingly, *XHRT1* is able to heterodimerize with *Hairy* proteins, suggesting that they may be biologically relevant partners. Although *XHRT1* responds to Notch and can inhibit *chd* and *bra*, it is unlikely to be involved in the notochord vs. FP switch because it appears after mid-gastrula, later than *hairy2a* (this work) and *hairy2b* (Taelman et al., 2004). However, the three transcripts ultimately co-localise in the FP precursors. This fact, together with the presence of *notch1* transcripts in FP at neural plate stages (López et al., 2003) and of *delta1* transcripts flanking the *hairy2a* FP domain (Fig. 1M of this work) support the idea of *XHRT1* having a role in preventing FP cells from adopting a neuronal fate (Taelman et al., 2004), perhaps in collaboration with *hairy2a/b*.

Thus, the FP fate would be specified and/or maintained in the course of at least two binary decisions involving Notch

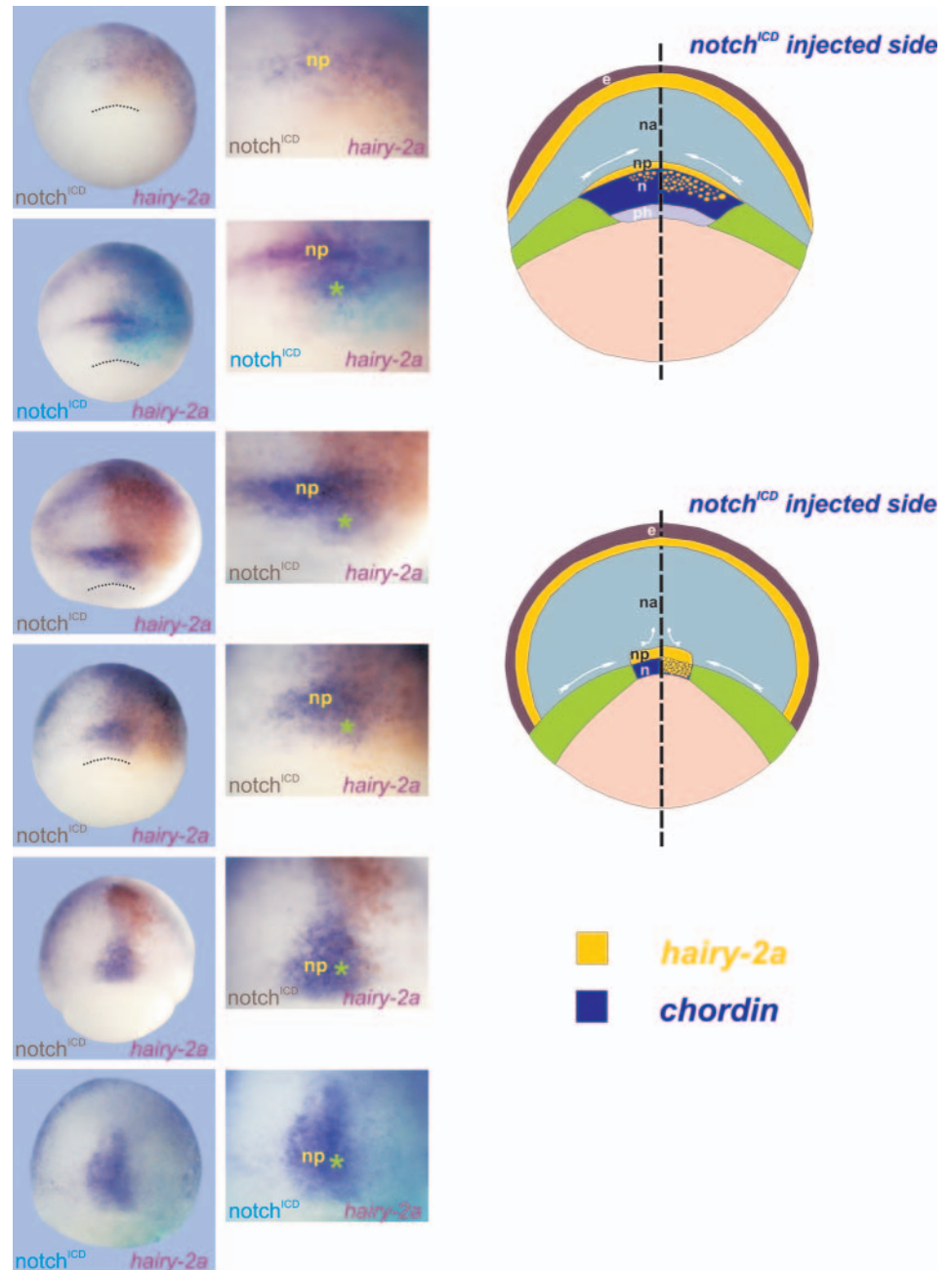


Fig. 7. Model interpreting the Notch-mediated cell-fate switch in the DML precursors. (Left) *hairy2a* expression (purple) in a developmental series of gastrula embryos injected with 1 ng of *notch1^{CD}* mRNA (brown or turquoise c-myc staining): left column, whole embryos; right column, higher magnification of the notochord region. In all embryos the injected side is oriented to the right. (Right) Scheme illustrating the molecular and cellular changes on DML precursors after *notch1^{CD}* mRNA injection. See text for details. e, prospective epidermis; na, neural anlage; np, notochord; n, notochord; ph, pharyngeal mesoderm; white arrows, convergent-extension movements.

signalling: (1) The early notochord/FP decision, which appears to take place before mid-gastrula (López et al., 2003). Two parallel mechanisms seem to contribute to stop this switch: (a) *notch1* and *delta1* transcripts disappear once the notochordal cells involute (Wittenberger et al., 1999; López et al., 2003) (this work), suggesting that they become refractory to divert to FP in response to Notch ligands, which, these cells do not

produce anymore; (b) the competence of the binary switch for responding to active Notch decreases throughout gastrulation (López et al., 2003). (2) The neuron/FP decision. In this event, Delta1 flanking the midline at neural plate stages would activate the Notch receptor in the neighbouring FP cells, which then would turn on *hairy2/XHRT1* bHLH-O genes, thus repressing the neuronal fate to maintain the FP phenotype.

Model interpreting the Notch-mediated cell-fate switch in the DML precursors

Fig. 7 (left column) shows the dynamics of *hairy2a* expression in *notch^{CD}*-injected embryos in a developmental series from early to late gastrula stages. Supernumerary *hairy2a*+ cells (asterisks) first appear on the DIMZ on the injected-side and gradually incorporate into the *hairy2a*-expressing arc on the DNIMZ that demarcates the prospective notoplate. We propose that, in normal embryogenesis, the early *Xenopus* organiser contains cells that have the potential to develop either as notochord or FP (Fig. 7 right column). *Delta1* expression starts at early gastrula in scattered cells on the organiser and interacts with the Notch receptor in the surrounding cells, leading to the activation of *hairy2a* and the repression of *chd* and *bra*. *hairy2a*, in turn, impedes the movement of involution, and *hairy2a*+ cells gradually incorporate into the growing arc on the DNIMZ. This arc ultimately converges and extends along the AP axis (white arrows), forming the notoplate (prospective FP). By this mechanism involving *notch* and *hairy2a*, Delta signalling executes a binary cell-fate switch that favours FP development at the expense of the notochord, leading to the specification of the different cell populations that contribute to the DML. This model reconciles the findings that the notoplate arises from the DNIMZ with the hypothesis that FP cells arise from the organiser (DIMZ). However, we cannot rule-out additional contributions to FP development from the neural ectoderm (more specifically, to the anterior and lateral FP) involving Shh as inducer, as it has been described for chicken and zebrafish embryos (Le Douarin and Halpern, 2000; Thibert et al., 2003; Charrier et al., 2002; Patten et al., 2003; Rebagliati et al., 1998; Sampath et al., 1998; Schauerte et al., 1998; Odenthal et al., 2000). Interestingly, the Axolotl homologue of *foxa4a*, unlike its *Xenopus* counterpart, has only a superficial expression in the early organiser and later, it is only detected in the FP but never in the notochord, thus resembling the expression of *hairy2a* in *Xenopus* (Whiteley et al., 1997). Whiteley et al. provide two possible explanations: (a) the superficial *foxa4a*+ cells in the axolotl organiser are future neural FP cells, programmed very early at gastrulation; (b) the expressing cells are a mixture of notochord and FP precursors, but later, *foxa4a* only persists in FP cells. We presume that both explanations may not be mutually exclusive and, as with *Xenopus hairy2a*, some cells may be representing anterior FP cells, programmed at very early stages of gastrulation, as those described in chicken (Patten et al., 2003), and other cells may be posterior FP precursors being specified from a bipotential population, which is also able to give rise to notochordal cells.

In this scenario, our proposal fits better with the induction and allocation model (see Introduction), where the inductive event (in charge of Delta-Notch signalling) takes place before the segregation of notochord and FP precursors (this work), with Shh expression (enhanced by Notch signalling in FP precursors) contributing to repress the notochordal fate and amplifying the

switch (López et al., 2003). It remains to be elucidated which are the molecules that pattern the scattered expression of *delta1* in the organiser, which initially defines the distribution of FP and notochord precursors according to our model.

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