Development 133, 2855-2864 (2006) doi:10.1242/dev.02466

A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in *Ciona* embryos

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The notochord is one of the defining features of chordates. The ascidian notochord is a rod like structure consisting of a single row of 40 cells. The anterior 32 'primary' notochord cells arise from the A-line (anterior vegetal) blastomeres of the eight-cell stage embryo, whereas the posterior 8 'secondary' notochord cells arise from the B-line (posterior vegetal) blastomeres of the eight-cell stage embryo. Specification of notochord precursors within these two lineages occurs in a spatially and temporally distinct manner. We show that specification of the secondary but not the primary notochord in *Ciona intestinalis* requires a relay mechanism involving two signalling pathways. First, we show evidence that acquisition of secondary notochord fate is dependent upon lateral Nodal signalling sources, situated in the adjacent b-line animal cells. Expression of the notochord specific gene *Ci-Brachyury* in the secondary notochord precursor was downregulated following selective inhibition of Nodal signal reception in B-line derivatives and also, strikingly, following selective inhibition of Nodal signal reception in A-line cell derivatives. Within the A-line, Nodal signals are required for localised expression of *Delta2*, which encodes a divergent form of Delta ligand. Using four distinct reagents to inhibit Delta2/Notch signals, we showed that Delta2 signalling from A-line cells, which activates the Notch/Su(H) pathway in adjacent B-line cells, is required for specification of the secondary notochord precursor. We propose a model whereby laterally produced Nodal acts to specify the secondary notochord precursor both directly in the B-line cells and via Delta2 induction in adjacent A-line cells.

KEY WORDS: Nodal, Delta, Notch, Notochord, Ascidian, *Ciona*, Tunicate, Signalling relay

INTRODUCTION

During development, fate specification of embryonic cells can be based on intrinsic or extrinsic properties. Intrinsic mechanisms dominate in embryos that use a mosaic strategy of development, whereas extrinsic mechanisms are used in embryos adopting regulative strategies (reviewed by Lemaire and Marcellini, 2003). Mosaic strategies rely on selective inheritance of localised cytoplasmic determinants. Regulative strategies employ extracellular signals to provide positional information so that each cell adopts its fate according to its position within an embryonic field. Extracellular signalling molecules can act at a distance, as morphogen gradients, locally during short-range signalling or they can generate relays of distinct signalling activities, whereby activation of one signalling pathway leads to the activation of a second signalling molecule.

We are using the invertebrate chordate embryos of *Ciona intestinalis* as a model system to study cell fate specification during embryogenesis. *Ciona* embryos develop with an invariant cell cleavage pattern and development proceeds with a small number of cells, such that gastrulation commences when the embryo consists of only 110 cells. Ascidian embryogenesis has been traditionally considered an example of mosaic development, as many embryonic territories are specified by the inheritance of cytoplasmic determinants (Conklin, 1905; Nishida, 2005). Although this strategy undoubtedly plays an important role, there are also an increasing number of examples in which cell-cell interactions are indispensable for cell-type specification in ascidians (reviewed by Nishida, 2002; Nishida, 2005).

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Accepted 30 May 2006

The notochord is one of the defining features of chordate embryos. It has important structural and signalling roles during chordate development (reviewed by Stemple, 2005). The ascidian larval notochord consists of 40 cells and is derived from two of the four founder cell lineages of the eight-cell stage embryo. The anterior 32 notochord cells come from the A-line (anterior-vegetal) founder lineage, whereas the posterior eight cells are generated from the B-line (posterior-vegetal) founder lineage (Nishida, 1987). The former is termed the primary notochord and the latter the secondary notochord. Specification of both primary and secondary notochord fate depends upon inductive cellular interactions (Nakatani and Nishida, 1994). FGF signalling during the 32- to 64-cell stages is required for specification of all the notochord precursors, but appears to act in a distinct manner in the two lineages (H.Y. and C.H., unpublished) (Darras and Nishida, 2001; Kim and Nishida, 2001; Minokawa et al., 2001; Nishida, 2003). Each primary notochord precursor becomes fate restricted at the 64-cell stage following a cell division that generates one notochord and one neural precursor. FGF signals are required for the specification of the fate restricted notochord precursors, which adopt a neural fate in the absence of FGF signalling. In the secondary lineage, the temporal requirement of FGF signalling during the 32- to 64-cell stages does not coincide with the fate restriction of notochord precursors. Rather, it is required for the formation of a precursor of mixed notochord and mesenchyme fate at the 64-cell stage, which subsequently divides to give one notochord and one mesenchyme precursor at the 76-cell stage. It remains to be fully understood how fate restricted secondary notochord precursors are specified in the secondary lineages.

We have recently identified a localised signalling source, situated in laterally positioned animal cells of the 32-cell embryo of *Ciona* (Hudson and Yasuo, 2005). Nodal ligand was shown to be responsible for this activity and to pattern the neural plate across its mediolateral axis. In this study, we show that Nodal signals are required for the specification of the secondary notochord precursor and that Nodal acts, in part, via a relay mechanism with a Delta ligand.

MATERIALS AND METHODS

In situ hybridisation and probes

In situ hybridisation was carried out as described previously, except that proteinase K treatment was carried out at room temperature and RNaseA treatment was omitted (Wada et al., 1995). DIG RNA probes were synthesised from the following cDNA clones: *Ci-AKR1a* (Tokuoka et al., 2004), *Ci-Bra* (Corbo et al., 1997), *Ci-Delta2* (Hudson and Yasuo, 2005), *Ci-Gataa* (Bertrand et al., 2003), *Ci-Hes-b* (Hudson and Yasuo, 2005; Imai et al., 2004), *Ci-Titf* (Ristoratore et al., 1999) and *Ci-Noto1* (Hotta et al., 2000). In the *C. intestinalis* genome, there are two *Twist-like 1* genes: *Ci-Twist-like 1a* and *Ci-Twist-like 1b* (Imai et al., 2004; Tokuoka et al., 2004). We used the cDNA ciclo29j13, which encodes *Ci-Twist-like 1a*, to synthesise in situ hybridisation probes. As *Ci-Twist-like 1a* and *Ci-Twist-like 1b* are 98% identical at the DNA level over more than 600 bp, it is likely that the in situ hybridisation probe used here would detect transcripts from both genes. Therefore, throughout this paper we refer to *Ci-Twist-like 1a/b* as *Ci-Twist-like 1*. Embryos were mounted in 50-80% glycerol and photographed with a Nikon D70 camera on an Olympus BX51.

mRNA injection constructs and morpholinos

Ci-Su(H)^{DBM} was generated by introducing mutations R218E, R220E, R227E and Y228S into the DNA-binding domain of Ci-Su(H) by overlap-extension PCR. Mutations at these four sites result in a DNA-binding mutant that acts in a dominant-negative manner (Wettstein et al., 1997). Three sets of PCR reactions were carried out using the following primers on the cDNA clone citb043k14: Su(H)-RI-F, 5′-ggaattccaccatgtatcacccccaccacctacc-3′; Su(H)-RI-R, 5′-ggaattcacgaggcagtacgaagcatgttc-3′; Su(H)DBM-F, 5′-caacGAActcGAAtcacagacagtgagcacaGAGTCCctgc-3′; Su(H)DBM-R, 5′-gcagGGACTCtgtgctcactgtctgtgaTTCgagTTCgttg-3′. Two primer pairs, Su(H)-RI-F/Su(H)DBM-R and Su(H)DBM-F/Su(H)-RI-R, were used in two independent PCRs. These two PCR products were used as a template for a third PCR using Su(H)-RI-F/ Su(H)-RI-R primer pairs. Amplified DNA fragments were subcloned into the *Eco*RI site of pRN3 vector (Lemaire et al., 1995). Removal of the intracellular domain of Delta ligands has been shown to result in a dominant-negative form (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996). *Ci-dnDelta2* (dnDel2) was generated by PCR using the following primer set on the cDNA clone cieg005o22: 05o22-RI-F, 5′-ggaattccaccatgagcatcaagcttatattacttc-3′; 05o22(1170)-RI-R, 5′-ggaattcaccgctgacgtaagttgctgc-3′. Amplified DNA was subcloned into the *Eco*RI site of pRN3. mRNA was synthesised using the mMessage mMachine kit (Ambion). The construct used to make *Ci-tALK4/5/7* mRNA has been previously described (Hudson and Yasuo, 2005). Delta2-Mo was purchased from Gene Tools (AGCTTGATGCTCATCGTTGTGTTTC), Control-Mo was the standard fluorescent control morpholino supplied by Gene Tools and Nodal-Mo has been described previously (Hudson and Yasuo, 2005).

Blastomere labelling

Blastomeres were labelled with CM-DiI (Molecular Probes) dissolved in colza oil at a concentration of 10 mg/ml. Embryos were treated with SB431542 from the 16-cell or DAPT from the 44-cell stage. When SB431542-treated embryos reached the 64-cell stage, an oil droplet containing CM-DiI was injected into B7.3 blastomeres on one side of the embryo under a Leica S8 APO stereomicroscope. Labelling of B8.6 blastomeres of DAPT-treated embryos was carried out when they reached to the 76-110 stages under a Zeiss upright miscroscope with a 25× objective. Following labelling, embryos were cultured in respective pharmacological inhibitors until the early tailbud stage, when they were fixed in 4% paraformaldehyde in 0.5 M NaCl/0.1 M MOPS for 20 minutes at room temperature. Fixed embryos were washed with PBS and then mounted in VECTASHIELD with DAPI (Vector Laboratories). Bright field and fluorescence images were captured with a Nikon D70 camera on an Olympus BX51 and processed using Photoshop (Adobe). Confocal images of the embryos were acquired with a Leica SP2 confocal microscope and processed using ImageJ (NIH) and Photoshop.

Embryo culture and manipulation

Blastomere names are those described by Conklin (Conklin, 1905) and lineages are described by Nishida (Nishida, 1987). Embryo culture, cytochalasin and SB431542 treatment and micro-injection are described previously (Hudson et al., 2003; Hudson and Yasuo, 2005). Unless stated otherwise, embryos were placed in SB431542 from the 16-cell stage until the time of fixation, except that cleaving embryos fixed at the early tailbud were washed at early gastrula stage and cultured in artificial sea water until fixation. DAPT was purchased from Calbiochem and used at a concentration of 100 μM. Embryos were placed in DAPT at the 44-cell stage, which is just prior to the onset of *Ci-Delta2* expression, until the time of fixation. Injections were carried out at the following concentrations: Control-Mo (1 mmol/l), Nodal-Mo (0.4 mmol/l), Delta2-Mo (0.125 mmol/l), Su(H)^{DBM} mRNA (1-1.5 μg/μl), dnDel2 mRNA (0.5 μg/μl), tALK4/5/7 mRNA (0.5 μg/μl) and GFP mRNA (0.5 μg/μl). In some cytochalasin B-treated embryos, the primary notochord was also perturbed following morpholino injection. However, *Ci-Noto1* expression in the primary notochord in cleaving embryos was not affected by Nodal or Delta inhibition (see Fig. 1 for SB431542 treatment; 24/25 positive, 1/25 weak expression with Nodal-Mo; and 61/65 positive, 3/65 reduced expression with DAPT treatment). Therefore, only embryos showing *Ci-Noto1* expression in the primary notochord were included in the analysis of cleavage-arrest experiments. For all data shown, data was pooled from at least two independent experiments.

RESULTS

Nodal signalling is not a generic inducer of mesoderm and endoderm in *Ciona* embryos

Ci-Nodal is expressed strongly in the laterally positioned b6.5 blastomeres from the 32-cell stage until mid-gastrula stage and weakly in the endoderm precursors at the 32-cell stage (Hudson and Yasuo, 2005; Imai et al., 2004; Morokuma et al., 2002). We have previously shown that Nodal signalling and the b6.5 blastomere are required to pattern the neural plate of *Ciona* embryos along the mediolateral axis (Hudson and Yasuo, 2005). We wanted to address if Nodal signalling was required for mesendoderm specification, as has been observed in vertebrate embryos (reviewed by Weng and Stemple, 2003). We thus treated embryos with a pharmacological reagent named SB431542, an inhibitor of the Nodal/Activin type I receptors ALK4, 5 and 7, of which there is one representative in the *Ciona* genome, *Ci-ALK4/5/7* (Hino et al., 2003; Inman et al., 2002). We found that both early and late markers for notochord (*Ci-Bra* and *Ci-Noto1* respectively), and endoderm (*Ci-Titf* and *Ci-Gataa*), remained expressed following treatment with SB431542 (Fig. 1). Thus, Nodal signalling is not required for formation of either primary notochord or endoderm, as shown previously for primary muscle (Hudson and Yasuo, 2005).

Nodal signalling and the b5.3 blastomere are required for secondary notochord fate

Despite the lack of a general requirement for Nodal for mesoderm and endoderm fates, we found that SB431542 treatment selectively abolished expression of *Ci-Bra* in the secondary notochord precursor, B8.6, when analysed at the early gastrula stage (Fig. 2A). Inhibition of Nodal signalling by injection of Nodal-Mo also caused a severe downregulation of *Ci-Bra* expression in B8.6. Furthermore, no expression of the notochord marker *Ci-Noto1* was detected in the secondary notochord lineages at tailbud stages. In order to identify the lineages expressing *Ci-Noto1* at late stages, embryos were treated with cytochalasin-B from the 110-cell stage to arrest cytokinesis and thus maintain the relative position of each cell from the 110-cell stage.

In order to test whether the Nodal signal responsible for secondary notochord induction derives from the b6.5 blastomere, we ablated the mother cell of this blastomere, b5.3. Ablation of b5.3 was previously shown to have a stronger effect on lateral neural plate marker gene expression than ablation of b6.5 itself (Hudson and Yasuo, 2005). We found that ablation of b5.3 resulted in a severe

reduction in *Ci-Bra* expression in the secondary notochord on the ablated side (Fig. 2B). In these experiments, 29% of embryos still showed some level of *Ci-Bra* expression in B8.6 on the ablated side. This may be due to the recovery of *Ci-Nodal* expression in other b-line cells on the ablated side, which we observed in 26% of cases when analysed at the 76-cell stage (*n*=87). This is consistent with a recent study showing that removal of embryonic parts can result in alterations in cell contacts, subsequently resulting in ectopic induction of gene expression (Tassy et al., 2006). We conclude that Ci-Nodal signalling from b-line cells plays a major role during the specification of secondary notochord fate.

The secondary notochord precursor forms following the division of B7.3, which gives rise to one notochord (B8.6) and one mesenchyme (B8.5) precursor. Of these two cells, the notochord precursor becomes positioned closest to where the Nodal expressing b6.5 blastomere was situated (black dots in Fig. 3B). To address whether this cell fate specification operates as a binary switch, with Nodal promoting notochord fate and repressing mesenchyme fate in B8.6, we tested whether the secondary notochord precursor adopts mesenchyme fate following Nodal inhibition by analysing expression of *Ci-Twist-like 1*. *Ci-Twist-like 1* encodes a bHLH transcription factor that is expressed in the A7.6 'trunk lateral cell' mesenchyme precursor and the B8.5 and B7.7 mesenchyme precursors and is required for them to adopt mesenchyme fate (Imai et al., 2003; Tokuoka et al., 2004). B8.6 did not express *Ci-Twist-like 1* following Nodal inhibition, indicating that there had not been a notochord-mesenchyme fate switch (Fig. 2C). Furthermore, expression of *Ci-Twist-like 1* was downregulated in both the A7.6 trunk lateral cell precursors and the B8.5 mesenchyme precursors, but not in B7.7 (Fig. 2C). This result was confirmed by analysing *Ci-AKR1a*, a late marker of mesenchyme fate at the early tailbud stage in SB431542-treated embryos incubated in cytochalasin B from the 110-cell stage (95% strong expression in B7.7; 32% strong expression and 16% weak expression in B8.5; 0% expression in A7.6; 0% expression in B8.6; *n*=38). These results imply that Nodal signalling is required for the correct specification of certain mesenchyme lineages as well as for notochord fate in B8.6. DiI labelling of B7.3 confirms that this blastomere generates mesenchyme and secondary notochord fate during normal development (5/6) (see Fig. S1 in the supplementary material) (Nishida, 1987). In SB431542-treated embryos B7.3 remains mitotically active and its derivatives can be observed at the early tailbud stage as a single cluster of cells in the interior of the embryo (7/7) (see Fig. S1 in the supplementary material). We do not know what cell type these blastomeres adopt following Nodal inhibition as neither endoderm nor muscle markers appeared to be expressed ectopically in B8.5 or B8.6 when Nodal signalling was inhibited (Fig. 1) (Hudson and Yasuo, 2005).

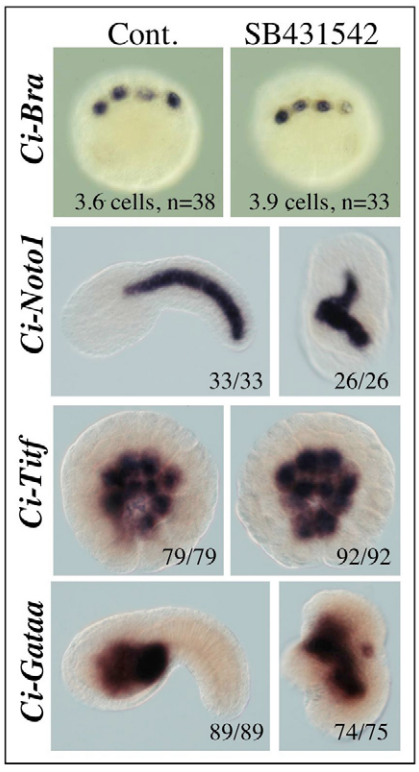

Fig. 1. Expression of notochord and endoderm markers following inhibition of ALK4/5/7 with SB431542. Embryo treatment is indicated at the top of the panels and the marker analysed is indicated at the left of the panels. *Ci-Bra* was analysed at the 64-cell stage, *Ci-Titf* at the early gastrula stage and *Ci-Noto1* and *Ci-Gataa* at the early tailbud stage. For the top panel, the average number of cells expressing *Ci-Bra* is indicated; *n*=number of embryos analysed. For the rest of the panels, the numbers indicate the number of embryos expressing a given gene/total number of embryos analysed.

In summary, Nodal signalling is required for both cell fates that are generated from the B7.3 lineage, suggesting that Nodal may be acting during the specification of this mixed-fate precursor cell, which forms at the 64-cell stage (Fig. 6B). We therefore tested when Nodal signalling was required for *Ci-Bra* expression in the secondary notochord precursor.

Secondary notochord precursor specification becomes independent of Nodal signalling by the 64-cell stage

In order to test when Nodal signalling was acting during secondary notochord specification, we placed embryos in the ALK4/5/7 inhibitor, SB431542, at different developmental time points. We found that *Ci-Bra* expression in the secondary notochord precursor was severely downregulated when embryos were placed in SB431542 at the 16- or late 32-cell stages, but became independent of Nodal signalling by the 64-cell stage (Fig. 3A). Although we do not know how long the inhibitor takes to penetrate the embryo and act, this timing fits well with the observation that the secondary notochord precursors (B6.2) are in direct contact with the Nodal-expressing cells during the 32-cell stage (Fig. 3B). Taken together with the observation that both notochord and mesenchyme fates are lost from the B7.3 lineage, this suggests that Nodal signalling is required during the 32- to 64-cell stages for specification of the mother cell of the notochord and mesenchyme precursors, which then cleaves to generate two different cell types by other, Nodal-independent, mechanisms.

Nodal signalling is required in both B- and A-line cells for correct specification of the secondary notochord

If Nodal signals are required directly to specify the mother cell (B7.3) of the notochord and mesenchyme precursors, selective inhibition of the reception of Nodal signalling in the B-line should be sufficient to block the formation of the secondary notochord precursor. For this purpose we injected, into the right hand B4.1 blastomere of the eight-cell stage embryo, a truncated form of the *Ciona* Nodal receptor (*Ci-tALK4/5/7*), which has been previously shown to inhibit Nodal signals in *Ciona* (Hudson and Yasuo, 2005). The B4.1 blastomere is the founder lineage of the secondary

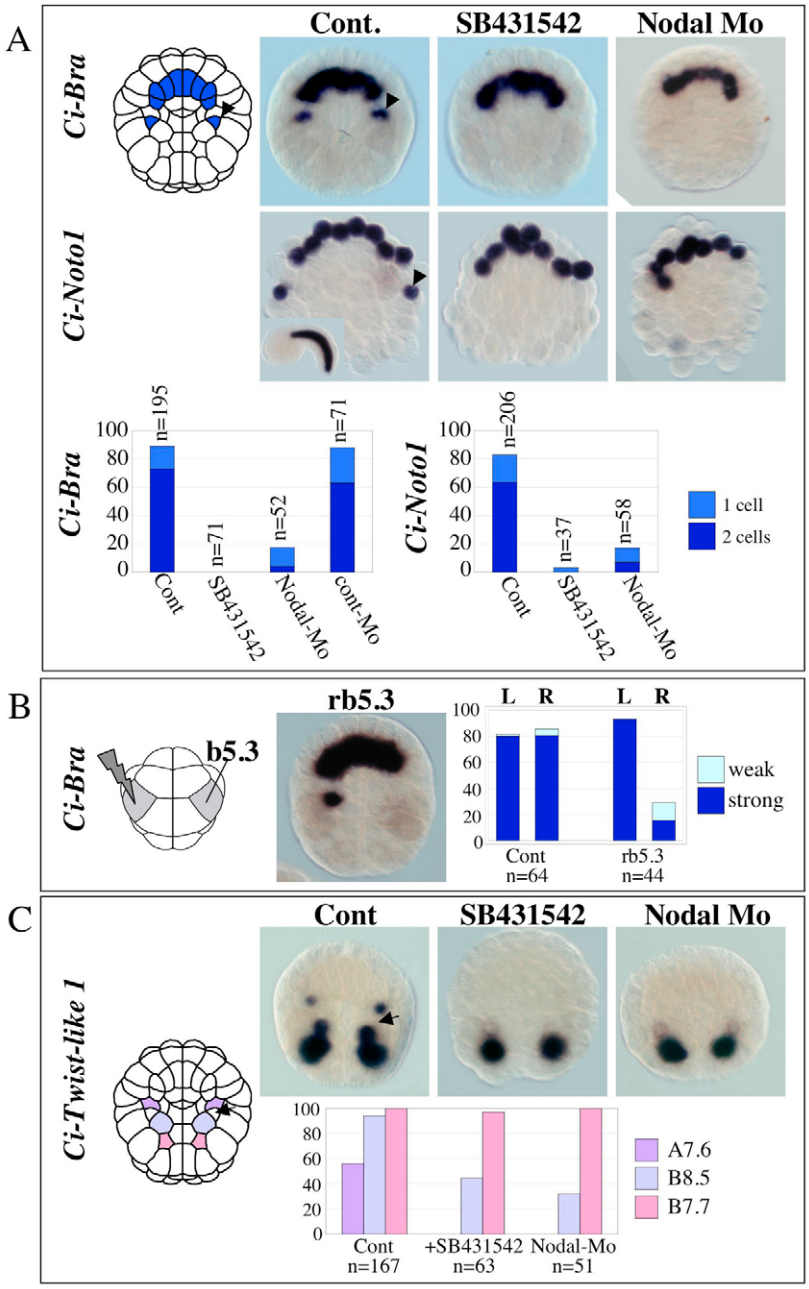

Fig. 2. Nodal signalling is required for secondary notochord fate. (A-C) Embryo treatment is indicated above the panels. The marker analysed is shown on the left of the panels. For the graphs, embryo treatment is indicated on the *x*-axis and percentage of embryos on the *y*-axis. *n*=total number of embryos analysed. (**A**) Expression of *Ci-Bra* at the early gastrula stage and *Ci-Noto1* at the early tailbud stage following inhibition of Nodal signalling. The schematic embryo, in a vegetal pole view, shows the positions of the primary and secondary notochord precursors (in blue) at the 110-cell stage. Arrowheads indicate the secondary notochord lineage. The insert in the control *Ci-Noto1* panel is a cleaving embryo to indicate the stage at which the analysis was carried out. The graphs show the percentage of embryos showing expression of the gene indicated on the left, in one or two secondary notochord precursors. (**B**) Ablation of b5.3 on the right hand side (rb5.3) leads to an inhibition of *Ci-Bra* expression in the secondary notochord precursor on the ablated side. The schematic drawing of a 16-cell stage embryo, in animal pole view, shows the position of the b5.3 blastomere. The graph shows the percentage of secondary notochord precursors expressing strong or weak *Ci-Bra* on the left- (L) or right- (R) hand side of the embryo at the early gastrula stage. (**C**) Expression of *Ci-Twist-like 1* at the early gastrula stage following inhibition of Nodal signalling. The graph shows the percentage of embryos expressing *Ci-Twist-like 1* in one or both blastomeres for the lineages indicated in the colour scheme (see key). The schematic embryo on the left shows the positions of these blastomeres at the 110-cell stage, using the same colour scheme. The arrows indicate the secondary notochord precursors.

notochord lineages. As a control, we injected *Ci-tALK4/5/7* into the right hand side A4.1 of the eight-cell stage embryo, which is the founder of the primary notochord lineages, the specification of which should not be affected by this treatment because the primary notochord is specified independently of Nodal. *GFP* mRNA was injected as a control to show that the injection process itself did not perturb gene expression. *Ci-Bra* expression was then analysed at the early gastrula stage. We found that injection of *Ci-tALK4/5/7* into B4.1 did indeed lead to a decrease in *Ci-Bra* expression on the injected side (Fig. 4). However, to our surprise, injection into A4.1, although having no effect on the primary notochord lineages, led to a severe downregulation of *Ci-Bra* expression in the secondary notochord precursor on the injected side (Fig. 4).

These results suggest that Nodal signalling acts in two ways during the specification of secondary notochord. First, it acts directly upon B-line cells, probably during the specification of the mother cell of the notochord and mesenchyme precursors. Second, it acts indirectly, via the A-line cells, indicating that Nodal signals are relayed by an, as yet, unknown signal, which subsequently acts upon the B-lineages in order to specify secondary notochord fate. We next investigated the nature of this inductive signal, which is predicted to originate from the A-line cells and to be dependent upon Nodal signalling.

Ci-Delta2 expression is induced in the A-lineages by Ci-Nodal

We have previously shown that *Ci-Delta2* is a transcriptional target of Nodal signalling at the early gastrula stage (Hudson and Yasuo, 2005). In this study, we characterised the initiation of *Ci-Delta2* expression, which could be detected at the 64-cell stage, in A7.6, b7.10, b7.9, and also weakly in some cases in A7.8 (Fig. 5A). Expression was variable in these different lineages from embryo to

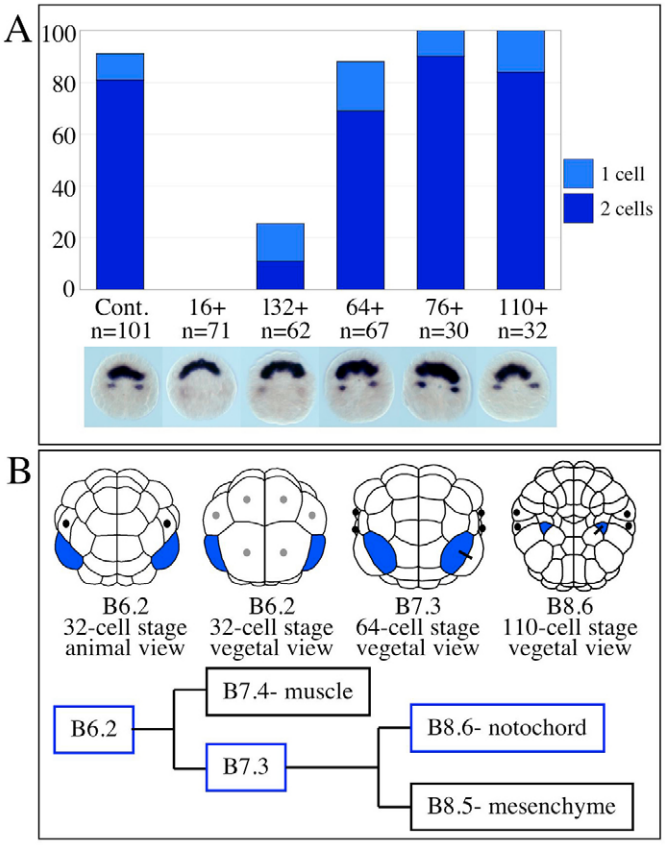

Fig. 3. The temporal requirement of Nodal signalling for *Ci-Bra* expression in the secondary notochord precursors at the early gastrula stage. (**A**) Embryos were treated with SB431542 at different developmental time points, indicated along the *x*-axis of the graph. l32 corresponds to the late 32-cell stage. The percentage of embryos expressing *Ci-Bra* in one or two B8.6 blastomeres is indicated on the *y*-axis. A representative embryo for each time point is shown below the graph. (**B**) Schematic drawings of embryos from the late 32-cell stage to the 110-cell stage. The secondary notochord lineages are shown in blue. The name of the secondary notochord precursor at the different stages and the stage and orientation of the embryos in the drawings is indicated below each drawing. Nodal-expressing blastomeres are marked with a black dot for those with strong expression and a grey dot for those with weak expression. Black bars connecting blastomeres indicate their sister cell relationship. Below the drawings is shown a cell lineage tree of the B6.2 presumptive secondary notochord precursor from the 32-cell stage until the generation of the secondary notochord precursor at the 110-cell stage. Blastomeres containing notochord fate are outlined in blue.

embryo, but the most robust expression was observed in the A7.6 trunk lateral cell precursor (Fig. 5A,B, graphs). The A7.6 blastomere is positioned adjacent to the B7.3 mother cell of the notochord and mesenchyme precursors, on the side on which the secondary notochord precursor will be specified (Fig. 5A). We found that expression of *Ci-Delta2* at the 64-cell stage was also dependent upon Nodal signalling. Treatment with SB431542 or injection of Nodal-Mo resulted in repression of *Ci-Delta2* expression in all lineages (Fig. 5A). Furthermore, selective inhibition of the reception of Nodal signalling in A4.1, or ablation of b5.3, the source of Nodal signals, was sufficient to abolish *Ci-Delta2* expression in A7.6 (and A7.8) on the treated side (Fig. 5B,C). Thus, the same treatments that block secondary notochord formation also inhibit *Ci-Delta2* expression in A-line cells. Taken together, these data indicate that Ci-Delta2 in A7.6 is an excellent candidate to relay Nodal signals during the specification of the secondary notochord.

The Delta2/Notch/Su(H) signalling pathway is required for secondary notochord fate

Membrane-bound Delta ligands act through Notch receptors in a cell-contact-dependent manner (for reviews, see Baron, 2003; Hansson et al., 2004; Lai, 2004). Ligand activation of the Notch receptor triggers two successive proteolytic cleavages of the receptor, mediated first by a metalloprotease and second by γ-secretase. The released Notch intracellular domain (NICD) then associates with a transcription factor, Suppressor of Hairless [Su(H)], converting it from a transcriptional repressor into an activator. *Ci-Delta2* encodes a more divergent form of Delta than that encoded by the ubiquitously expressed *Ci-Delta* (Imai et al., 2004). The domain responsible for ligand binding (DSL for Delta Serrate Lag domain), is only weakly conserved in Ci-Delta2 (Fig. 6A). However, we believe that it may be acting as a Notch ligand because of the expression of *Ci-Hes-b. Ci-Hes-b* is a member of the *Hairy/Enhancer of split* family, genes that often act as transcriptional targets of the Delta/Notch/Su(H) pathway in other systems (Baron, 2003; Hansson et al., 2004). We found that *Ci-Hes-b* is expressed precisely in and around the *Ci-Delta2*-expressing cells, in the A8.16 (neural/muscle fate), A8.8 (primary notochord) and B8.6 (secondary notochord) blastomeres at the early gastrula stage (Fig. 6B). The presence of two Su(H) consensus-binding sites in tandem in the putative upstream regulatory sequences of *Ci-Hes-b* (H.Y., unpublished) further supports the idea that Ci-Delta2 might act as a Notch ligand during activation of *Ci-Hes-b*.

In order to investigate the role of Ci-Delta2 during secondary notochord formation, we inhibited Notch-Delta at various levels of the signalling pathway. In order to inhibit the Delta2 ligand, we used an antisense morpholino oligonucleotide against *Ci-Delta2* (Del2-Mo) or a version of Ci-Delta2 lacking the intracellular domain (dnDel2). The removal of the intracellular domain of Delta ligands has previously been shown to convert them into dominant-negative forms (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996). As the DSL domain of Ci-Delta2 is not well conserved, we wanted to ascertain whether Ci-Delta2 was acting through the canonical Notch-Delta signalling pathway during secondary notochord induction. Therefore, we also made use of a pharmacological reagent, DAPT, which inhibits γ-secretase. Application of DAPT has previously been shown to inhibit Notch-Delta signalling in zebrafish embryos (Geling et al., 2002). Finally, we constructed a DNA-binding mutant of *Ciona Suppressor of Hairless* [Ci-Su(H)^{DBM}]. This mutant form of Su(H) still binds to NICD, but not to its target DNA sequences, thus interfering with the ability of NICD to interact with endogenous Su(H) proteins (Wettstein et al., 1997).

Following inhibition at the level of Delta2 ligand or γ-secretase, *Ci-Hes-b* expression was lost, including that in the secondary notochord precursor (Fig. 6B). Moreover, *Ci-Bra* expression in the secondary notochord precursors was lost when Delta/Notch/Su(H) signalling was inhibited by any of the four reagents, and *Ci-Noto1* expression in the secondary notochord was abolished at later stages (Fig. 7A). Furthermore, ablation of A6.3, the precursor of A7.6, which expresses *Ci-Delta2* and is in direct contact with the secondary notochord precursor, resulted in an inhibition of *Ci-Bra* expression in the secondary notochord precursor on the ablated side (Fig. 7B). Taken together, these results suggest that Ci-Delta2 activity, derived from the A7.6-lineage and acting via the canonical

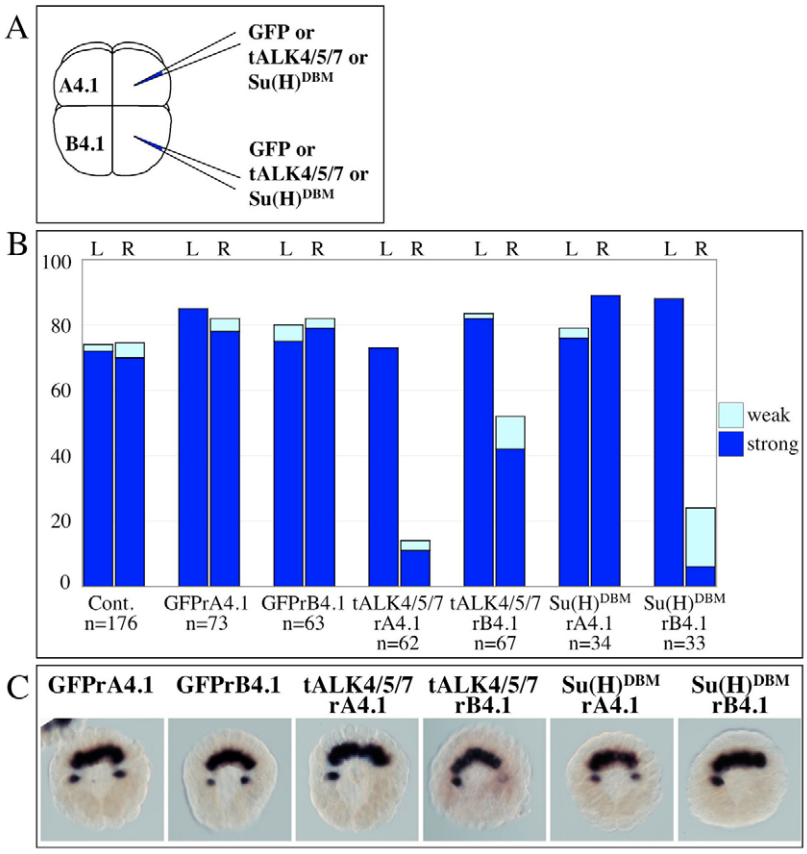

Fig. 4. Selective inhibition of the reception of either Nodal or Delta signals in one of the four founder cells of the eight-cell embryo. (**A**) Experimental design showing a vegetal pole view of the eight-cell stage embryo; eight-cell stage embryos were injected with *tALK4/5/7* mRNA to block Nodal signal reception, *Su(H)^{DBM}* mRNA to block Delta/Notch signal transduction or *GFP* mRNA as a control. mRNAs were selectively injected into either the A4.1 or the B4.1 blastomere on the right hand side and then analysed for *Ci-Bra* expression at the early gastrula stage. (**B**) A graph showing the percentage of embryos (*y*-axis) with strong or weak *Ci-Bra* expression in the B8.6 blastomere on the left- (L) or right- (R) hand side of the embryo. Injected RNAs and injected blastomere names are indicated on the *x*-axis. *n*=total number of embryos analysed. (**C**) A representative embryo for each of the treatments.

Delta/Notch/Su(H) signalling pathway, is required for the specification of secondary notochord precursor. DiI labelling of B8.6 shows that, during normal embryogenesis, this blastomere gives rise to four secondary notochord cells (4/5) (see Fig. S1 in the supplementary material) (Nishida, 1987). However, in DAPT-treated embryos, B8.6 gives rise to many more smaller cells that remain inside the embryo by the early tailbud stage (4/4) (see Fig. S1 in the supplementary material). This suggests that the cell cycle control of B8.6 may be coupled with its fate specification. During secondary notochord fate specification, Ci-Delta2 signalling does not appear to be operating as a simple binary cell-fate switch because in the Delta2/Notch-inhibited embryos the secondary notochord precursor did not adopt mesenchyme fate, as assessed by *Ci-Twist-like 1* expression (Fig. 7C). Similarly, in early tailbud stage embryos treated with DAPT from the 44-cell stage and with cytochalasin from the 110-cell stage, *Ci-AKR1a* expression was rarely observed in B8.6 (3/30 embryos showed expression, on one side, in a blastomere that was in a position consistent to be B8.6). B8.6 also does not appear to be adopting endoderm or muscle fate following DAPT treatment (data not shown). Thus, it is not clear what fate these cells adopt following inhibition of Delta/Notch signals.

Finally, to verify that the cells responding to Delta2 indeed originated in the B lineages, we injected *Ci-Su(H)^{DBM}* mRNA into A4.1 or B4.1 blastomeres at the eight-cell stage. Only injection into B4.1 resulted in inhibition of *Ci-Bra* expression in the secondary notochord precursor on the injected side (Fig. 4). We conclude that while Nodal signalling is required for secondary notochord formation both in B-line cells and (indirectly) in A-line cells, the Notch signalling pathway, activated by A-line *Ci-Delta2*-expressing cells, is required only in the B-lineages.

DISCUSSION

Based on our findings, we propose the following model for the roles of Nodal and Delta2 during induction of secondary notochord fate in *Ciona* embryos (Fig. 8). Ci-Nodal signals appear to play a dual role during this process. First, Nodal signals are required within the B6.2 lineage for the correct specification of the notochord (B8.6) and mesenchyme (B8.5) precursors following two rounds of cell division. Second, Nodal is required in the A6.3 lineage to induce expression of *Ci-Delta2*. Nodal signals are no longer required for the induction of secondary notochord fate from the 64-cell stage, when *Ci-Delta2* starts to be expressed in the A7.6 'trunk lateral' mesenchyme precursor (one of the daughters of A6.3). A7.6 is in direct contact with the B7.3 blastomere, which is the mother cell of the secondary notochord (B8.6) and mesenchyme (B8.5) precursors. Ci-Delta2 signalling from A7.6, activating the canonical Notch pathway in B-line cells, induces notochord fate in the B8.6 blastomere. Thus, Nodal signals are relayed via Delta2 during secondary notochord specification. It should be noted that, during secondary notochord induction, Ci-Delta2 does not appear to be operating a simple binary switch. In the absence of Delta2/Notch signalling, the B8.6 blastomere does not adopt the fate of its sister blastomere, that of mesenchyme. This suggests that additional mechanisms are involved in the differential fate specification of the B8.5 and B8.6 sister cells.

In addition to the role of Nodal in secondary notochord induction, we have observed that Nodal is also required for expression of *Ci-Twist-like 1*, a causal regulator of mesenchyme fate (Imai et al., 2003; Tokuoka at al, 2004), in the A7.6 and B8.5 lineages. Nodal signals emanating from the b6.5 blastomere may contribute to the proposed signal derived from animal cells during the 16-32 cell

stages, which was shown to be required for trunk lateral cell (A7.6) fate in *Halocynthia* (Kawaminani and Nishida, 1997). Taken together with our previous observations that Nodal signalling is required for patterning across the mediolateral axis of the neural plate and for specification of the secondary muscle formation from the A6.4 lineage, as well as recent evidence for the role of Nodal during patterning the dorsal epidermis, it is transpiring that Nodal plays a broad patterning role during ascidian development, across all the embryonic germ layers (this study) (Hudson and Yasuo, 2005; Pasini et al., 2006).

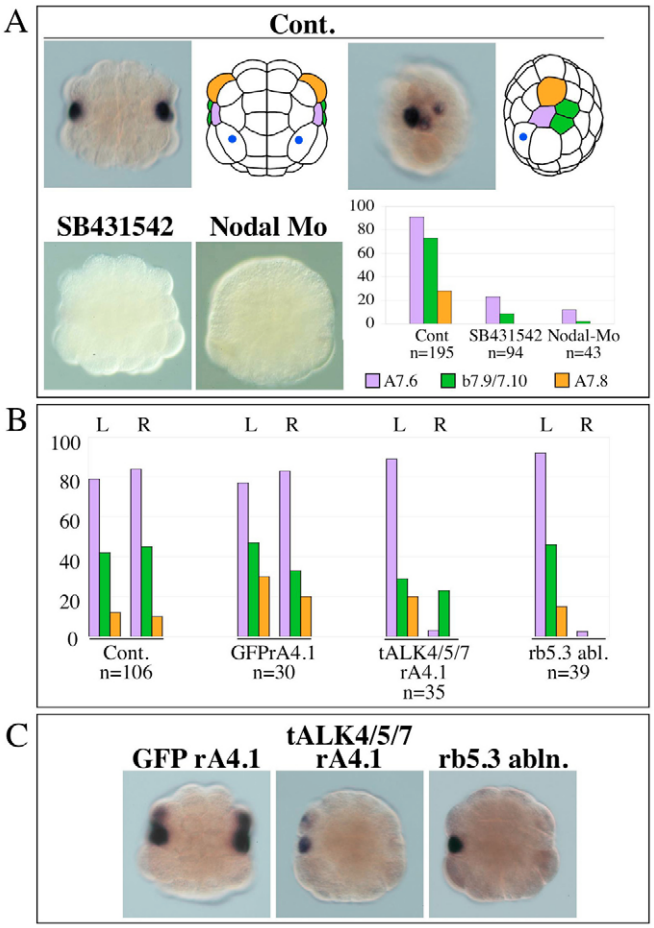

Fig. 5. *Ci-Delta2* expression at the 64-cell stage is a target of Nodal signalling. (A-C) Embryo treatment is indicated above the panels. *n*=total number of embryos analysed. (**A**) Expression of *Ci-Delta2* at the 64-cell stage. The expression pattern is represented by schematic drawings on the right of each embryo panel. *Ci-Delta2* is expressed in A7.8 (orange), A7.6 (mauve) and b7.10/b7.9 (green). On the schematic drawings, the secondary notochord precursor is marked by a blue dot. The graph shows the percentage of embryos (*y*-axis) showing expression in at least one blastomere for each of the different lineages (indicated by the colour scheme), following the treatment shown on the *x*-axis. (**B**) A graph showing the percentage of embryos (*y*-axis) in which *Ci-Delta2* expression was detected in the different lineages on the left- (L) or right- (R) hand sides on the embryo following the treatments indicated on the *x*-axis. The colour scheme is the same as in A. Consistent with the observation that *Ci-Bra* and *Ci-Nodal* was sometimes recovered following b5.3 ablation, we observed expression of *Ci-Delta2* in the trunk lateral cell precursor on the ablated side in 11% of embryos when analysed at the 76-cell stage (*n*=53). (**C**) A representative embryo for each of the treatments shown in B.

Notochord specification in ascidian embryos

During the specification of ascidian notochord, two seemingly parallel pathways have been implicated, both of which are initially activated by maternal β-catenin in the vegetal cells. The first pathway begins with transcriptional activation of *FGF9/16/20* in the vegetal cells and the second pathway begins with the transcriptional activation of *FoxD,* a transcription factor (Imai et al., 2002a; Imai et al., 2002b).

For primary notochord development, FGF/MEK/ERK1/2 activity is required during the 32-64 cell stages for the A-line notochord precursors to adopt notochord fate and to repress neural fate (H.Y. and C.H., unpublished) (Hudson et al., 2003; Imai et al., 2002a; Kim and Nishida, 2001; Minokawa et al., 2001). In the secondary notochord lineages, FGF signalling appears to play a dual role. First, it is required to suppress muscle fate in the mother cell of the notochord and mesenchyme precursors at the 64-cell stage (Darras and Nishida, 2001; Imai et al., 2002a; Kim and Nishida, 1999; Kim et al., 2000; Kim and Nishida, 2001). Second, the FGF/MEK/ERK1/2 pathway activates *Ci-Nodal* expression in the b6.5 blastomere at the 32-cell stage (Hudson and Yasuo, 2005). Ci-Nodal is then required, both directly and via *Ci-Delta2* gene

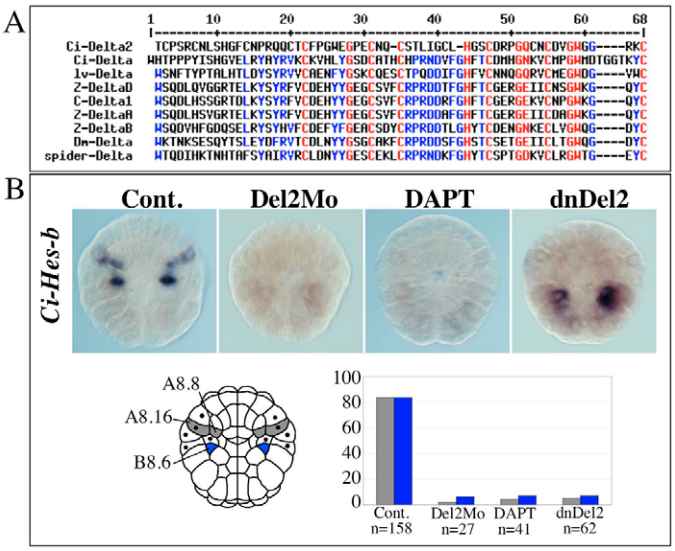

Fig. 6. *Ci-Delta2* encodes a Delta-like molecule. (**A**) Alignment of the putative DSL domain of Ci-Delta2 with DSLs from other Delta proteins. Ci, *Ciona intestinalis*; lv, *Lytechinus variegatus* (green sea urchin); Z, zebrafish (*Danio rerio*); C, chick; Dm, *Drosophila melanogaster*; spider, *Cupiennius salei.* The DSL domain of Ci-Delta2 was identified using the SMART programme, but gave a score less significant than the required threshold to be confidently predicted as a DSL domain using this programme (Letunic et al., 2004; Schultz et al., 1998). (**B**) *Ci-Hes-b* expression at the early gastrula stage is a target of Ci-Delta2. Expression of *Ci-Hes-b* is shown following the treatment indicated above the panels. Ectopic expression was sometimes observed in the mesenchyme lineages following injection of *dnDel2* (far right). The graph shows the percentage of embryos (*y*-axis) showing expression in at least one cell of the A-line (A8.16, A8.8, in grey) and B-line (B8.6, in blue) following the treatments indicated on the *x*-axis. *n*=total number of embryos examined. The schematic drawing shows the positions of these blastomeres. Dots indicate the blastomere derivatives that were expressing *Ci-Delta2* at the 64-cell stage. At the early gastrula stage, *Ci-Delta* expression is downregulated in A7.6, while that in A8.15/A8.16 (daughters of A7.8) becomes stronger (Hudson and Yasuo, 2005).

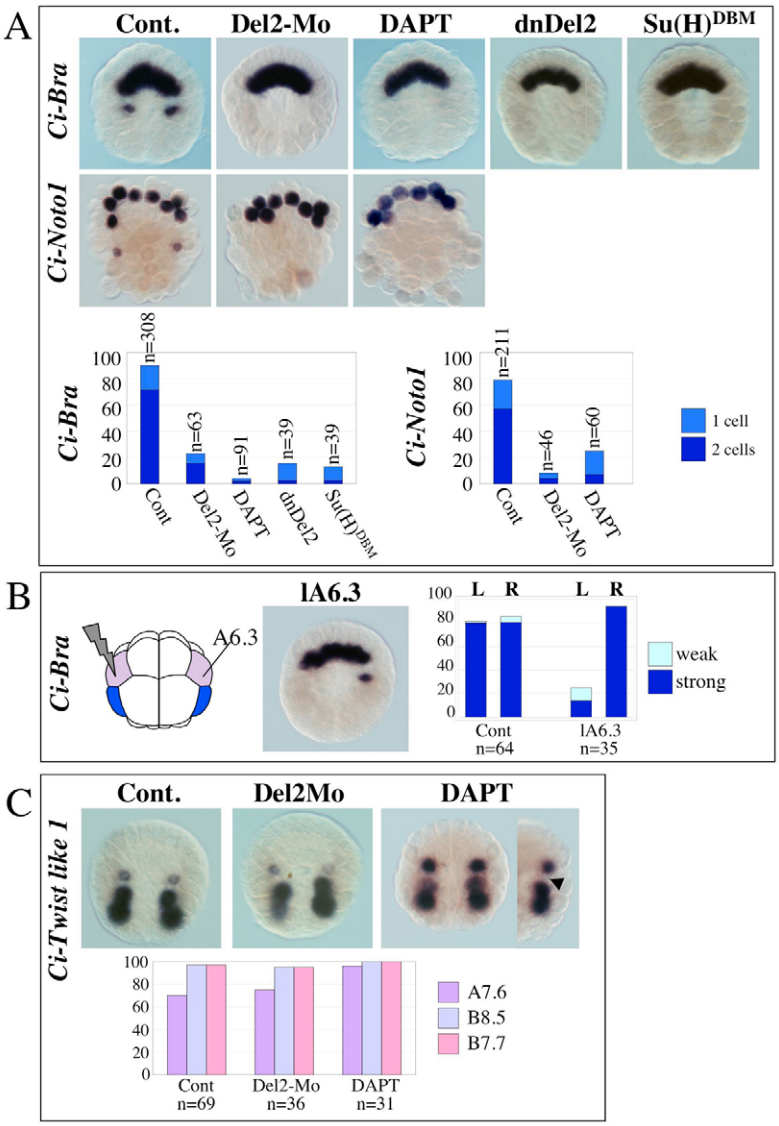

Fig. 7. Delta2/Notch signalling is required for secondary notochord induction. (A-C) The embryonic treatment is indicated above the panels and the marker analysed on the left. *n*=total number of embryos examined. (**A**) Expression of Ci-Bra at the early gastrula stage and Ci-Noto1 at the early tailbud stage following inhibition of Delta2/Notch signalling. The graphs show the percentage of embryos (*y*-axis) expressing *Ci-Bra* or *Ci-Noto1* in one or two B8.6 blastomeres following the treatments indicated on the *x*-axis. (**B**) Ablation of A6.3 on the left-hand side (lA6.3) leads to a loss of *Ci-Bra* expression in the secondary notochord precursor on the ablated side. The schematic drawing of a vegetal pole view 32-cell stage embryo shows the A6.3 blastomere (dark pink) relative to the presumptive secondary notochord precursor (blue). The graph shows the percentage of embryos (*y*-axis) expressing strong or weak *Ci-Bra* in B8.6 on the left- (L) or right- (R) hand side of the embryo, following ablation of A6.3 on the left-hand side. (**C**) Expression of *Ci-Twist-like 1* at the early gastrula stage following inhibition of Delta signalling. The DAPT-treated embryo shown is tilted slightly (far right) in order to show more clearly the B8.6 blastomere (arrowhead), which is not expressing *Ci-Twist-like 1*. The graph shows the percentage of embryos (*y*-axis) expressing *Ci-Twist-like 1* in at least one cell of the different lineages following the treatment indicated on the *x*-axis.

activation, for the specification of the secondary notochord precursor (this study). Delta2 most probably activates the Notch signalling pathway and *Ci-Bra* expression directly in B8.6. Evidence supporting this includes the observation that *Ci-Hes-b* is activated in the B8.6 blastomere in a Delta2/Notch-dependent manner and that Su(H) activity is required within the B-line lineages for expression of *Ci-Bra* in B8.6 (this study). In addition, Su(H)-binding sites, to which Ci-Su(H) has been shown to bind in vitro, are present in the upstream regulatory sequences of *Ci-Bra* (Corbo et al., 1997; Corbo et al., 1998). Notch-Delta signalling has previously been implicated in both primary and secondary notochord formation in *Ciona* embryos. Mutation or deletion of the Su(H)-binding sites in a *Ci-Bra* minimal promoter was shown to abolish reporter gene activity in all notochord cells (Corbo et al., 1997; Corbo et al., 1998). However, using a DNA-binding mutant of Su(H), which should attenuate Notch activation of endogenous Su(H), as well as inhibiting the pathway at the level of the Delta2 ligand or Notch receptor processing, we observed a downregulation of endogenous *Ci-Bra* expression only in the secondary notochord lineage. This is consistent with the observation that widespread activation of Notch signalling, by injection of a constitutively active Notch receptor, leads to ectopic *Ci-Bra* activation in B-line cells much more readily than in A-line cells (Imai et al., 2002b). It is possible that the mutations and deletions in the Su(H) binding sites in the *Ci-Bra* minimal promoter also resulted in the disruption of additional binding sites. Another possibility is that Su(H) acts independently of Notch in the primary notochord. Indeed, Su(H) is able to act as a transcriptional activator independently of Notch during maintenance of its own expression in *Drosophila* adult socket cells (part of the mechanosensory bristles), although the initial activation of this expression depends on Notch signalling (Barolo et al., 2000). In light of our findings, the *Ci-Bra* regulatory sequences require further investigation to understand precisely the role of Su(H) during transcriptional control of *Ci-Bra* expression in the primary notochord lineages.

FoxD is a target of vegetally activated β-catenin and is required for formation of primary and secondary notochord (Imai et al., 2002b). FoxD acts in part through a second transcription factor, ZicL, which directly binds and activates the *Ci-Bra* promoter (Imai et al., 2002b; Imai et al., 2002c; Wada and Saiga, 2002; Yagi et al., 2004). It is not yet clear at what level the FoxD/ZicL and FGF9/Nodal/Delta2 pathways interact during secondary notochord formation.

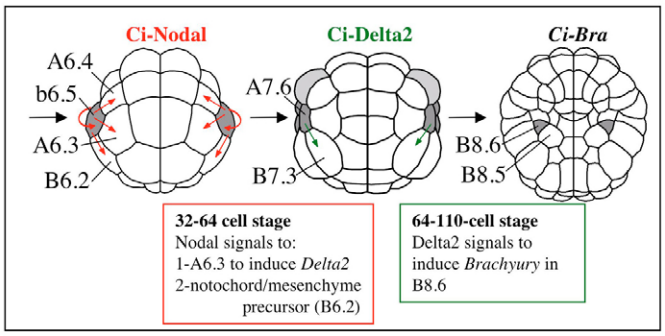

Fig. 8. The role of Nodal and Delta2 during secondary notochord induction in *Ciona* embryos. The positions of blastomeres that are discussed in the text are indicated. Ci-Nodal signals are indicated by red arrows and Ci-Delta2 signals by green arrows. For each schematic drawing, the expression pattern of the gene indicated above it is shown by grey coloured blastomeres. Schematic drawings are vegetal pole views of an early 32-cell stage embryo, a 64-cell stage embryo and a 110-cell stage embryo, from left to right. Although *Ci-Nodal* expression is not detected until the late 32-cell stage, an early 32-cell stage embryo is shown so that the b6.5 blastomere can be seen on the vegetal pole view of the embryo (compare with Fig. 3B). The roles played by Ci-Nodal and Ci-Delta2 signals during secondary notochord induction during these stages is indicated below the drawings.

It has been reported in *Halocynthia*, that BMP2/4 signalling from the anterior endoderm precursors, together with FGF-signalling, is required for induction of both primary and secondary notochord (Darras and Nishida, 2001). By contrast, BMP2/4 signalling does not appear to play a major role in *Ciona* notochord specification. Injection of *Xenopus Chordin* mRNA does not result in any obvious defects in notochord formation and the *Ciona* orthologue of *BMP2/4* is not expressed in the anterior endoderm precursors (H.Y., unpublished) (Imai et al., 2004). It thus appears that there are real differences in the mechanisms used to specify secondary notochord in *Halocynthia* and *Ciona* embryos, two distantly related ascidian species (Cameron et al., 2000; Swalla et al., 2000; Wada, 1998), despite extensive similarities in their developmental mode and cleavage patterns.

Distinct inductive mechanisms can govern specification of similar tissue types

This study highlights an interesting aspect of ascidian development, which is that the same cell fate can be specified in distinct cell lineages by largely independent mechanisms. We have shown that neither inhibition of Nodal or Delta signalling nor ablation of the signalling sources (b5.3 or A6.3) resulted in inhibition of the primary notochord, while appearing crucial for secondary notochord.

Muscle cells also arise from different embryonic origins, the primary lineage from the B-line and the secondary lineages from the b- and A-lines and these different lineages are also specified by distinct mechanisms. The primary lineage is specified cell-autonomously by the inheritance of cytoplasmic determinants, including the zinc-finger transcription factor Macho-1, whereas the secondary muscle is specified by inductive cellular interactions (Deno et al., 1984; Meedel et al., 1987; Meedel et al., 2002; Nishida, 1990; Nishida and Sawada, 2001; Satou et al., 2002). We have recently shown that Nodal signalling is required for the specification of muscle fate in one of the secondary muscle lineages (Hudson and Yasuo, 2005). In addition, the mesenchyme fates, even within the B-line, also appear to be dependent on different strategies. We have shown in this study that B8.5 and A7.6 depend upon Nodal signals in order to express *Ci-Twist-like 1*, whereas B7.7 does not.

Fate specification by different molecular strategies is not restricted to the mesoderm germ layer. It is also observed in the central nervous system (CNS) in which FGF signalling is required for neural fate in a-line cells but not in A-line cells (Bertrand et al., 2003; Minokawa et al., 2001). Finally, in *Halocynthia*, it has been shown that anterior endoderm specification occurs cell autonomously, whereas posterior endoderm specification requires FGF and BMP signalling (Kim and Nishida, 2001; Kondoh et al., 2003).

Although it is clear that distinct mechanisms can generate similar tissue types in the different lineages of ascidian embryos, cell-type specific transcription factors have been identified that promote tissue-type fate specification irrespective of the lineage. Examples in the mesoderm lineages include *Brachyury* for notochord formation (Takahashi et al., 1999; Yasuo and Satoh, 1998), *Ci-Twist-like 1* for mesenchyme (Imai et al., 2003), and *Ci-Tbx6* family members for muscle (Yagi et al., 2005). It thus appears that different cell fate specification strategies converge at the transcriptional level to activate genes encoding these cell-type specific transcription factors.

If the upstream mechanisms governing the expression of effector genes that drive cell-type fate specification are indeed under relatively little constraint, it would not be surprising if we observe with increasing frequency that a variety of cell-autonomous and cell signalling strategies are used in different species and even within individual embryos to generate cells of seemingly identical tissue type.

We thank A. Spagnuolo (*Ci-Titf*), Y. Satou and N. Satoh (*Ci-Noto1*), and Nori Satoh and colleagues (*Ciona* Gene Collection plates) for kindly providing material. We thank Andrea Pasini for helpful discussions and for suggesting the use of DAPT; Evelyn Houliston for her careful reading of the manuscript and many helpful suggestions; and the staff of the UMR7009 for their help and support. This work was funded by the Centre National de la Recherche Scientifique (CNRS), including ATIP and ATIP-plus grants; the Université Paris VI; the Association Française contre les Myopathies (AFM); the Association pour la Recherche sur le Cancer (ARC, grant no. 3885); and the Agence Nationale de la Recherche (ANR).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/15/2855/DC1

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