Developmental Biology 403 (2015) 172–179

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

# Snail mediates medial–lateral patterning of the ascidian neural plate

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ARTICLE INFO

*Article history:*
Received 13 March 2015
Received in revised form
20 April 2015
Accepted 24 April 2015
Available online 8 May 2015

*Keywords:*
Ascidian
Neural patterning
Snail
Nodal
CNS

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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http://dx.doi.org/10.1016/j.ydbio.2015.04.026
0012-1606/

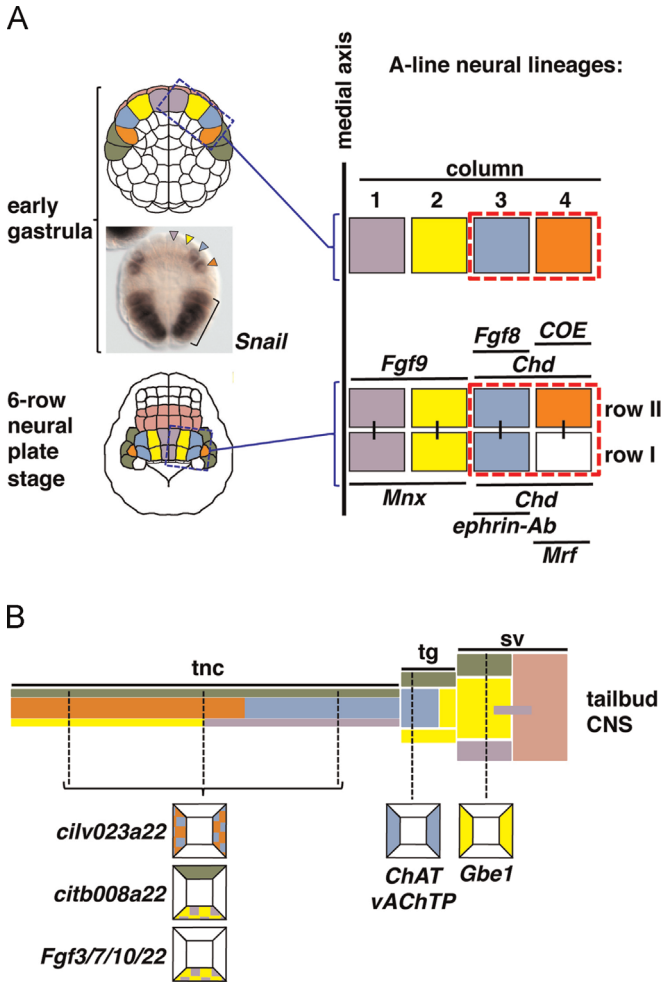


**Fig. 1.** Cell lineages and gene expression in the A-line derived part of the CNS. (A) Adapted from Hudson et al. (2007). The drawing at the top represents an early gastrula stage embryo with the different CNS lineages coloured: b-line (green); a-line (pink); A-line -column 1 (A8.7 lineage, lilac), -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.