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Divergent mechanisms specify chordate motoneurons: evidence from ascidians

Clare Hudson[1,2,*], Moly Ba[1,2], Christian Rouvière[1,2] and Hitoyoshi Yasuo[1,2,*]

SUMMARY
Ascidians are members of the vertebrate sister group Urochordata. Their larvae exhibit a chordate body plan, which forms by a highly accelerated embryonic strategy involving a fixed cell lineage and small cell numbers. We report a detailed analysis of the specification of three of the five pairs of motoneurons in the ascidian *Ciona intestinalis* and show that despite well-conserved gene expression patterns and embryological outcomes compared with vertebrates, key signalling molecules have adopted different roles. We employed a combination of cell ablation and gene manipulation to analyse the function of two signalling molecules with key roles in vertebrate motoneuron specification that are known to be expressed equivalently in ascidians: the inducer Sonic hedgehog, produced ventrally by the notochord and floorplate; and the inhibitory BMP2/4, produced on the lateral/dorsal side of the neural plate. Our surprising conclusion is that neither BMP2/4 signalling nor the ventral cell lineages expressing hedgehog play crucial roles in motoneuron formation in *Ciona*. Furthermore, BMP2/4 overexpression induced ectopic motoneurons, the opposite of its vertebrate role. We suggest that the specification of motoneurons has been modified during ascidian evolution, such that BMP2/4 has adopted a redundant inductive role rather than a repressive role and Nodal, expressed upstream of BMP2/4 in the dorsal neural tube precursors, acts as a motoneuron inducer during normal development. Thus, our results uncover significant differences in the mechanisms used for motoneuron specification within chordates and also highlight the dangers of interpreting equivalent expression patterns as indicative of conserved function in evo-devo studies.

KEY WORDS: Motoneuron, Ascidian, Evolution, Spinal cord, BMP, Shh, Nodal, Midline, Dorsal ventral pattern

INTRODUCTION
One of the major mechanisms for generating diversity during evolution is thought to involve changes in the regulation of the genes controlling embryonic development (Duboule and Wilkins, 1998; Davidson and Erwin, 2006). According to this paradigm, conservation of the expression profile of developmental regulatory genes between species should pinpoint areas of evolutionary stability. Despite an evolutionary distance of over 500 million years (Shu et al., 2001), the tadpole larvae of ascidians has a body plan remarkably similar to that of vertebrate embryos, suggesting a degree of conservation in the developmental mechanisms used to generate this body plan. It is, thus, not surprising that some studies have revealed highly similar expression territories for developmental regulatory genes, acting in equivalent steps during embryogenesis [e.g. brachyury in notochord formation (Yasuo and Satoh, 1998) and MyoD for muscle formation (Meedel et al., 2007)]. There is increasing evidence, however, that equivalent morphological features can arise by distinct developmental routes, a phenomenon termed 'developmental systems drift' (True and Haag, 2001). For example, among nematodes, the formation of the vulva involves a surprisingly diverse array of developmental strategies to produce an equivalent structure (Kiontke et al., 2007). Similarly, interactions between equivalent cells, but using different signalling molecules, are involved in the fate determination of a particular muscle precursor in two different species of ascidian (Hudson et al., 2007; Hudson and Yasuo, 2008; Tokuoka et al., 2007). This means that during evolution, developmental mechanisms can be 'rewired' to produce a conserved phenotypic outcome.

Recently, a systematic large-scale comparison of whole-mount in situ hybridisation patterns of *Ciona intestinalis* and the zebrafish found an unexpectedly high level of divergence in the gene expression profiles between these two chordate species (Sobral et al., 2009), suggesting that the use of different developmental mechanisms to produce a similar morphological output may be surprisingly common. Interestingly, this comparison revealed that conservation of orthologous gene expression between the two species is highest in tissues involved in locomotion, including muscle, notochord and central nervous system. Here, we examine the mechanisms used to generate three out of the five pairs of somatic motoneurons in the ascidian *Ciona intestinalis* (hereafter referred to as *Ciona*) and compare them with the mechanisms used in vertebrates.

One of the hallmarks of the chordate body plan is the presence of a dorsal hollow central nervous system, which contains distinct types of neurons along the anteroposterior and dorsoventral axis. In vertebrates, the somatic motoneurons form at a precise position in the ventral spinal cord, close to the ventral midline (Fig. 1D) (Briscoe and Ericson, 2001).

A number of signalling pathways have been shown to be crucial for the formation of motoneurons in vertebrates. Sonic hedgehog (*Shh*) is expressed in the underlying notochord and in the ventral midline of the neural tube, the floor plate, and promotes motoneuron fate (Briscoe and Ericson, 2001; Ulloa and Briscoe, 2007; Wilson and Maden, 2005). Retinoids from the lateral somites are also required for motoneuron formation (Wilson and Maden, 2005). BMP and Wnt signals from the overlying ectoderm and roof plate, the dorsal-most structure of the spinal cord, are required for

[1]UPMC University of Paris 06, UMR7009, Developmental Biology Unit, Observatoire Océanologique de Villefranche-sur-mer, BP28, 06230, Villefranche-sur-mer, France. [2]CNRS, UMR7009, Developmental Biology Unit, Observatoire Océanologique de Villefranche-sur-mer, 06230, BP28, Villefranche-sur-mer, France.

*Authors for correspondence (hudson@obs-vlfr.fr; yasuo@obs-vlfr.fr)

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the formation of the dorsal spinal cord and repress formation of the ventral spinal cord, including motoneurons (Lewis and Eisen, 2003; Liu and Niswander, 2005). Antagonists of BMP signalling expressed in the axial and paraxial mesoderm, and in the dorsal spinal cord itself (roof plate) restrict the activity of BMP dorsally (McMahon et al., 1998; Mekki-Dauriac et al., 2002; Liem et al., 2000; Jessell, 2000; Liu and Niswander, 2005). Thus, BMP and Shh signals act broadly across the neural tube in a mutually restrictive fashion with BMP limiting the range of Shh activity and vice versa to establish the dorsal-ventral pattern of the vertebrate spinal cord.

Ascidian larvae have a chordate body plan of central notochord, dorsal neural tube and lateral muscles in miniature, consisting of only about 3000 cells (Lemaire et al., 2008). Ascidian development proceeds with a fixed cell cleavage pattern enabling the cell lineages to be described (Conklin, 1905; Nishida, 1987). In *Ciona*, the motoneurons arise from the A8.15 lineage of the early gastrula stage embryo or column 3 of the neural plate (Fig. 1A) (Cole and Meinertzhagen, 2004). By hatching stage, the five pairs of motoneurons along the anteroposterior axis are A12.239, A13.474, A11.118, A11.117 and A10.57. These motoneurons finally reside in a ventrolateral position of the larval trunk ganglion, considered to be the equivalent of the vertebrate spinal cord, and project axons that terminate in characteristic terminal endplates on the larval muscle (Fig. 1A-C) (Cole and Meinertzhagen, 2004; Dufour et al., 2006; Imai and Meinertzhagen, 2007; Horie et al., 2010). Thus, the position of the motoneurons within the neural tube and in relation to other embryonic tissues such as notochord and muscle in *Ciona* is equivalent to that in vertebrates (Fig. 1B-D). However, unlike in vertebrates, the tiny size of the ascidian trunk ganglion means that the motoneurons are in close proximity to both the dorsal and ventral cells of the neural tube. Moreover, motoneurons are initially formed in the lateral neural tube directly in between the dorsal and ventral cells, before shifting into their final ventrolateral position of the thickened trunk ganglion (Cole and Meinertzhagen, 2004).

In *Ciona*, *hh-2*, a homologue of vertebrate hedgehog, is expressed in the ventral-most cells of the neural tube, the positional equivalent of the vertebrate floor plate (Takatori et al., 2002), while *BMP2/4* expression has been reported in the lateral neural plate (future dorsal and lateral neural tube) in another ascidian species [*Halocynthia roretzi* (Miya et al., 1997)]. The conserved expression pattern of these molecules strongly suggests that their crucial role in spinal cord patterning has been conserved during evolution of the chordate lineage. The aim of this study was to investigate this possibility.

MATERIALS AND METHODS

Embryo experiments

Adult *Ciona intestinalis* were purchased from the Roscoff Marine Biological Station (France). Blastomere names, lineage and fate mapping are as described in previous studies (Conklin, 1905; Cole and Meinertzhagen, 2004; Nicol and Meinertzhagen, 1988a; Nicol and Meinertzhagen, 1988b; Nishida, 1987). Embryo culture, ablation, DiI labelling, microinjection, Nodal-morpholino, Smad1/5-morpholino (at 0.5 mM) and SB431542 treatment have been described previously (Hudson et al., 2003; Hudson and Yasuo, 2005; Pasini et al., 2006; Sardet et al., 2011). *Xenopus chordin* mRNA was injected into unfertilised eggs at a concentration of 0.6 μg/μl (Sasai et al., 1994). pSPE3-Chordin vector for RNA synthesis was made by taking the *Ciona Chordin* full-length construct (pENTR-Chordin, from the Unigene collection plates, purchased from Cogenics) (U. Rothbächer, M. J. Gilchrist and P. Lemaire et al., unpublished) and cloning into pSPE3 by Gateway LR reaction (Invitrogen) (Roure et al., 2007). *Ciona Chordin* RNA was injected at concentrations of 0.5 to 0.75 μg/μl.

Electroporation was carried out as described previously with modifications (Corbo et al., 1997; Bertrand et al., 2003). FOG::Nodal, FOG::Noggin and FOG::BMP2/4 and pvAChTP::EGFP constructs have been previously described (Pasini et al., 2006; Yoshida et al., 2004). FOG::Chordin was constructed by Gateway cloning pENTR-Chordin into pFOG::RfA (Pasini et al., 2006). A DNA solution (50 μg in 250 μl) was used in all experiments using FOG::Noggin, FOG::BMP2/4 and FOG::Nodal, except those in Fig. 5B and Fig. 7D where 25 μg of each plasmid was used to give a maximum total of 50 μg of DNA per electroporation. FOG::Chordin was toxic to embryos at high concentrations, so 15 to 25 μg of DNA per electroporation was used. Embryos were selected for normal development at the gastrula stage. Problems with development prior to and during gastrulation were not observed following injection of *Ci-Chordin* mRNA into eggs and therefore were most probably non-specific effects. For all experiments, data were pooled from at least two independent experiments.

In situ hybridisation and probes

Except for *BMP2/4*, all expression patterns have been described previously (Hudson et al., 2003; Hudson et al., 2007; Hudson and Yasuo, 2005; Ikuta and Saiga, 2007; Imai et al., 2004; Imai et al., 2006; Shi et al., 2009; Takamura et al., 2002) (http://ghost.zool.kyoto-u.ac.jp). In situ hybridisation was carried out as described previously (Hudson and Yasuo, 2006; Wada et al., 1995). For fluorescent in situ hybridisation, the first 2 days of the protocol was the same as above, after which the protocol described by Takatori et al. was used and adapted for double fluorescent in situ hybridisation (Takatori et al., 2010). Embryos were mounted in Vectashield DAPI (Vector Laboratories).

Confocal analysis and statistics

Confocal analysis was performed with a Leica SP5 microscope and images were processed using Image J software. For the comparative expression analyses of motoneuron markers (Fig. 1; see Fig. S1 in the supplementary material), all in situ hybridisations were carried out on the same batch of embryos. For ablation experiments in Fig. 3, one experiment gave no staining in notochord-ablated embryos. As this was observed only once in six independent notochord-ablation experiments, this entire data set was removed from the analysis. Sometimes embryos displayed more than three motoneurons on one side and fewer than three on the other side. In order to represent these cases and to distinguish, for example, embryos displaying six cells on one side and none on the other from those with three on each side, we decided to consider each half of the embryo independently for our analyses. The level of staining was not taken into consideration during cell counts, rather the number of nuclei with detectable levels of signal. For statistical analysis, Microsoft Excel, Excel Box-and-whiskers plots V2 (http://www.coventry.ac.uk/ec//~nhunt/boxplot.htm) and Mann-Whitney test V5 (Excel) (Siegel and Castellan, 1988) software were used.

RESULTS

Three motoneuron pairs defined by expression of *Ciona vAChTP*, *ChAT* and *Mnx*

In order to carry out an analysis of motoneuron formation, we chose three markers: *Ciona vAChTP*, which encodes a vesicular acetylcholine transporter; *Ciona ChAT*, which encodes choline acetlytransferase; and *Ciona Mnx*, an orthologue of vertebrate somatic motoneuron markers *Hb9* and *Mnr2* (Takamura et al., 2002; Wada et al., 2003; Tanabe et al., 1998; Thaler et al., 1999; Ikuta and Saiga, 2007). Comparing fluorescent in situ hybridisation analysis with neural maps previously published (Cole and Meinertzhagen, 2004), we confirmed expression of these markers in the motoneurons (Fig. 1B,C,E,F; see Fig. S1 in the supplementary material). At the late tail bud stage (13 hours of development), all three markers were co-expressed in three of the five pairs of motoneurons: A10.57, A11.117 and A11.118. Expression of *Mnx* was weak in A11.117 and this gene was also detected in cells of the tail neural tube, probably the descendents

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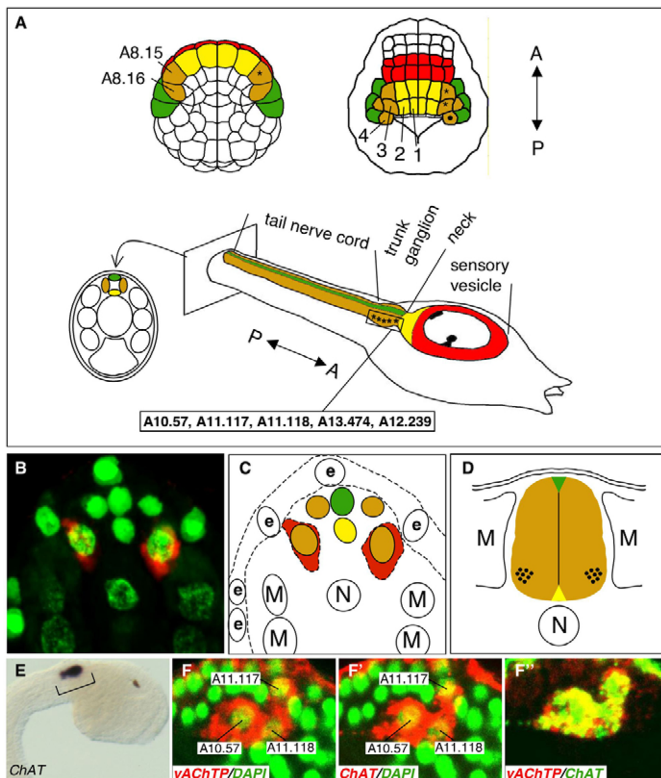

Fig. 1. The larval central nervous system of *Ciona intestinalis*. (**A**) Cell lineages of the ascidian larval central nervous system traced back to the early gastrula. The embryo is bilaterally symmetrical and all labelling refers to both sides. Nomenclature refers to the four founder lineages identified at the eight-cell stage, A- and B-lines vegetally and a- and b-lines animally. The cell lineages contributing to the central nervous system are coloured as follows: a-line in red (anterior sensory vesicle precursors); b-line in green (dorsal neural tube precursors); A-line in yellow (for posterior sensory vesicle and ventral midline precursors) or tan (for lateral neural tube precursors). The names of the lateral neural precursors are labelled on the gastrula fate map and the four different columns on the neural plate map. Asterisks mark the lineage from which the motoneurons arise (column 3) on the right side of the embryo drawings. A dot marks the muscle precursor that arises from the neural plate. On the larval stage drawing, asterisks mark the position of the motoneurons, with their cell names. During development, the A11.118 cell rotates ventrally and posteriorly around A11.117 (Cole and Meinertzhagen, 2004). (**B**) Confocal stack showing a transverse section of the trunk ganglion of a 13-hour *Ciona* embryo (at 19°C) following fluorescent in situ hybridisation with *vAChTP* probe and DAPI staining, at the level of the A10.57 motoneuron. (**C**) Schematic representation of B. Labelling as follows: neural tube nuclei are coloured with the same scheme as in A; the cell boundary of A10.57, estimated by the in situ hybridisation signal, is marked by a broken line and coloured red; broken lines indicate estimated boundaries of the epidermis. e, epidermis nuclei; N, notochord nucleus; M, muscle nuclei. (**D**) Schematic representation of the vertebrate spinal cord (tan) showing motoneurons as black dots, roof plate in green and floor plate in yellow. M, muscle (somite); N, notochord. (**E**) In situ hybridisation with *ChAT* probe at 13 hours of development (19°C). The bracket indicates the approximate region shown in the confocal image stacks in F. (**F-F″**) Confocal image stacks of a lateral view of an embryo following double fluorescent in situ hybridisation and DAPI staining, as indicated in the panels. Cell nuclei of motoneurons are labelled.

of A12.230 (not shown). Expression in the anterior two motoneurons A12.239 and A13.474, which form later than the three posterior motoneurons, probably starts later in development; however, at later stages of development non-specific staining of the tunic obscured the analysis.

Thus, *vAChTP*, *ChAT* and *Mnx* are expressed in three of the five pairs of motoneurons and these markers were used to investigate motoneuron formation in *Ciona* embryos. We will refer to these three pairs of motoneurons as *vAChTP*$^+$ motoneurons.

Ascidian motoneuron specification in the absence of ventral midline structures

In vertebrates, signals from the midline tissues, the notochord and the neural tube floorplate play a pivotal role in spinal cord patterning, a patterning process beginning as early as gastrula stages (Beattie et al., 1997; Placzek et al., 1991; Yamada et al., 1991; Yamada et al., 1993). These midline tissues express both *Shh* (which is required for formation of ventral spinal cord cell types, including motoneurons) and BMP antagonists (which act to protect the ventral neural tube from dorsalising BMP signals) (McMahon et al., 1998; Jessell, 2000; Lewis and Eisen, 2001). Correspondingly, *hh-2* in *Ciona* is expressed in the ventralmost cells of the tail nerve cord (Takatori et al., 2002), while the BMP antagonist *Chordin* is expressed in the notochord (and lateral neural tube) from gastrula to tailbud stages (Imai et al., 2004). In order to test the requirement for midline signals in motoneuron formation in *Ciona* embryos, we performed cell ablations at the 64-cell stage, prior to gastrulation, to prevent formation of notochord and/or 'floorplate' tissues (Fig. 2A, Fig. 3). We monitored motoneuron specification using *ChAT* probe for conventional in situ hybridisation and *vAChTP* for fluorescent in situ hybridisation. We first prevented formation of the ascidian 'floor plate', the ventral row of cells in the tail nerve cord and trunk ganglion, by ablating the A7.4 pair of cells (removing all the yellow cells in Fig. 1A) (Cole and Meinertzhagen, 2004; Nishida, 1987; Taniguchi and Nishida, 2004). Ablation of these cells had no effect on *ChAT* expression. We noticed, however, that ventral neural tissue was not entirely absent in these embryos, as assayed by *Ciona FGF3* expression (Shi et al., 2009), with a small domain of expression persisting in the anterior part of the central nervous system (Fig. 2A). This remaining ventral tissue of the neural tube derives from the a7.10 cells of the 64-cell stage embryo (Cole and Meinertzhagen, 2004). Removal of a7.10 together with A7.4 ('FP-ablated') resulted in a complete loss of *FGF3* expression, but *ChAT* expression still persisted. We quantified the effect of ablations by counting the number of *vAChTP*$^+$ cells following fluorescent in situ hybridisation (Fig. 3), analysing each half of the embryo independently. In 65% of cases the normal number of three motoneurons could be detected per embryo half. The remaining embryo halves had 1 to 2 motoneurons, to give an average of 2.5 motoneurons each side. Thus, we conclude that the ascidian 'floor plate' cells are not essential for the formation of *vAChTP*$^+$ motoneurons.

We next addressed whether the axial mesoderm plays a role in formation of motoneurons. *ChAT* expression was not affected following ablation of the four anterior notochord precursors ('N-ablation') (Fig. 2A). Confocal analysis revealed an average of 2.3 *vAChTP*$^+$ cells each side, with 39% of embryo halves displaying the correct number of motoneurons (Fig. 3). When we ablated both primary (A-line) and secondary (B-line) notochord lineages ('allN ablation'), which also prevents development of some mesenchymal cell fates, embryo halves had an average of 2.4 motoneurons and

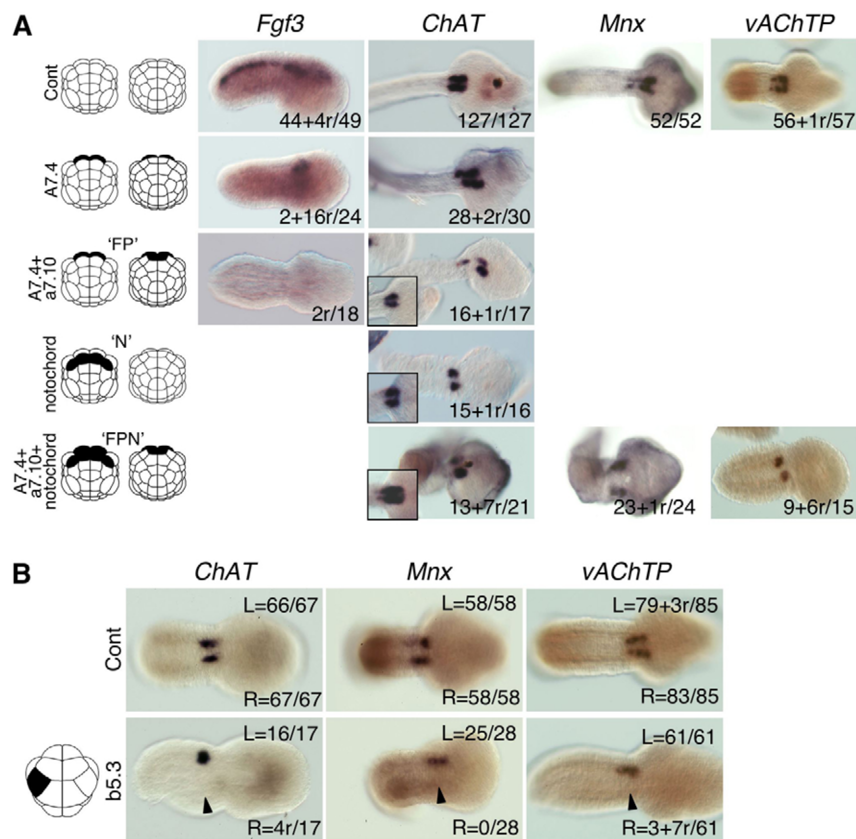

Fig. 2. Specification of motoneuron fate in the absence of midline structures. (**A**) Schematic drawings of 64-cell stage embryos show the ablated blastomeres in black (vegetal pole views in left column and animal pole views in right column). The ablated tissue or cell name is indicated on the left of the drawings, summarised as 'FP' (floor plate), 'N' (notochord) and 'FPN' (floorplate plus notochord). The marker analysed is indicated above the columns. For *ChAT*, the control shown is from the A7.4 ablation experiment. As the size of the expression domain of *ChAT* varies between batches, an inset showing the control expression of the corresponding batch of embryos is presented in each experimental panel. Numbers indicate the number of embryos showing expression over the total number of embryos analysed. 'r' indicates severely reduced expression or expression on only one side of the embryo. (**B**) The ablated cell is indicated in the schematic of the 16-cell stage embryo (animal pole view) and the marker analysed above the panels. Expression on the left-hand side (L) or right-hand side (R) is indicated at the top and bottom of each panel, respectively. The ablated side is indicated by arrowheads. Expression of low levels of *ChAT* and *vAChTP* on the ablated side may be due to the recovery of lateral Nodal expression that is seen occasionally following ablation (Hudson and Yasuo, 2006).

71% displayed the correct number of three (Fig. 3). These results show that the majority of motoneurons form following notochord ablation and suggest that the notochord is not crucial for *vAChTP*$^+$ motoneuron formation in *Ciona* embryos.

Similar results were found following FP+N-ablation, with an average of 2.4 cells expressing *vAChTP* each side and 28% of embryos displaying the correct number of motoneurons (Fig. 3). Furthermore, expression of all three motoneuron markers was detected in N+FP ablated embryos by conventional in situ hybridisation (Fig. 2A). The reduction and increase in variability in the number of motoneurons formed on each side of the embryo following ablations may indicate some role for midline structures, particularly the notochord, in controlling the precise number of motoneurons formed. Alternatively, it might be a non-specific consequence of the general morphological disorganisation caused by the ablation of these structures. The fact that motoneurons do form in most cases indicates strongly, however, that there is no absolute requirement for either the floorplate, the notochord or a combination of these tissues for the specification of *vAChTP*$^+$ motoneurons in *Ciona* embryos. This is clearly different to the situation in vertebrates where these tissues are essential for the formation of somatic motoneurons.

Expression of *Ciona BMP2/4* and *Chordin* in the dorsal/lateral neural tube, downstream of Nodal

In vertebrates, dorsally derived TGFβ family signals play a prominent role in specifying cell fates in the dorsal part of the neural tube, in particular, members of the BMP family (Lee and Jessell, 1999). In *Ciona* embryos, a TGFβ ligand from a distinct class, *Nodal*, is expressed lateral to the neural plate precursors and is required for expression of the motoneuron markers *Mnx* and *ChAT* (Hudson and Yasuo, 2005). Consistently, ablation of b5.3, the mother cell of the *Nodal*-expressing b6.5 cell, resulted in loss of motoneuron markers on the ablated side (Fig. 2B). We hypothesised that Nodal may act via BMPs in ascidians as *BMP2/4* expression has been reported in the lateral neural plate precursors of *Halocynthia* embryos and promotes motoneuron fate (Miya et al., 1997; Katsuyama et al., 2005). We confirmed that the one *BMP2/4* orthologue in the *Ciona* genome (Hino et al., 2003) was also expressed laterally in the neural plate (Fig. 4A). In gastrula embryos, expression was detected in the lateral A-line neural precursors A8.15 (progenitor of all the larval motoneurons) and A8.16, as well as b-line (b8.19) neural precursors and some B-line muscle precursors (Fig. 4A). By neural plate stages, *BMP2/4* expression was detected in lateral b-line cells bordering the neural plate and a9.49 (Fig. 4A). Expression of *BMP2/4*, initially variable and often patchy (see Fig. 4B, control), became robust by this stage. By the time the neural plate consists of six rows of cells, *BMP2/4* expression was restricted to b9.37, b9.38 and the ventral epidermis precursors (Fig. 4A and data not shown). During neurula stages, expression was restricted to the ventral epidermis (Imai et al., 2004).

We confirmed that *BMP2/4* expression in the lateral neural plate precursors is downstream of Nodal by using a pharmacological Nodal type I receptor (ALK4/5/7) inhibitor (SB431542), by injection of Nodal-morpholino and by electroporating the FOG::Nodal overexpression construct, which drives expression throughout the animal cells from the 16-cell stage (Inman et al., 2002; Hudson and Yasuo, 2005; Pasini et al., 2006). Both methods used to inhibit Nodal signalling resulted in loss of *BMP2/4* expression in the A- and b-line cells (Fig. 4B). Overexpression of Nodal resulted in ectopic expression of *BMP2/4* throughout the A-

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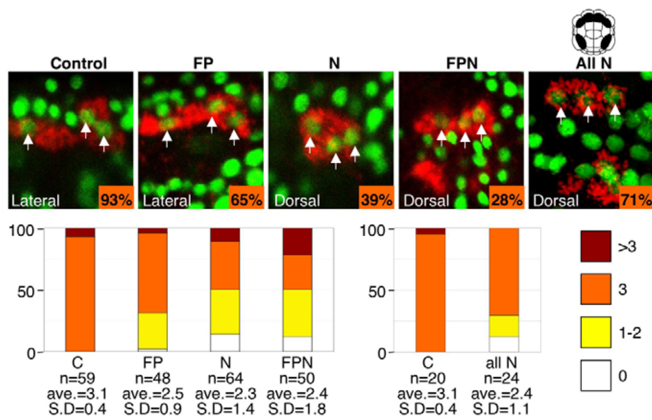

Fig. 3. *vAChTP*⁺ cell counts following cell ablation. Images show confocal image stacks following *vAChTP* in situ hybridisation and DAPI staining. Arrows indicate cell nuclei of *vAChTP*⁺ cells. Position of the embryo is indicated, anterior towards the right. Graphs show the percentage of embryo halves with the number of *vAChTP*⁺ cells indicated in the key (right). Total number (*n*) of embryos halves analysed, mean average number (ave.) of *vAChTP*⁺ cells per half embryo and the standard deviation (s.d.) are shown. The schematic drawing of a 64-cell stage embryo (top right) shows the ablated cells in 'all N' (all notochord) ablation. These 'all N' ablations were carried out independently of the other ablation series and are thus shown on a separate graph.

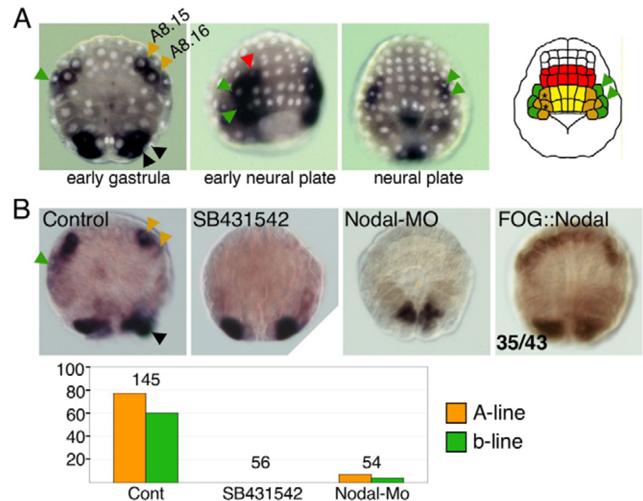

Fig. 4. *BMP2/4* expression in the lateral neural precursors is a target of Nodal. (**A**) Expression pattern of *BMP2/4*. Cell lineages are indicated by arrowheads: a-line in red (anterior sensory vesicle precursors); b-line in green (dorsal neural tube precursors); A-line in yellow (for posterior sensory vesicle and ventral midline precursors) or tan (for lateral neural tube precursors). B-line muscle precursors are indicated by black arrowheads. (**B**) *BMP2/4* expression in early gastrula stage embryos in which Nodal signalling has been modified. Graph shows percentage of embryos with expression in at least one A-line (A8.15 and A8.16) or b-line (b8.19) cell, following the colour code indicated on the right. The number of embryos analysed is indicated above the graphs. For the FOG::Nodal experiment, numbers indicate the number of embryos with ectopic (medial) *BMP2/4* expression over the total number of embryos analysed. The remaining embryos had lateral expression only. In this experiment, the in situ hybridisation was stopped before any *BMP2/4* expression in A- or b-line cells could be seen in the controls.

line neural precursors, as well as ectopic expression of its antagonist *Chordin* (Fig. 4B, Fig. 5A). The expression of *Chordin* in a broad lateral domain encompassing the lateral neural plate and the epidermal cells bordering the neural plate has previously been shown to be a target of Nodal signals, although *Chordin* expression in the presumptive notochord is Nodal independent (Hudson and Yasuo, 2005; Imai et al., 2006).

We conclude that, in *Ciona*, both *BMP2/4* and its antagonist *Chordin* are expressed in the lateral neural plate, directly or indirectly downstream of Nodal, and so are well placed to participate in the patterning of the neural tube.

A non-essential role for BMP signalling in neural plate patterning in *Ciona*

As *BMP2/4* expression begins at the early gastrula stage, we investigated its role in patterning the A-line neural precursors at the gastrula and neural plate stages. We found that overexpression of *BMP2/4* had effects very similar to overexpression of *Nodal*, with expression of lateral markers expanded into medial neural plate cells at the expense of medial marker expression (Fig. 5; see Fig. S2 in the supplementary material). Thus, as previously shown for Nodal, BMP2/4 appears capable of transforming medial neural plate into lateral neural plate fates (Hudson and Yasuo, 2005; Imai et al., 2006; Hudson et al., 2007). We ruled out the possibility that the lateralising effect of BMP2/4 was indirect via the ectopic activation of the Nodal signalling pathway by comparing the effects of different combinations of treatments on the expression of *Snail*, an early target of Nodal signalling in *Ciona* (Hudson and Yasuo, 2005). *Snail* expression was totally abolished when Nodal signalling was inhibited using the ALK4/5/7 inhibitor SB431542, even in the presence of excess Nodal ligand (Fig. 5Bi-iv). However, overexpression of BMP2/4 in the presence of SB431542 was able to restore *Snail* expression to significant levels (Fig. 5Bvii).

Given that BMP2/4 was clearly able to lateralise the neural plate independently of Nodal signals, we were surprised to find that the antagonists Noggin and Chordin had little effect on any of the neural plate marker genes analysed (Fig. 5A; see Fig. S2 in the supplementary material). We confirmed that Noggin overexpression effectively inhibited BMP2/4 but not Nodal in *Ciona* embryos, as it had no effect on *Snail* expression induced ectopically by Nodal, but reduced to one-fifth its ectopic expression induced by BMP2/4 (compare Fig. 5iii,v,vi,viii).

We conclude that although BMP2/4 has the ability to lateralise the neural plate independently of Nodal signals, BMP2/4 signals do not play a major role in neural plate patterning during normal development. These findings are not contradictory but suggest a redundant role for BMP2/4 in this process, possibly with Nodal itself.

BMP signalling is required for sensory but not motoneuron formation

It has previously been shown that modification of BMP signalling in ascidians caused a number of characteristic morphological defects, including rounding and fusion of the palps and a gain or loss of epidermal sensory neurons (Miya et al., 1997; Pasini et al., 2006; Darras and Nishida, 2001). We observed the same effects following introduction of the FOG::Noggin, FOG::Chordin and FOG::BMP2/4 constructs, and by injection of *Ciona* and *Xenopus Chordin* mRNA and Smad1/5-morpholino (Fig. 6; see Fig. S3 in

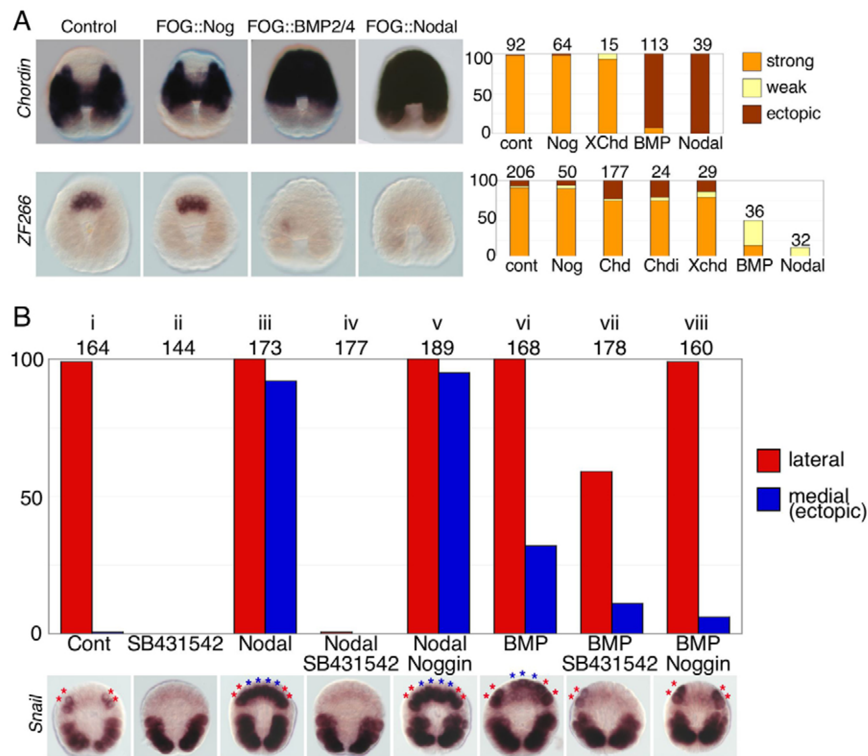

Fig. 5. Effect of BMP2/4 and Nodal signal modification on neural gene expression. (**A**) Analysis of *Chordin* and *ZNF266* expression at the neural plate stage. Graphs show the percentage of embryos with strong, weak or ectopic expression, as indicated in the key. Embryos were scored 'ectopic' if at least one additional cell showed expression. For expression of *ZNF266,* embryos were scored strong if at least half the medial cells showed control level expression. The number of embryos analysed is indicated above the graphs. cont, control; Nog, FOG::Noggin; Chd, FOG::Chordin; Chdi, *Chordin* RNA injection; XChd, *Xenopus Chordin* RNA injection; BMP, FOG::BMP2/4; Nodal, FOG::Nodal. (**B**) Expression of *Snail* at the early gastrula stage. Graphs showing the percentage of embryos with lateral (red) or ectopic (blue) expression of *Snail* following the treatments indicated. A representative embryo is shown for each treatment below the graph. Asterisks mark cells with medial or lateral expression following the same colour code. FOG::Nodal, FOG::Noggin and FOG::BMP2/4 are abbreviated to Nodal, Noggin and BMP, respectively. The number of embryos analysed is indicated above the graph. Scoring was as follows: lateral expression (red) was scored positive when one to four cells showed expression regardless of level. The vast majority of cases had all four cells positive except for BMP/SB431542 treatment, where most embryos were positive in only one or two cells. Ectopic (medial) expression (blue) was scored positive when one to four cells showed expression regardless of the level.

the supplementary material). In addition, following BMP signalling the tail bent up dorsally, sometimes forming a corkscrew curl (Fig. 6; see Fig. S3 in the supplementary material). Inhibition of BMP signalling, however, had no effect on motoneuron gene expression (Fig. 6; see Fig. S3 in the supplementary material), with no difference in the number of *vAChTP*$^+$ cells formed per half embryo following Chordin or Noggin overexpression (Fig. 7A,B). By contrast, overexpression of BMP2/4 resulted in a marked increase in the expression domains of the three motoneuron markers (Fig. 6) as well as the previously reported upregulation in epidermal sensory neurons (Fig. 6) (Pasini et al., 2006). The average number of *vAChTP*$^+$ cells per whole embryos increased from 7.2 to 19 following BMP2/4 overexpression (Fig. 7C). Nodal overexpression had similar effects on the expression of motoneuron markers, but resulted in loss or severe reduction of all epidermal sensory neurons (Fig. 6).

The formation of excess motoneurons following Nodal or BMP2/4 overexpression is probably a result of the transformation of medial into lateral neural plate, as observed at gastrula and neural plate stages (Fig. 5; see Fig. S2 in the supplementary material). Indeed, DiI labelling of the medial pair of blastomeres at the 64-cell stage (A7.4 pair) demonstrated that medial cells produced motoneurons following BMP2/4 or Nodal overexpression, but never in unmanipulated embryos (Fig. 7D). Consistently, markers of tissues derived from the medial neural plate, *08C09* (which marks the posterior sensory vesicle) and *Fgf3* (which marks the ventral neural tube) were lost following BMP2/4 or Nodal overexpression (see Fig. S4 in the supplementary material).

We conclude that BMP2/4 is capable, like Nodal, of transforming medial into lateral neural plate fates, thereby inducing excess *vAChTP*$^+$ motoneuron formation in *Ciona* embryos, but that during normal development BMP2/4 does not play an essential role in this process.

DISCUSSION

The evolutionary split between the vertebrate and urochordate lineages took place more than 500 million years ago. Although the morphology of their common ancestor is not certain, it appears likely that in the ascidian lineage evolutionary pressures favouring rapid development have driven a remarkable acceleration of the early developmental programme. Fate specification of key chordate features such as notochord and neural tube therefore occur in a very different cellular context, typically with signalling occurring between one or two defined cells rather than across fields of hundreds of cells (Lemaire, 2009). Comparisons of the developmental programmes, cellular interactions and regulatory

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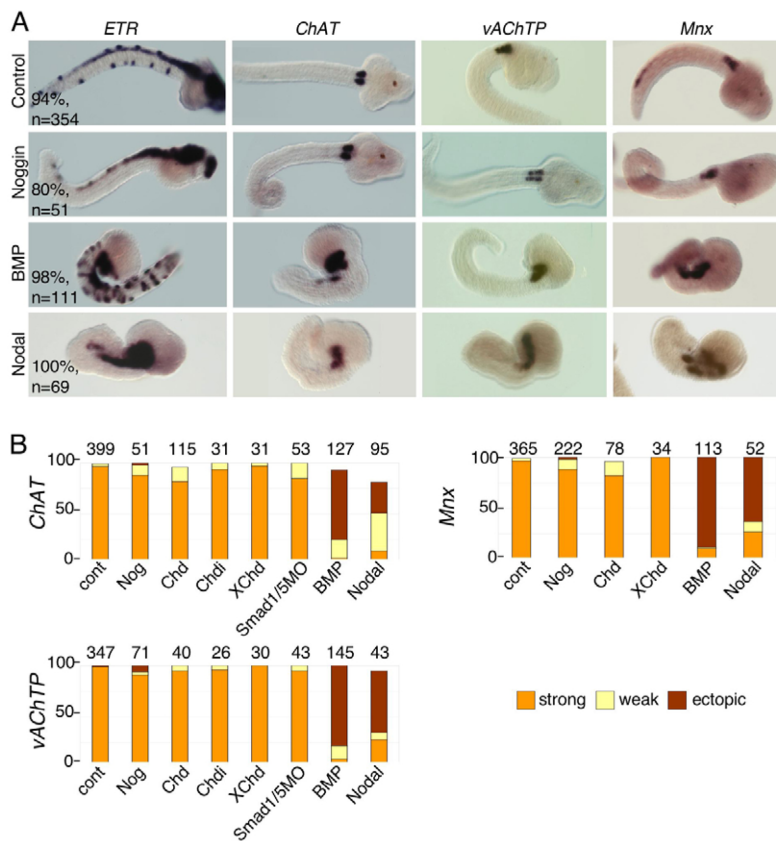

Fig. 6. BMP signalling is required for sensory neuron but not for motoneuron formation. (**A**) Embryo treatment is indicated on the left of the panels and the marker analysed is indicated above. For *ETR*, the numbers show the total number of embryos analysed (*n*) and the percentage of embryos that the panel represents; for control, the numbers show the percentage of embryos with staining in both dorsal and ventral epidermal sensory neurons; for Noggin, the numbers show the percentage of embryos with expression in the dorsal but not the ventral epidermal sensory neurons; for BMP, the numbers show the percentage of embryos with ectopic epidermal sensory neurons; for Nodal, the numbers show the percentage of embryos with no or with severely reduced numbers of epidermal sensory neurons. (**B**) The graphs show the percentage of embryos showing expression of the three motoneuron markers, following the key. Numbers indicate the total number of embryos analysed. cont, control; Nog, FOG::Noggin; Chd, FOG::Chordin; Chdi, *Chordin* RNA injection; XChd, *Xenopus Chordin* RNA injection; Smad1/5MO, Smad1/5-morpholino injection; BMP, FOG::BMP2/4; Nodal, FOG::Nodal.

gene expression, between vertebrates and ascidians, can thus be informative both about the ancestral developmental strategy and the degree of evolutionary flexibility, allowing mechanistic rewiring of developmental processes (Lemaire et al., 2008). There is now ample evidence that changes in gene expression drive changes in development and the final body plan (Davidson and Erwin, 2006; Erwin and Davidson, 2009). Here, we highlight an example where the gene expression patterns of key regulatory genes and the final overall organisation of the resulting structure appear to be well conserved; a *Hh* gene is expressed in the ventral midline of the neural tube, *BMP2/4* is expressed in the lateral/dorsal neural plate and the final position of the motoneurons in a ventrolateral position all appear to be conserved. However, we nonetheless find evidence for divergence in the mechanisms that control the specification of this important cell type. Future studies should aim to identify the changes in the cis-regulatory control sequences within the gene regulatory network of motoneuron specification that underlie this signal deployment change.

Midline cells and Shh signalling

In vertebrate embryos, the formation of somatic motoneurons in ventrolateral regions of the neural tube depends on Shh signals emanating from the notochord and floor plate (Beattie et al., 1997; Placzek et al., 1991; Weinstein et al., 1994; Yamada et al., 1991; Lewis and Eisen, 2001; Wilson and Maden, 2005). Similarly, expression of a *Ciona* homologue of *hedgehog*, *hh-2*, is restricted to the ventral midline of the neural tube during tailbud stages (Takatori et al., 2002). Although this expression occurs later than that observed in vertebrates, in which Shh begins to pattern the neural tube at gastrula and neural plate stages (Beattie et al., 1997; Yamada et al., 1993), it is nonetheless well placed for a role in motoneuron development. However, our experiments involving ablation of the *hh-2*-expressing 'floor plate' argue against a crucial role for Hedgehog in *Ciona vAChTP*$^+$ motoneuron formation. Although we cannot rule out that some Hedgehog activity remains in these embryos, it is likely to be significantly reduced. The fact that the majority of *Ciona vAChTP*$^+$ motoneurons form following ablation of the *hh-2* expression territory strongly suggests that the floor plate and Hedgehog signalling are not acting in the same way during vertebrate and *Ciona* motoneuron formation. Consistently, injection of hh-2 morpholino or a dominant-negative form of mouse Patched (mPtcΔloop) (Briscoe et al., 2001) in *Ciona* did not disrupt motoneuron formation (Imai et al., 2009) (H.Y., unpublished). Similarly, in *Halocynthia* embryos, attempts to activate or inhibit the Shh signalling pathway had no effects on the formation of motoneurons (Katsuyama et al., 2005). Furthermore, ablations causing the entire loss of both notochord and floorplate failed to prevent the formation of *vAChTP*$^+$ motoneurons (Figs 3, 4). Although we cannot rule out some role for midline structures and *hh-2* in controlling precisely the number and position of motoneurons, our data indicate that these midline tissues are not playing the same role during the induction of motoneuron fate in the ascidian and vertebrate spinal cord.

On the other hand, despite Shh having been assigned a pivotal role in vertebrate motoneuron specification, there is some evidence for parallel Shh-independent pathways (Eggenschwiler et al., 2001; Litingtung and Chiang, 2000; Ruiz i Altaba et al., 2003; Wijgerde et al., 2002; Wilson and Maden, 2005). This evidence comes from embryos carrying double mutations, in *Shh* itself (or its receptor) as well as in a negative regulator of the Shh signalling pathway such as *Rab23* or *Gli3* (Eggenschwiler et al., 2001; Litingtung and Chiang, 2000; Wijgerde et al., 2002). In these double mutant embryos, motoneurons still form, as do some other types of ventral neurons that are normally dependent on Shh signalling, although the

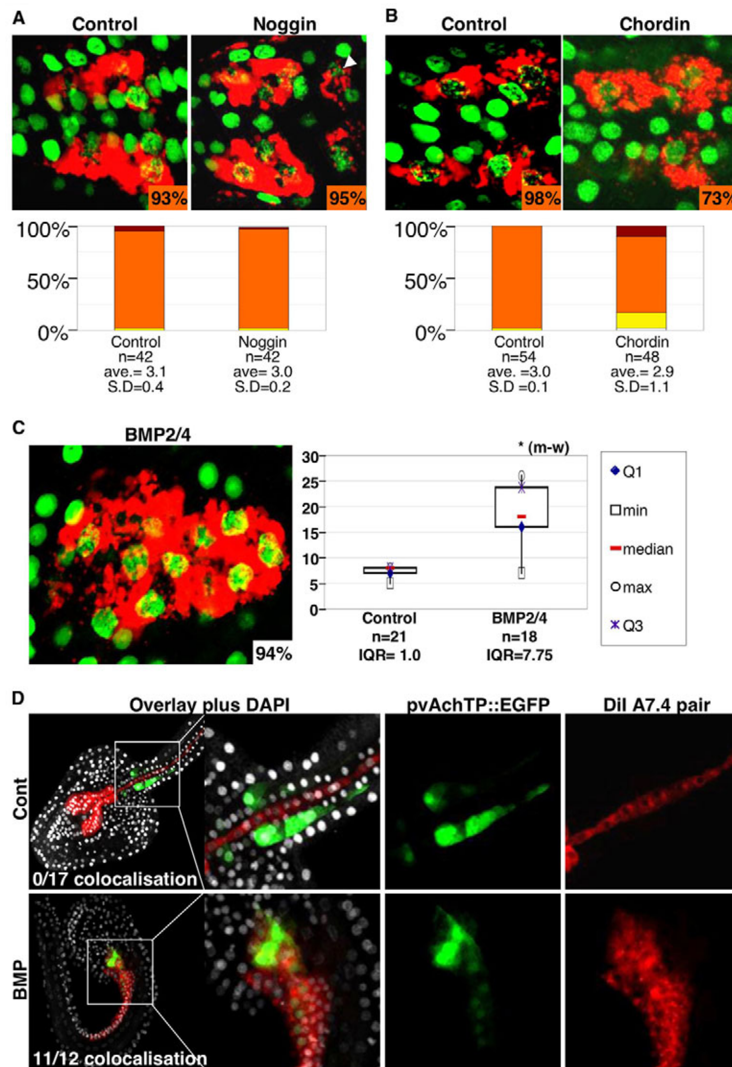

Fig. 7. *vAChTP*+ cell counts following BMP activation and inhibition. (**A-C**) Panels show confocal image stacks of dorsal views of embryos, anterior towards the right, following *vAChTP* in situ hybridisation and DAPI staining. (A,B) Graphs show the percentage of embryo halves with the number of *vAChTP*+ cells indicated in the key in Fig. 3. Total number (*n*) of embryo halves analysed, mean average number (ave.) of *vAChTP*+ cells per half embryo and the s.d. are shown. (A) Although not included in the cell counts, in these experiments we could detect expression of *vAChTP* in A12.239 (arrowhead) in 64% of controls and 52% of Noggin-overexpressed embryos, suggesting this motoneuron may also form independently of BMP signalling. (C) In this experiment A12.239 could also be detected and was included in the cell counts. The total number of *vAChTP*+ cells per whole embryo was counted as each side of the embryo could not be distinguished in FOG::BMP2/4 embryos. The graph shows a boxplot of the total number of number of *vAChTP*+ cells in control and FOG::BMP2/4 embryos. Box indicates the interquartile range (25-75%) of values (Q3 to Q1) along with the maximum (circle) and minimum (square) number of cells counted and the median average (red bar), following the key on the right. As the number of samples (*n*) was relatively low, the interquartile range (instead of the s.d.) is indicated below, to give an idea of the variation. The percentage of embryos with more than eight *vAChTP*+ cells is indicated on the panel. The number of *vAChTP*+ cells was significantly different between the two samples (*Mann-Whitney test; m-w). (**D**) Electroporation of pvAChTP::EGFP (Yoshida et al., 2004) alone, with FOG::BMP2/4 or with FOG::Nodal, as indicated. The medial pair of neural precursors of the 64-cell stage embryo (A7.4 pair) were labelled with DiI (red). Panels on the far left show the entire embryo overlay with DiI in red, nuclei (DAPI) in white and motoneurons in green. Subsequent panels show a higher magnification of the labelled territory. Not all motoneurons become GFP positive, owing to the mosaic distribution of the electroporated plasmid. Numbers indicate the number of embryos in which motoneurons were found colocalised with DiI. For Nodal, eight out of eight embryos showed colocalisation.

positioning of these neurons within the spinal cord is perturbed. This suggests that a Shh-independent patterning mechanism exists that is sufficient for the formation of ventral neuronal cell types. It is possible that the patterning system that is operating in ascidian embryos is the same as the Shh-independent system uncovered in vertebrate double mutant embryos, and that Shh was recruited to refine the neural tube patterning process during evolution of the vertebrate lineage. Alternatively, a more crucial role for Hedgehog signalling may have been lost or reduced in the ascidian lineage. This may have been a consequence of the drastic reduction in cell number during evolution of the ascidian neural plate/tube, which brought the motoneuron precursors into closer proximity to and therefore into the influence of dorsal signals (Fig. 1). This in turn may have allowed dorsal signals to take on a more prominent and positive role during motoneuron induction and ventral signals to be dispensed with. Functional data from Amphioxus, which belongs to the cephalochordates, the basal group of the chordate phylum and in which *Hedgehog* is expressed in both the floor plate and notochord from neural plate stages (Shimeld, 1999), would shed light on which of these scenarios is likely to be the most plausible.

It remains possible that Hedgehog signalling is involved in other aspects of ascidian neural tube patterning, including the specification of particular neuronal cell types. Recently, putative cholinergic and glycinergic cells have been identified in the tail nerve cord of *Ciona*, which was previously thought to be devoid of neurons (Imai and Meinertzhagen, 2007; Horie et al., 2010). It will be interesting in future studies to test the role of the ventral midline and Hedgehog in the specification of these cell types.

Despite the differences in the initial induction mechanisms of motoneurons between ascidian and vertebrates, a shared set of transcription factors are expressed in the trunk ganglion of ascidians, including *Lhx*, *Mnx* and *Islet* (Katsuyama et al., 2005; Ikuta and Saiga, 2007). Thus, distinct inductive mechanisms may converge on a common gene regulatory cassette, although thorough functional studies would be required in ascidians to support this hypothesis.

Dorsal TGFβs and neural tube patterning

In vertebrates, two other types of signal act in parallel with Shh in spinal cord patterning: retinoids and BMPs (Litingtung and Chiang, 2000; Ruiz i Altaba et al., 2003; Wilson and Maden, 2005). Retinoids from lateral somites are vital for the formation of vertebrate motoneurons (Novitch et al., 2003). Although not yet fully investigated in ascidians, treatment of embryos with retinoic acid does not seem to affect the expression of motoneuron markers (Katsuyama and Saiga, 1998).

In vertebrates, BMPs appear to act over the entire neural plate, promoting dorsal and repressing ventral cell types, including motoneurons (Helms and Johnson, 2003; Wilson and Maden,

2005; Barth et al., 1999; Lewis and Eisen, 2001). Indeed, BMPs are involved in dorsoventral neural patterning in many species (Denes et al., 2007; Katsuyama et al., 2005; Lowe et al., 2006; Mizutani et al., 2006; Rusten et al., 2002). In both *Halocynthia* and *Ciona* embryos, *BMP2/4* expression is detected in the lateral/dorsal neural plate, as in vertebrates (this study) (Miya et al., 1997). However, our experiments revealed no role for BMP signalling during formation of *Ciona vAChTP*$^{+}$ motoneurons, a conclusion supported by BMP2/4 morpholino knockdown experiments (Imai et al., 2009). This was somewhat surprising, given that, in *Halocynthia*, inhibition of BMP signalling resulted in loss of motoneurons, suggesting that BMPs may promote motoneuron formation in ascidians rather than inhibiting their formation as in vertebrates (Katsuyama et al., 2005). Correspondingly, overexpression of BMP2/4 in *Ciona* (this study) and *Halocynthia* (Katsuyama et al., 2005) resulted in an increase in motoneurons. This may point to a recent loss of a requirement for BMP signalling in motoneuron formation in *Ciona* embryos, with Nodal a good candidate to have replaced BMP as the principal motoneuron inducer. The role of Nodal itself is also different from that in vertebrates, where Nodal is required for formation of the ventral most part of the spinal cord: the floor plate (Strähle et al., 2004). Intriguingly, *Ciona Nodal* is expressed in a bilateral pair of cells, b6.5, ablation of which leads to loss of early lateral neural plate markers and of motoneurons (Fig. 2B) (Hudson and Yasuo, 2005). This cell will give rise to the dorsal epidermis and dorsal neural tube. Thus, the embryonic origin of the Nodal signal in *Ciona* is equivalent to that of the dorsal signalling centre that patterns the spinal cord of vertebrates (epidermis and roof plate). This may be an example where geometric conservation of cellular interactions is accompanied by divergence in the signalling molecules deployed. We recently highlighted another example during the induction of a specific muscle precursor in *Ciona* and *Halocynthia.* Although the geometric identity of the interacting cells during the induction of this cell type in these two ascidian species is remarkably similar, the signalling molecules involved are not the same (Hudson and Yasuo, 2008). Surprisingly, it may be a common theme that, during evolution, the use of cellular interactions can be apparently conserved, while the underlying molecular mechanisms have been modified. A parallel conclusion from this study with important implications for evo-devo studies is that conservation of gene expression patterns does not necessarily point to an equivalent role for these molecules during embryonic patterning.

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Competing interests statement
The authors declare no competing financial interests.

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References

Barth, K., Kishimoto, Y., Rohr, K., Seydler, C., Schulte-Merker, S. and Wilson, S. (1999). Bmp activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.
Beattie, C. E., Hatta, K., Halpern, M. E., Liu, H., Eisen, J. S. and Kimmel, C. B. (1997). Temporal separation in the specification of primary and secondary motoneurons in zebrafish. *Dev. Biol.* **187**, 171-182.
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.
Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.
Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001). A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol. Cell* **7**, 1279-1291.
Cole, A. G. and Meinertzhagen, I. A. (2004). The central nervous system of the ascidian larva: mitotic history of cells forming the neural tube in late embryonic Ciona intestinalis. *Dev. Biol.* **271**, 239-262.
Conklin, E. G. (1905). The organisation and cell lineage of the ascidian egg. *J. Acad. Natl. Sci. (Philadelphia)* **13**, 1-119.
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.
Darras, S. and Nishida, H. (2001). The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Dev. Biol.* **236**, 271-288.
Davidson, E. H. and Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science* **311**, 796-800.
Denes, A. S., Jékely, G., Steinmetz, P. R. H., Raible, F., Snyman, H., Prud'homme, B., Ferrier, D. E. K., Balavoine, G. and Arendt, D. (2007). Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell* **129**, 277-288.
Duboule, D. and Wilkins, A. S. (1998). The evolution of 'bricolage'. *Trends Genet.* **14**, 54-59.
Dufour, H. D., Chettouh, Z., Deyts, C., de Rosa, R., Goridis, C., Joly, J. and Brunet, J. (2006). Precraniate origin of cranial motoneurons. *Proc. Natl. Acad. Sci. USA* **103**, 8727-8732.
Eggenschwiler, J. T., Espinoza, E. and Anderson, K. V. (2001). Rab23 is an essential negative regulator of the mouse Sonic hedgehog signalling pathway. *Nature* **412**, 194-198.
Erwin, D. H. and Davidson, E. H. (2009). The evolution of hierarchical gene regulatory networks. *Nat. Rev. Genet.* **10**, 141-148.
Helms, A. W. and Johnson, J. E. (2003). Specification of dorsal spinal cord interneurons. *Curr. Opin. Neurobiol.* **13**, 42-49.
Hino, K., Satou, Y., Yagi, K. and Satoh, N. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. VI. Genes for Wnt, TGFbeta, Hedgehog and JAK/STAT signaling pathways. *Dev. Genes Evol.* **213**, 264-272.
Horie, T., Nakagawa, M., Sasakura, Y., Kusakabe, T. G. and Tsuda, M. (2010). Simple motor system of the ascidian larva: neuronal complex comprising putative cholinergic and GABAergic/glycinergic neurons. *Zool. Sci.* **27**, 181-190.
Hudson, C. and Yasuo, H. (2005). Patterning across the ascidian neural plate by lateral Nodal signalling sources. *Development* **132**, 1199-1210.
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. and Yasuo, H. (2008). Similarity and diversity in mechanisms of muscle fate induction between ascidian species. *Biol. Cell* **100**, 265-277.
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.
Hudson, C., Lotito, S. and Yasuo, H. (2007). Sequential and combinatorial inputs from Nodal, Delta2/Notch and FGF/MEK/ERK signalling pathways establish a grid-like organisation of distinct cell identities in the ascidian neural plate. *Development* **134**, 3527-3537.
Ikuta, T. and Saiga, H. (2007). Dynamic change in the expression of developmental genes in the ascidian central nervous system: revisit to the tripartite model and the origin of the midbrain-hindbrain boundary region. *Dev. Biol.* **312**, 631-643.
Imai, J. H. and Meinertzhagen, I. A. (2007). Neurons of the ascidian larval nervous system in Ciona intestinalis: I. Central nervous system. *J. Comp. Neurol.* **501**, 316-334.
Imai, K. S., Hino, K., Yagi, K., Satoh, N. and Satou, Y. (2004). Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. *Development* **131**, 4047-4058.

DEVELOPMENT

Imai, K. S., Levine, M., Satoh, N. and Satou, Y. (2006). Regulatory blueprint for a chordate embryo. *Science* **312**, 1183-1187.
Imai, K. S., Stolfi, A., Levine, M. and Satou, Y. (2009). Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. *Development* **136**, 285-293.
Inman, G. J., Nicolás, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. and Hill, C. S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* **62**, 65-74.
Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Katsuyama, Y. and Saiga, H. (1998). Retinoic acid affects patterning along the anterior-posterior axis of the ascidian embryo. *Dev. Growth Differ.* **40**, 413-422.
Katsuyama, Y., Okada, T., Matsumoto, J., Ohtsuka, Y., Terashima, T. and Okamura, Y. (2005). Early specification of ascidian larval motor neurons. *Dev. Biol.* **278**, 310-322.
Kiontke, K., Barrière, A., Kolotuev, I., Podbilewicz, B., Sommer, R., Fitch, D. H. A. and Félix, M. (2007). Trends, stasis, and drift in the evolution of nematode vulva development. *Curr. Biol.* **17**, 1925-1937.
Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.
Lemaire, P. (2009). Unfolding a chordate developmental program, one cell at a time: invariant cell lineages, short-range inductions and evolutionary plasticity in ascidians. *Dev. Biol.* **332**, 48-60.
Lemaire, P., Smith, W. C. and Nishida, H. (2008). Ascidians and the plasticity of the chordate developmental program. *Curr. Biol.* **18**, R620-R631.
Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.
Lewis, K. E. and Eisen, J. S. (2003). From cells to circuits: development of the zebrafish spinal cord. *Prog. Neurobiol.* **69**, 419-449.
Liem, K. F., Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.
Litingtung, Y. and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985.
Liu, A. and Niswander, L. A. (2005). Bone morphogenetic protein signalling and vertebrate nervous system development. *Nat. Rev. Neurosci.* **6**, 945-954.
Lowe, C. J., Terasaki, M., Wu, M., Freeman, R. M., Runft, L., Kwan, K., Haigo, S., Aronowicz, J., Lander, E., Gruber, C. et al. (2006). Dorsoventral patterning in hemichordates: insights into early chordate evolution. *PLoS Biol.* **4**, e291.
McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438-1452.
Meedel, T. H., Chang, P. and Yasuo, H. (2007). Muscle development in Ciona intestinalis requires the b-HLH myogenic regulatory factor gene Ci-MRF. *Dev. Biol.* **302**, 333-344.
Mekki-Dauriac, S., Agius, E., Kan, P. and Cochard, P. (2002). Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord. *Development* **129**, 5117-5130.
Miya, T., Morita, K., Suzuki, A., Ueno, N. and Satoh, N. (1997). Functional analysis of an ascidian homologue of vertebrate Bmp-2/Bmp-4 suggests its role in the inhibition of neural fate specification. *Development* **124**, 5149-5159.
Mizutani, C. M., Meyer, N., Roelink, H. and Bier, E. (2006). Threshold-dependent BMP-mediated repression: a model for a conserved mechanism that patterns the neuroectoderm. *PLoS Biol.* **4**, e313.
Nicol, D. and Meinertzhagen, I. A. (1988a). Development of the central nervous system of the larva of the ascidian, Ciona intestinalis L. I. The early lineages of the neural plate. *Dev. Biol.* **130**, 721-736.
Nicol, D. and Meinertzhagen, I. A. (1988b). Development of the central nervous system of the larva of the ascidian, Ciona intestinalis L. II. Neural plate morphogenesis and cell lineages during neurulation. *Dev. Biol.* **130**, 737-766.
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.
Novitch, B. G., Wichterle, H., Jessell, T. M. and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40**, 81-95.
Pasini, A., Amiel, A., Rothbächer, U., Roure, A., Lemaire, P. and Darras, S. (2006). Formation of the ascidian epidermal sensory neurons: insights into the origin of the chordate peripheral nervous system. *PLoS Biol.* **4**, e225.
Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T. and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development* **Suppl. 2**, 105-122.
Roure, A., Rothbächer, U., Robin, F., Kalmar, E., Ferone, G., Lamy, C., Missero, C., Mueller, F. and Lemaire, P. (2007). A multicassette Gateway vector set for high throughput and comparative analyses in ciona and vertebrate embryos. *PLoS One* **2**, e916.
Ruiz i Altaba, A., Nguyên, V. and Palma, V. (2003). The emergent design of the neural tube: prepattern, SHH morphogen and GLI code. *Curr. Opin. Genet. Dev.* **13**, 513-521.
Rusten, T. E., Cantera, R., Kafatos, F. C. and Barrio, R. (2002). The role of TGF beta signaling in the formation of the dorsal nervous system is conserved between Drosophila and chordates. *Development* **129**, 3575-3584.
Sardet, C., McDougall, A., Yasuo, H., Chenevert, J., Pruliere, G., Dumollard, R., Hudson, C., Hebras, C., Le Nguyen, N. and Paix, A. (2011). Embryological methods in ascidians: the Villefranche-sur-Mer protocols. *Methods Mol. Biol.* (in press).
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
Shi, W., Peyrot, S. M., Munro, E. and Levine, M. (2009). FGF3 in the floor plate directs notochord convergent extension in the Ciona tadpole. *Development* **136**, 23-28.
Shimeld, S. M. (1999). The evolution of the hedgehog gene family in chordates: insights from amphioxus hedgehog. *Dev. Genes Evol.* **209**, 40-47.
Shu, D. G., Chen, L., Han, J. and Zhang, X. L. (2001). An early cambrian tunicate from China. *Nature* **411**, 472-473.
Siegel, S. and Castellan, N. J. (1988). *Nonparametric Statistics for the Behavioral Sciences*. 2nd edn. New York and Montréal: McGraw-Hill.
Sobral, D., Tassy, O. and Lemaire, P. (2009). Highly divergent gene expression programs can lead to similar chordate larval body plans. *Curr. Biol.* **19**, 2014-2019.
Strähle, U., Lam, C. S., Ertzer, R. and Rastegar, S. (2004). Vertebrate floor-plate specification: variations on common themes. *Trends Genet.* **20**, 155-162.
Takamura, K., Egawa, T., Ohnishi, S., Okada, T. and Fukuoka, T. (2002). Developmental expression of ascidian neurotransmitter synthesis genes. I. Choline acetyltransferase and acetylcholine transporter genes. *Dev. Genes Evol.* **212**, 50-53.
Takatori, N., Satou, Y. and Satoh, N. (2002). Expression of hedgehog genes in Ciona intestinalis embryos. *Mech. Dev.* **116**, 235-238.
Takatori, N., Kumano, G., Saiga, H. and Nishida, H. (2010). Segregation of germ layer fates by nuclear migration-dependent localization of Not mRNA. *Dev. Cell* **19**, 589-598.
Tanabe, Y., William, C. and Jessell, T. M. (1998). Specification of motor neuron identity by the MNR2 homeodomain protein. *Cell* **95**, 67-80.
Taniguchi, K. and Nishida, H. (2004). Tracing cell fate in brain formation during embryogenesis of the ascidian Halocynthia roretzi. *Dev. Growth Differ.* **46**, 163-180.
Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J. and Pfaff, S. L. (1999). Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* **23**, 675-687.
Tokuoka, M., Kumano, G. and Nishida, H. (2007). FGF9/16/20 and Wnt-5alpha signals are involved in specification of secondary muscle fate in embryos of the ascidian, Halocynthia roretzi. *Dev. Genes Evol.* **217**, 515-527.
True, J. R. and Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evol. Dev.* **3**, 109-119.
Ulloa, F. and Briscoe, J. (2007). Morphogens and the control of cell proliferation and patterning in the spinal cord. *Cell Cycle* **6**, 2640-2649.
Wada, S., Katsuyama, Y., Yasugi, S. and Saiga, H. (1995). Spatially and temporally regulated expression of the LIM class homeobox gene Hrlim suggests multiple distinct functions in development of the ascidian, Halocynthia roretzi. *Mech. Dev.* **51**, 115-126.
Wada, S., Tokuoka, M., Shoguchi, E., Kobayashi, K., Di Gregorio, A., Spagnuolo, A., Branno, M., Kohara, Y., Rokhsar, D., Levine, M. et al. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. II. Genes for homeobox transcription factors. *Dev. Genes Evol.* **213**, 222-234.
Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.
Wijgerde, M., McMahon, J. A., Rule, M. and McMahon, A. P. (2002). A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes Dev.* **16**, 2849-2864.
Wilson, L. and Maden, M. (2005). The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Dev. Biol.* **282**, 1-13.
Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.
Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.
Yasuo, H. and Satoh, N. (1998). Conservation of the developmental role of Brachyury in notochord formation in a urochordate, the ascidian Halocynthia roretzi. *Dev. Biol.* **200**, 158-170.
Yoshida, R., Sakurai, D., Horie, T., Kawakami, I., Tsuda, M. and Kusakabe, T. (2004). Identification of neuron-specific promoters in Ciona intestinalis. *Genesis* **39**, 130-140.