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Snail mediates medial–lateral patterning of the ascidian neural plate

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ABSTRACT

The ascidian neural plate exhibits a regular, grid-like arrangement of cells. Patterning of the neural plate across the medial–lateral axis is initiated by bilateral sources of Nodal signalling, such that Nodal signalling induces expression of lateral neural plate genes and represses expression of medial neural plate genes. One of the earliest lateral neural plate genes induced by Nodal signals encodes the transcription factor Snail. Here, we show that Snail is a critical downstream factor mediating this Nodal-dependent patterning. Using gain and loss of function approaches, we show that Snail is required to repress medial neural plate gene expression at neural plate stages and to maintain the lateral neural tube genetic programme at later stages. A comparison of these results to those obtained following Nodal gain and loss of function indicates that Snail mediates a subset of Nodal functions. Consistently, overexpression of Snail can partially rescue a Nodal inhibition phenotype. We conclude that Snail is an early component of the gene regulatory network, initiated by Nodal signals, that patterns the ascidian neural plate.

Introduction

A dorsal hollow central nervous system (CNS) is one of the hallmarks of the chordate body plan, together with pharyngeal slits and notochord (Satoh et al., 2014). The chordate phylum (or superphylum, see Satoh et al., 2014), consists of cephalochordates, urochordates (including ascidians) and vertebrates. Despite a high degree of conservation of chordate larval body plans, the mechanisms used to generate these body plans do not always seem to be well conserved in distantly related species. For example, a comparison of the mechanisms used to pattern the neural tube along the dorsal–ventral axis reveals significant divergence between ascidians and vertebrates. In vertebrates, the neural tube is patterned by Shh and BMP signals from ventral and dorsal signalling sources, respectively (Wilson and Maden, 2005). Conversely, in ascidians, Nodal signalling from lateral sources (including the future dorsal neural tube itself) appears to play a pivotal role in dorsal–ventral patterning of the neural tube (Hudson and Yasuo, 2005; Imai et al., 2006; Mita and Fujiwara, 2007). There is little evidence of a major role for Shh in this process while BMP2/4, itself transcriptionally induced by Nodal signals, may be acting in a similar way to Nodal (Hudson et al., 2011; Katsuyama et al., 2005).

The ascidian CNS derives from three of the four founder lineages, such that both the a- and b-animal lineages as well as the A-vegetal lineage contribute to the CNS, whereas the B-vegetal lineage does not (Nicol and Meinertzhagen, 1988a, 1988b; Nishida, 1987). This study focuses on the A-line derived part of the CNS. At neural plate stages, when the neural plate consists of 6 rows of cells, the A-line cells form the two posterior-most rows of eight cells (Fig. 1). Rows I and II are differentially specified along the anterior–posterior axis by FGF/ERK signals active in the row I but not in the row II cells (Hudson et al., 2007). Along the medial–lateral axis, neural plate cells are arranged into four columns at each side of the midline (Fig. 1). Column 1 is the medial-most column and column 4 the lateral-most. Patterning of these columns of cells along the medial–lateral axis of the neural plate is initiated by Nodal signals (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007). *Nodal* starts to be expressed in a bilateral pair of b-line neural precursors that are in direct contact with A-line column 3 and 4 precursors. Nodal signals are required for the differential gene regulatory states of lateral versus medial columns. In the absence of Nodal signals, all genes normally expressed in the lateral columns 3 and 4 are no longer expressed and genes normally restricted to the medial columns 1 and 2 are ectopically expressed in columns 3 and 4. *Snail* and *Delta2* are early transcriptional targets of Nodal signalling and are both expressed at early gastrula stages in columns 3 and 4 precursors (Corbo et al., 1997; Hudson and Yasuo, 2005; Wada and Saiga, 1999). The role of Delta2, a Notch ligand, in neural plate patterning has been previously investigated (Hudson et al., 2007). Delta2/Notch refines the initial medial–lateral pattern

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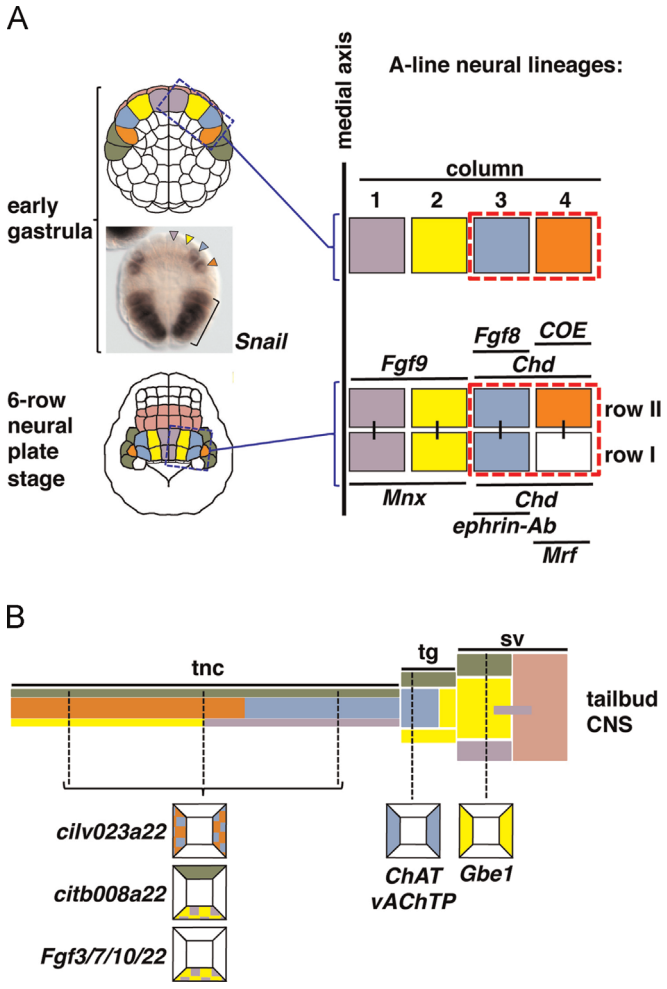

Fig. 1. Cell lineages and gene expression in the A-line derived part of the CNS. (A) Adapted from Hudson et al. (2007). The drawing at the top represents an early gastrula stage embryo with the different CNS lineages coloured: b-line (green); a-line (pink); A-line -column 1 (A8.7 lineage, lilac), -column 2 (A8.8 lineage, yellow), -column 3 (A8.15 lineage, blue), -column 4 (A8.16 lineage, orange). Below the early gastrula stage drawing is an early gastrula stage embryo following *Snail* in situ hybridisation. The different A-line neural lineages are marked by arrowheads on the right hand side of the embryo following the same colour code described above. The black bracket indicates the B-line muscle lineages. At the bottom left hand side is an embryo drawing of a 6-row neural plate stage following the same colour code. Compared to the early gastrula stage, at this stage, the A-line neural lineages have divided once along the anterior–posterior axis to give rise to two rows of eight cells. The cell in row I/column 4 is a muscle cell and therefore does not contribute to the CNS. On the right are schematic representations of the A-line neural lineages to highlight their respective columns. Since ascidian embryos are bilaterally symmetrical, only the right-hand half is shown, with the medial axis indicated. The area boxed with a red dotted line is the *Snail* expression territory. On the schematic representation of the A-line neural lineages at neural plate stages, the expression pattern of the genes analysed in this study are indicated. Gene expression in row II cells is indicated by bars above the schematic and gene expression in row I cells is indicated by bars below the schematic. *Fgf9=Fgf9/16/20*; *Fgf8=Fgf8/17/18*; *Chd=Chordin*; *ephrin-Ab=Efna.b*; *COE=Ebf*. (B) Lineages of the larval CNS based on Cole and Meinertzhagen (2004). The schematised drawing represents a lateral view of the larval CNS, showing along the anterior-to-posterior axis the sensory vesicle (sv), trunk ganglion (tg, also called visceral ganglion) and tail nerve cord (tnc). The contribution of each lineage is indicated following the same colour code in (A). The horizontal lilac bar represents part of the ventral floor of the neurocoel. In summary, the medial neural plate derivatives (columns 1 and 2; lilac and yellow) contribute to the posterior sensory vesicle and the ventral row of cells in the tail nerve cord, while the lateral cells (columns 3 and 4; blue and orange) contribute to the lateral trunk ganglion and lateral tail nerve cord. Below the CNS depiction are 'cross-sections' of the neural tube showing the expression patterns of the various genes analysed in this study. Each cross section is divided into dorsal, ventral and lateral segments with the segments coloured if the respective gene is expressed. For example, *Gbe1* is expressed in the lateral posterior part of the sensory vesicle, *ChAT* and *vAChTP* are expressed in the lateral column 3-derived part of the trunk ganglion, *cilv023a22* is expressed along the tail nerve cord in the lateral cells derived from both columns 3 (blue) and 4 (orange), and so on.

established by Nodal signals, inducing the specific gene expression states of columns 2 and 4. *Snail* encodes a C2H2 zinc finger transcription factor that is generally considered to act as a repressor, although it also has an activating function in certain developmental contexts (Hemavathy et al., 2000; Nieto, 2002; Reece-Hoyes et al., 2009; Rembold et al., 2014; Sakai et al., 2006). Snail is implicated in formation of many different tissue types, particularly during mesoderm formation and in cells that undergo the epithelial–mesenchyme transition, such as the premigratory neural crest (Nieto, 2002). Here, we investigate the role of Snail as a component of the gene regulatory network, triggered by Nodal signals, for medial–lateral patterning of the ascidian neural plate.

Materials and methods

Embryo experiments and constructs

Adult *Ciona intestinalis* were purchased from the Station Biologique de Roscoff (France). Cell nomenclature, lineage and the fate maps are previously described (Cole and Meinertzhagen, 2004; Conklin, 1905; Nishida, 1987). Ascidian embryo culture and injection have been described (Sardet et al., 2011). SB431542 (Tocris) was added to artificial seawater at 5 µM and washed at early gastrula stage, as described previously (Hudson and Yasuo, 2005). Snail-MO1 is described in Imai et al. (2006), Snail-MO2 sequence is: AAAGCATGGGCTCGACGGAGGTCAT. Both morpholinos were injected at 0.75 mM with a volume of approximately one-quarter egg diameter. pFOG > Nodal has been previously described (Pasini et al., 2006). pETR > Snail was generated as follows: The *ETR* upstream sequences were PCR-amplified from genomic DNA of *Ciona intestinalis* using the following primers (ETR-up-attB3-F3 ATAAAGTAGGCTCCGTCACGTGATCCCGTTTCC, ETR-up-attB5-R3 GAAAAGTTGGGTGTCGTTTATCCCCGCTTTGGC) and subcloned into pDONR 221 P3-P5 (Roure et al., 2007) to generate pENTR-L3-ETR-L5. The ORF of *Snail* was PCR-amplified from the cDNA (Corbo et al., 1997) using the following primers (Snail-F-attB1 AAAAAGCAGGCTACCATGACCTCCGTCGAGCCCATG, Snail-R-attB2 AGAAAGCTGGGTTTAGGATGCTGTCTTGCGTTGTGC) and subcloned into pDONR 221 P1–P2 to generate pENTR-L1-Snail-L2. These entry clones were mixed with pSP1.72BSSPE-R3-ccdB/cmR–R5::RfA (Roure et al., 2007) in a LR reaction to generate pETR > Snail. Electroporation was carried out with 50 µg of plasmid DNA per electroporation, as described (Christiaen et al., 2009). All data was pooled from at least two independent experiments.

In situ hybridisation

In situ hybridisation was carried out as previously described (Hudson and Yasuo, 2006; Wada et al., 1995). Dig-labelled probes were synthesized from the following, previously described, *Ciona* cDNA clones: *Chordin, COE, ephrin-Ab, Mnx, Mrf, Fgf8/17/18, Fgf9/16/20, ZnF266* (*ciad008m15*, previously referred to as *ZnF(C2H2)-33*) (Hudson et al., 2007; Imai et al., 2004), *cilv023a22* (Mochizuki et al., 2003), *Fgf3/7/10/22* (Imai et al., 2002), *citb008a22* (Hudson et al., 2003), *Gbe1* (clone number 008zc09, encoding 1,4-alpha-glucan-branching enzyme-like gene) (Hudson et al., 2003), *ChAT* (Takamura et al., 2002) and *vAChTP* (Ikuta and Saiga, 2007). According to recent nomenclature guidelines (Stolfi et al., 2015), the correct gene name for *ephrin-Ab* is *Efna.b* and for *COE* it is *Ebf*. We use *ephrin-Ab* and *COE* in this manuscript for ease of recognition.

Results

Snail is required for repression of medial neural plate genes

In order to investigate the function of Snail during medial–lateral A-line neural plate patterning, we inhibited Snail function using two different antisense morpholino oligonucleotides (Fig. 2). We analysed expression of both lateral and medial neural plate genes, the expression of which is described in Fig. 1A. Following injection of Snail morpholinos, lateral genes were expressed correctly, except for *ephrin-Ab (Efna.b),* which was down-regulated (Fig. 2). Loss of *ephrin-Ab* expression concurs with previous results (Imai et al., 2009). We occasionally saw ectopic expression of *Fgf8/17/18* in row I/column 4, as was also reported previously, although we observed this only rarely (Fig. 2) (Imai et al., 2009). The medial genes showed a marked upregulation following Snail knockdown, with strong ectopic expression observed in the lateral neural plate of most embryos (Fig. 2), consistent with previous data (Imai et al., 2006, 2009). Comparing these results to those obtained following Nodal inhibition (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007), we conclude that, while Nodal is required for induction of all lateral genes and for repression of medial genes at neural plate stages, Snail is predominantly required to repress medial genes in the lateral columns of neural plate cells.

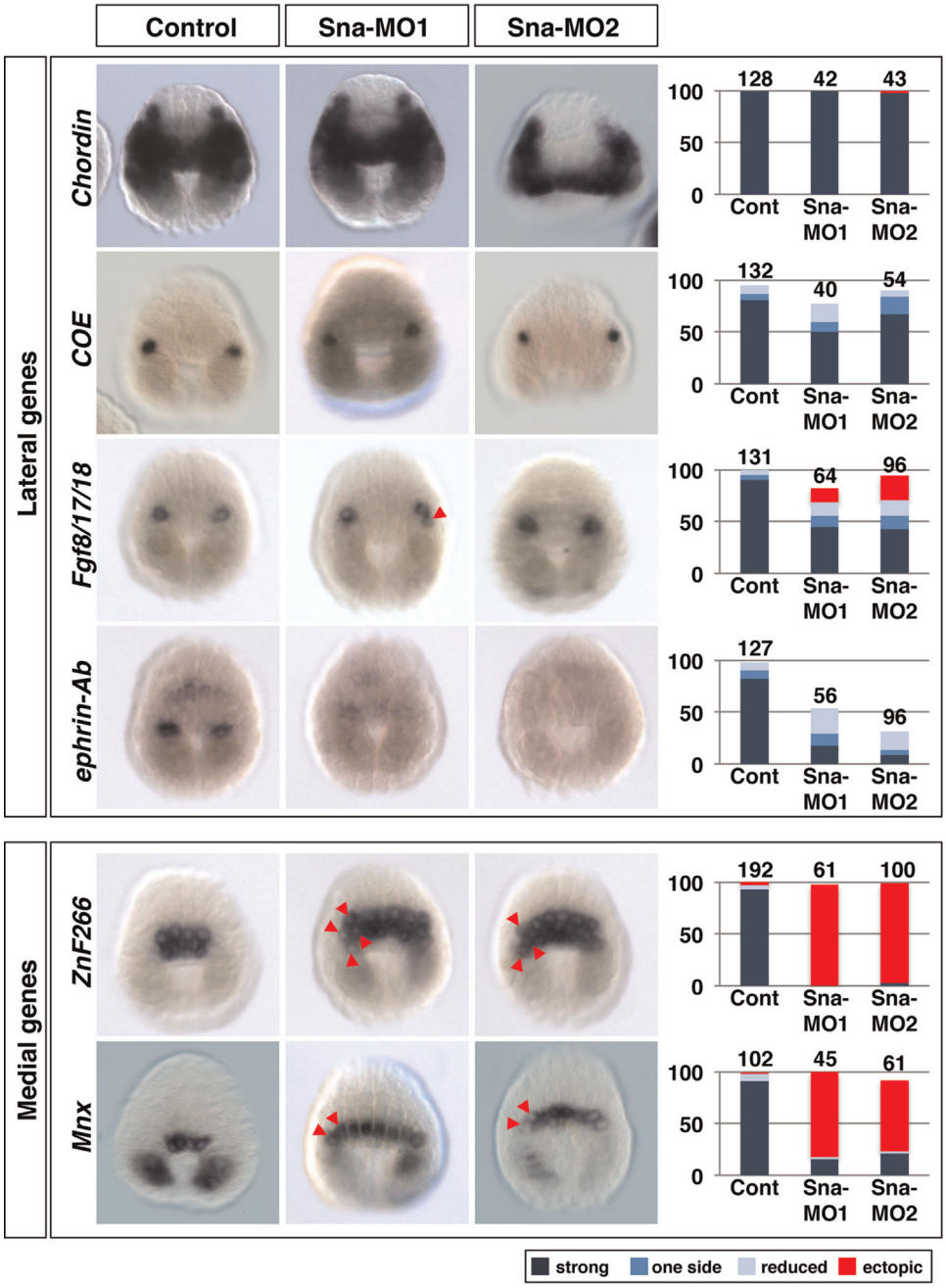

Fig. 2. The role of Snail during neural plate patterning. Genes expressed in the lateral neural plate are shown in the upper box and genes expressed in the medial neural plate in the lower box for this and all subsequent figures. Embryo treatment is indicated along the top of the panels and the gene analysed by in situ hybridisation to the left of the panels. Red arrowheads mark ectopic expression. The graphs depict the proportion of embryos with 'strong' (more than half of control expression domain), 'one-side', 'reduced' (less than half of control expression domain or lower levels of expression) or 'ectopic' neural plate expression, following the key indicated below the panels. The number above each bar on the graph indicates the number of embryos analysed.

Snail is required for maintenance of lateral neural genes

Expression of the lateral neural plate genes was largely unaffected by Snail knockdown, except for *ephrin-Ab* (Fig. 2). At tailbud stages, we analysed genes expressed in distinct territories of the caudal CNS, as described in Fig. 1B. In Snail morphants, we observed a strong repression of genes expressed in lateral neural plate derivatives (Fig. 3). Genes normally expressed in medial neural plate derivatives continued to be expressed, though it was difficult to assess if ectopic expression occurred, except for *Gbe1* in which an ectopic domain of expression was detected in the region of the trunk ganglion (Fig. 3). We analysed expression of the same gene set in embryos in which Nodal signalling was inhibited by treating embryos with SB431542, a pharmacological inhibitor of Nodal type 1 receptors (ALK4, 5 and 6). Loss of the cholinergic markers of the motor ganglion following Nodal inhibition have previously been reported (Hudson and Yasuo, 2005). Similarly to Snail inhibition, SB431542-treatment resulted in loss of genes that are expressed in derivatives of the lateral neural plate, with little effect on genes that are expressed in derivatives of the medial neural plate (Fig. 4) (Hudson and Yasuo, 2005). The only difference observed was for *Gbe1* expression, which is lost when Nodal signalling was inhibited, but not following Snail knockdown. This is most likely due to loss of *Delta2* expression following Nodal signal inhibition (Hudson and Yasuo, 2005). Delta2 is required for genes expressed in column 2 cells, which give rise to the posterior sensory vesicle where *Gbe1* is expressed (Fig. 1) (Cole and Meinertzhagen, 2004; Hudson et al., 2007). We conclude that Snail is required for maintenance of the lateral neural plate genetic programme.

Snail is sufficient to repress medial neural plate genes and induce ephrin-Ab

We next addressed the consequences of ectopic *Snail* expression. We analysed lateral and medial genes following overexpression of *Snail* in neural cells. This was achieved by using the upstream regulatory sequences of *ETR* to drive *Snail* expression throughout the a- and A-line neural cells from early gastrula stages (*pETR > Snail*). The results were compared to overexpression of *Nodal*, driven by the *FOG* upstream sequences, which drives expression in all animal cells from the 16-cell stage (Rothbächer et al., 2007). In *pETR > Snail*-electroporated embryos, lateral genes generally remained expressed in the correct domain (Fig. 5). However, *ephrin-Ab* expression was ectopically induced in other row-I cells following *Snail* overexpression (Fig. 5). In contrast to *Snail* overexpression, *Nodal* overexpression was able to induce ectopic expression of all lateral markers tested within their correct row of cells (Fig. 5) (Hudson et al., 2011; Mita and Fujiwara, 2007). All medial genes tested were strongly repressed by overexpression of either *Snail* or *Nodal* (Fig. 5). At tailbud stages, embryos in which *Snail* or *Nodal* was overexpressed exhibited a similar phenotype with repression of genes expressed in medial neural plate derivatives (Fig. 6). Genes normally expressed in lateral neural plate derivatives continued to be expressed (Fig. 6). It was difficult to assess if overexpression resulted in ectopic domains of

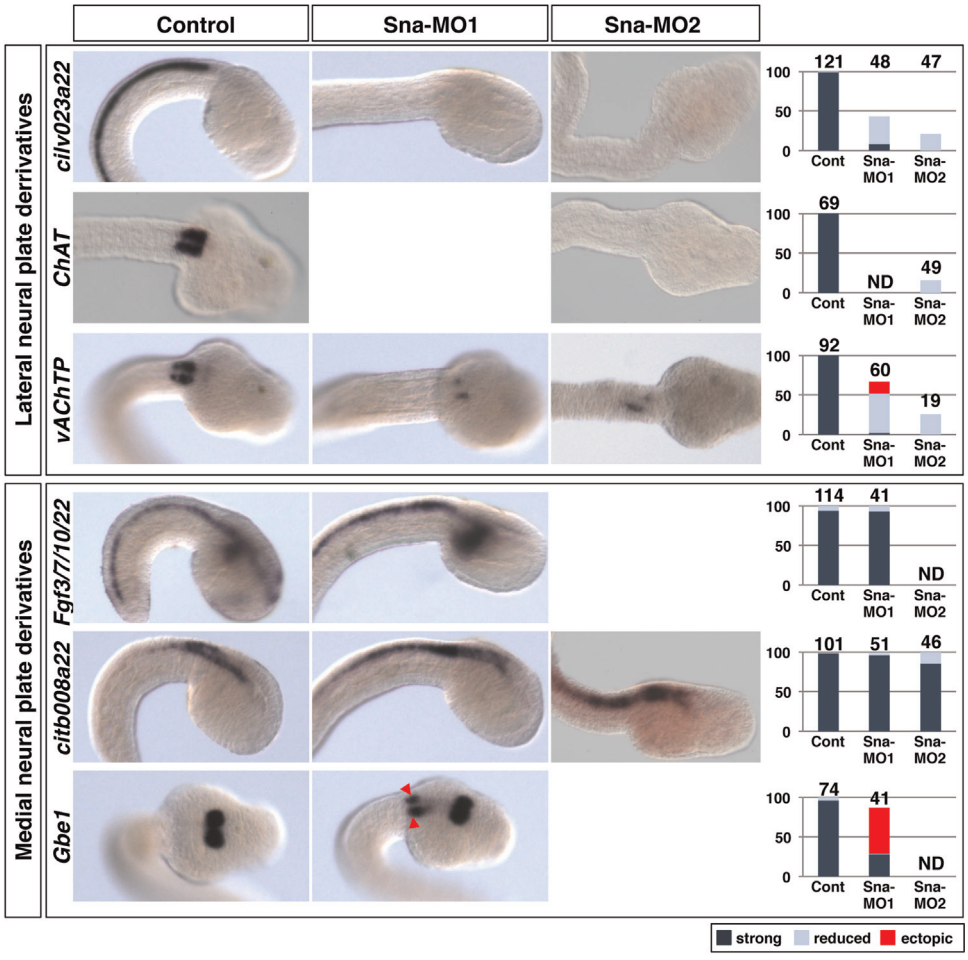

Fig. 3. Snail-knockdown affects neural tube patterning. Embryo treatment is indicated along the top of the panels and the gene analysed by in situ hybridisation to the left of the panels. The red arrowheads mark ectopic expression of *Gbe1* in the trunk ganglion region. The graphs depict the proportion of embryos with expression following the key indicated below the panels. The number above each bar on the graph indicates the number of embryos analysed. ND= not done.

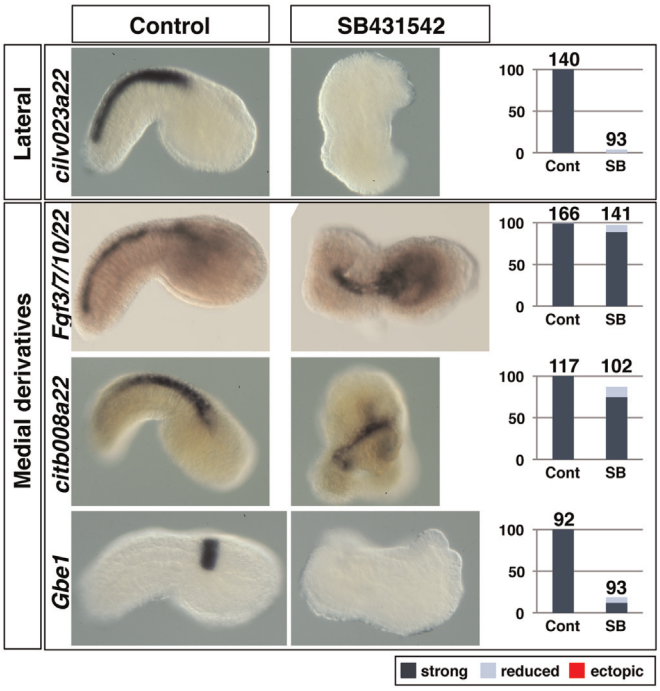

Fig. 4. Inhibition of Nodal signals affects neural tube patterning. Embryo treatment is indicated along the top of the panels and the gene analysed by in situ hybridisation to the left of the panels. The graphs depict the proportion of embryos with expression following the key indicated below the panels. The number above each bar on the graph indicates the number of embryos analysed.

lateral gene expression in these perturbed embryos, particularly since the electroporation procedure itself also affected gene expression to some extent (Fig. 6, cE). We conclude that overexpression of *Snail* has the opposite effect to knockdown of *Snail* and is sufficient to repress medial neural plate genes and to induce expression of *ephrin-Ab* at neural plate stages.

Snail overexpression can rescue ephrin-Ab expression and medial neural plate gene repression in Nodal signal-inhibited embryos

When Nodal signals are inhibited, all lateral neural plate genes are repressed and medial gene expression is expanded into the lateral neural plate (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007). Part of this phenotype is likely due to loss of *Snail* expression. In order to address to what extent Snail mediates Nodal-dependent medial–lateral neural patterning, we attempted to rescue Nodal inhibition by *Snail* overexpression. This was carried out by electroporating embryos with *pETR > Snail* and treating them with SB431542 (Fig. 5). Consistent with previous results, SB431542-treatment alone resulted in loss of all lateral gene expression and ectopic expression of medial genes in the lateral neural plate (Fig. 5) (Hudson and Yasuo, 2005; Hudson et al., 2007). However, in SB431542-treated embryos that were also electroporated with *pETR > Snail,* expression of *eprhin-Ab*, but not other lateral genes, was recovered, and medial neural plate genes were repressed (Fig. 5). Thus, in the gene regulatory network of medial–lateral neural plate patterning that is triggered by Nodal signals, Snail appears to mediate induction of *ephrin-Ab* expression and repression of medial neural plate genes.

Discussion

Nodal signalling plays a major role during medial–lateral A-line neural plate patterning where it is required for all lateral fates and to restrict medial neural plate fates (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007). Here, we show that Snail, acting within the lateral neural plate, mediates Nodal-dependent repression of medial neural plate fates as well as induction of *ephrin-Ab* in the column 3/row I cell (A9.29) (Fig. 7). The transcriptional repression of medial genes and positive regulation of *ephrin-Ab* by Snail suggest that ascidian Snail may act as both a repressor and activator as it does in other systems (Hemavathy et al., 2000; Nieto, 2002; Reece-Hoyes et al., 2009; Rembold et al., 2014; Sakai et al., 2006). Alternatively Snail could activate *ephrin-Ab* indirectly, via the repression of a gene that encodes a repressor of *ephrin-Ab*. Further work is required to ascertain if any of these ascidian targets are regulated directly or indirectly by Snail. The observation that overexpressed Snail binds directly to the cis-regulatory elements of two medial genes, *Mnx* and *FoxAa*, suggests that at least some of these transcriptional regulations may be direct (Imai et al., 2009; Kubo et al., 2010). Later in development, lateral neural tube genes are not expressed in Snail-morphant embryos, indicating that Snail has a role in maintaining lateral neural identity. This failure to maintain lateral neural identity may be an indirect consequence of the ectopic expression of medial genes in Snail-knockdown embryos, since medial genes themselves may encode repressors of lateral genes. One such candidate gene could be *Mnx*, which encodes a transcription factor and has been shown to have a repressive role during neural patterning in vertebrates (Thaler et al., 1999; William et al., 2003). The role of transcriptional repressors during neural patterning would be particularly interesting to address in future studies since cross-repressive interactions between transcription factors play an important role during patterning of the vertebrate neural tube (Briscoe and Novitch, 2008).

Evolution of the role of Snail during neural patterning

In both vertebrates and invertebrates, Snail is involved in mesoderm formation and in tissues undergoing epithelial–mesenchyme transition, including the neural crest (Hemavathy et al., 2000; Manzanares et al., 2001; Nieto, 2002). However, many other domains of expression are also observed in a species specific manner. In terms of early CNS development, in most vertebrates, *Snail* expression is restricted to the neural crest precursors situated at the neural plate border. The expression of *Snail* in the ascidian lateral neural plate is much broader than the neural crest specific expression observed in vertebrates, so it is probably unlikely that in *Ciona* the *Snail* expression domain delimits a pre-crest territory in its entirety. The expression pattern in ascidians appears more similar to that observed in amphioxus and lamprey in which *Snail* is also detected within the neural plate (Langeland et al., 1998; Rahimi et al., 2009). Interestingly, *Snail* is also expressed in the pigment cell precursors of *Ciona*, in the a-line derived part of the neural plate (Imai et al., 2006). These cells have recently been described as a potential evolutionary precursor to the vertebrate neural crest (Abitua et al., 2012) and it will be important in future studies to address the function of Snail in this cell type. One possible evolutionary scenario is that Snail was broadly employed during neural plate patterning, becoming restricted to a role in neural crest development in the vertebrate lineage. Alternatively, Snail may have been independently recruited in the neural plate of ascidians. It is clear that a much wider species sampling would be required to resolve this issue.

The gene regulatory network for A-line neural cell patterning

This study indicates that the Snail transcription factor mediates one of the first fate subdivisions that take place across the medial–lateral axis of the ascidian neural plate. Nodal signals subdivide

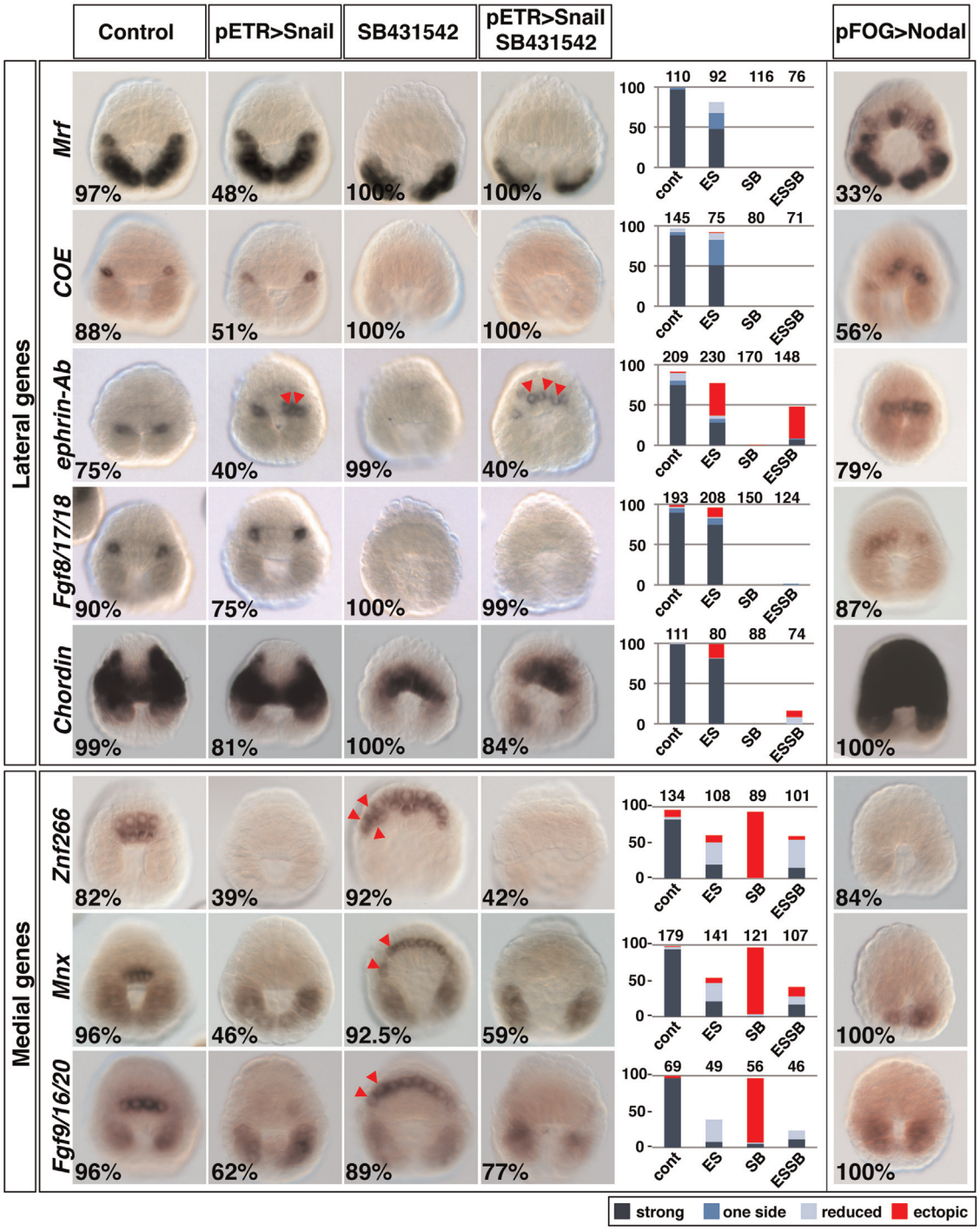

Fig. 5. Effects of *Snail* overexpression on medial–lateral neural plate patterning. Embryo treatment is indicated along the top of the panels and gene analysed by in situ hybridisation to the left of the panels. The percentages on the panels indicate the proportion of embryos that the panel represents. Red arrowheads indicate ectopic expression. The graphs depict the proportion of embryos with expression following the key indicated below the panels, which is described in Fig. 2 (cont=control; ES=pETR > Snail; SB=SB431542-treatment; ESSB=pETR > Snail plus SB431542-treatment). The number above each bar on the graph indicates the number of embryos analysed. On the right are *pFOG > Nodal*-electroporated embryos. The percentages on the panels indicate the proportion of embryos that the panel represents. The graphs showing the embryo counts for this data have been previously published (Hudson et al., 2011), except for *Znf266* and *Chordin*. The data for these genes is as follows: *Chordin*- control embryo 100% positive ($n=28$), pFOG > Nodal embryo 100% ectopic ($n=39$); *ZnF266*- control embryo 100% positive ($n=39$), pFOG > Nodal embryo 84% no expression in neural plate and 16% reduced expression ($n=32$).

the neural plate into a broad medial (column 1/2) and lateral (column 3/4) fate by promoting column 3/4 fates and repressing column 1/2 fates. Snail is largely responsible for the repressive function of Nodal signals, being required and sufficient to repress column 1/2 (medial) fates. Other factors immediately downstream of Nodal, including Delta2, refine the initial pattern established by Nodal, leading to the subdivision of the neural plate into its four columns (Hudson et al., 2007). Superimposed upon this medial–lateral patterning, FGF/ERK signals pattern the A-line neural plate cells along the anterior–posterior axis. FGF/ERK signals are differentially activated in the A-neural lineage with only row I cells exhibiting ERK1/2 activation, although the mechanism governing the differential response to FGF between row I and row II cells remains to be addressed (Hudson et al., 2007). The patterning

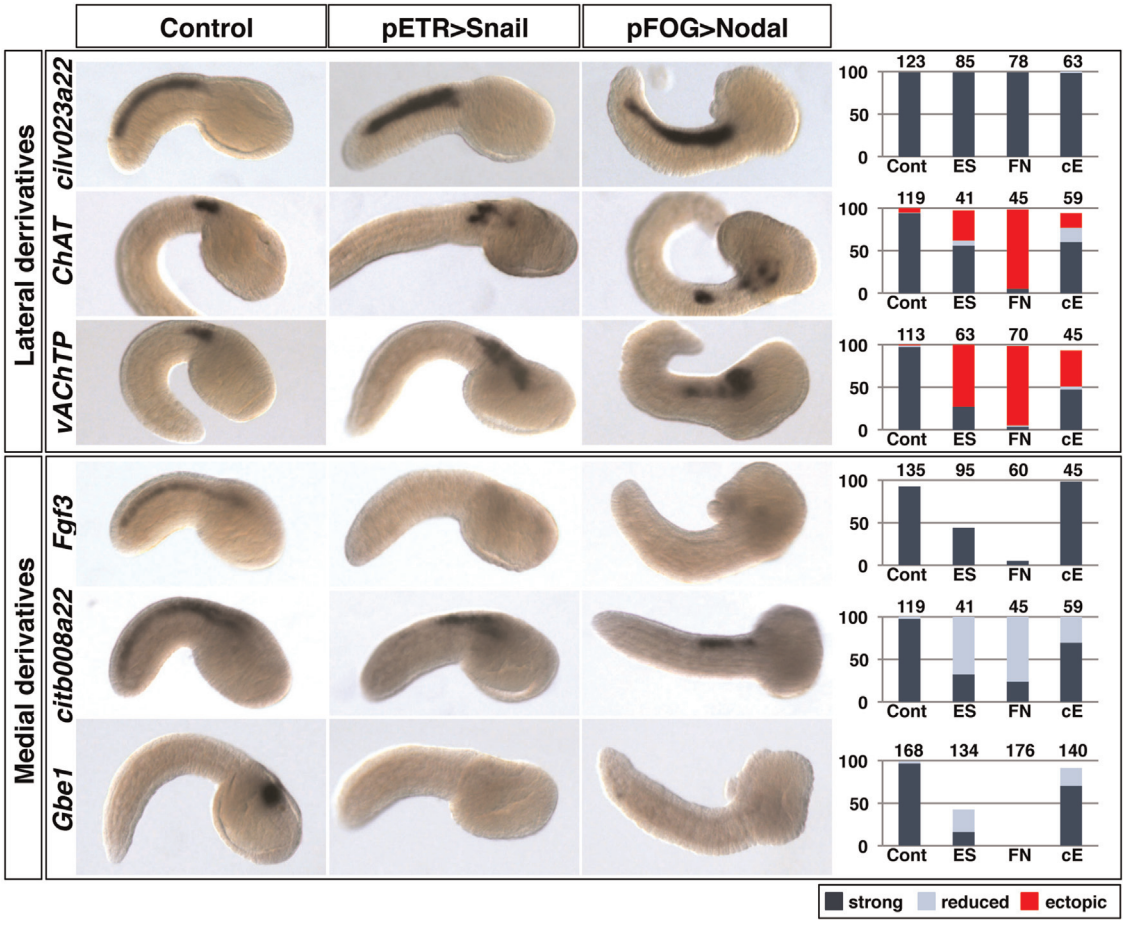

Fig. 6. Effects of overexpression of *Snail* or *Nodal* on neural tube genes. Embryo treatment is indicated along the top of the panels and the gene analysed by in situ hybridisation to the left of the panels. *Fgf3*=*Fgf3/7/10/22*. The graphs depict the proportion of embryos with expression following the key indicated below the panels (Cont=Control; ES=pETR > Snail; FN=pFOG > Nodal; cE=control electroporation with pETR > lacZ). The number above each bar on the graph indicates the number of embryos analysed.

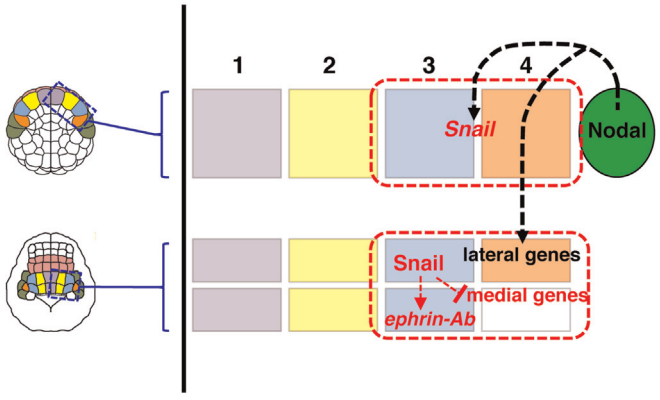

Fig. 7. Summary of the role of Snail during neural plate patterning. *Snail* is induced by Nodal signals in the lateral neural plate lineage and is required to repress medial neural plate genes as well as induce expression of *ephrin-Ab* in row I/column 3. The area boxed with the red dotted line represents the *Snail* expression territory. The genetic interactions are represented by dotted lines to indicate that the interaction could be direct or indirect. See text for details.

mechanisms operating along the two axes collectively lead to a unique gene expression code for each of the 8 distinct cell identities present in the posterior neural plate (Hudson et al., 2007; Imai et al., 2004, 2006). These factors themselves go on to pattern further the neural lineages (Imai et al., 2009) and so on, ultimately leading to the myriad of cell types present in the simplest of chordate central nervous systems.

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