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Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in *Ciona* embryos

Vincent Picco, Clare Hudson and Hitoyoshi Yasuo*

Asymmetric cell divisions produce two sibling cells with distinct fates, providing an important means of generating cell diversity in developing embryos. Many examples of such cell divisions have been described, but so far only a limited number of the underlying mechanisms have been elucidated. Here, we have uncovered a novel mechanism controlling an asymmetric cell division in the ascidian embryo. This division produces one notochord and one neural precursor. Differential activation of extracellular-signal-regulated kinase (ERK) between the sibling cells determines their distinct fates, with ERK activation promoting notochord fate. We first demonstrate that the segregation of notochord and neural fates is an autonomous property of the mother cell and that the mother cell acquires this functional polarity via interactions with neighbouring ectoderm precursors. We show that these cellular interactions are mediated by the ephrin-Eph signalling system, previously implicated in controlling cell movement and adhesion. Disruption of contacts with the signalling cells or inhibition of the ephrin-Eph signal results in the symmetric division of the mother cell, generating two notochord precursors. Finally, we demonstrate that the ephrin-Eph signal acts via attenuation of ERK activation in the neural-fated daughter cell. We propose a model whereby directional ephrin-Eph signals functionally polarise the notochord/neural mother cell, leading to asymmetric modulation of the FGF-Ras-ERK pathway between the daughter cells and, thus, to their differential fate specification.

KEY WORDS: ephrin-Eph, ERK, Asymmetric cell division, *Ciona*, Notochord, Neural

INTRODUCTION

Embryogenesis is a process during which a variety of cell types are produced in a spatially and temporally coordinated manner. Certain cell divisions during embryogenesis, termed asymmetric cell divisions, result in the production of two daughter cells specified to adopt distinct developmental fates, thus contributing to the generation of cell diversity (Horvitz and Herskowitz, 1992; Knoblich, 2001; Roegiers and Jan, 2004; Betschinger and Knoblich, 2004). Although many such divisions have been described, the underlying molecular mechanisms are currently understood in only a few cases analysed in *Drosophila melanogaster* and *Caenorhabditis elegans*. For the fly sensory organ precursors (SOPs) to generate two distinct siblings, an asymmetrically distributed protein called Numb plays a crucial role by attenuating Notch signals in one of the daughter cells and thus creating differential transduction of the signalling pathway between the daughter cells (Rhyu et al., 1994; Guo et al., 1996). A different mechanism controls the asymmetric cell division of the *Drosophila* larval neuroblasts, in which the transcription factor Prospero is asymmetrically inherited between the daughter cells and acts as a binary switch between the self-renewal and differentiation fates adopted by the daughters (Hirata et al., 1995; Knoblich et al., 1995; Choksi et al., 2006). In both these cases, segregation of these factors in mother cells appears to take place intrinsically (Gho and Schweisguth, 1998; Siegrist and Doe, 2006). In another example, in *C. elegans*, during the cell division of an embryonic cell named EMS, extrinsic cues, provided by a Wnt ligand, govern the binary choice between endoderm and mesoderm fates of the resultant daughter cells (Thorpe et al., 1997; Rocheleau et al., 1997). This extrinsic and directional Wnt signal, acting on the mother cell, results in a differential nuclear accumulation of β-catenin between the daughter cells, and thus to their differential fate specification (Goldstein et al., 2006; Nakamura et al., 2005; Lin et al., 1998). These examples reveal that a variety of molecular strategies can be used to generate two distinct cell fates following an asymmetric cell division.

One model system that could be particularly amenable to the study of asymmetric cell division is the invertebrate chordate embryos of ascidians. Ascidian embryogenesis is characterised by a fixed cell-cleavage pattern and a small number of cells, which undergo fate restriction during the early cleavage stages. These characteristics have led to a complete documentation of cell lineages up to the onset of gastrulation (Nishida, 1987). The cell lineages have revealed that a number of cell divisions are coupled with the differential segregation of cell fates between the sibling cells. We are studying one such example, the generation of notochord and neural precursors, from two pairs of mother cells named A6.2 and A6.4, located in an anterior-marginal position of the 32-cell-stage embryo (Fig. 1). Following cleavage of these cells, the daughter cells that reside on the animal-pole side become neural precursors and give rise to a part of the larval central nervous system, whereas daughter cells arising on the vegetal-pole side become precursors of the larval notochord (Fig. 1). It has been shown that the fibroblast growth factor (FGF) pathway – involving the small GTPase Ras, MAPK/ERK kinase (MEK) and extracellular-signal-regulated kinase (ERK) – plays a crucial role during this differential fate specification (Nakatani and Nishida, 1997; Kim and Nishida, 2001). When FGF-ERK1/2 signals are inhibited, each mother cell generates two neural precursors, instead of one notochord and one neural precursor (Minokawa et al., 2001; Yasuo and Hudson, 2007). Conversely, if isolated mother cells are treated with exogenous FGF, both daughters adopt a notochord fate (Minokawa et al., 2001; Nakatani et al., 1996). Consistent with these results, activation of ERK1/2 is detected in notochord/neural mother cells but remains active only in the notochord precursors following cell division (Yasuo and Hudson, 2007). Thus, differential ERK activation

Developmental Biology Unit, Université Pierre et Marie Curie (Paris VI) and CNRS, Observatoire Océanologique, 06230 Villefranche sur mer, France.

*Author for correspondence (e-mail: yasuo@obs-vlfr.fr)

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between these daughter cells, which is the first known sign of an asymmetry between them, is the determinative event driving their binary cell fate choice. It has been proposed that the differential activation of ERK1/2, and thus differential fate specification of the two daughter cells, might be explained by a directional FGF signal coming from the vegetal region (neighbouring endoderm precursors) (Minokawa et al., 2001). However, transcripts of *FGF9/16/20*, encoding the FGF ligand responsible for ERK1/2 activation and early notochord induction, are detected widely in vegetal cells, including in the notochord/neural mother cells themselves, suggesting that it is unlikely to provide an asymmetric positional cue (Imai et al., 2002; Bertrand et al., 2003).

In this study, we first present evidence that the notochord/neural mother cell acquires, during its cell cycle, the autonomous capacity to divide asymmetrically and give rise to one notochord and one neural precursor. The acquisition of this functional polarity by the mother cell depends on cellular interactions between the mother cell and neighbouring epidermal precursors, positioned on the animal side. The molecular nature of this signal is a GPI-membrane-bound form of ephrin ligand, signalling via the Eph receptor (ephrin-Eph signalling). Disruption of contacts with the signalling cells or inhibition of the ephrin-Eph signals results in the symmetric division of the mother cell and the generation of two notochord precursors. Finally, we demonstrate that the ephrin-mediated signal acts to attenuate ERK activation in the neural precursor, and is thus responsible for creating the differential ERK activation between sibling cells.

MATERIALS AND METHODS

Embryo culture and manipulations of *Ciona intestinalis*

Adult *Ciona intestinalis* (*Ciona*) were purchased from the Roscoff Marine Biological Station (Roscoff, France). Embryo culture, microinjection of unfertilised eggs, UO126-treatment, and isolation and recombination of blastomeres have been described previously (Hudson et al., 2003; Hudson and Lemaire, 2001). All data presented in this study were collected from at least two independent experiments.

In situ hybridisation, immunohistochemistry and western blots

In situ hybridisation of *Ciona* embryos was carried out as described previously (Hudson and Yasuo, 2006). Dig-probes were synthesised from the following cDNA clones: *Ci-Bra* (Corbo et al., 1997); *Ci-ETR* (Hudson et al., 2003); *Ci-ephrin-Ad* (ciad008n17 from the Kyoto Gene Collection Plates) (Satou et al., 2002). For immunohistochemistry, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in artificial sea water for 30 minutes at room temperature, washed with PBS, 0.1% Triton X-100 (PBST), blocked in PBST, 5% goat serum, 1% Roche Blocking Reagent, and incubated overnight in anti-dpERK1/2 (Sigma, 1:1000) at 4°C. After washing in PBST, embryos were incubated overnight in anti-mouse-HRP (Molecular Probes, 1:100) at 4°C and then washed prior to signal detection, which was performed using TSA Plus Fluorescence Systems (PerkinElmer) with cyanine 5 as fluochrome. Western blot analyses were carried out following standard protocols with anti-dpERK1/2 (Cell Signaling Technology) and anti-rabbit-HRP (Jackson ImmunoResearch) used at a dilution of 1:350 and 1:3000, respectively. A total of 20 embryos dissolved in lysis buffer were used for each analysis.

Reagents

Ephrin-Ad-MO (5′-GGTAGTAGGTAAATTGAGTTGCCAT-3′) was purchased from GeneTools LLC and injected at a concentration of 0.5 mM. A dominant-negative form of Eph3 was constructed using a cDNA clone, cieg009e01, from the Kyoto Gene Collection Plates. cDNA fragments corresponding to the entire extracellular domain, transmembrane domain and a part of intracellular domain were PCR-amplified. For ephrin-Ad RNA, cDNA fragments corresponding to the entire ORF was PCR-amplified from ciad008n17. Amplified cDNA fragments were subcloned in pRN3 (Lemaire et al., 1995) and used to synthesise RNAs. dnEph3 and ephrin-Ad RNAs were injected at a concentration of 0.125 and 0.25 mg/ml, respectively.

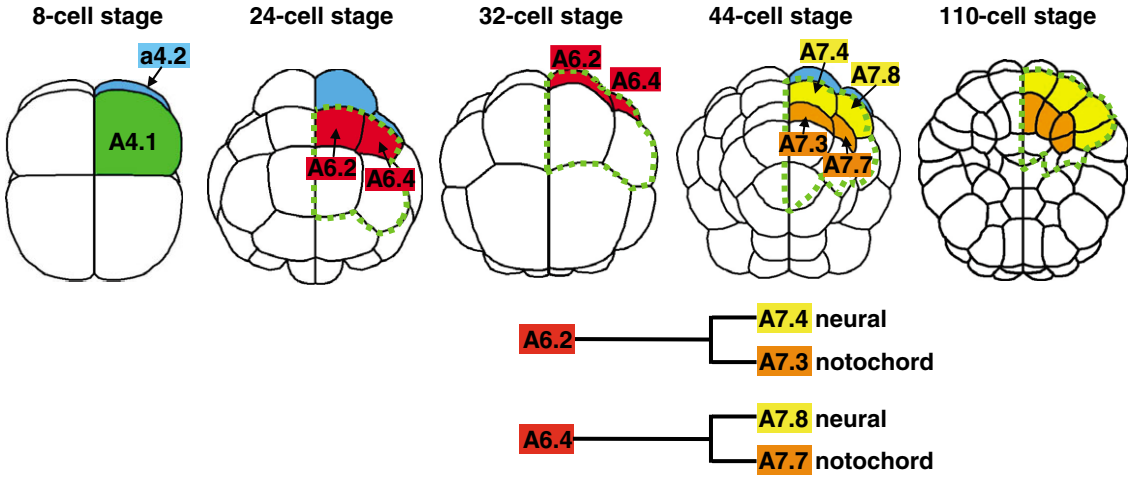

Fig. 1. A binary choice between notochord and neural fates in ascidian embryos. All embryos are viewed from the vegetal pole with the anterior side up. Because ascidian embryos are bilaterally symmetrical, pairs of blastomeres will be referred to by their blastomere name (e.g. A6.4), rather than as 'a pair of'. A4.1 (green), located in an anterior-vegetal position of the eight-cell-stage embryo, divides twice to generate four cells (outlined by green dotted line) in the 24-cell-stage embryo. These four cells consist of two mother cells of notochord/neural precursors, named A6.2 and A6.4 (red), one endoderm/mesenchyme mother cell and one endoderm precursor. From the 24- to 32-cell stages, no cell divisions take place in the A4.1-lineage. At the late 32-cell stage, the cells in the A4.1 lineage enter mitosis and each of the A6.2 and A6.4 mother cells divides along the anterior-posterior axis to give rise to one notochord (orange) and one neural (yellow) precursor at the 44-cell stage. Each of the precursors then divides in the medial-lateral direction to generate four notochord and four neural precursors in the 110-cell-stage embryo. Therefore, the A4.1 cell generates four notochord and four neural precursors at the 110-cell stage, whereby each of the A6.2 and A6.4 mother cells gives rise to two notochord and two neural precursors. The anterior-animal cell, a4.2, of the eight-cell-stage embryo, and its descendents, are coloured in blue.

RESULTS

Cell interactions functionally polarise the notochord/neural mother cell

Because the expression pattern of *FGF9/16/20* does not explain differential ERK1/2 activation between notochord and neural precursors (see above), we speculated that an inhibitory mechanism may exist to attenuate ERK1/2 activation in the neural precursors. To test for inhibitory influences from neighbouring cells, we isolated A4.1 blastomeres (green in Fig. 1) – the progenitors of notochord and neural precursors – from eight-cell-stage embryos, cultured them until control embryos reached the 110-cell stage and analysed them for the number of notochord and neural precursors (Fig. 2). The presence of notochord and neural precursors was monitored by expression of *Ci-Bra* (Corbo et al., 1997) and *Ci-ETR* (Hudson et al., 2003), respectively. The mean number of cells expressing *Ci-Bra* in the A4.1-derived partial embryos was significantly higher than that predicted from the cell lineage (6.4 versus 4; Fig. 1 and Fig. 2B,K). By contrast, *Ci-ETR*-positive cells rarely formed (average of 0.8 cells), despite the fact that the cell lineage predicts four neural precursors (Fig. 1 and Fig. 2G,K). Thus, when A4.1 progenitor cells are isolated, additional notochord precursors form at the expense of neural precursors, indicating that interactions with other cells are required for correct neural fate specification.

To address the timing of these cellular interactions, we performed similar isolation experiments on A6.2 and A6.4 mother cells at the start or at the end of their cell cycle. According to the cell lineage, each of these mother cells should generate two notochord and two neural precursors at the 110-cell stage (Fig. 1). When isolated shortly before their division (at the late 32-cell stage), these cells showed equivalent fate attribution, expressing *Ci-Bra* and *Ci-ETR* in an average of 2.0 and 1.8 cells, respectively (Fig. 2E,J,L and Fig. 1). By contrast, when they were isolated early in the cell cycle (at the 24-cell stage), the resultant partial embryos had an average of 3.2 cells positive for *Ci-Bra* and 1.3 cells positive for *Ci-ETR* (Fig. 2D,I,L). These results show that the cellular interactions required for the suppression of notochord fate and promotion of neural fate are still occurring at the 24-cell stage but that they have terminated before the A6.2 and A6.4 mother cells begin cytokinesis. Thus, the mother cells acquire the autonomous capacity to divide asymmetrically during their cell cycle. These results indicate, rather unexpectedly, that the notochord/neural fate decision is not dependent on differential signalling to the daughter cells but on the prior polarisation of the mother cell.

Interaction with anterior-animal cells is required to repress notochord fate and induce neural fate in A4.1 lineages

We hypothesised that the inhibitory signals implicated in suppressing notochord fate in the neural lineage may arise from the anterior-animal lineage derived from a4.2 (blue in Fig. 1), because these cells are in contact with the A6.2 and A6.4 mother cells on the side on which the neural precursors will form. Using the recently reported bioinformatics tool, 3D Virtual Embryo version 1.0 (Tassy et al., 2006), we identified contacts between animal cells and notochord-neural mother cells at the 24-cell stage. A6.2 contacts a5.3 with a contact area of 23.8% relative to the entire cell surface of A6.2, whereas the A6.4 blastomere contacts a5.3 (10.9%), a5.4 (16.4%) and b5.3 (15.5%) with a total area of contact of 42.8%. a5.3 and a5.4 cells originate from the a4.2 anterior-animal cell of the eight-cell-stage embryo. We demonstrated that animal cells are

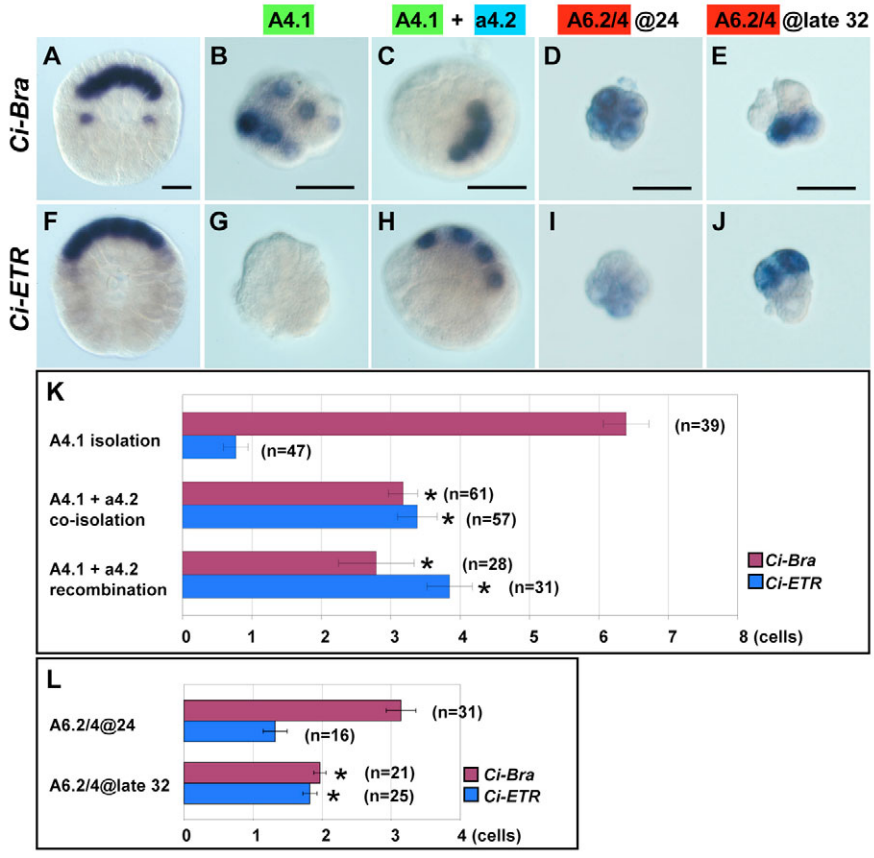

Fig. 2. Signals from the anterior-animal cells induce neural fate and suppress notochord fate. (**A-J**) Expression of a notochord-marker, *Ci-Bra* (A-E), and a neural marker, *Ci-ETR* (F-J), analysed at the 110-cell stage by in situ hybridisation in embryos and partial embryos derived from isolated cells, as indicated. (**K**,**L**) Histograms showing the average numbers of cells positive for *Ci-Bra* (magenta) and *Ci-ETR* (blue) in partial embryos as indicated on the left of the graphs. The data are expressed as mean±s.e.m. Significant differences compared to the results obtained by A4.1 isolation (for K) or by A6.2 or A6.4 isolation at the 24-cell stage (for L) were evaluated by the Student's *t*-test and indicated with asterisks ($P<0.01$). *n*, total number of samples analysed. Scale bars: 25 μm.

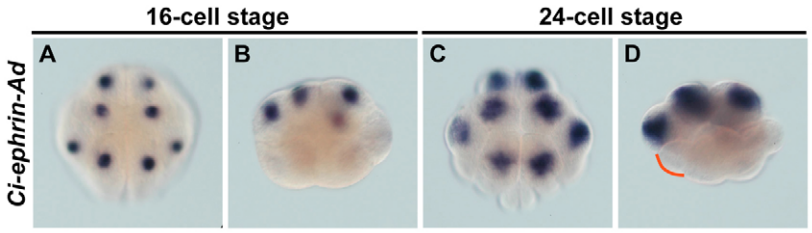

Fig. 3. Spatial expression-profile of *ephrin-Ad*. (**A-D**) The expression pattern of the *Ci-ephrin-Ad* gene is shown in animal-pole (A and C; anterior up) and lateral (B and D; anterior to the left) views at the stages indicated. The position of the A6.2 blastomere is indicated by a red line in D.

indeed the source of the notochord-inhibitory (neural inducing) signals by cell co-isolation and recombination experiments. In A4.1 cells co-isolated with a4.2 cells, the average number of *Ci-Bra*-positive cells at the equivalent of the 110-cell stage was reduced to 3.2 cells (Fig. 2C,K) compared with 6.4 cells in A4.1-derived partial embryos. Concomitantly, expression of *Ci-ETR* was recovered with an average of 3.4 positive cells compared with 0.8 cells in A4.1-derived partial embryos (Fig. 2H,K). Similar results were obtained when A4.1 and a4.2 cells were isolated separately and then recombined (Fig. 2K) or when A4.1 was recombined with b4.2 (mean of 3.1 cells positive for *Ci-ETR* expression, n=16), but not when A4.1 was recombined with the vegetal cells A4.1 or B4.1 (mean of 0.0 and 0.0 cells positive for *Ci-ETR* expression; n=15 and n=10, respectively). These results indicate that cell contact between mother cells and animal cells is required to promote neural fate and suppress notochord fate in the neural precursors.

Selective attenuation of ERK activation via ephrin-Ad in neural precursors accounts for the segregation of notochord and neural fates

We next sought to identify the molecular nature of the animal-derived signals. Candidate molecules are predicted to be expressed in the animal cells at around the 8- to 32-cell stages and to be secreted or membrane-bound ligands capable of modulating the FGF signalling pathway (see Introduction). The ephrin-Eph signalling molecules provide ideal candidates. Activation of Eph receptor tyrosine kinases (RTKs) has been shown to inhibit the Ras-ERK1/2 pathway in a range of cell lines (Elowe et al., 2001; Miao et al., 2001). In particular, it has been shown that activation of EphA leads to a rapid attenuation of ERK1/2 activity induced by other RTKs, including those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Miao et al., 2001). Eph receptors are activated by ephrin ligands. As ephrins are membrane-bound proteins, ephrin-Eph signals are mediated in a cell-contact-dependent manner. We first analysed the expression profiles, during early embryogenesis, of all five *ephrin* genes annotated in the *Ciona* genome (Satou et al., 2003). Among the profiles obtained, the only appropriate spatial and temporal expression was observed for *Ci-ephrin-Ad*, which encodes a GPI-anchored form of ephrin ligand. *Ci-ephrin-Ad* starts to be expressed in all animal cells at the 16-cell stage and its expression is maintained through the 24- and 32-cell stages (Fig. 3A-D).

To address the potential role of Ci-ephrin-Ad in directing the notochord-neural binary cell fate choice, we first overexpressed it by injecting in vitro synthesised RNA into *Ciona* eggs. Injected eggs were subsequently fertilised and cultured to the 64- and 110-cell stages for the analysis of *Ci-Bra* and *Ci-ETR* expression, respectively. Consistent with the idea that ephrin-Eph signals inhibit notochord fates, expression of *Ci-Bra* was blocked following Ci-ephrin-Ad injection (Fig. 4A-C). Furthermore, notochord precursors now expressed the neural marker *Ci-ETR*, suggesting that both of the daughter cells of A6.2 and A6.4 cells had adopted a neural fate (Fig. 4E,F,G). This effect of Ci-ephrin-Ad overexpression phenocopied that of the inhibition of FGF-MEK signals (Minokawa et al., 2001; Yasuo and Hudson, 2007), suggesting that activation of ephrin-Eph signals can inhibit the FGF-Ras-MEK-ERK pathway in ascidian embryonic cells. This was directly addressed by using an antibody recognising the activated, diphosphorylated form of ERK1/2, for which there is a single representative in the *Ciona* genome (Satou et al., 2003). Strikingly, activation of ERK1/2 in Ci-ephrin-Ad-injected embryos was blocked as efficiently as in sibling embryos treated with UO126, a pharmacological inhibitor for MEK (Favata et al., 1998) (Fig. 5A). Furthermore, immunohistochemistry on whole embryos showed that ERK1/2 activation was inhibited throughout the embryo following Ci-ephrin-Ad injection (Fig. 5C).

Ephrin-Eph signals are known to act bi-directionally; receptor signalling is referred to as forward signalling and ephrin-ligand signalling as reverse signalling (Cowan and Henkemeyer, 2002; Murai and Pasquale, 2003). We thus asked whether the effect of Ci-ephrin-Ad on ERK1/2 activity was mediated via forward or reverse signalling. Transcripts of two of the five *Ciona* Eph receptor genes,

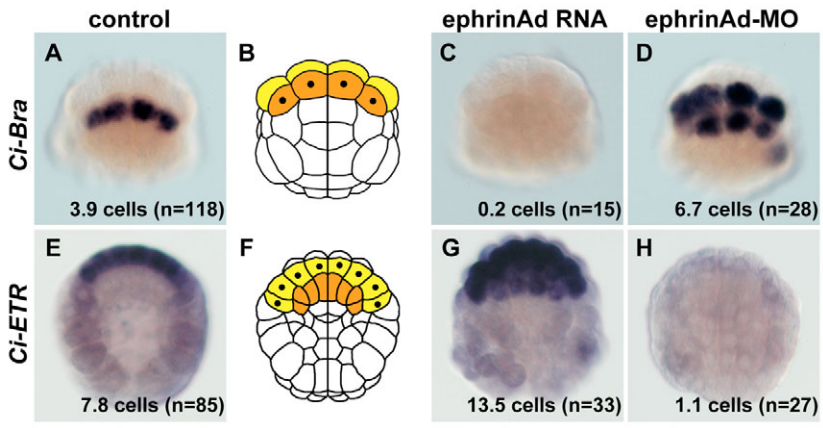

Fig. 4. Ephrin-Ad provides the notochord-suppressing signals from anterior-animal cells. Expression of *Ci-Bra* (**A-D**) and *Ci-ETR* (**E-H**) at the 64- and 110-cell stages, respectively, following the treatments indicated above the panels. The average number of cells positive for each marker gene is indicated in each panel, together with the total number of samples analysed. Embryos in A,C and D are slightly tilted in order to place the notochord and neural precursors in the same focal plane. Schematics in B and F represent vegetal-pole views of the 64- and 110-cell-stage embryos, respectively, with notochord in orange and neural cells in yellow. Black dots mark cells positive for *Ci-Bra* (in B) and *Ci-ETR* (in F) in the control embryo. dnEph3-RNA injection gave similar results to ephrin-Ad-MO injection (7.2 cells for *Ci-Bra*, n=34; 1.2 cells for *Ci-ETR*, n=22).

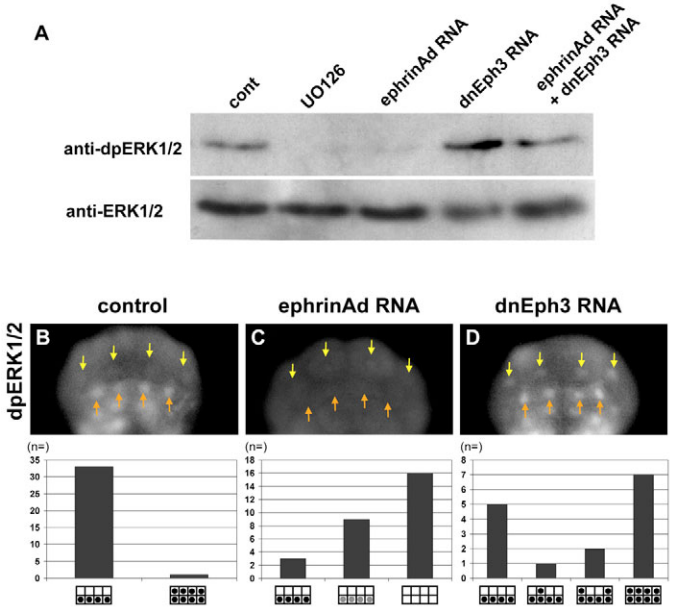

Fig. 5. Forward ephrin-Ad-Eph signals attenuate activation of ERK1/2. (**A**) Western blot analysis to detect activated ERK1/2 using anti-dpERK1/2 (top panel) in 44-cell-stage embryos following the treatment indicated. Lower panel shows the total amount of ERK1/2 protein, as a loading control. (**B-D**) Activation of ERK1/2 was detected by immunohistochemistry with the same antibodies in 44-cell-stage embryos following the treatments indicated above the panels. Orange arrows point to notochord precursors and yellow arrows to neural precursors. Histograms below each panel show the number of embryos obtained exhibiting the pattern schematised in the grid below. The grid represents the four neural (upper) and four notochord (lower) precursors. ERK activation is represented by a black dot, with very weak detection indicated by a grey dot.

namely *Ci-Eph2* and *Ci-Eph3*, showed a broad distribution during the early cleavage stages. We generated a dominant-negative form of Ci-Eph3 (dnEph3) by removing most of its intracellular domain. Co-injection of RNAs for dnEph3 and Ci-ephrin-Ad completely suppressed the ephrin-mediated inhibition of ERK1/2 activation (Fig. 5A), demonstrating that Ci-ephrin-Ad attenuates ERK1/2 activation via Eph receptors.

To address whether ephrin-Ad–Eph signalling is required in the embryo to suppress notochord fate and promote neural fate in neural precursors, we inhibited it by antisense morpholino (MO)-mediated knockdown of Ci-ephrin-Ad, and by the dominant-negative form of Eph3. In embryos injected with either ephrin-Ad-MO or dnEph3 RNA, expression of *Ci-ETR* was repressed, whereas *Ci-Bra* was expressed ectopically in the neural precursors (Fig. 4D,H). Thus, in the absence of ephrin-Ad–Eph signalling, the neural precursors transform into a notochord fate. Concomitantly, an ectopic activation of ERK1/2 was observed immunohistochemically in the neural lineages of dnEph3-injected embryos (Fig. 5D). These results show that ephrin-Ad signalling via Eph receptors is required to inhibit ERK, and thus to repress notochord fate and to induce neural fate in the neural precursors. Thus, ephrin-Ad is a strong candidate to be the animal-derived signal identified from our embryological studies.

Ephrin-Ad is required for the asymmetric cell division of isolated mother cells

The studies described above identified ephrin-Ad as a major factor controlling the binary fate choice of the notochord and neural precursors in the context of whole embryos, but did not reveal whether it does so by acting on the mother cell or on the neural-fated daughter cell. To address this issue, we isolated notochord/neural mother cells from uninjected or ephrin-Ad-MO-injected embryos at the late 32-cell stage, and monitored *Ci-Bra* and *Ci-ETR* expression at the equivalent of the early gastrula stage (Fig. 6A). Similar to the result shown in Fig. 2L, mother cells isolated from control late 32-cell-stage embryos developed into four-cell partial embryos expressing *Ci-Bra* and *Ci-ETR* in average of 1.6 and 1.9 cells, respectively (Fig. 6B). By contrast, the resultant partial embryos developed from mother cells isolated from ephrin-Ad-MO-injected embryos produced more notochord cells (2.9 cells) at the expense of neural cells (0.5 cells) (Fig. 6B). These results show that ephrin-Ad is indeed acting on the notochord/neural mother cells and is required for their autonomous capacity to generate two distinct daughter cells, with ephrin-Ad promoting the neural daughter cell fate.

DISCUSSION

Asymmetric cell divisions are intensively studied as a mechanism used to generate cell diversity during development, and also to control the self-renewal versus differentiation choice during stem-cell divisions. Two basic types of mechanisms can generate two daughter cells that adopt distinct cell fates. The first, generally referred to as asymmetric cell division, relies on the polarisation of the mother cell, which intrinsically gives rise to distinct daughter cells (Knoblich, 2001; Roegiers and Jan, 2004; Betschinger and Knoblich, 2004) (see also Horvitz and Herskowitz, 1992; Morrison and Kimble, 2006). The second type does not involve polarisation of the mother cell but involves the asymmetric placement of equivalent daughter cells relative to extrinsic instructive cues, thus resulting in their adoption of distinct fates (Horvitz and Herskowitz, 1992; Morrison and Kimble, 2006). We have shown that the cell division of the notochord/neural mother cell in *Ciona* embryos can be considered as truly ‘asymmetric’ because, in isolation, this cell is able to divide autonomously to generate two daughter cells with distinct fates. This asymmetric cell division is dependent on cellular interactions between the mother cell and adjacent animal cells, and requires ephrin-Eph signalling. We have found that Ci-ephrin-Ad, present in animal cells, signals via Eph receptors to the mother cells, which is required for them to divide into two distinct daughter cells, with ephrin-Ad promoting the neural daughter cell fate. Our study also demonstrates that the ephrin-Eph signal acts via attenuation of ERK activation in the neural precursor. Based on these findings, we propose the following model for the asymmetric cell division of notochord/neural mother cells. While broadly expressed FGF9/16/20 activates the Ras-MEK-ERK pathway in the mother cell, directional ephrin-Ad signals from the animal cells act in parallel to functionally polarise the mother cell. Local activation of the ephrin-Eph signal in the mother cell becomes manifest, following cell division, as the selective attenuation of ERK activation in the neural-fated daughter cell, thus resulting in the differential fate specification of the daughter cells as notochord (ERK on) and neural (ERK off) precursors.

It will be of great interest in future studies to unravel the precise molecular cascade acting downstream of ephrin-Ad-Eph signalling in order to understand how this activity becomes spatially restricted within the mother cell prior to its division, and how this leads to the differential attenuation of ERK activation between the daughter cells

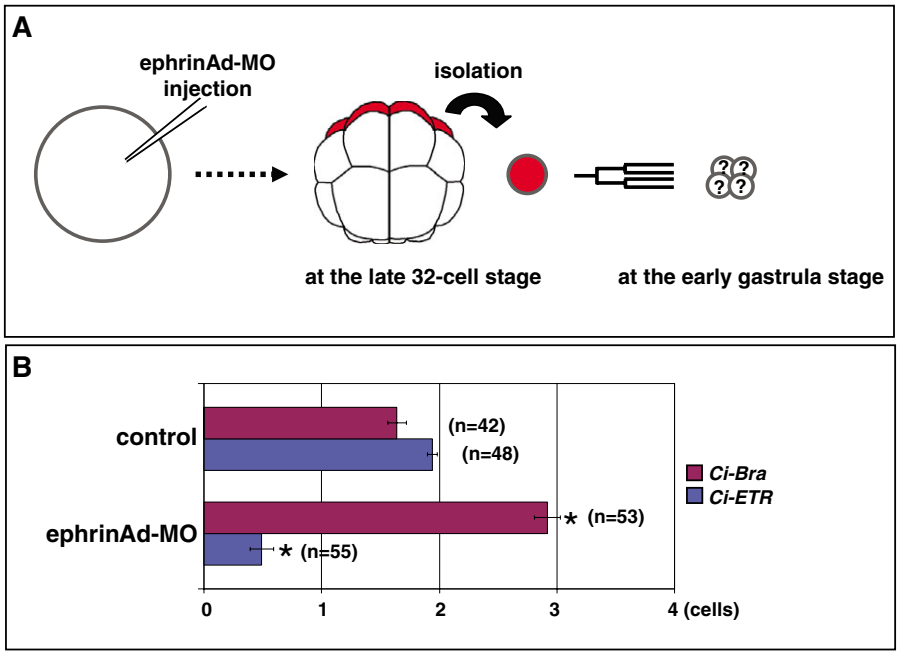

Fig. 6. Ephrin-Ad acts upon the notochord/neural mother cells. (**A**) Experimental strategy. Eggs were injected with ephrin-Ad-MO, fertilised and cultured until the late 32-cell stage, when the notochord/neural mother cells were isolated. The isolated mother cells were further cultured until the equivalent of the early gastrula stage, when they were fixed for in situ hybridisation analyses for the expression of *Ci-Bra* and *Ci-ETR*. At this stage, the resultant partial embryos consist of four cells. (**B**) Histograms showing the average numbers of cells positive for *Ci-Bra* (magenta) and *Ci-ETR* (blue) in partial embryos as indicated on the left of the graphs. The data are expressed as mean±s.e.m. Significant differences compared to the results obtained from the control were evaluated by the Student's *t*-test and indicated with asterisks ($P \leqslant 0.01$). *n*, total number of samples analysed.

following division. RTKs, including Eph, commonly signal through cytoplasmic proteins with SH2 domains, which bind either directly to phosphotyrosine sites on the activated receptor or to phosphorylated docking proteins. A variety of SH2 proteins have been identified as potential Eph receptor-binding partners (Hock et al., 1998; Holland et al., 1997). Among them is the p120-Ras GTPase-activating protein (p120-RasGAP), which is a negative regulator of the Ras small G protein (Hock et al., 1998; Holland et al., 1997) and has been shown to be required for EphB2- and EphA2-mediated attenuation of ERK1/2 in NG108 neuronal cell lines and mouse embryonic fibroblasts, respectively (Elowe et al., 2001; Tong et al., 2003). It is tempting to speculate that activation of ephrin-Ad-Eph signals results in the recruitment of p120-RasGAP to a sub-membrane domain on the animal-pole side of the A6.2 and A6.4 mother cells, and that the subsequent cytokinesis of the mother cells results in the partitioning of the RasGAP-enriched sub-membrane domain only to the future neural daughter cells.

During animal development, ephrin-Eph signals are well-known for their ability to control cell behaviours such as attraction/repulsion, adhesion/de-adhesion and migration in a range of developmental contexts (Poliakov et al., 2004). Our current study adds to the list a new role for ephrin-Eph signals – a role in cell fate specification coupled with an asymmetric cell division. The current study also provides compelling evidence that activation of the ephrin-Eph signalling pathway can result in attenuation of ERK1/2 activation in the context of embryonic development, in addition to the examples in cell lines and during *C. elegans* oocyte maturation (Elowe et al., 2001; Miao et al., 2001; Miller et al., 2003). RTK-mediated ERK1/2 activation is responsible for the control of a wide variety of developmental processes requiring spatial and temporal precision (Christen and Slack, 1999; Corson et al., 2003; Eblaghie et al., 2003; Gabay et al., 1997; Sawada et al., 2001; Shinya et al., 2001). Thus, it will be important to determine whether ephrin-Eph signalling acts more generally, in additional developmental contexts, as a potent local regulator of the RTK-ERK signalling pathway.

In conclusion, we have revealed a novel mechanism of asymmetric cell division based on ephrin-Eph signalling and selective ERK attenuation, broadening our knowledge of the diverse mechanisms used to control these specialised cell divisions. It will now be important to address whether ephrin-Eph signalling is playing similar roles in additional developmental and cellular contexts.

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References

Bertrand, V., Hudson, C., Caillol, D., Popovici, C. and Lemaire, P. (2003). Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. *Cell* **115**, 615-627.
Betschinger, J. and Knoblich, J. A. (2004). Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. *Curr. Biol.* **14**, R674-R685.
Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., de Wit, E., Fischer, B. E., van Steensel, B., Micklem, G. and Brand, A. H. (2006). Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. *Dev. Cell* **11**, 775-789.
Christen, B. and Slack, J. M. (1999). Spatial response to fibroblast growth factor signalling in Xenopus embryos. *Development* **126**, 119-125.
Corbo, J. C., Levine, M. and Zeller, R. W. (1997). Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, Ciona intestinalis. *Development* **124**, 589-602.
Corson, L. B., Yamanaka, Y., Lai, K. M. and Rossant, J. (2003). Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development* **130**, 4527-4537.
Cowan, C. A. and Henkemeyer, M. (2002). Ephrins in reverse, park and drive. *Trends Cell Biol.* **12**, 339-346.
Eblaghie, M. C., Lunn, J. S., Dickinson, R. J., Munsterberg, A. E., Sanz-Ezquerro, J. J., Farrell, E. R., Mathers, J., Keyse, S. M., Storey, K. and Tickle, C. (2003). Negative feedback regulation of FGF signaling levels by Pyst1/MKP3 in chick embryos. *Curr. Biol.* **13**, 1009-1018.
Elowe, S., Holland, S. J., Kulkarni, S. and Pawson, T. (2001). Downregulation of the Ras-mitogen-activated protein kinase pathway by the EphB2 receptor tyrosine kinase is required for ephrin-induced neurite retraction. *Mol. Cell. Biol.* **21**, 7429-7441.
Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273**, 18623-18632.
Gabay, L., Seger, R. and Shilo, B. Z. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. *Development* **124**, 3535-3541.

Gho, M. and Schweisguth, F. (1998). Frizzled signalling controls orientation of asymmetric sense organ precursor cell divisions in Drosophila. *Nature* **393**, 178-181.
Goldstein, B., Takeshita, H., Mizumoto, K. and Sawa, H. (2006). Wnt signals can function as positional cues in establishing cell polarity. *Dev. Cell* **10**, 391-396.
Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.
Hirata, J., Nakagoshi, H., Nabeshima, Y. and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. *Nature* **377**, 627-630.
Hock, B., Bohme, B., Karn, T., Feller, S., Rubsamen-Waigmann, H. and Strebhardt, K. (1998). Tyrosine-614, the major autophosphorylation site of the receptor tyrosine kinase HEK2, functions as multi-docking site for SH2-domain mediated interactions. *Oncogene* **17**, 255-260.
Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D. and Pawson, T. (1997). Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J.* **16**, 3877-3888.
Horvitz, H. R. and Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. *Cell* **68**, 237-255.
Hudson, C. and Lemaire, P. (2001). Induction of anterior neural fates in the ascidian Ciona intestinalis. *Mech. Dev.* **100**, 189-203.
Hudson, C. and Yasuo, H. (2006). A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in Ciona embryos. *Development* **133**, 2855-2864.
Hudson, C., Darras, S., Caillol, D., Yasuo, H. and Lemaire, P. (2003). A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian Ciona intestinalis. *Development* **130**, 147-159.
Imai, K. S., Satoh, N. and Satou, Y. (2002). Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in Ciona savignyi embryos. *Development* **129**, 1729-1738.
Kim, G. J. and Nishida, H. (2001). Role of the FGF and MEK signaling pathway in the ascidian embryo. *Dev. Growth Differ.* **43**, 521-533.
Knoblich, J. A. (2001). Asymmetric cell division during animal development. *Nat. Rev. Mol. Cell Biol.* **2**, 11-20.
Knoblich, J. A., Jan, L. Y. and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* **377**, 624-627.
Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.
Lin, R., Hill, R. J. and Priess, J. R. (1998). POP-1 and anterior-posterior fate decisions in C. elegans embryos. *Cell* **92**, 229-239.
Miao, H., Wei, B. R., Peehl, D. M., Li, Q., Alexandrou, T., Schelling, J. R., Rhim, J. S., Sedor, J. R., Burnett, E. and Wang, B. (2001). Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway. *Nat. Cell Biol*. **3**, 527-530.
Miller, M. A., Ruest, P. J., Kosinski, M., Hanks, S. K. and Greenstein, D. (2003). An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in Caenorhabditis elegans. *Genes Dev.* **17**, 187-200.
Minokawa, T., Yagi, K., Makabe, K. W. and Nishida, H. (2001). Binary specification of nerve cord and notochord cell fates in ascidian embryos. *Development* **128**, 2007-2017.
Morrison, S. J. and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441**, 1068-1074.
Murai, K. K. and Pasquale, E. B. (2003). 'Eph'ective signaling: forward, reverse and crosstalk. *J. Cell Sci.* **116**, 2823-2832.
Nakamura, K., Kim, S., Ishidate, T., Bei, Y., Pang, K., Shirayama, M., Trzepacz, C., Brownell, D. R. and Mello, C. C. (2005). Wnt signaling drives WRM-1/beta-catenin asymmetries in early C. elegans embryos. *Genes Dev.* **19**, 1749-1754.
Nakatani, Y. and Nishida, H. (1997). Ras is an essential component for notochord formation during ascidian embryogenesis. *Mech. Dev*. **68**, 81-89.
Nakatani, Y., Yasuo, H., Satoh, N. and Nishida, H. (1996). Basic fibroblast growth factor induces notochord formation and the expression of As-T, a Brachyury homolog, during ascidian embryogenesis. *Development* **122**, 2023-2031.
Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121**, 526-541.
Poliakov, A., Cotrina, M. and Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev. Cell* **7**, 465-480.
Rhyu, M. S., Jan, L. Y. and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* **76**, 477-491.
Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell* **90**, 707-716.
Roegiers, F. and Jan, Y. N. (2004). Asymmetric cell division. *Curr. Opin. Cell Biol.* **16**, 195-205.
Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y. et al. (2002). A cDNA resource from the basal chordate Ciona intestinalis. *Genesis* **33**, 153-154.
Satou, Y., Sasakura, Y., Yamada, L., Imai, K. S., Satoh, N. and Degnan, B. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. V. Genes for receptor tyrosine kinase pathway and Notch signaling pathway. *Dev. Genes Evol.* **213**, 254-263.
Sawada, A., Shinya, M., Jiang, Y. J., Kawakami, A., Kuroiwa, A. and Takeda, H. (2001). Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. *Development* **128**, 4873-4880.
Shinya, M., Koshida, S., Sawada, A., Kuroiwa, A. and Takeda, H. (2001). Fgf signalling through MAPK cascade is required for development of the subpallial telencephalon in zebrafish embryos. *Development* **128**, 4153-4164.
Siegrist, S. E. and Doe, C. Q. (2006). Extrinsic cues orient the cell division axis in Drosophila embryonic neuroblasts. *Development* **133**, 529-536.
Tassy, O., Daian, F., Hudson, C., Bertrand, V. and Lemaire, P. (2006). A quantitative approach to the study of cell shapes and interactions during early chordate embryogenesis. *Curr. Biol.* **16**, 345-358.
Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.
Tong, J., Elowe, S., Nash, P. and Pawson, T. (2003). Manipulation of EphB2 regulatory motifs and SH2 binding sites switches MAPK signaling and biological activity. *J. Biol. Chem.* **278**, 6111-6119.
Yasuo, H. and Hudson, C. (2007). FGF8/17/18 functions together with FGF9/16/20 during formation of the notochord in Ciona embryos. *Dev. Biol.* **302**, 92-103.