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This article was published in DEVELOPMENT :

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Development 2016 : doi: 10.1242/dev.142307

Patterning of brain precursors in ascidian embryos

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Short title: Ascidian brain patterning

Key words: Ascidian, Ciona, brain, sensory vesicle, neural patterning

SUMMARY

In terms of their embryonic origins, the anterior and posterior parts of the ascidian central nervous system (CNS) are associated with distinct germ layers. The anterior part of the sensory vesicle, or brain, originates from ectoderm lineages following a neuro-epidermal binary fate decision. In contrast, a large part of the remaining posterior CNS is generated following neuro-mesodermal binary fate decisions. Here, we address the mechanisms that pattern the anterior brain precursors along the medial-lateral axis (future dorsal-ventral) at neural plate stages. Our functional studies show that Nodal signals are required for induction of lateral genes including *Delta-like, Snail, Msxb* and *Trp*. Delta-like/Notch signalling induces intermediate (*Gsx)* over medial (*Meis)* gene expression in intermediate cells, while the combinatorial action of Snail and Msxb prevents the expression of *Gsx* in lateral cells. We conclude that despite the distinct embryonic lineage origins within the larval CNS, the mechanisms that pattern neural precursors are remarkably similar.

INTRODUCTION

The chordate super-phylum is characterised by a well patterned dorsal tubular central nervous system (CNS) (Satoh et al., 2014). Ascidians belong to the urochordates, or tunicates, a phylum of invertebrate chordates closely related to vertebrates (Delsuc et al., 2006; Satoh et al., 2014). Ascidian embryos develop with very few numbers of cells and a fixed cell lineage, features enabling the step-by-step analysis of developmental cell fate choices with a single-cell level of precision (Hudson, 2016).

Founder cell lineages of the ascidian embryo are established at the 8-cell stage, when the embryo divides along the animal-vegetal axis to produce two pairs of animal cells (the a- and b-lineages) and two pairs of vegetal cells (the A- and B-lineages). The CNS arises from the a-, b- and A-lineages (Nicol and Meinertzhagen, 1988a; Nicol and Meinertzhagen, 1988b; Nishida, 1987). The anterior-most part of the sensory vesicle, including the pigmented cells, has an a-lineage origin and thus shares a common origin with anterior epidermis. The dorsal most cells of the remaining CNS arise from the b-lineage with the rest of the CNS arising from the A-lineage cells, which share a common lineage origin with mesoderm (notochord). At mid-gastrula stages, A- and a-lineage CNS precursors are arranged in a neural plate that consists of six rows of cells along the anterior-posterior axis, such that row I is the most posterior and row VI the most anterior (Figure 1A). The posterior-most two rows (I-II) of cells are A-lineage, and the anterior four rows (III-VI) of cells are a-lineage. Cells are aligned in columns along the medial-lateral axis, with column 1 the medial-most pair of columns and column 3 the lateral-most, though the A-lineage has an additional forth column. The b-lineage cells are positioned lateral to this grid-like array. Of the four rows of a-lineage cells, only rows III and IV will actually contribute to the CNS, generating the anterior part of the sensory vesicle, the ascidian ‘brain’, and contributing to the oral siphon primordium (Christiaen et al., 2007; Cole and Meinertzhagen, 2004; Nishida, 1987; Taniguchi and Nishida, 2004; Veeman et al., 2010). Rows V and VI will form a specialised region of anterior epidermis, including a placode-like territory and the palps (Abitua et al., 2015; Nishida, 1987).

Patterning of the A-lineage derived neural plate involves combinatorial inputs of FGF/ERK, Nodal, and two temporally separable Delta/Notch signals (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007). Each cell, present on both sides of the bilaterally symmetrical embryo, receives a unique combination of these three signalling pathways, which determine the eight distinct cell types (Hudson et al., 2007). Like the A-lineage derived neural plate, differential FGF/ERK signalling also patterns the a-lineage derived neural plate along its anterior-posterior axis. Specifically, FGF/ERK signalling is required to promote row III over row IV cell identities (Haupaix et al., 2014; Racioppi et al., 2014). Similarly, like in the A-lineage neural plate, Nodal signalling is implicated in specification of the lateral part of the a-lineage neural plate, as lateral gene expression is lost in the a-lineage cells when Nodal signalling is inhibited (Hudson and Yasuo, 2005; Imai et al., 2006; Ohtsuka et al., 2014). In this study, we investigate in detail the mechanisms responsible for patterning of the a-lineage row III brain precursors of *Ciona* embryos.

RESULTS AND DISCUSSION

Nodal is required for medial-lateral patterning of the a-lineage derived neural plate

In order to investigate patterning of the ascidian brain precursors, we used a set of three genes, *Trp*, *Gsx* and *Meis*, which label row III cells in columns 3 (lateral), 2 (intermediate), and 1 (medial), respectively, at neurula stages. The expression of *Trp* and *Meis* was analysed at the neurula stage (approximately 8.25 hours of development at 18°C), when all of the 6-row neural plate cells have divided along the A-P axis (Figure 1A). *Trp* is expressed in column 3, with stronger expression in the posterior cell, a10.97, while *Meis* is expressed in column 1, with stronger expression in the posterior cell, a10.73 (Figures 1A, 2A). *Gsx* expression was analysed in slightly earlier neurula stage embryos (7.5 hours of development at 18°C), when it is expressed in both row IIIa and row IIIp (a10.66 and a10.65 respectively), because at 8+ hours of development *Gsx* also starts to be expressed in column 1.

We first investigated the role of Nodal during medial-lateral patterning of the a-lineage derived neural plate. From the 32-cell stage Nodal is expressed in cells that contact the lateral-most a-lineage neural precursors (Figure 1B). To inhibit Nodal activity, we treated embryos with a pharmacological inhibitor of TGFβ type I receptors ALK4, 5 and 6 (SB431542) or inhibited *Nodal* mRNA translation by injection of anti-sense morpholino oligonucleotides (Nodal-MO) (Figure 2A). These treatments resulted in loss of *Trp* expression from column 3. *Gsx* expression in column 2 was also strongly reduced following Nodal signal inhibition. However, in many embryos, while expression of *Gsx* was lost from column 2, we observed its ectopic expression in column 3 (Figure 2A). Thus, Nodal is required both to promote *Gsx* expression in column 2 as well as inhibit its expression in column 3. In Nodal-inhibited embryos, *Meis* was ectopically expressed in column 2 of most embryos (88% Nodal-MO; 96% SB431542-treated) and in column 3 in a proportion of embryos (18% Nodal-MO; 27% SB431542-treated). Overexpression of *Nodal* had the opposite effect to inhibition of Nodal (Figure 2A). We overexpressed Nodal using the upstream regulatory sequences of *FOG* (*pFOG>Nodal*) to drive expression of Nodal throughout the animal hemisphere from the 16-cell stage of development (Hudson et al., 2015; Pasini et al., 2006; Rothbächer et al., 2007). This led to ectopic expression of *Trp* throughout the row III daughters and loss of both *Gsx* and *Meis* expression (Figure 2A). Thus Nodal promotes column 3 identity and represses column 1 and 2 identity. Taken together, we conclude that Nodal signals are required for the correct specification of both columns 2 and 3 and to repress medial column gene expression in lateral cells.

Delta/Notch specifies column 2 over column 1 fates.

One of the transcriptional targets of Nodal signals, *Delta-like* (previously *Delta2*), is expressed in b-lineage neural precursors as well as a vegetal A-lineage cell at the 64-cell stage (Figure 1B). At the early gastrula stage, *Delta-like* is expressed in the lateral A-lineage neural precursors and b-line cells and later, at neural plate stage, it is expressed in the lateral borders of the neural plate (Figure 1B). Thus, from the 64-cell stage, *Delta-like* expressing cells are in contact with lateral a-lineage precursors. Notch receptor transcripts are present ubiquitously during early cleavage stages with expression detected from the late gastrula stage in the developing nervous system (Imai et al., 2004). Consistent with a role for Notch signalling during patterning of the a-lineage derived neural plate, *Hesb*, a transcriptional target of Delta-like/Notch signals, is expressed in both column 2 and 3 of row III (Hudson et al., 2007). To inhibit Delta-like/Notch signalling, we treated embryos from the 76-cell stage with DAPT, an inhibitor of gamma-secretase, an enzyme required for Notch receptor processing. Alternatively, we injected mRNA encoding a dominant negative form of Suppressor of Hairless, a transcription factor known to mediate Notch signalling. Either of these treatments resulted in a strong reduction in *Gsx* expression and concomitantly, ectopic expression of *Meis* in column 2 (Figure 2B). Overexpression of *Delta-like,* by electroportation of *pFOG>Delta-like*, had the opposite effect; expression of *Meis* was lost and ectopic expression of *Gsx* was observed in column 1 (Figure 2B). This data indicates that Delta-like-Notch signals promote column 2 fates at the expense of column 1 fates in the a-lineage neural plate.

Snail and Msxb repress *Gsx* in column 3

So far we have shown that Nodal signals are required for the correct specification of the column 2 and 3 cells and to repress medial gene expression in the lateral neural plate, whereas Notch signalling specifies column 2 over column 1 cell identity. Based on *Hesb* expression, column 3 cells also respond to Delta-like/Notch signalling, yet they do not express *Gsx*. We hypothesised that a factor, induced by Nodal in column 3 cells, acts to repress *Gsx* expression in response to Notch signals. Snail, which encodes a transcription factor that can act as a repressor (Nieto, 2002) would be a good candidate for the repression of *Gsx* transcription in column 3. Indeed, Snail has been shown to mediate Nodal-dependent repression of medial genes in the A-lineage derived neural plate (Hudson et al., 2015; Imai et al., 2006). Furthermore, *Snail* is expressed downstream of Nodal in the row III/column 3 precursor at the 6-row neural plate stage (Figure 1B; S1). In order to address the role of Snail, we knocked it down using Snail-MO or overexpressed it throughout the neural plate using the *ETR* promoter (*pETR>Snail*) (Figure 3A) (Hudson et al., 2015). Overexpression of Snail resulted in down-regulation of both *Meis* and *Gsx* (Figure 3A). Knockdown of *Snail* resulted in a down-regulation of *Trp*, but only a very occasional ectopic expression of *Gsx* in column 3 (Figure 3A). However, we saw strong ectopic expression of *Gsx* in column 3 of Snail-MO injected embryos when analysed at the 6-row neural plate stage (Figure 3B). This suggests that Snail represses *Gsx* in column 3 at the 6-row neural plate stage, but that other factors act, during later neurula stages, to repress *Gsx* in column 3. One candidate is *Msxb*, which is expressed a little later than *Snail* in a9.49 (row III/column 3) (Figure 1B). *Msxb* expression in a-lineage column 3 is also downstream of Nodal (Figure S1), as has been shown previously for b-lineage *Msxb* expression (Roure et al., 2014). Using Msxb-MOs, we found that while knockdown of *Msxb* alone had no effect on *Gsx* expression, combined inhibition of both *Msxb* and *Snail* resulted in strong ectopic expression of *Gsx* in column 3 at the neurula stage (Figure

3C; Figure S2A). Thus, Snail and Msxb both act, downstream of Nodal, to repress *Gsx* expression in the column 3 cells.

Conclusion

Our data are consistent with the following model (Figure 4A). Medial-lateral patterning of the a-lineage neural plate, much like medial-lateral patterning of the A-lineage neural plate, depends upon patterning mechanisms initiated by Nodal signals. Nodal is required for correct specification of columns 2 and 3 and to prevent ectopic expression of medial genes in the lateral neural plate. Nodal induces expression of *Delta-like*, and Notch signals are required to specify column 2 over column 1 fates. In column 3, Nodal-dependent expression of *Snail* and *Msxb* is required to repress *Gsx* expression in column 3. We conclude that despite the distinct lineage origins of the anterior and posterior nervous system, these cells are subsequently patterned by very similar mechanisms (Figure 4B).

Patterning across the medial-lateral (future ventral-dorsal) axis of the neural plate in ascidians involves distinct signalling molecules compared to vertebrates (Dessaud et al., 2008; Hudson et al., 2007; Hudson et al., 2011; Urbach and Technau, 2008). Nonetheless, for many genes, the order of transcription factor gene expression along this axis appears well conserved (e.g dorsal *Snail* and *Msx*, intermediate *Gsx*, ventral *FoxAa*) (Corbo et al., 1997). Indeed, for some genes, their relative dorsal-ventral expression order may be traceable to the bilaterian ancestor (Buresi et al., 2016; Cornell and Ohlen, 2000; Denes et al., 2007; Urbach and Technau, 2008; Winterbottom et al., 2010).

MATERIALS AND METHODS

Overexpression and knockdown tools

Mopholinos for *Snail* (MO1), *Nodal* and *Msxb* and *Ciona* Su(H)^{DBM} are described previously (Hudson and Yasuo, 2005; Hudson and Yasuo, 2006; Hudson et al., 2015; Imai et al., 2006; Roure and Darras, 2016). SB431542 (Tocris) and DAPT (Calbiochem) treatments have been described previously (Hudson and Yasuo, 2005; Hudson and Yasuo, 2006). SB431542 was added to embryos at the 16 or 32 cell stage and DAPT at the 76-cell stage. Although previously DAPT gave consistent results (Hudson et al., 2007), recent lots purchased did not give consistent phenotypes among different batches of embryos. We therefore treated batches of embryos and analysed them at the 6-row stage for *Ebf* (previously *COE*) expression (should be lost) and *Foxb* (previously *FoxB*) expression (should be ectopically expressed in column 2) (Hudson et al., 2007). Only batches of embryos that gave the expected result were processed further. Su(H)^{DBM} on the other hand, gave consistent results in all experiments. The electroporation constructs *pFOG>Nodal*, *pFOG<Delta-like* and *pETR>Snail* have been previously described (Hudson et al., 2007; Hudson et al., 2015; Pasini et al., 2006).

Embryological experiments.

Adult *Ciona intestinalis* were purchased from the Station Biologique de Roscoff (France) or from Stazione Zoologica Anton Dohrn (Italy). Blastomere names, lineage and the fate maps are previously described (Conklin, 1905; Nishida, 1987). Ascidian embryo culture and microinjection have been described (Sardet et al., 2011). All microinjections were carried out in unfertilised eggs. The electroporation protocol was based on (Christiaen et al., 2009). All data were pooled from at least two independent experiments (i.e. on different batches of embryos). For data shown in Figure S2, embryos were first injected with Snail-MO or Snail+Msxb-MO. Uninjected, or MO-injected embryos were then split into two groups and one group injected with *Ciona* Su(H)^{DBM} RNA. After fertilisation and culturing to neurula stages, the uninjected and MO-injected embryos were further divided into two groups for *Meis* and *Gsx* analysis.

In situ hybridisation

Gene markers used for in situ hybridisation have been described (Aniello et al., 1999; Hudson and Lemaire, 2001; Imai et al., 2004) (http://ghost.zool.kyoto-u.ac.jp) and named according to recent guidelines (Stolfi et al., 2015). The *Ciona TRP* (*L-dopachrome tautomerase*) used corresponds to the Genebank entry reported previously (Hudson et al., 2003). In situ hybridisation was carried out and photographed as described (Hudson and Yasuo, 2006; Hudson et al., 2013; Hudson et al., 2016; Wada et al., 1995).

ACKNOWLEDGEMENTS

We thank Nori Satoh and colleagues for the *Ciona* gene collection plates and Sébastian Darras for the Msxb-MO. The group of H.Y is supported by the Centre National de la Recherche Scientifique (CNRS), the Université Pierre et Marie Curie, and the Agence Nationale de la Recherche (ANR-09-BLAN-0013-01). Work by RE in the laboratory of HY was supported by an EMBO short term fellowship (ASTF 534-2014). Work by RE and AP in the laboratory of A.S has been supported by SZN PhD fellowships.

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

CH, HY, AS, RE conception and design of the project. CH, HY, RE, AP, CS, AS, acquisition analysis and interpretation of data. CH drafting and all authors revising the article.

REFERENCES

Abitua, P. B., Gainous, T. B., Kaczmarczyk, A. N., Winchell, C. J., Hudson, C., Kamata, K., Nakagawa, M., Tsuda, M., Kusakabe, T. G. and Levine, M. (2015). The pre-vertebrate origins of neurogenic placodes. *Nature*.

Aniello, F., Locascio, A., Villani, M. G., Di Gregorio, A., Fucci, L. and Branno, M. (1999). Identification and developmental expression of Ci-msxb: a novel homologue of Drosophila msh gene in Ciona intestinalis. *Mech. Dev.* **88**, 123–126.

Buresi, A., Andouche, A., Navet, S., Bassaglia, Y., Bonnaud-Ponticelli, L. and Baratte, S. (2016). Nervous system development in cephalopods: How egg yolk-richness modifies the topology of the mediolateral patterning system. *Dev. Biol.* **415**, 143–156.

Christiaen, L., Jaszczyszyn, Y., Kerfant, M., Kano, S., Thermes, V. and Joly, J.-S. (2007). Evolutionary modification of mouth position in deuterostomes. *Semin. Cell Dev. Biol.* **18**, 502–511.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009). Electroporation of transgenic DNAs in the sea squirt Ciona. *Cold Spring Harb. Protoc.* **2009**, pdb.prot5345.

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 Phila.* 1–119.

Corbo, J. C., Erives, A., Di Gregorio, A., Chang, A. and Levine, M. (1997). Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. *Dev. Camb. Engl.* **124**, 2335–2344.

Cornell, R. A. and Ohlen, T. V. (2000). Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* **10**, 63–71.

Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965–968.

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.

Dessaud, E., McMahon, A. P. and Briscoe, J. (2008). Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* **135**, 2489–2503.

Haupaix, N., Abitua, P. B., Sirour, C., Yasuo, H., Levine, M. and Hudson, C. (2014). Ephrin-mediated restriction of ERK1/2 activity delimits the number of pigment cells in the Ciona CNS. *Dev. Biol.* **394**, 170–180.

Hudson, C. (2016). The central nervous system of ascidian larvae. *Wiley Interdiscip. Rev. Dev. Biol.* **doi: 10.1002/wdev.239**,.

Hudson, C. and Lemaire, P. (2001). Induction of anterior neural fates in the ascidian Ciona intestinalis. *Mech. Dev.* **100**, 189–203.

Hudson, C. and Yasuo, H. (2005). Patterning across the ascidian neural plate by lateral Nodal signalling sources. *Dev. Camb. Engl.* **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. *Dev. Camb. Engl.* **133**, 2855–2864.

Hudson, C., Darras, S., Caillol, D., Yasuo, H. and Lemaire, P. (2003). A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian Ciona intestinalis. *Dev. Camb. Engl.* **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. *Dev. Camb. Engl.* **134**, 3527–3537.

Hudson, C., Ba, M., Rouvière, C. and Yasuo, H. (2011). Divergent mechanisms specify chordate motoneurons: evidence from ascidians. *Dev. Camb. Engl.* **138**, 1643–1652.

Hudson, C., Kawai, N., Negishi, T. and Yasuo, H. (2013). β-Catenin-Driven Binary Fate Specification Segregates Germ Layers in Ascidian Embryos. *Curr. Biol. CB* **23**, 491–495.

Hudson, C., Sirour, C. and Yasuo, H. (2015). Snail mediates medial-lateral patterning of the ascidian neural plate. *Dev. Biol.* **403**, 172–179.

Hudson, C., Sirour, C. and Yasuo, H. (2016). Co-expression of Foxa.a, Foxd and Fgf9/16/20 defines a transient mesendoderm regulatory state in ascidian embryos. *eLife* **5**,.

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. *Dev. Camb. Engl.* **131**, 4047–4058.

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. *Dev. Camb.*

Engl. **136**, 285–293.

Longabaugh, W. J. R., Davidson, E. H. and Bolouri, H. (2005). Computational representation of developmental genetic regulatory networks. *Dev. Biol.* **283**, 1–16.

Mita, K. and Fujiwara, S. (2007). Nodal regulates neural tube formation in the Ciona intestinalis embryo. *Dev. Genes Evol.* **217**, 593–601.

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.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155–166.

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.

Ohtsuka, Y., Matsumoto, J., Katsuyama, Y. and Okamura, Y. (2014). Nodal signaling regulates specification of ascidian peripheral neurons through control of the BMP signal. *Dev. Camb. Engl.* **141**, 3889–3899.

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.

Racioppi, C., Kamal, A. K., Razy-Krajka, F., Gambardella, G., Zanetti, L., di Bernardo, D., Sanges, R., Christiaen, L. A. and Ristoratore, F. (2014). Fibroblast growth factor signalling controls nervous system patterning and pigment cell formation in Ciona intestinalis. *Nat. Commun.* **5**, 4830.

Rothbächer, U., Bertrand, V., Lamy, C. and Lemaire, P. (2007). A combinatorial code of maternal GATA, Ets and beta-catenin-TCF transcription factors specifies and patterns the early ascidian ectoderm. *Dev. Camb. Engl.* **134**, 4023–4032.

Roure, A. and Darras, S. (2016). Msxb is a core component of the genetic circuitry specifying the dorsal and ventral neurogenic midlines in the ascidian embryo. *Dev. Biol.* **409**, 277–287.

Roure, A., Lemaire, P. and Darras, S. (2014). An otx/nodal regulatory signature for posterior neural development in ascidians. *PLoS Genet.* **10**, e1004548.

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. Clifton NJ* **770**, 365–400.

Satoh, N., Rokhsar, D. and Nishikawa, T. (2014). Chordate evolution and the three-phylum system. *Proc. Biol. Sci.* **281**, 20141729.

Stolfi, A., Sasakura, Y., Chalopin, D., Satou, Y., Christiaen, L., Dantec, C., Endo, T., Naville, M., Nishida, H., Swalla, B. J., et al. (2015). Guidelines for the nomenclature of genetic elements in tunicate genomes. *Genes. N. Y. N 2000* **53**, 1–14.

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.

Urbach, R. and Technau, G. M. (2008). Dorsoventral patterning of the brain: a comparative approach. *Adv. Exp. Med. Biol.* **628**, 42–56.

Veeman, M. T., Newman-Smith, E., El-Nachef, D. and Smith, W. C. (2010). The ascidian mouth opening is derived from the anterior neuropore: reassessing the mouth/neural tube relationship in chordate evolution. *Dev. Biol.* **344**, 138–149.

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.

Winterbottom, E. F., Illes, J. C., Faas, L. and Isaacs, H. V. (2010). Conserved and novel roles for the Gsh2 transcription factor in primary neurogenesis. *Dev. Camb. Engl.* **137**, 2623–2631.

FIGURE LEGENDS

Figure 1. Expression patterns of genes analysed in this study. A) Schematic drawings of 6-row neural plate stage and mid-neurula stage highlighting the different columns of row III. *Trp, Gsx* and *Meis* are expressed within distinct columns of row III. *Trp* and *Gsx* expression begins at the 6-row neural plate stage, whereas *Meis* expression is first detected at the neurula stage. B) Sequential activation of *Nodal*, *Delta-like*, *Snail* and *Msxb* during the 32-cell stage to 6-row neural plate stage, based on data in Figure S1 and (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2009; Roure et al., 2014). The stage and orientation of the embryo in each drawing is indicated below each column. Animal pole is to the right for the 32-, 64- and 112-cell stage embryo drawings. Gene expression is indicated by black dots, with weaker expression represented by grey dots. The a-lineage neural plate cells that generate the CNS are coloured in red.

Figure 2. Nodal and Notch pattern the a-lineage CNS precursors. A-B) Marker analysed is indicated to the left, embryo treatment indicated above the columns. All embryos are at neurula stage in dorsal view. Red arrowheads or brackets indicate ectopic expression. Some embryos are stained with DAPI to confirm cell identification. The graphs show the percentage of embryos in each category of expression following the key below. The hashed blue/red bars for *Gsx* expression in (A) indicate that at least one column 2 and one column 3 cell exhibited detectable *Gsx* expression (i.e. we did not distinguish strong or weak levels of expression for this category). n= total number of embryos analysed.

Figure 3. ***Snail*** **and** ***Msxb*** **repress** ***Gsx*** **expression in column 3.** A-C) Marker analysed is indicated to the left, embryo treatment indicated above the columns. Embryos were analysed at neurula stage (A, C) or 6-row neural plate stage (B, C-upper graph) and shown in dorsal view. Red arrowheads indicate ectopic expression. Some embryos were stained with DAPI to confirm cell identification. The graphs show the percentage of embryos in each category of expression following the key below. n= total number of embryos analysed.

Figure 4. Model for patterning of the a-line derived brain precursors in ***Ciona*****.** A) A gene regulatory network constructed using Biotapesty (Longabaugh et al., 2005). Genetic interactions may be direct or indirect. Nodal signals from lateral b-line cells induce *Msxb*, *Snail*, *Delta-like* and *Trp* in the lateral column (col. 3). Delta-like/Notch induces *Gsx* and represses *Meis* in col. 2. Col. 1 receives neither Nodal nor Notch signals and expresses *Meis*. In col. 3 *Msxb* and *Snail* prevent col. 3 cells expressing *Gsx* in response to Notch signalling. Snail repression of *Meis* in column 3 is based on overexpression data (Figure 3A). However, simultaneous inhibition of Snail/Msxb and Notch did not result in ectopic expression of *Meis* in column 3 of the majority of embryos (Figure S2). This suggests that other factor(s) (Gene X?, in red) prevent *Meis* expression in column 3 of Notch-inhibited embryos. B) a- and A-lineage neural plate are pattered by very similar mechanisms. Nodal is required for the entire lateral domain where it induces *Snail* expression. Snail (together with Msxb in a-line) represses medial gene expression in lateral cells. *Delta-like* is induced by Nodal and Notch signalling promotes column 2 over column 1 gene expression, as well as inducing column 4 gene expression (A-line only).

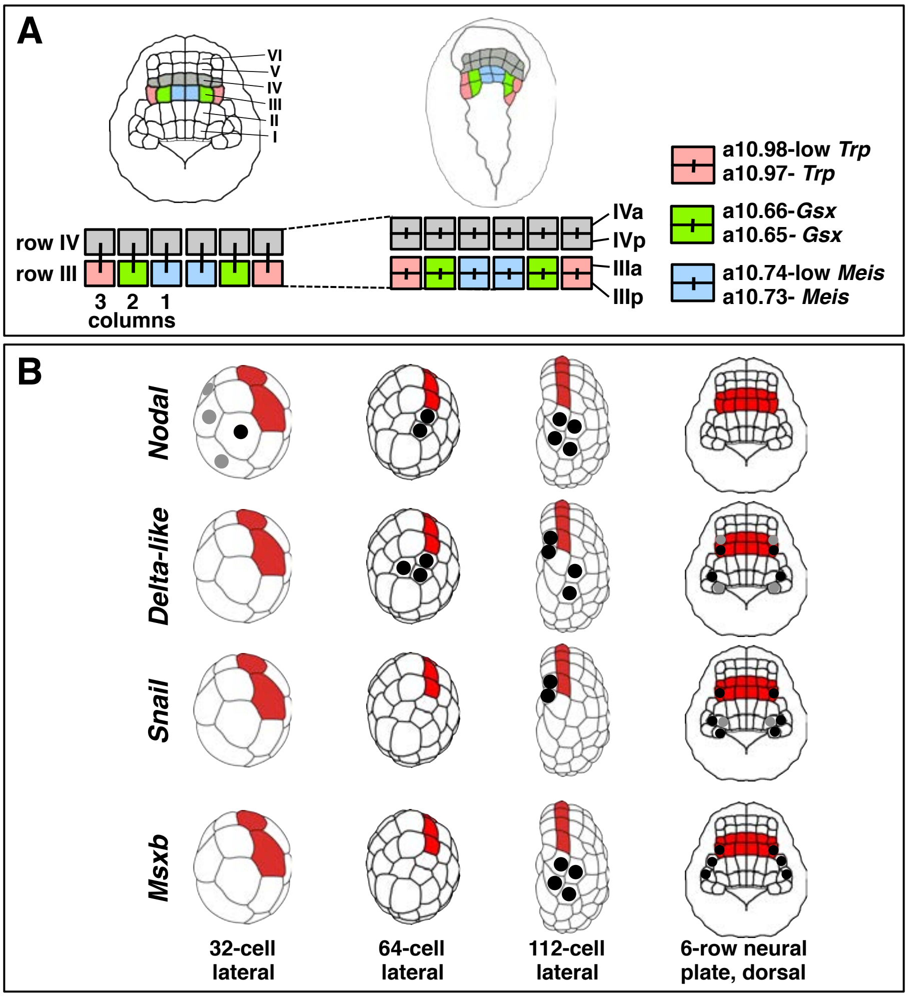

A
VI
V
IV
III
II
I
row IV
row III
3 2 1
columns
IVa
IVp
IIIa
IIIp
a10.98-low Trp
a10.97- Trp
a10.66-Gsx
a10.65- Gsx
a10.74-low Meis
a10.73- Meis
B
Nodal
Delta-like
Snail
Msxb
32-cell lateral
64-cell lateral
112-cell lateral
6-row neural plate, dorsal

Figure 1

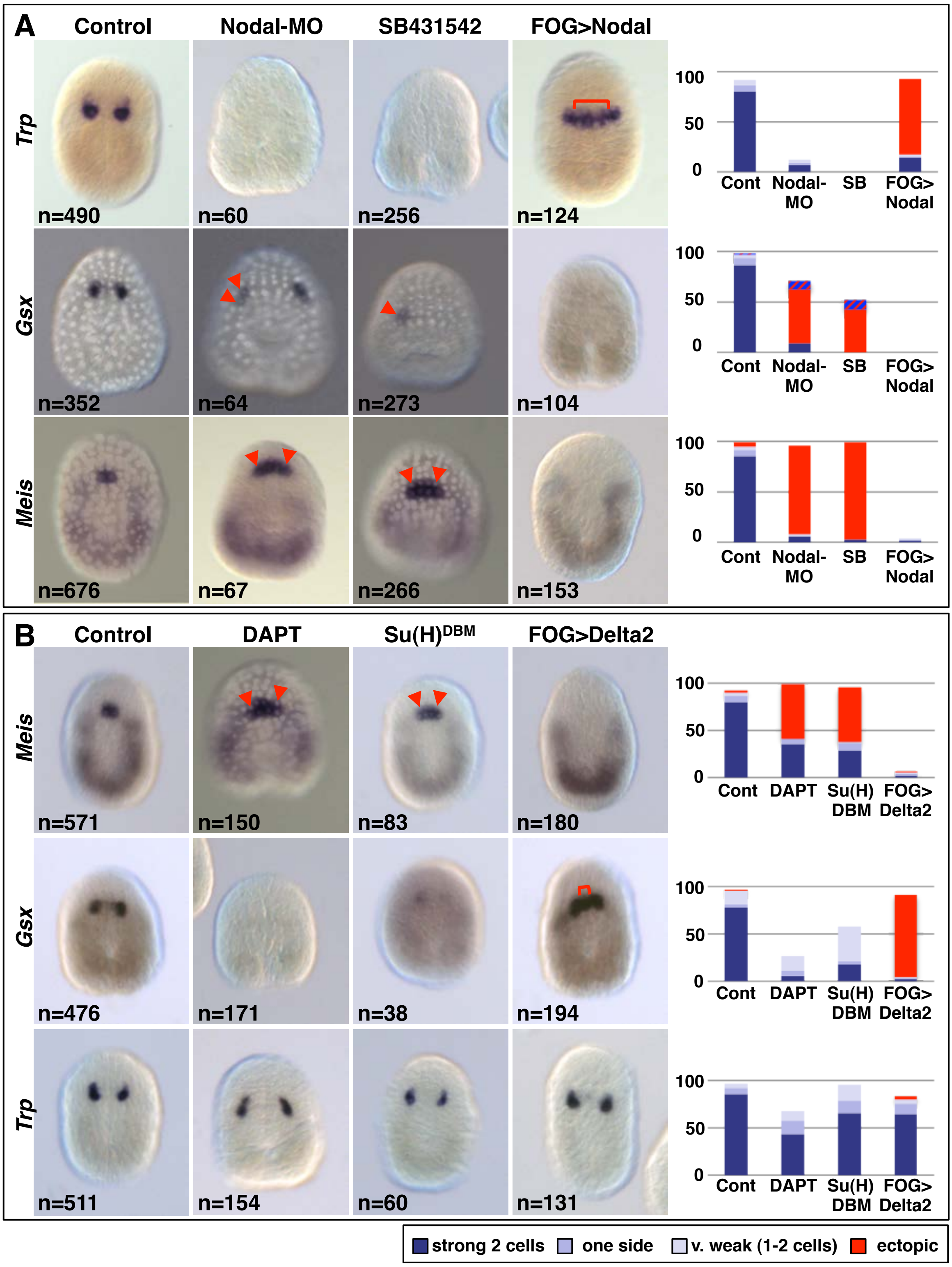
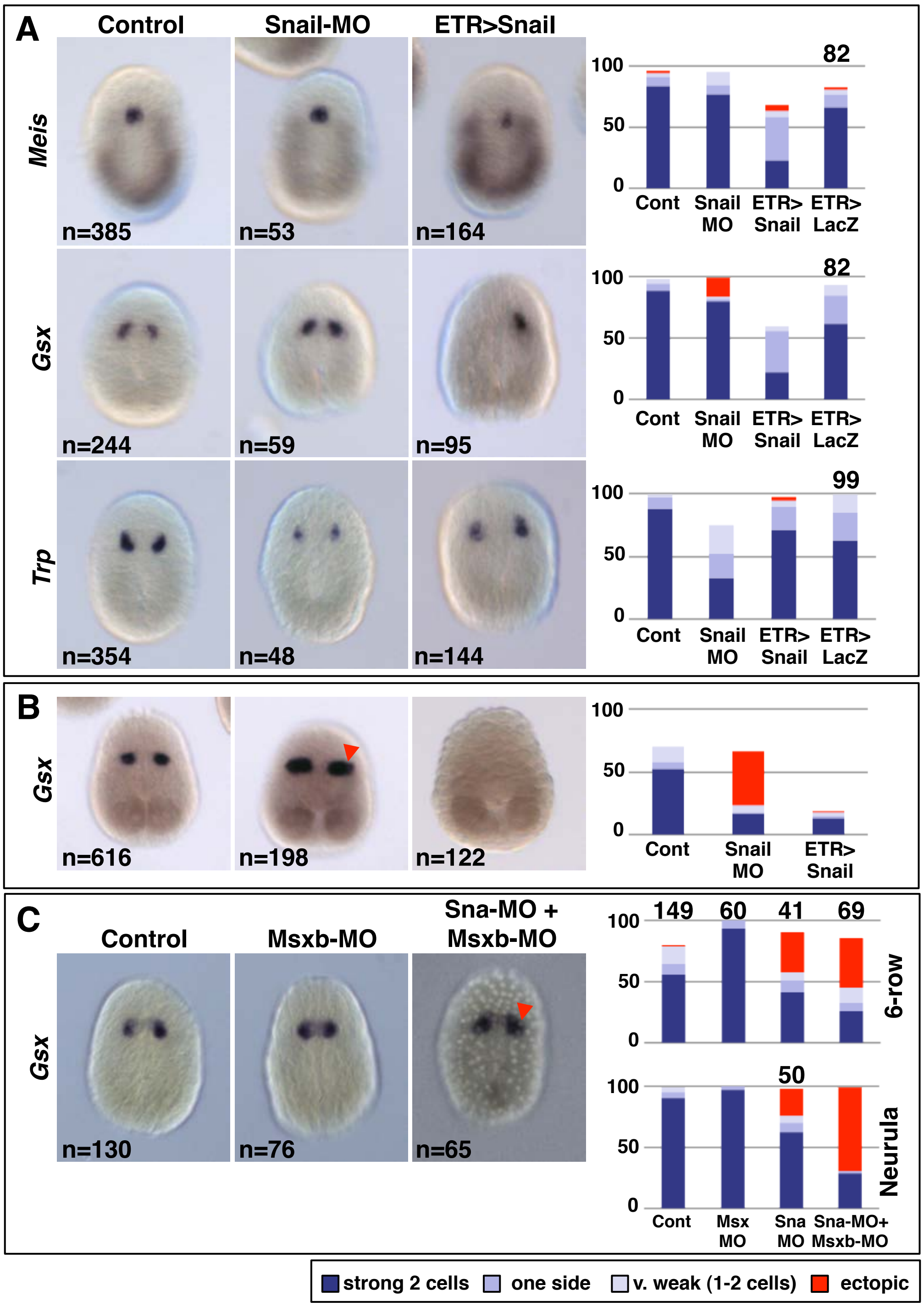
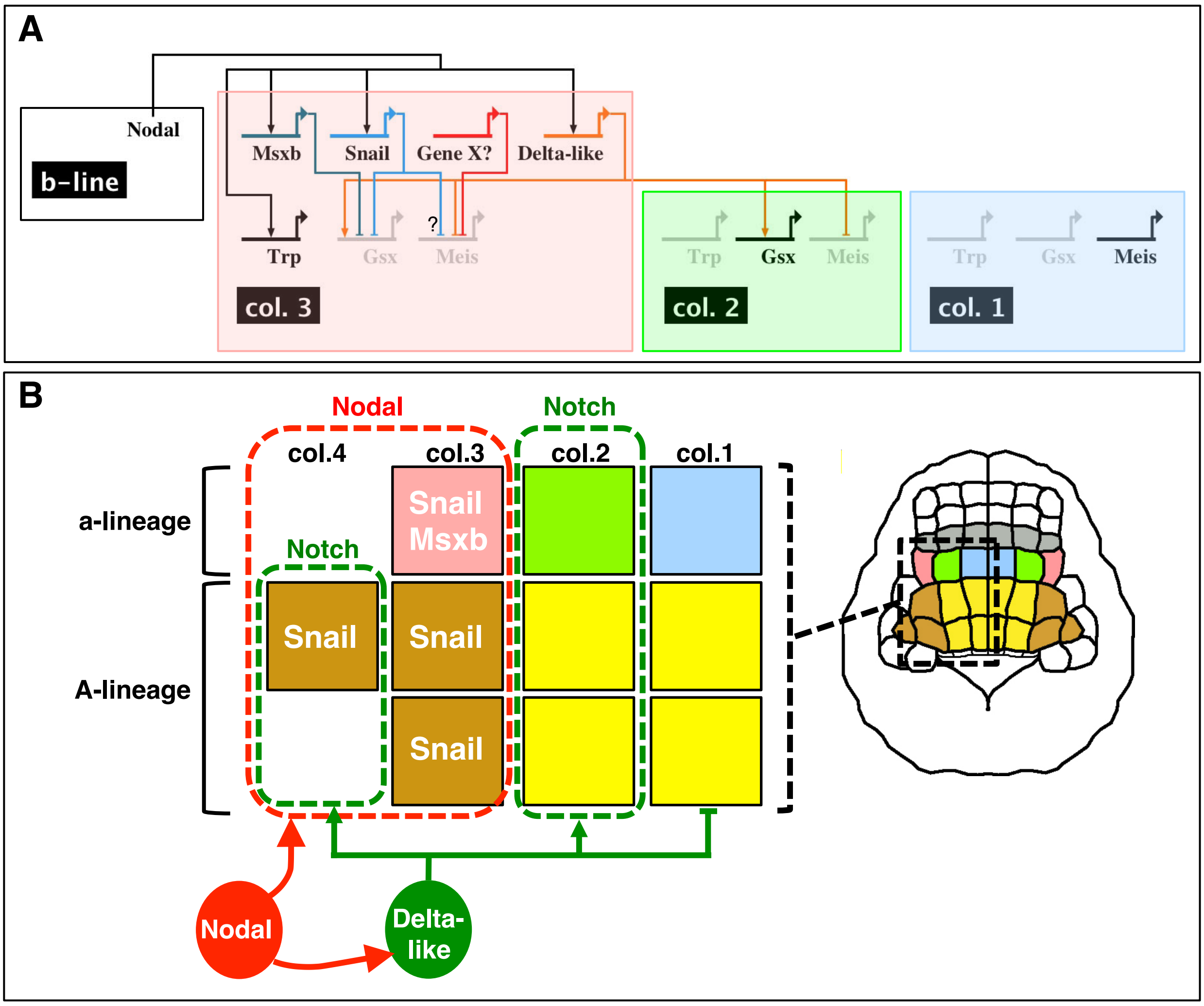

A
Control
Nodal-MO
SB431542
FOG>Nodal
Trp
Gsx
Meis
n=490
n=60
n=256
n=124
n=352
n=64
n=273
n=104
n=676
n=67
n=266
n=153
100
50
0
Cont
Nodal-MO
SB
FOG>Nodal
B
Control
DAPT
Su(H)DBM
FOG>Delta2
Meis
Gsx
Trp
n=571
n=150
n=83
n=180
n=476
n=171
n=38
n=194
n=511
n=154
n=60
n=131
Cont
DAPT
Su(H) DBM
FOG> Delta2
strong 2 cells
one side
v. weak (1-2 cells)
ectopic

Figure 2



A
Control
Snail-MO
ETR>Snail
Meis
n=385
n=53
n=164
Gsx
n=244
n=59
n=95
Trp
n=354
n=48
n=144
82
82
99
100
50
0
Cont
Snail MO
ETR> Snail
ETR> LacZ
B
Gsx
n=616
n=198
n=122
Cont
Snail MO
ETR> Snail
C
Control
Msxb-MO
Sna-MO + Msxb-MO
Gsx
n=130
n=76
n=65
149
60
41
69
6-row
50
Neurula
Cont
Msx MO
Sna MO
Sna-MO+ Msxb-MO
strong 2 cells
one side
v. weak (1-2 cells)
ectopic

Figure 3



A
Nodal
b-line
Msxb
Snail
Gene X?
Delta-like
Trp
Gsx
?
Meis
col. 3
Trp
Gsx
Meis
col. 2
Trp
Gsx
Meis
col. 1
B
Nodal
Notch
col.4
col.3
col.2
col.1
a-lineage
Snail
Msxb
Notch
A-lineage
Snail
Snail
Snail
Nodal
Delta-like

Figure 4

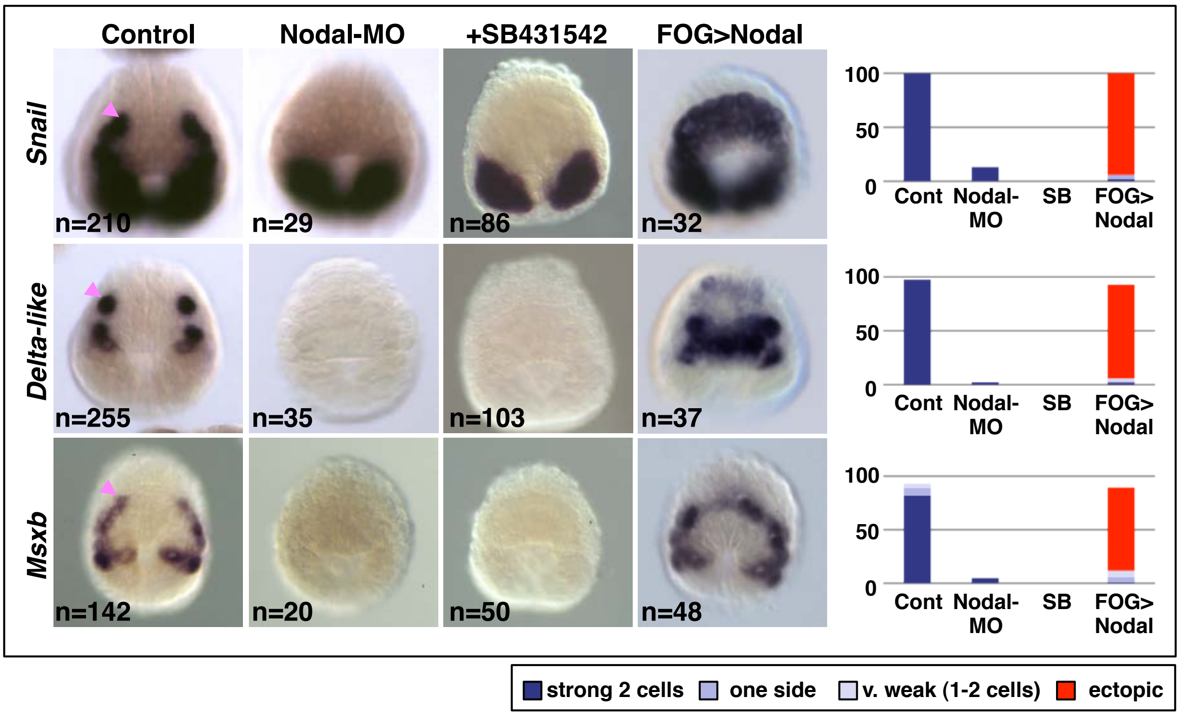


Figure S1: *Delta-like*, *Snail* and *Msxb* are targets of Nodal. A-C) Marker analysed is indicated to the left, embryo treatment indicated above the columns. All embryos are at approximately 6-row neural plate stage. Pink arrowheads indicate column 3 expression (a9.49). The graphs show the percentage of embryos in each category of expression following the key below. n= total number of embryos analysed.

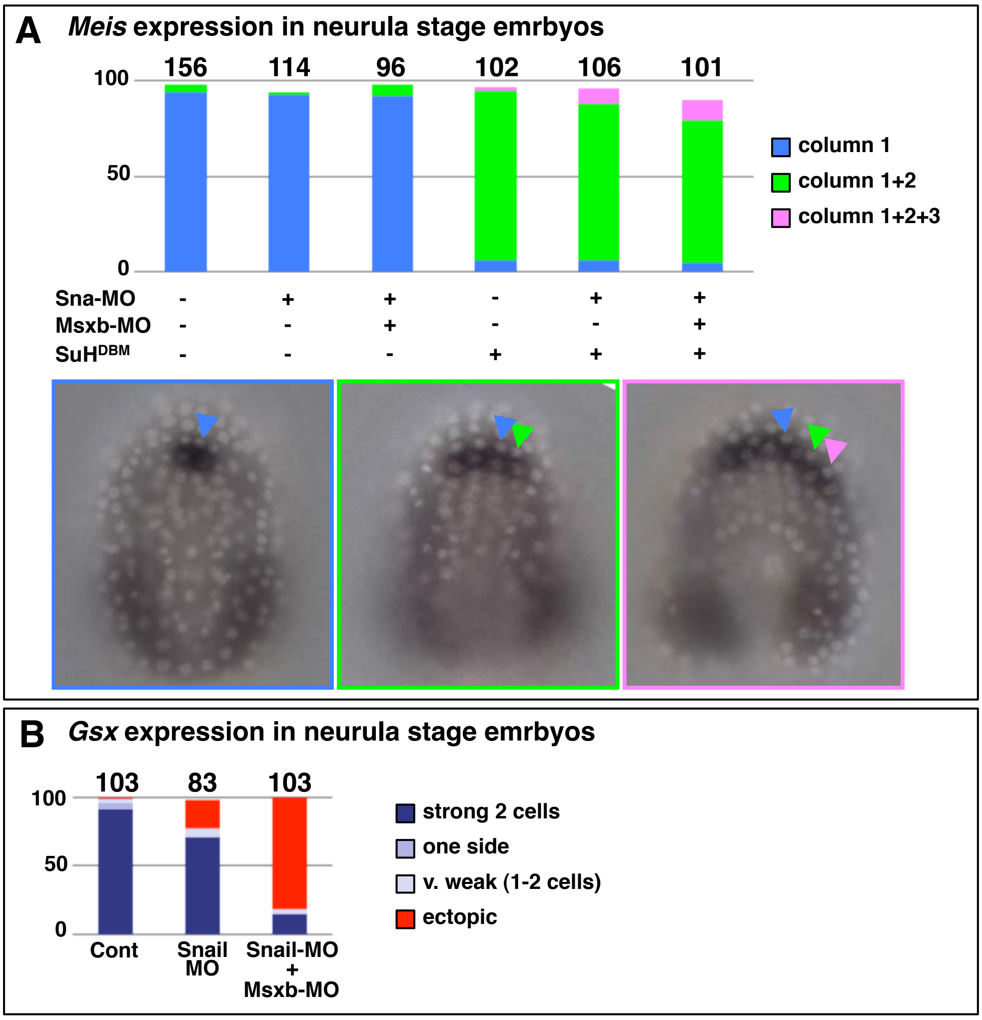


Figure S2. Simultaneous inhibition of Snail, Msxb and Notch signals. A) Embryos were injected with Snail-MO, Msxb-MO and SuH^{DBM} RNA as indicated below the graph. Embryos were scored at the neurula stage for expression of *Meis*. The graph shows the proportion of embryos with expression in column 1 alone, column 1 and 2, or column 1, 2 and 3, as indicated by the key. Every embryo was mounted and stained with nuclear dye to confirm expression. Below the graph are examples of embryos falling into the categories indicated by the colour code. Arrowheads indicate expression in column 1 (blue), 2 (green) and 3 (pink). While the effectiveness of SuH^{DBM} could be ascertained by ectopic *Meis* expression, the effectiveness of the MO- injections was confirmed by analysing ectopic *Gsx* expression in a proportion of embryos from each experiment (B). The numbers above the graphs indicate the total number of embryos analysed.