ABSTRACT
Bilaterian neurogenesis is characterized by the generation of diverse neural cell types from dedicated neural stem/progenitor cells (NPCs). However, the evolutionary origin of NPCs is unclear, as neurogenesis in representatives of the bilaterian sister group, the Cnidaria, occurs via interstitial stem cells that also possess broader, non-neural, developmental potential. We address this question by analysing neurogenesis in an anthozoan cnidarian, *Nematostella vectensis*. Using a transgenic reporter line, we show that *NvSoxB(2)* – an orthologue of bilaterian SoxB genes that have conserved roles in neurogenesis in an anthozoan cnidarian, *Nematostella vectensis*. Transgenic analysis of a *NvSoxB(2)*::mOrange transgenic line, demonstrates that cells express *NvSoxB(2)* before mitosis and identifies asymmetric behaviours of sibling cells within *NvSoxB(2)*+ lineages. Morpholino-mediated gene knockdown of *NvSoxB(2)* blocks the formation of all three neural cell types, thereby identifying *NvSoxB(2)* as an essential positive regulator of nervous system development. Our results demonstrate that diverse neural cell types derive from an *NvSoxB(2)*-expressing population of mitotic cells in *Nematostella* and that SoxB genes are ancient components of a neurogenic program. To our knowledge this is the first description of a lineage-restricted, multipotent cell population outside the Bilateria and we propose that neurogenesis via dedicated, SoxB-expressing NPCs predates the split between cnidarians and bilarians.

KEY WORDS: Cnidaria, Neurogenesis, Neural progenitor cells, *Nematostella*, SoxB genes

INTRODUCTION
The complex central nervous systems of bilaterians develop from a relatively small pool of multipotent neural progenitor cells (NPCs). These cells undergo a choreographed program of lineage expansion and diversification to generate an impressively diverse array of specialized cell types in a strict spatio-temporal pattern. A characteristic feature of NPC development is the pattern of cell divisions within a lineage. Symmetric cell divisions can expand a progenitor pool or produce two terminally differentiated neurons, whereas asymmetric divisions produce unlike daughters with varying capacities for self-renewal or differentiation [reviewed by Huttner and Kosodo (2005)]. Well-studied examples, such as *Drosophila* neuroblasts and mammalian radial glial cells, demonstrate stereotypic lineage progressions, during which they take on distinct spatial identities and generate diverse daughter cells [reviewed by Kohwi and Doe (2013)]. The regulated differentiation of NPCs is thus considered part of the shared cellular framework of bilaterian neurogenesis. However, the evolutionary origin of NPCs is obscure, as a similar NPC-type population has not been identified in the bilaterian sister group, the Cnidaria (Fig. 1A).

Cnidarians (sea anemones, corals, jellyfish and hydroids) possess relatively simple nervous systems, consisting of sensory cells, ganglion cells (the morphological equivalent to interneurons) and nematocytes (stinging cells), which are usually considered to be a third, highly specialized, neural cell type [Fig. 1B; reviewed by Galliot et al. (2009)]. Although their neurons are not uniformly distributed, cnidian polyps lack brain-like nervous system centralization and their nervous system is often described as a nerve net (Galliot et al., 2009; Watanabe et al., 2009). The cellular origin of neurons has mainly been described in hydrozoans (e.g. *Hydra*), in which a pluripotent, endodermally derived interstitial stem cell population gives rise to most neurons and nematocytes, as well as to gland cells and gametes (Bosch and David, 1987; Bosch, 2009; David, 2012; Müller et al., 2004). This origin of neurons differs significantly from bilaterian systems, but interstitial stem cells might represent a derived condition, as they have not been described outside hydrozoans.

The sea anemone *Nematostella vectensis* is an anthozoan cnidarian, and we have previously shown that its neurons derive from both germ layers, i.e. ectoderm and endoderm (Nakanishi et al., 2012). At the molecular level, *Nematostella* possesses many genes related to conserved bilaterian neural genes (Putnam et al., 2007; Watanabe et al., 2009), and subsets of its nervous system have been examined using antibodies against conserved neuropeptide precursor molecules [e.g. Marlow et al. (2009)]. The generation of neurons in *Nematostella* has previously been examined through studies of conserved bilaterian transcription factors. However, whereas many of these genes display evocative ‘salt-and-pepper’ expression patterns, and, in the case of the proneural orthologue *NveAsh4*, can regulate the expression of putative neural markers (Layden et al., 2012), thus far none of these expression patterns has been definitively linked to the production of a mature neural cell type [e.g. Marlow et al. (2009); Matus et al. (2007)]. Here, we address the developmental origins of neural cell types in anthozoans by employing a transgenic approach based on the putative neural regulator *NvSoxB(2)* (Magie et al., 2005).

*NvSoxB(2)* [also known as *SoxB2*, see Magie et al. (2005); *NveSoxBa*, see Royo et al. (2011)] is one of five *Nematostella* SoxB genes closely related to the bilaterian HMG-box-containing SoxB transcription factor family. The five *Nematostella* SoxB genes are expressed in overlapping patterns during embryogenesis, with *NvSoxB(2)* being expressed in a scattered pattern of individual cells in the ectoderm and endoderm (Magie et al., 2005) that is coincident with the timing of neurogenesis in these layers. In bilaterians, *SoxB* genes are expressed in the neural primordia of diverse organisms.
NPCs and are downregulated prior to terminal differentiation. Based on sequence similarity, bilaterian SoxB genes have been classed into two groups, SoxB1 and SoxB2, although phylogenies commonly only resolve a monophyletic SoxB1 group, which resides within an unresolved group of the other SoxB genes (designated SoxB2, see Bowles et al. (2000); Jager et al. (2011); Wilson and Dearden (2008)). The precise phylogenetic affinities of cnidian SoxB genes are currently uncertain (e.g. Jager et al. (2011)).

Thus far, functional studies of SoxB genes have been limited to investigations in classic model organisms. In vertebrates, SoxB1 genes have an important role in the maintenance of NPCs (Bylund et al., 2003; Graham et al., 2003; Kishi et al., 2000). The best-described example is the chick neural tube, in which SoxB1 genes (Sox1-3) suppress neurogenesis by maintaining neuroepithelial cells in an undifferentiated state, and SoxB2 genes (Sox21, Sox14) promote neuronal differentiation by counteracting SoxB1 activity (Bylund et al., 2003; Graham et al., 2003; Holmberg et al., 2008; Sandberg et al., 2005). In Drosophila, functional studies have focused on two SoxB genes, soxNeuro (a SoxB1 gene) and dichaete (probably a SoxB2 gene), that are expressed in the neuroectoderm and are involved in the formation of neuroblasts (Buescher et al., 2002; Overton et al., 2007; Zhao and Skeath, 2002). However, their modes of activity do not bear direct comparison to vertebrate SoxB1 and SoxB2 genes, as both genes can positively regulate neural development by promoting neuroblast formation.

In this study, we present an NvSoxB(2)::mOrange transgenic reporter line, which we use to investigate the origin of neural cells and the function of NvSoxB(2) during early neurogenesis in Nematostella. We find that NvSoxB(2)-expressing cells give rise to three major neural cell types: sensory cells, ganglion cells and nematocytes. Using 5-Ethynyl-2′-deoxyuridine (EdU) labelling, we show that NvSoxB(2)+ cells are mitotic, and that there are asynchronies in the division of sibling cells in NvSoxB(2)+ lineages, which might underpin the generation of neural cell type diversity. With knockdown experiments we demonstrate that NvSoxB(2) regulates development of the nervous system but not ectodermal patterning. Together, these observations reveal the existence of dedicated NPCs in an anthozoan cnidarian and identify SoxB activity as an ancient regulator of neural progenitor populations.

RESULTS

Neurons and nematocytes are generated from a pool of NvSoxB(2)+ cells

NvSoxB(2) was initially classified as a SoxB2 gene, but has subsequently been shown to be a SoxB gene that cannot be grouped into the B1 or B2 subfamilies (e.g. Jager et al. (2011); Shinzato et al. (2008)). NvSoxB(2) is expressed in scattered cells from blastula stage on, first in the ectoderm and later also in the endoderm (Magie et al., 2005). To test whether NvSoxB(2)-expressing cells contribute to the nervous system, we generated a stable transgenic line [NvSoxB(2)::mOrange], in which a promoter region of NvSoxB(2) drives the expression of the fluorescent reporter mOrange (Renfer et al., 2010; Shaner et al., 2004). This technique allows the identification of NvSoxB(2)-expressing cells and their progeny via the expression and inheritance of the mOrange reporter protein. Co-labelling of transgenic embryos with probes for both mOrange and NvSoxB(2) mRNA demonstrated strong (ca. 75-85%) correspondence between the expression of the reporter gene and that of endogenous NvSoxB(2) (supplementary material Fig. S1). Although we cannot unequivocally exclude that a specific sub-population of NvSoxB(2)-expressing cells is not represented in the transgenic line, we did not observe anything...
that would support this suggestion – e.g. no systematic lack of colocalization of NvSoxB(2) and mOrange during a particular time point or in a particular region of the embryo. Subsequent analysis of reporter protein localization enabled us to identify that cells expressing NvSoxB(2) early in their development go on to become sensory, ganglion and nematocyte cells within the Nematostella ectodermal and endodermal nervous systems (Fig. 2; supplementary material Fig. S2).

In gastrula stages, mOrange is localized in scattered ectodermal cells – some are dividing at an apical position within the epithelium (Fig. 2A-F; Meyer et al., 2011). By early planula stage, mOrange localization highlights the development of the basi-epithelial ectodermal nerve net, located predominantly in the aboral two-thirds of the larva, but absent from directly under the aboral pole (where the larval apical organ is located; Fig. 2G-I). The nerve net is composed of neurites extending from the basal side of ectodermal neural cells; the primary orientation of these neurites is lateral, encircling the oral-aboral axis, but this is not strictly uniform (Fig. 2I). In addition to sensory neurons with medially located nuclei and apical cilia (Fig. 2J), NvSoxB(2)::mOrange also labels ganglion neurons, identified by their basal location in the ectoderm (Fig. 2K). In the early planula, activation of the NvSoxB(2) promoter becomes detectable in the endoderm (Fig. 2L). By mid-planula, many cells in the endoderm are positive for mOrange (M-O). Some of the mOrange+ cells are positive for NvElav1::cerulean, in the ectoderm (arrows in P,Q) and endoderm (arrows in R,S). Others contain nematocysts, as labelled by anti-NvNcol3 (white) (arrows in T,U). (A,D,G,M,R) Lateral medial cross-sections, whole embryos; (C,F,J,K,O,Q,T) lateral medial cross-sections, higher magnification; (B,E,U) surface views/superficial sections; (I,P) aboral views; (L,N,S) sections through endoderm; (H,I) projections of half an embryo. Asterisks mark the oral pole. Dashed lines demarcate ectoderm and endoderm. Scale bars: 100 μm in A,D,G-I,M,P,R; 10 μm in all others.

Fig. 2. The NvSoxB(2) promoter drives mOrange expression in cells of the developing nervous system. In gastrulae (A-F), mOrange is present in many ectodermal cells, some of which are dividing (arrows in B,E). By late gastrula, the differentiation of mOrange+ ganglion cells is evident by their more basally located cell body (e.g. arrows in F,K). In early planula, many mOrange+ cells have developed basal processes, forming the ectodermal nerve net (G-I); many of these cells are ciliated (arrows in J). Cells in the endoderm also begin to express mOrange (arrow in L). By mid-planula, many cells in the endoderm are positive for mOrange (M-O). Some of the mOrange+ cells are positive for NvElav1::cerulean, in the ectoderm (arrows in P,Q) and endoderm (arrows in R,S). Others contain nematocysts, as labelled by anti-NvNcol3 (white) (arrows in T,U). (A,D,G,M,R) Lateral medial cross-sections, whole embryos; (C,F,J,K,O,Q,T) lateral medial cross-sections, higher magnification; (B,E,U) surface views/superficial sections; (I,P) aboral views; (L,N,S) sections through endoderm; (H,I) projections of half an embryo. Asterisks mark the oral pole. Dashed lines demarcate ectoderm and endoderm. Scale bars: 100 μm in A,D,G-I,M,P,R; 10 μm in all others.
In comparison to the previously characterized line \textit{NvElav1::mOrange}, in which a promoter sequence of a marker of differentiated neurons drives \textit{mOrange} expression in a subset of sensory and ganglion cells (Nakanishi et al., 2012), we found a greater number and diversity of cells labelled with \textit{mOrange} in the \textit{NvSoxB2} line (supplementary material Fig. S2I-L). In double-transgenic animals (\textit{NvSoxB2::mOrange} × \textit{NvElav1::cerulean}), the \textit{NvElav1} cell population is also labelled by \textit{NvSoxB2::mOrange} transgene (Fig. 2P-S). We speculated that \textit{NvSoxB2} cells that were \textit{NvElav1} might represent nematocytes, the third putative neural cell type of cnidarians. Thus, we performed an immunostaining on the \textit{NvSoxB2::mOrange} line using an antibody against Minicollagen3 (\textit{NvNcol3}), the major component of the capsule (nematocyst) of nematocytes (Zenkert et al., 2011). This experiment showed that a subset of the \textit{NvSoxB2::mOrange} cells contained nematocysts, thus unambiguously identifying these cells as nematocytes (Fig. 2T,U). These data further support the classification of nematocytes as a bona fide neural cell type of cnidarians.

To better visualize the morphology and neurite projections of individual neurons we used a mosaic analysis based on the injection of an \textit{NvSoxB2::EGFP} plasmid into eggs of the stable \textit{NvSoxB2::mOrange} line (mosaicism of \textit{EGFP} in the F0 population is due to spatio-temporal differences in construct incorporation/expression within and between embryos). These experiments confirmed the heterogeneity of the \textit{NvSoxB2}+ population, with regard to both cell shape and neurite projections arising from individual cells (supplementary material Fig. S2M-V). We found that \textit{NvSoxB2}+ cells are multipolar and that they display variation in the number, orientation, length and branching patterns of their basal projections. Variation is also apparent in the number and shape of varicosities found at the base of cells and along neurites. These observations hint at a considerable level of complexity in the neural architecture of this simple nerve net-based nervous system.

\textbf{\textit{NvSoxB2} is expressed in a scattered, proliferating cell population}

Although the \textit{NvSoxB2} transgenic line, through the inheritance of the stable reporter protein \textit{mOrange}, reveals the contribution of \textit{NvSoxB2}+ cells to the three principal neural cell populations in \textit{Nematostella}, it does not show when \textit{NvSoxB2} is transcribed during the development of a neural cell. We thus extended the previous analysis of \textit{NvSoxB2} transcription across a more detailed time course and tested for co-localization of \textit{NvSoxB2} with neural differentiation markers. We found that the earliest expression of \textit{NvSoxB2} is in the hollow coeloblastula, where it is expressed in discrete groups of cells distributed throughout the epithelium (Fig. 3A,J). The neural marker \textit{anthoRFamide} [\textit{NvRFamide}: sensory and ganglion neurons, Marlow et al. (2009)] is expressed in a few scattered single cells at this stage (Fig. 3D). By late blastula, the expression of \textit{NvSoxB2}, \textit{NvRFamide} and the nematocyte marker \textit{minicollagen 3} [\textit{NvNcol3}, Zenkert et al. (2011)], is in scattered single cells throughout the ectoderm (Fig. 3B,E,H), but fluorescent double \textit{in situ} hybridization shows that there is no co-localization between \textit{NvSoxB2} and either of the neural markers (Fig. 3L,N). In the gastrula, all three genes maintain scattered ectodermal expression patterns, with \textit{NvSoxB2} being expressed in a greater number of cells (Fig. 3C,F,I). Again, we found no evidence for co-localization of \textit{NvSoxB2} with \textit{NvNcol3}; however, we observed some rare instances of co-localization with \textit{NvRFamide} (Fig. 3M,O). By counter-staining for acetylated tubulin, we identified that some of the \textit{NvSoxB2}-expressing cells possess a mitotic spindle and are thus undergoing cell division (also evidenced by their rounded shape and apical localization within

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{\textbf{\textit{NvSoxB2} is expressed in proliferative ectodermal cells, but does not co-localize with markers of differentiating neural cells.} In early blastulae, \textit{NvSoxB2} is expressed in groups of cells (A,J), this resolves to a scattered single-cell pattern in late blastulae (B) and gastrula (C). (K) Some of these cells are dividing (green, \textit{NvSoxB2}; white, anti-acetylated tubulin). The expression of markers for sensory neurons, \textit{NvRFamide} (D-F), and for nematocytes, \textit{NvNcol3} (G-I), is detected from early blastula and late blastula stages, respectively. There is limited overlap between \textit{NvSoxB2} (red) and \textit{NvRFamide} (green) expression (arrowhead in M), and no overlap between \textit{NvSoxB2} (red) and \textit{NvNcol3} (green) (L-O). (A-I) Surface views; insets in B,E,H, optical sections at mid-level. (J-O) Confocal images of FISH; blue, DAPI nuclear staining. (M-O) Whole embryo mid-sections. (J-L,N) Higher magnification of surface sections.}
\end{figure}
the ectodermal epithelium, Fig. 3K). At later stages, *NvSoxB(2)* expression continues to be seen in scattered ectodermal cells, some of which are dividing, and in the invaginating pharynx (supplementary material Fig. S3). Expression in endodermal cells is first detected in the early planula stage; a subset of these cells is also undergoing division (supplementary material Fig. S3).

Together with the transgenic analysis, these gene expression data suggest that *NvSoxB(2)* is transiently expressed in mitotically active cells that give rise predominantly or exclusively to neural cell types. This is in line with data from Bilateria, in which the timing of expression of SoxB genes is commonly restricted to specific phases of neurogenesis [e.g. Bylund et al. (2003); Kerner et al. (2009)].

**Proliferating *NvSoxB(2)* NPCs are present throughout development and in both germ layers**

To further investigate proliferation within the *NvSoxB(2)*-expressing cell population, we incubated wild-type embryos for 30 min in the presence of the thymidine analogue EdU and then immediately fixed them for in situ hybridization with a probe for *NvSoxB(2)*. Note that, due to the relatively short EdU incubation period and the lack of a chase period prior to fixation, these data present only a snapshot of the total proliferation that will be occurring within the *NvSoxB(2)*+ population. We have used this protocol to ensure that the cells labelled by EdU are those which are in a pre-mitotic state only. For analysis, we selected a 100 μm × 100 μm area of ectoderm on the mid-lateral side of each of six embryos/stage and scored the number of *NvSoxB(2)*-expressing cells with and without EdU incorporation; we also estimated the total number of cells within the area (Fig. 4A-D). These experiments revealed that the number of *NvSoxB(2)*+ cells in the sampled area remains relatively constant over the course of development, from early blastula to mid-planula. However, due to the increase in the total number of cells in the ectoderm during this time, the *NvSoxB(2)*+ population makes up a larger proportion of the sampled ectoderm in earlier stages (e.g. compare 10% in early blastula with 2.5% in mid-planula). Similarly, whereas there is always a subset of *NvSoxB(2)*+ cells incorporating EdU, this proportion is highest in early development and diminishes over time (e.g. compare 30% in early blastula with 10% in mid-planula). Overall, the level of EdU incorporation in the *NvSoxB(2)*+ population is lower than that calculated for the rest of the ectoderm (supplementary material Fig. S4E). However, we cannot consider slow cell-cycling to be a definitive characteristic of the *NvSoxB(2)*+ population, as our measure of cell proliferation in the rest of the ectoderm encompasses a mixture of cell types.

**EdU labelling of *NvSoxB(2)*::mOrange+ cells indicates asymmetries in sibling lineages**

To examine cell division within the progeny of *NvSoxB(2)*+ cells, we treated *NvSoxB(2)*::mOrange transgenic animals with a 30 min EdU pulse and fixed them immediately afterwards (Fig. 5A). As in the previous experiment, the mid-lateral ectoderm of embryos was examined. We scored for the presence of EdU incorporation in mOrange+ cells and counted the number of contiguous mOrange+ cells clustered around each EdU+ mOrange+ cell. As we did not observe expression of *NvSoxB(2)* mRNA in directly adjacent cells at the stages of analysis, we assume that contiguous transgenic cells predominantly represent sibling daughter cells produced via cell division of a common mother progenitor (supplementary material Fig. S5A). Notably, we recorded a range of differently sized mOrange+ cell clusters (2–7 cells), indicating that mitoses of *NvSoxB(2)*+ cells and their progeny can be asynchronous (synchronous cell division would result in even-numbered cell clusters only). As the majority of mOrange+ cell clusters did not contain EdU+ cells (a likely consequence of the short EdU incubation time, see note above), in the subsequent section we refer exclusively to clusters which contained at least one EdU+ cell.

Within the mOrange+ EdU+ clusters, we commonly observed that only one cell was EdU positive, a further indication of asymmetries in the behaviours of sibling cells in the *NvSoxB(2)*::mOrange line. We next tested whether the cells in an EdU+ cluster after the short incubation of 30 min would remain EdU+ after a longer pulse. As the time from S phase to mitosis at gastrula stage is ca. 2–4 h (supplementary material Fig. S5B), we labelled gastrula stages with EdU for either 30 min or 2 h; a 2 h limit was chosen to ensure that EdU labelling captured pre-mitotic cells only. We further restricted our analysis to clusters containing 2–3 cells, as larger clusters might represent daughters from multiple founder cells, and we wanted to analyse sibling clones only. Our results show that, even when incubated with EdU for longer periods, in the majority of *NvSoxB(2)*::mOrange+ two-cell clusters (97%) across both stages only one cell had incorporated EdU (Fig. 5B). Similarly, the number of cells labelled in the three-cell clusters did not change markedly between the two pulses within a stage; however, in early gastrulae a majority (90%) of three-cell clusters showed one EdU+ cell, in contrast to two positive cells (73%) in gastrulae. Taken together, these findings identify differential cell cycling in the daughters of individual NPCs. Specifically, at a given time point, one daughter from an *NvSoxB(2)*+ progenitor can be in S phase whereas the other is not. The cell not in S phase may be quiescent, post-mitotic or residing in

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**Fig. 4. EdU labelling reveals dynamics of *NvSoxB(2)* cell proliferation during development.** Over time, the number of cells expressing *NvSoxB(2)* in a 100 μm × 100 μm patch of mid-lateral ectoderm remains roughly equivalent (A), with between 10% (mid-planula) to 25% (early blastula) of these cells also incorporating EdU (B). The total number of cells in the sampled area (C) increases from ∼250 (early blastula) to ∼1250 (mid-planula); thus, the relative proportion of cells expressing *NvSoxB(2)* within the ectoderm is fourfold higher in earlier stages (D). Average values from six embryos/stage; error bars: s.d.
Marker genes was decreased in RT-PCR (RT-qPCR) confirmed that the expression of neural Martindale et al., 2004; Matus et al., 2007b; Rentzsch et al., 2008; and NvWnt2 and by injecting the morphants by immunostaining against NvRFamide and NvNcol3, we assessed the presence of neurons and nematocytes in expression on the morphology of the larval nervous system, stages (Fig. 6G). To determine the effect of this change in gene stages (Fig. 6G). To determine the effect of this change in gene

G1/G2, with all of these cases representing asymmetry in the developmental progression of daughter cells from NvSoxB(2)+ progenitors.

NvSoxB(2) regulates neurogenesis

To identify the function of NvSoxB(2), we used a translation-inhibiting morpholino [NvSoxB(2)/MO1] that targeted the 5′UTR of NvSoxB(2). NvSoxB(2)/MO1 caused a reduction in the expression of the neural markers NvElav1, NvRFamide and NvNcol3 in the ectoderm of early planulae (Fig. 6A-F), but had no effect on the expression of the regional ectodermal markers NvFkh, NvFGFa1 and NvWnt2 (Fritzenwanker et al., 2004; Kusserow et al., 2005; Martindale et al., 2004; Matus et al., 2007b; Rentzsch et al., 2008; see also supplementary material Fig. S6H-M). Quantitative RT-PCR (RT-qPCR) confirmed that the expression of neural marker genes was decreased in NvSoxB(2) morphants, relative to control MO-injected embryos, at both the gastrula and planula stages (Fig. 6G). To determine the effect of this change in gene expression on the morphology of the larval nervous system, we assessed the presence of neurons and nematocytes in morphants by immunostaining against NvRFamide and NvNcol3, and by injecting the NvSoxB(2)/MO1 into the NvElav1:mOrange transgenic line. In all cases, neural cells were strongly reduced or absent in the NvSoxB(2) morphants (Fig. 6H-P; for additional control experiments see supplementary material Fig. S6A-G). We conclude that NvSoxB(2) plays an important role in the development of three major neural cell types in Nematostella.

DISCUSSION

Together with observations in the NvSoxB(2)::mOrange transgenic line, our gene expression analyses suggest the existence of a population of dedicated NPCs in Nematostella. To our knowledge, this is the first description outside the Bilateria of a cell population restricted in fate (in this case neural) but able to generate multiple different cell types (i.e. sensory cells, ganglion cells and nematocytes). Our data are in contrast to studies on a different lineage of cnidarians, the Hydrozoa, in which neurons are mainly derived from pluripotent interstitial stem cells that give rise to both neural and non-neural cells (Bosh and David, 1987; Bosch, 2009; David, 2012; Muller et al., 2004). These observations suggest that

Nematostella neural development is more akin to the epithelial progenitor-based neurogenesis of bilaterians than that of hydrozoans, although the levels and extent of homology between these processes remain to be analysed in detail. Indeed, with current data we cannot determine whether the NPCs of Nematostella and bilaterians are homologous, or whether they represent independent events of lineage restriction from an ancestral stem cell population which possessed broader, non-neural potential. Plasticity in the evolution of NPC lineages has previously been observed in the independent acquisition of neural stem cells in the arthropod and vertebrate lineages (Erikkson and Stollewerk, 2010).

By tracing the fate of NvSoxB(2)-expressing cells, we have revealed that sensory and ganglion neurons and nematocytes are linked via a common activation of the NvSoxB(2) promoter during their ontogeny (Fig. 7A). Following on from this discovery, it will be important to determine the potency of the NvSoxB(2) population – whether it is homogenous [in that all NvSoxB(2)+ cells have equal developmental potential] or whether it consists of multiple classes of neural progenitors that will go on to generate different numbers and types of neural cells (Fig. 7B). One possibility is that the NPC pool might be subdivided into nematocyte-producing cells and into cells that produce both sensory and ganglion neurons. Certainly at the molecular and morphological level, sensory and ganglion neurons have more in common with each other than either has with nematocytes [e.g. Marlow et al. (2009); Nakamishii et al. (2012)]. Such putative subsets of NPCs might in turn be defined by specific gene expression signatures, similar to the transcription factor coding of neural fate seen in bilaterian neurogenesis (Guillenot, 2007).

Intriguingly, our analysis of S phase labelling within sibling cells in the NvSoxB(2)::mOrange line demonstrated asymmetric cell behaviours within neural lineages of Nematostella (Fig. 5; supplementary material Fig. S5). In addition to identifying clusters of transgenic cells containing uneven cell numbers [most likely indicating asynchronous cell division from an initial NvSoxB(2)+ progenitor], we observed that only one cell in a pair of sibling transgenic cells incorporated EdU, even after a period of prolonged EdU incubation. This probably indicates that either one of the two cells is already post-mitotic (G0), or that it is in an extended G1 phase and thus lagging behind the cell cycle of its sibling. These observations are noteworthy, as, both in mammals and Drosophila, changes in the length of cell cycle phases have been found to mark changes in the future trajectories of NPCs (Bayraktar et al., 2010; Bowman et al., 2008; Calegari et al., 2005; Takahashi et al., 1995).

The cell cycle asymmetries we have documented in Nematostella might reflect differential self-renewal and/or the adoption of distinct fates by sibling cells within NvSoxB(2)+ lineages. To understand the significance of these asymmetries, and their relationship to the generation of neural diversity in Nematostella, will require the application of live-imaging techniques and/or detailed clonal analysis, in combination with the development of an improved suite of neural cell type-specific markers.

An unexpected finding was that the absolute number of NvSoxB(2)+ cells in our sampled area of ectoderm remained roughly the same from blastula to planula stage (Fig. 4). This could indicate that the NvSoxB(2)+ population is a stable pool of stem cells residing in the ectoderm, from which differentiated neural cell types [no longer expressing NvSoxB(2)] emerge over time. Inconsistent with this scenario is that NvSoxB(2)+ cells labelled with EdU generally display lower levels of NvSoxB(2) mRNA than other NvSoxB(2)-expressing cells. Furthermore, in the transgenic line we did not observe an accumulation of the reporter protein in cells labelled with

Fig. 5. Cell division is asynchronous in NvSoxB(2)::mOrange+ lineages. (A) Incubation of NvSoxB(2)::mOrange transgenic embryos in EdU identifies S phase nuclei, and thus presumptive cell division events, within NvSoxB(2)+ cells and their progeny (arrowhead). (B) In gastrula stages, within two-cell clusters, one cell is in S phase after a 30 min EdU pulse (23/24 clusters). After a 2 h pulse, it remains that only one cell per cluster is in S phase (32/32). In three-cell clusters, early gastrulae tend to show one labelled cell per cluster (9/10), whereas gastrulae display two per cluster (11/15); this pattern is unaffected by the length of EdU treatment. Data shown for each treatment are compiled from ten embryos/stage. EdU-incorporating cells, green; mOrange+ cells, red.
EdU, as would be expected if this was a stable population of cells in which NvSoxB(2) was being continuously expressed. We therefore favour a scenario in which NvSoxB(2)+ progenitors are constantly being produced, either from an unidentified pool of neural stem cells, or directly from ectodermal cells. In the latter case, their number could be kept constant over time by restrictive patterning mechanisms, such as lateral inhibition.

Among bilaterians there is no simple correlation between the orthology and function of specific SoxB family members. Similarly, in cnidarians SoxB genes can be expressed in stem cell populations and/or differentiating cells, in different neural cell types and at different developmental stages (Jager et al., 2011). This suggests that, whereas SoxB function in regulating the developmental progression of neural lineages has been conserved over evolutionary time, considerable variation has arisen in the specific roles that each family member might take during the course of neurogenesis.

The expression and functional data presented here indicate that NvSoxB(2) is required for the proper development of NPCs in Nematostella. NvSoxB(2) may either function as a positive regulator of NPC fate or act in the initiation of a neural differentiation program in an already formed NPC population. In the former scenario, we envisage that the broad expression of NvSoxB(B1), NvSoxB1 and NvSoxB3 (Magie et al., 2005) provides neural potential to ectodermal cells and that the scattered expression pattern of NvSoxB(2) is indicative of the promotion or stabilization of NPC fate. This scenario might further require that NvSoxB(2) be then downregulated for neural differentiation to proceed, akin to the requirement for SoxB1 downregulation in the differentiation of the chick CNS (Bylund et al., 2003; Sandberg et al., 2005). The latter scenario, in which NvSoxB(2) promotes a neural differentiation program, resembles the function of the SoxB2 group gene Sox21 in chick (Sandberg et al., 2005). Sox21 counteracts the function of the SoxB1 group genes Sox1, Sox2 and Sox3, which maintain NPCs in an undifferentiated state without promoting their proliferative activity (Bylund et al., 2003). Whether a similar antagonism between SoxB genes exists in Nematostella is unknown. The broad expression patterns of NvSoxB(B1), NvSoxB1 and NvSoxB3 are compatible with a role in maintaining an undifferentiated state that is counteracted by NvSoxB(2), although they do not suggest a role that is specific to neural stem/progenitor cells. We thus favour the first scenario, in which NvSoxB(2) acts in the promotion/stabilization of NPC fate; however, more refined analyses will be required to determine the precise role of NvSoxB(2) in NPC generation and development.

Fig. 6. Morpholino knockdown of NvSoxB(2) inhibits the expression of neural markers and the development of the nervous system. In NvSoxB(2) morphants, there is a reduction in expression of the neuron markers NvElav1 and NvRFamide (C,D) and of the nematocyte marker NvNcol3 (E,F) at early planula stage. (G) RT-qPCR shows that this downregulation is evident in gastrulae, but more striking by planula stage. NvElav1::mOrange+ (red: H,I,N) and NvRFamide+ neurons (white: J,K,O), as well as NvNcol3+ nematocytes (white: L,M,P), do not develop in NvSoxB(2) morphants. (O,P) Embryos were scored ‘positive’ if displaying a wild-type pattern of immunostaining (Nakanishi et al., 2012; this study) and ‘negative’ if possessing few or no immunostained cells. NvElav1::mOrange animals are derived from an incross of a heterozygous line; thus, 25% of the animals are expected to be mOrange+. n=28-41 in O; n=5 in P; n=12-15 in Q. All images have oral pole up. (A-F) In situ hybridizations; (H-M) immunostainings with DAPI (nuclei: blue) and phalloidin (F-actin: green). (A,B,E,F,L,M) Lateral cross-sections; (C,D) surface views; (H,K) projections of half an embryo.
progenitors. 8 mediated transgenesis as described by Renfer et al. (2010). The NvSoxB(2)::mOrange blastula, 18 h; early gastrula, 20 h; gastrula, 24 h; late gastrula, 30 h; early NM. Embryos were raised at 21°C. Early blastula, 12 h; blastula, 16 h; late blastula, 18 h; early gastrula, 20 h; gastrula, 24 h; late gastrula, 30 h; early planula, 48 h; mid-planula, 72 h; late planula, 96 h; primary polyp, 10 days.

MATERIALS AND METHODS
Nematostella culture
Animals were maintained in 0.3× filtered seawater (NM) and induced to spawn as described by Fritztenwanker and Technau (2002). Fertilized eggs were removed from jelly packages by incubation for 25 min in 3% cysteine/ NM. Embryos were raised at 21°C. Early blastula, 12 h; blastula, 16 h; late blastula, 18 h; early gastrula, 20 h; gastrula, 24 h; late gastrula, 30 h; early planula, 48 h; mid-planula, 72 h; late planula, 96 h; primary polyp, 10 days.

Transgenics
The NvSoxB(2)::mOrange transgenic line was generated by meganuclease-mediated transgenesis as described by Renfer et al. (2010). The NvSoxB(2) promoter sequence (genomic coordinates: minus strand on scaffold 84; 792,491-793,895) was cloned in front of mOrange (Shaner et al., 2004), with the addition of a membrane-tethering CAAX domain to help visualize the boundaries and morphology of cells expressing the reporter protein. Animals used for experiments were progeny from either crossing of F1 heterozygotes or crossing F2 homozygotes with wild type. In all images, expression of mOrange was visualized via immunostaining with anti-DsRed (rabbit, Clontech, 632496; 1:100).

Morpholino injection
Microinjections were carried out as described by Rentzsch et al. (2008). Fertilized eggs were injected with 250-500 μM morpholino (Gene Tools) and 40 μg/ml Alexa Fluor-conjugated dextran (Invitrogen) in TAE buffer. Control injections used either a generic control or specific mismatch control [NvSoxB(2)Mm] morpholino. For morpholino sequences see supplementary material Table S2.

Cell proliferation assays
Embryos were incubated with 100 μM EdU/DMSO in NM for either 30 min or 2 h at 21°C and then fixed (see methods in the supplementary material) immediately for either ICC or FISH. After ICC or FISH protocols were completed, EdU incorporation was visualized using the Click-it EdU Alexa Fluor 488 imaging kit (Molecular Probes) following the manufacturer’s instructions. For counting NvSoxB(2)+ neurons and cell clusters, a 100 μm×100 μm sampling area was defined in the mid-lateral region of the ectoderm, and serial 1 μm sections through the layer of nuclei within this region were scanned via confocal microscopy. For total cell number estimates, nuclei were counted within multiple 10 μm×10 μm subsamples of the 100 μm×100 μm region; these data were then extrapolated to the whole area.

RT-qPCR
RNA from embryos from three independent injections was extracted using the RNAsqueous kit (Ambion) and DNAase-treated using TurboDNase (Ambion). Quality and quantity of RNA was assessed using a Bioanalyzer (Agilent). cDNA was reverse-transcribed using Superscript III (Invitrogen) primed with random hexamers (Roche). Control reactions without the addition of reverse transcriptase were prepared for all samples. Primer pairs with PCR efficiencies of 90-105% were used for qPCR. Two technical replicates were performed for each of the three biological replicates. Relative expression was calculated using the 2-ΔΔCT method; control gene stability was assessed using RefFinder (http://www.leoncixie.com). with NvAtPas1 and NvELF1B being selected as most stable. Mean relative expression and s.e.m. of three biological replicates are plotted. For primer sequences see supplementary material Table S1.

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Competing interests
The authors declare no competing financial interests.

Author contributions
F.R. generated the transgenic line and G.S.R. performed the experiments and analysis. Both authors contributed to the design of the study and the preparation of the manuscript.


SUPPLEMENTARY MATERIAL

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   Table S2: Morpholino sequences

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   Figure S6: Control experiments for NvSoxB(2)MO1.
1. Supplementary Materials and Methods

Fixation

Embryos were fixed in 3.7% formaldehyde/0.25% glutaraldehyde/NM for 2 min on ice, then in 3.7% formaldehyde/PTW (PBS+0.1%Tween20) for 1 h at 4°C. Embryos for in situ hybridization were washed in PTW, H₂O and then stored in methanol at -20°C. Embryos for immunocytochemistry were washed in PTW and stored at 4°C.

In situ hybridization (ISH)

The following protocol is based on published in situ hybridization methods (Nakanishi et al., 2012; Rentzsch et al., 2006; Rentzsch et al., 2008). Samples were rehydrated in PTW, and then incubated in 20μg/ml Proteinase K for 10 min at room temperature (RT) followed by washes in 4mg/ml Glycine/PTW. They were then washed in 1% triethanolamine in PBS, followed by the addition of 0.25%, then 0.5% acetic anhydride. Samples were next washed in PTW and refixed in 3.7% formaldehyde/PTW, followed by washes in PTW. Pre-hybridization in hybridization buffer (HB: 50% formamide, 5X SSC, 1% SDS, 50 μg/ml heparin, 100 μg/ml salmon sperm DNA, 9.25 mM citric acid, 0.1X Tween20) was for at least 2 h at 60°C. Digoxigenin-labeled riboprobes were synthesized from PCR templates (MEGAscript Kit, Ambion) and incubated with the samples at a final concentration of 0.1-1 ng/μl for at least 60 h at 60°C. Unbound probe was removed via a series of 60°C washes of HB/2X SSC solutions [75/25, 50/50, 25/75, 0/100 (v/v)], then 0.2X SSC, 0.1XSSC. This was followed by RT washes of SSC/PTW solutions [75/25, 50/50, 25/75, 0/100 (v/v)]. Samples were then blocked in blocking solution [1% Block (Roche)/Maleic acid buffer (100mM maleic acid, 150mM NaCl)] for 2 h at RT and incubated overnight with 1:5000 anti-digoxigenin alkaline phosphatase (Roche)/blocking solution. Unbound antibody was removed with 10 x 15 min washes of PBTxBSA (PBS /0.2% TritonX100 /0.1% bovine serum albumin); samples were
then washed with staining buffer (100mM Trs pH 9.5, 100mM NaCl, 50mM MgCl$_2$, 0.1%Tween20) before color was developed via the addition of 1:200 NBT/BCIP solution (Roche) in staining buffer. When the staining reaction was judged to be complete, samples were washed as follows – staining buffer, PTW, H$_2$O, ethanol, H$_2$O, PTW – and then post-fixed for 30 min with 3.7% formaldehyde/PTW before being washed with PTW and then cleared via overnight incubation in 87% glycerol at 4°C.

**Immunocytochemistry (ICC)**

Immunostainings were carried out based on (Nakanishi et al., 2012). Fixed samples were washed for 2 h with PBTx (PBS/0.3%TritonX100) and then incubated in block (5% normal goat serum/PBTx) for 1 h at RT before overnight incubation in primary antibodies at 4°C. Primary antibodies and concentrations were: anti-DsRed 1:100 (rabbit, Clontech 632496); anti-GFP 1:200 (mouse, abcam 1218); anti-NCol3 1:100 (Zenkert et al. 2011); anti-acetylated tubulin 1:100 (mouse, Sigma T6793); anti-FMRFamide 1:500 (rabbit, Millipore AB15348). Samples were then washed for 2 h with PBTx, incubated for 1 h at RT in block and then overnight at 4°C in secondary antibodies and Alexa Fluor 488-conjugated phalloidin 1:50 (Molecular Probes). Alexa Fluor conjugated secondary antibodies (Molecular Probes) were used at 1:200. Samples were then washed for 2 h with PBTx, incubated for 30 min in DAPI 1:1000 (Molecular Probes) then mounted in ProLong Gold antifade reagent (Molecular Probes).

**Fluorescent in situ hybridization (FISH)**

Fixed samples were incubated in 2% hydrogen peroxide in methanol to quench endogenous hydrogen peroxidase activity. Samples were then rehydrated in PTW and the ISH protocol (see above) was followed from the Proteinase K incubation step until the end of the SSC/PTW
RT washes. During hybridization, samples were incubated with either digoxigenin or fluorescein-labelled riboprobes (MEGAscript Kit, Ambion) at a final concentration of 1 ng/μl. After the SSC/PTW RT washes, samples were washed in TNT (0.1M Tris-HCl pH 7.5/0.15M NaCl/0.5% Triton X-100) and then blocked in TNTblock [0.5% blocking reagent (PerkinElmer)/TNT] for 1 h at RT before overnight incubation with anti-digoxigenin (1:100) or anti-fluorescein (1:250) horseradish peroxidase (Roche) together with other primary antibodies (if the FISH was to be followed by ICC). Unbound antibodies were removed by 10 x 15 min TNT washes, and samples were then incubated in fluorophore tyramide amplification reagent (TSA Plus Kit, PerkinElmer). After the TSA reaction, samples were washed in TNT and then were either incubated with DAPI 1:1000 and mounted in ProLong Gold antifade reagent, or, treated with one of the following protocols.

- For double labelling, samples were washed in 0.1M glycine pH 2.0 and then incubated 1 h in TNTblock before overnight incubation with anti-digoxigenin or anti-fluorescein horseradish peroxidase (Roche). Post-antibody washing and the TSA reaction were repeated as for the first probe; samples were then washed in TNT, incubated with DAPI 1:1000 and mounted in ProLong Gold antifade reagent.

- For immunolabelling, samples were washed in PBTx and incubated in block (5% normal goat serum/PBTx) for 1 h at RT before overnight incubation at 4°C in secondary antibodies. Post-incubation, samples were washed for 2 h with PBTx and then incubated with DAPI 1:1000 and mounted in ProLong Gold antifade reagent.
### Table S1: qPCR primers

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### Table S2: Morpholino sequences

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<tr>
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<td>(generic ControlMO)</td>
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MO: morpholino; Mm: mismatch morpholino
2. Supplementary References


3. Supplementary Figures and Figure Legends

Figure S1: Co-localization of NvSoxB(2) and mOrange mRNA in transgenic embryos.

Double fluorescent in situ hybridization (FISH), using probes for NvSoxB(2) and mOrange, demonstrates that reporter gene expression closely mimics endogenous NvSoxB(2) expression in embryos from the NvSoxB(2)::mOrange transgenic line. A correspondence estimate of ca. 75-85% between the expression of NvSoxB(2) and mOrange was determined via visual examination of confocal stacks of embryos across a representative developmental timecourse. We consider the incomplete overlap of the probes to largely be a technical issue - it is challenging to get optimal signal from both probes in double fluorescent in situ hybridization experiments, as probes differ in signal strength and signal to background ratios, as do the fluorescent labels used to tag the hybridized probes. A-B, in gastrula stages, co-localized expression is seen in ectodermal cells, including dividing cells (D), and cells with sensory morphology (E). In planulae (C) co-expression of both transcripts is additionally seen in the invaginating pharynx, and endodermal cells (F). Arrowheads and arrows indicate representative double-labelled ectodermal and endodermal cells respectively. Green, NvSoxB(2) FISH; red, mOrange FISH; blue, DAPI nuclear stain. A, D: lateral surface view; B-C, E-F: lateral medial cross-sections. Row 2 shows higher magnification images of features from embryos in row 1. Asterisks mark the oral pole; dashed line demarcates ectoderm and endoderm.
Figure S2: Further description of the *Nematostella* nervous system and neural cell types.

(A-H) Activation of *NvSoxB(2)* is localized within the nervous system of the late planula and primary polyp. In late planula (A-D) and primary polyps (E-H), *NvSoxB(2)::mOrange* expression highlights the development of the endodermal nervous system, as can be seen by the differentiation of ciliated endodermal...
neurons (arrow in C) and the condensation of neurites and soma (arrows in B, F along the developing muscle fibers (arrow heads in B, F, G). Expression of mOrange also persists in the neurons of the ectodermal nerve net (arrows in D, H). A, C, E, G: lateral medial cross-sections; B, F: lateral mid-endodermal sections; D, H: lateral cross-sections close to the base of the ectoderm. Asterisks mark the oral pole. Dashed line demarcates ectoderm and endoderm. Scale bars - A, E, 100μm; B-D, F-H, 20μm. (I-L) NvSoxB(2)::mOrange is expressed earlier and more broadly than NvElav1::mOrange. At gastrula stage mOrange is not expressed in the NvElav1::mOrange line, yet it is already expressed in many cells in the NvSoxB(2)::mOrange line (I-J). In the early planula both lines express mOrange, but in the NvSoxB(2) line there are more mOrange+ cells and these cells have more varied morphologies than in the NvElav1 line (K-L). (M-V) Mosaic analysis of NvSoxB(2)::EGFP injected into the NvSoxB(2)::mOrange line highlights the heterogeneity of individual cells within the NvSoxB(2)+ population. M-T, double labelled cells in the ectoderm of early planula larvae. U-V, two sides of the same embryo, mid planula stage, neurites in (V) originate from the somata in (U) and thus extend around the circumference. All images are confocal projections around the limits of the EGFP-labelled neurons. Arrowheads, cell bodies; arrows, neurites; asterisks mark the oral pole.
Figure S3: *NvSoxB(2)* mRNA expression across a developmental timecourse.

*NvSoxB(2)* is expressed in single cells rather than broad domains; initially in the ectoderm, then predominantly in the endoderm and pharynx during planula development. In gastrulae and early planulae (A-I), *NvSoxB(2)* is expressed in single, scattered ectodermal cells (arrows in B, E, H), some of which possess mitotic spindles (arrowheads in C, F, I). In the early planula expression in the endoderm is first evident (G). Endodermal expression increases in the planula and late planula stages (J-O), relative to the level of ectodermal expression (arrow in K); some of the endodermal cells are also dividing (arrowhead in L). In late planulae there is strong expression of *NvSoxB(2)* in the pharynx (N), as well as in scattered single cells within the endoderm (arrow in O). Green, *NvSoxB(2)* FISH; white, anti-acetylated tubulin immunostaining; blue, DAPI nuclear stain. A: cross-section, oral view; B-O: lateral medial cross-sections. Columns 2 and 3 show higher magnification images of embryos in column 1. B-C, E-F, G-H, K-L, N-O: higher magnification images of embryos A-M respectively. Asterisks mark the oral pole; dashed line demarcates ectoderm and endoderm.
Figure S4: EdU incorporation of NvSoxB(2) expressing cells.

(A) Example images of NvSoxB(2) expressing cells in S-phase. The incorporation of EdU (green) by cells expressing NvSoxB(2) (red) can be seen in confocal scans through the apical (nuclear) section of the ectoderm. Arrows, NvSoxB(2)+ cells; arrowheads, NvSoxB(2)+ EdU+ cells.

(B-D) Raw data from scoring EdU+ and NvSoxB(2)+ cells in a 100μm x 100μm area of lateral ectoderm. Graphs show data from 6 embryos scored/stage; Figure 5A-D contains the same information presented as the mean and standard deviation for each timepoint.

E. Summary of all cells in S-phase in sampled area. Results are subdivided to differentiate cells that are expressing NvSoxB(2) from cells which are not expressing NvSoxB(2). Means and standard deviations of 3 embryos per stage (NvSoxB(2) negative) or 6 embryos/stage (NvSoxB(2) positive) are shown.
Figure S5: EdU labelling of the NvSoxB(2)::mOrange transgenic line.

(A) EdU incorporation within clusters of cells expressing mOrange. A timecourse of embryos was incubated in EdU and then fixed immediately. 100µm x 100µm sections of the mid-lateral ectoderm were then scored for the presence of EdU⁺/mOrange⁻ cells, and the number of contiguous mOrange⁺ cells (EdU⁺ or EdU⁻) was also noted. Single EdU⁺/mOrange⁻ cells were rarely observed. There was no bias towards cell clusters of even numbered sizes, indicating a lack of synchrony in cell division within a cluster. Similarly, it was common for only one, or a minority, of the cells within a cluster to be in S-phase. 6 embryos were scored for each stage. EdU incorporating cells, green; mOrange⁺ cells, red. Note that the depictions of cluster shape, including the location of the S-phase nuclei within each cluster, has been stylized for uniform presentation of the data.  

(B) Pulse-chase EdU labelling to estimate the time between S-phase and mitosis. Gastrula stage embryos were incubated with EdU for 15 min and then subject to a chase period of between 30 min and 4 h, during which time they were incubated in NM. After fixation and labelling, embryos were examined for the presence of EdU incorporation in mitotic spindles (arrowheads) – which are easily identified by the elongated shape of the Hoechst nuclear staining, and their apical position in the ectoderm, within rounded cells. No EdU labelling of spindles was seen after the 30 min chase, but a few spindles had weak labeling after 1 h. A larger subset of spindles were labelled after 2 h, and most spindles were labelled after 4 h. From this we estimate that the time between S-phase and mitosis is minimally 1 h, but more commonly 2-4 h.
Figure S6: Control experiments for *NvSoxB(2)*MO1.

(A-D) *NvSoxB(2)*MO1 binds to the 5'UTR of *NvSoxB(2)*. Injection of (A) ControlMO or (B) *NvSoxB(2)*Mm - a morpholino with 5 base mismatch to *NvSoxB(2)*MO1 – had no effect on the expression of mOrange as driven by the *NvSoxB(2)* promoter region. In contrast, mOrange expression was downregulated in embryos injected with (C) *NvSoxB(2)*MO1. None of the *NvSoxB(2)*MO1 injected animals were positive for mOrange expression, in comparison to 100% of animals injected with ControlMO or >95% with *NvSoxB(2)*Mm (D) \( n = 25-30 \) larvae/treatment. Images A-C are projections of 10 x 1μm confocal sections through the center of early planula larvae; the oral pole is to the top. DAPI (nuclei: blue), Phalloidin (F-actin: green).

(E-G) An ATG targeted morpholino, *NvSoxB(2)*MO2, induces the loss of the *NvElav1::mOrange* + nervous system. When compared to control MO injected animals (E), embryos injected with *NvSoxB(2)*MO2 (F) displayed the same phenotype as in *NvSoxB(2)*MO1 injections (Figure 5) in that a decrease in the mOrange + nervous system was observed.
Almost 60% (n=43) of control injected animals possessed mOrange⁺ neurons, compared to 20% (n=39) of animals injected with NvSoxB(2)MO2 (G). Note that the NvElav1::mOrange transgenic line is heterozygous, i.e. 75% of the embryos derived from an incross are transgenic. E-F: confocal projections showing the surface to the base of the ectoderm of early planulae; oral pole up. **(H-M) Expression of regional ectodermal markers is unchanged in NvSoxB(2) morphants.** The oral marker NvFkh (H-I)(Fritzenwanker et al., 2004; Martindale et al., 2004), aboral marker NvFGFa1 (J-K)(Matus et al., 2007b) and mid-body marker NvWnt2 (L-M)(Kusserow et al., 2005) show similar expression in NvSoxB(2)MO1 injected animals as compared with ControlMO injections. All images are early planula larvae with the oral pole to the top.