Neuronal subtype specification in the spinal cord of a protovertebrate
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There was an error published in Development 138, 995-1004.

On p. 1000, it was incorrectly stated that Islet can repress Vsx in A13.474 but not in A11.117. The reverse is true, as stated in the legend to Fig. 6B. The corrected text appears below.

Vsx can actually induce ectopic Islet expression (Fig. 6C), while Islet can repress Vsx in A11.117, but not in A13.474 (Fig. 6B), hinting at a more complicated interaction between these two genes.

The authors apologise to readers for this mistake.
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Alberto Stolfi and Michael Levine*

SUMMARY
The visceral ganglion (VG) comprises the basic motor pool of the swimming ascidian tadpole and has been proposed to be homologous to the spinal cord of vertebrates. Here, we use cis-regulatory modules, or enhancers, from transcription factor genes expressed in single VG neuronal precursors to label and identify morphologically distinct moto- and interneuron subtypes in the Ciona intestinalis tadpole larva. We also show that the transcription factor complement present in each differentiating neuron correlates with its unique morphology. Forced expression of putative interneuron markers Dmbx and Vsx results in ectopic interneuron-like cells at the expense of motoneurons. Furthermore, by perturbing upstream signaling events, we can change the transcription factor expression profile and subsequent identity of the different precursors. Perturbation of FGF signaling transforms the entire VG into Vsx+Pitx+ putative cholinergic interneurons, while perturbation of Notch signaling results in duplication of Dmbx+ decussating interneurons. These experiments demonstrate the connection between transcriptional regulation and the neuronal subtype diversity underlying swimming behavior in a simple chordate.

KEY WORDS: Ciona, Ascidian, Gene regulatory network, Spinal cord, Motoneurons

INTRODUCTION
Ascidians belong to the urochordates, or tunicates, which comprise the sister group to the vertebrates within the chordate phylum (Delsuc et al., 2006). Thus, ascidians (or sea squirts) are the extant invertebrates most closely related to vertebrates. The sea squirt Ciona intestinalis (to which we will now refer in this text as simply Ciona) has emerged as a model system for studying the regulation of chordate developmental processes (Satoh, 2003). Their small size, rapid development and determinist cell lineages have long been appreciated by classical embryologists (Chabry, 1887; Conklin, 1905), while their compact genome and suitability to molecular perturbation and imaging have propelled them into the post-genome era (Dehal et al., 2002).

Although adult sea squirts feature numerous morphological adaptations to a life of sessile filter-feeding, their free-swimming tadpole larvae possess a typical chordate body plan. This includes a dorsally located central nervous system (CNS) derived from a neural plate that rolls up to form a hollow neural tube (Nicol and Meinertzhagen, 1988). The Ciona larval CNS comprises ~335 cells, of which roughly one third are neurons (Nicol and Meinertzhagen, 1991). They arise through evolutionarily conserved, invariant cell lineages that have been described in detail (Cole and Meinertzhagen, 2004). The CNS is divided along the anterior-posterior axis into distinct anatomical regions. At its most anterior lies a sensory vesicle containing melanized pigment cells and associated sensory cells that sense light and gravity (Dilly, 1964; Dilly, 1962; Sato and Yamamoto, 2001). The majority of neurons of the CNS are located in a tight cluster associated with this sensory vesicle. Immediately posterior to the sensory vesicle and associated neurons lies the ‘neck’, which comprises a few quiescent precursors in larvae (Nicol and Meinertzhagen, 1991). After metamorphosis, these precursors differentiate into the branchial basket motoneurons of the adult that are thought to be homologous to the cranial motoneurons of the vertebrate hindbrain (Dufour et al., 2006).

Further posterior is a ‘visceral ganglion’ (VG), which consists of cholinergic neurons innervating the longitudinal muscle bands on either side of the tail. These muscles contract in left/right alternation to produce the vigorous swimming behavior that aids in the dispersal of the larvae (Brown et al., 2005; Horie et al., 2010; Ohmori and Sasaki, 1977; Takamura et al., 2002). These neurons are thought to receive inputs from the sensory vesicle, as well as from the peripheral nervous system (Horie et al., 2008a; Takamura, 1998). It has been shown that swimming behavior is modulated by light and gravity (Horie et al., 2008b; Jiang et al., 2005; Tsuda et al., 2003), and that the response of the larvae to these stimuli can change over time (Kajiwara and Yoshida, 1985). Thus, the CNS of the ascidian larva can be seen as a central pattern generator for swimming, modulated by photo- and geotropically sensitive systems (Goulding, 2009).

Gene expression studies suggest that many of the molecular mechanisms underlying the patterning of the ascidian larval CNS are shared with vertebrates (Meinertzhagen et al., 2004; Meinertzhagen and Okamura, 2001). Some of these conserved mechanisms, such as dorsoventral patterning involving Pax3/7 (Wada et al., 1997), anteroposterior patterning by retinoic acid (Nagatomo and Fujiwara, 2003) and organizer activity of FGF8/17/18 (Imai et al., 2009), have so far been observed only in chordates. As such, Ciona has the potential to serve as a model system for studying chordate-specific gene regulatory networks underlying the development of the CNS.

Recently, preliminary gene regulatory networks have been described at single-cell resolution for each cell in the vegetally derived neural plate and neural tube in the embryo up to the tailbud stage (Imai et al., 2006; Imai et al., 2009). In other studies, the morphological diversity of CNS neurons in swimming larvae has
been described using fluorescence microscopy (Imai and Meinertzhagen, 2007; Okada et al., 2001; Takamura et al., 2010; Yoshida et al., 2004). However, because pan-neural or neurotransmitter-related fusion genes were used to visualize the neurons, as opposed to individually labeling them, the identities and lineages of these cells remain uncertain. Thus, there is a gap in information between the cell lineages and gene regulatory networks in the embryo, and the final morphology of the differentiated neurons of the tadpole.

In this study, we use fusion genes containing cis-regulatory elements (‘enhancers’) from several regulatory genes to label unique pairs of cells in the developing VG and visualize them in their final differentiated state in swimming larvae. Included in this analysis are enhancers from Ciona orthologs of transcription factors known to play a role in neuronal specification and differentiation in the spinal cord of vertebrates, such as Dmbx1, Vxs, Chx10, Islet1, Nk6, Pitx2 and Onecut/Hnf6. These fusion genes (hereafter termed ‘reporter constructs’) revealed morphological traits that are specific to single pairs of VG neurons. Furthermore, we present evidence that the morphology of distinct VG neuronal subtypes is regulated by these transcription factors, and that the FGF and Notch signaling pathways establish the unique expression profiles of each differentiating VG neuron.

MATERIALS AND METHODS

Molecular cloning

Cis-regulatory regions of Dmbx, Vxs, Nk6, Coe and Onecut were obtained by PCR off genomic DNA template isolated from California C. intestinalis adults using the following primers (all sequences indicated as 5’ to 3’ from left to right): Dmbx –3.5 kb F, CACTCCTGCTCATTAGGC; Dmbx –1 start R, TATGCGTATAAGGACCTGG; Vxs exons introns F, GGTCCTAAATGCAACTTGG; Vxs exons introns R, ATTCCTCTCCTGGACCCAG; Nk6 intron1 F, TGGC�AAAAATGCCCGCAACAAG; Nk6 intron2 R, TGGCA�CAAACCTACATTGGC; Coe –2.6 kb, GTTCATATTTGTGTCTGACAG; Coe +8 start mutATG R, TCCCGGATCCGGTTACCG; Onecut –4.2 kb F, TGTCGTTCTCCTCTAAGAGG; Onecut +105 R, CATACTGATAAAGCTGCAGT. Restriction enzyme sites were designed to the 5’ end of these oligonucleotides in order to clone the fragments into reporter expression plasmids. Dmbx, Coe and Onecut regulatory regions contained their respective endogenous basal promoters. In the case of Dmbx, the reverse primer was just 5’ of the putative start codon, while for Coe, the start codon was mutated. Vxs and Nk6 exonic/intronic fragments were cloned upstream of the basal promoter from the friend of GATA gene (bpFOG) (Rohrbacher et al., 2007), fused in frame to the reporter gene. For double-electroporation with Dmbx reporter construct, a smaller fragment containing DNA –3.5 kb to –2.1 kb upstream of the Dmbx transcription start site was amplified using Dmbx –3.5 kb F and Dmbx –2.1 kb R (AGTACTAGTCGCTTCACT), and cloned upstream of bpFOG in order to minimize reporter construct crosstalk. A smaller 279 bp fragment within the 3rd intron of Vxs was found to be sufficient to recapitulate Vxs expression, and was sometimes used in place of the larger fragment. This smaller piece was amplified using Vxs intron3 F (GTATGCTCTCTCTTTTGGG) and Vxs intron 3 R (AGAAGAATCTACGGAATTC) and cloned upstream of bpFOG. Plasmids were constructed using pCESA backbone (Corbo et al., 1997). Previously published drivers used in this study include Fg8/17/18 (Imai et al., 2009), Islet (Stolfi et al., 2010) and Pitx proximal fragment (Christiaen et al., 2005). To minimize promoter crosstalk, a smaller –3.4 kb fragment including the ‘P3’ and ‘P1’ elements from the Pitx proximal regulatory regions was amplified using Pitx P3 F (CTTAGATCGGGAATGCG) and Pitx P1 R (GTTCGTCGTACCTGGTTC) and cloned upstream of bpFOG.

The reporter genes used were lacZ with a nuclear localization signal or unc-76-tagged fluorescent proteins that label axons more efficiently than untagged versions (Dynes and Nglai, 1998). In a few instances, F-actin-binding GFP::Moesin fusion was used (Edwards et al., 1997).

In situ hybridization probes

Dmbx and Vxs probe template was prepared by cloning the coding sequence of either into PBSKM, the plasmid backbone of Ciona intestinalis gene collection library clones, linearized with KpnI and NotI. A 1 kb fragment from the coding sequence of Nk6 was amplified with Nk6 cDNA N F primer and Nk6 1 kb C R primer (ATCACCCTCTATGGTCCCG) and cloned in the same backbone with KpnI and NotI. Antisense ribonucleotide probe was synthesized in vitro using T7 polymerase (Roche), cleaned up with RNase-free DNase I (Roche) and purified using RNeasy Mini Kit columns (Qiagen).

Embryo handling, manipulation and analysis

Embryos (Hs/DMM/HsS/0808c12) template were retrieved from said gene collection. All template plasmids were linearized with NotI or SpeI and digoxigenin-UTP-labeled probe made by in vitro transcription with T7 RNA polymerase (Roche). Adult Ciona intestinalis were obtained from Pillar Point marina (Half Moon Bay, CA) or purchased from M-Rep (San Diego, CA). Fertilization, dechorionation, electroporation, fixation, counterstaining and mounting of embryos was carried out as established (Christiaen et al., 2009). Each electroporation used 50-100 μg of plasmid. Histone2B-fluorescent protein fusions are very stable and bright, and thus only 5-10 μg of these plasmids was needed per electroporation. The technique of ‘consecutive electroporation’ consisted of electroporating embryos with one plasmid mix, rinsing in sea water by transferring to new dishes and gentle swirling, then electroporation with a second plasmid mix. Fluorescent in situ hybridization coupled to β-galactosidase immunodetection was performed using a TSA-plus fluorescein tyramide signal amplification kit (Perkin-Elmer) and monoclonal mouse anti-β-galactosidase antibody (Promega F7278A) as previously described (Beth et al., 2007). Embryos were imaged using Nikon or Zeiss upright microscopes. Staging was estimated in hours after fertilization at 16°C, and confirmed by counting A9.30 lineage cells according to the mitotic history and staging nomenclature established for the neural tube (Cole and Meinertzhagen, 2001; Cole and Meinertzhagen, 2004).

RESULTS

Detailed descriptions of the mitotic history of the neural tube of Ciona have identified the precise lineages of the five pairs of cholinergic neurons comprising the VG (Cole and Meinertzhagen, 2004). Hereafter, we will refer to them as single cells on either side of the embryo: the four anteriormost neurons are descended from the A9.30 blastomere (in order, from anterior to posterior: A12,239,
A13.474, A11.118, A11.117), while the posterior-most 5th neuron (A10.57) is the posterior daughter cell of the A9.29 blastomere (summarized in Fig. 1C). We have previously shown that electroporation of 4.8 kb of genomic DNA located immediately upstream of the Fgf8/17/18 transcription start site fused to a reporter gene preferentially labels cells descended from the A9.30 blastomere, as Fgf8/17/18 is strongly expressed in A9.30 at the late gastrula stage (Fig. 1A,B) (Imai et al., 2009).

**Cell-specific reporter gene expression reveals neuronal subtypes in the VG**

By coupling fluorescent in situ hybridization to immunofluorescence-based detection of β-galactosidase driven by the Fgf8/17/18 enhancer, we visualized, with single-cell resolution, the expression patterns of three homeodomain-containing transcription factors (TFs): Dmbx, Vsx (also known Chx10) and Islet (Fig. 2A-C). These were described in previous studies as being exclusively expressed in a single pair of cells in the developing VG. Double in situ hybridization/antibody stains confirmed previous reports that, at the tailbud stage, Dmbx is expressed only in the A12.239 pair (Ikuta and Saiga, 2007; Takahashi and Holland, 2004), and Islet is expressed only in the A10.57 pair, in addition to being expressed in other tissues such as notochord, palps, pharyngeal mesoderm and bipolar tail neurons (Giuliano et al., 1998; Stolfi et al., 2010). In the distantly related ascidian *Halocynthia roretzi*, Islet has been shown to be transiently expressed in the A9.30 lineage, but maintained late only in A10.57 (Katsuyama et al., 2005). Similarly, we confirmed that Islet expression in the VG is eventually restricted to A10.57 (Imai et al., 2009). Vsx was found to be expressed by two pairs of neurons, initially being expressed in A11.117, and later in A13.474. This difference in the onset of Vsx expression is consistent with the difference in specification of these two pairs of neurons: A11.117 ceases mitotic activity at a time when A13.474 has not yet been born.

We next searched for enhancers in or surrounding these three genes that would be sufficient to recapitulate their cell-specific patterns. A fusion of 3.5 kb of Dmbx upstream DNA with unc-76-tagged enhanced green fluorescent protein (GFP) recapitulated strong expression in A12.239, with slight expression in its sister cell, A12.240 (Fig. 2D). Similarly, a DNA sequence spanning the entire Vsx transcribed region recapitulated expression in A11.117 and A13.474 (Fig. 2E, Fig. 3B), suggesting the presence of an intronic enhancer. Finally, we have previously described an upstream enhancer of Islet that drives reporter gene expression in A10.57 (Islet>GFP) (Stolfi et al., 2010), which we have also used in this study (Fig. 2F).

**Dmbx in A12.239**

When embryos electroporated with such reporter constructs were allowed to develop until hatching, we were able to visualize individual terminally differentiated neurons. A12.239, as revealed by Dmbx>mCherry, shows a thin axon projecting down the tail. However, it does not form the conspicuous, frondose endplates at the base of the tail revealed by electroporation of pan-neural reporter constructs (Imai and Meinertzhagen, 2007). Furthermore,
Despite a contralateral projection that could in theory help establish left/right coordination of muscle contraction during swimming, A12.239 expresses cholinergic markers and is not believed to be an inhibitory interneuron (Ikuta and Saiga, 2007). GABAergic interneurons that arise from a different lineage are situated at the base of the tail and contact the motoneuron axon bundles in a contralateral manner (Brown et al., 2005; Horie et al., 2010). These are more likely to serve as inhibitory interneurons for modulating oscillatory left/right motoneuron firing.

**Vsx in A13.474 and A11.117, Pitx in only A11.117**

Neurons A13.474 and A11.117, as revealed by Vsx>GFP or mCherry (Fig. 3B), have thin axons and do not form conspicuous endplates, but do not appear to project contralaterally. Their axons never cross the midline as they project down the tail. The two pairs are distinguished by cell body size. A13.474 has a smaller cell body than that of A11.117 and the other neurons (Fig. 3B,E). Furthermore, Vsx>GFP revealed neurites emanating from the soma of A11.117 but not of A13.474, possibly representing dendrites (Fig. 3E). These dendrites were not seen on any of the other neurons.

The two Vsx-expressing pairs of neurons were separately labeled by co-electroporation with Pitx reporter constructs. A proximal genomic DNA fragment that drives the expression of the homeodomain TF Pitx in the visceral ganglion has been previously described (Christiaen et al., 2005). Electroporation of embryos with Pitx>mCherry specifically labeled A11.117 but not A13.474 (Fig. 3F,G). Co-labeling with Vsx>GFP and Pitx>mCherry also shows that A13.474 lacks the putative dendrites that are specific to A11.117 (Fig. 3G,H). Pitx reporter gene expression was not seen prior to hatching (data not shown), suggesting Pitx is activated in A11.117 downstream of Vsx.

Recently, Pitx2 was identified as a novel marker of cholinergic spinal interneurons in mouse (Enijn et al., 2010). A11.117 is also cholinergic and its lack of endplates further suggests a cholinergic interneuron identity. By contrast, Vsx orthologs are more broadly associated with interneurons arising in different nervous tissues and expressing different neurotransmitters (Kimura et al., 2006; Svendsen and McGhee, 1995). Therefore, the combination of Vsx and Pitx could be important for the specification of a cholinergic spinal interneuron identity.

**Islet in A10.57**

A10.57, as revealed by Islet>mCherry or GFP and in accordance with previous studies, displays a cell body more elongated along its anterior-posterior axis than the other VG neurons (Imai and Meinertzhagen, 2007; Okada et al., 2002), but does not form prominent endplates (Fig. 3C,D). Putative motor endplates labeled by the Islet reporter were consistently smaller than the frondose endplates revealed by pan-neural reporter constructs (see Fig. S1 in the supplementary material).

**Nk6 in A11.118**

The preceding reporter constructs revealed the morphology of four out of five pairs of neurons in the VG, yet the frondose endplates still escaped cell-specific labeling. A fourth reporter construct, composed of the transcribed region of the Nk6 gene fused to GFP, stained frondose endplates in ~50% of transfected embryos (Fig. 4A; see Fig. S2 in the supplementary material). This indicated the presence of an intrinsic enhancer. In half these cases, the staining was associated mainly with one cell body (see Fig. S2 in the supplementary material). Upon co-electroporation of
Nk6\textsuperscript{mCherry} and Vsx\textsuperscript{GFP}, this cell body was shown to be situated between A13.474 and A11.117 (Fig. 4D) and probably corresponds to A11.118. This staining by the Nk6 reporter construct was unexpected, because, at the tailbud stage, Nk6 is expressed throughout the developing VG. This preferential labeling of A11.118 might be due to maintenance of Nk6 in this cell later in development. In situ hybridization of Nk6 coupled to immunodetection of β-galactosidase in embryos electroporated with Vsx\textsuperscript{lacZ}, which labels A13.474 and A11.117 (Fig. 4B). Co-electroporation of Nk6\textsuperscript{GFP} and Dmbx\textsuperscript{mCherry} or Islet\textsuperscript{mCherry} further supported our conclusion that this late Nk6\textsuperscript{+} cell is A11.118 and that it is the only motoneuron to form the frondose endplates contacting the lateral surfaces of the anterior tail muscle cells (Fig. 4C,E). In fact, co-electroporation with Islet\textsuperscript{mCherry} suggested that A10.57 might modulate A11.118.

Fig. 3. Reporter constructs label individual differentiated VG neurons in the swimming tadpole. (A) Dorsal view of A12.239 pair labeled with Dmbx\textsuperscript{mCherry}. (B) Lateral view of A13.474 (arrow) and A11.117 (arrowhead) on left side labeled with Vsx\textsuperscript{mCherry} (red). (C) Lateral view of A10.57 labeled with Islet\textsuperscript{mCherry}. (D-D\textsuperscript{+}) Successive electroporation results in a rare tadpole with mutually exclusive left-right mosaic uptake of Dmbx\textsuperscript{GFP} + Islet\textsuperscript{GFP} and Dmbx\textsuperscript{mCherry} + Islet\textsuperscript{mCherry} plasmid combinations. (D) Cell bodies of A12.239 and A10.57 on the right side of the embryo labeled with GFP. (D\textsuperscript{+}) Cell bodies of A12.239 and A10.57 on the left side of the embryo labeled with mCherry. When red and green channels are merged (D\textsuperscript{+}), the axon from GFP-labeled A12.239 on the right traverses the midline (dotted line) to associate with the mCherry-labeled axon of A10.57 on the left side, demonstrating the contralateral projections of the A12.239 pair. (E) Embryos electroporated with Vsx\textsuperscript{GFP}, which labels A13.474 and A11.117 pairs. (F) Ptx\textsuperscript{mCherry}, by contrast, labels only A11.117. (G) Merged image of E and F. A13.474 does not appear to have dendrites. (H) Magnified view of inset in F, showing putative dendrites belonging to A11.117 (arrows). (I) Dorsal view of an embryo electroporated with Ptx\textsuperscript{YFP}, showing labeling of both left and right cells in the A11.117 pair. The dendrites are oriented towards the midline, and the axons do not project contralaterally.

Fig. 4. Nk6 reporter reveals frondose motor endplates of A11.118. (A) Nk6\textsuperscript{GFP} reporter construct labels frondose motor endplates in 50% of electroporated embryos (see text for details). (A') Magnified view of boxed area in A. (B) Late Nk6 expression (green) seen in stage E90 embryos in between cells A13.474 and A11.117 as revealed by antibody staining for β-gal in embryos electroporated with Vsx\textsuperscript{lacZ}. (B') Magnified view of boxed area in B. (C) Larva electroporated with Nk6\textsuperscript{mCherry} (green) and Vsx\textsuperscript{GFP} (red). False-color scheme is inverted for consistency in the presentation of the data. (E) Larva electroporated with Nk6\textsuperscript{GFP} (green) and Islet\textsuperscript{mCherry} (red). (C'-E') Magnified or different focal planes of areas boxed in C-E to highlight frondose endplates always labeled by the Nk6 reporter construct, but not by Dmbx, Vsx or Islet reporters.
presynaptically, by what appears to be contacts onto the frondose endplates themselves (Fig. 4E; see Fig. S1B in the supplementary material).

None of the neurons exhibit an axon trajectory along the middle or ventral bands of the tail muscle, as was observed in the larvae of Halocynthia (Okada et al., 2002) and another ascidian species, Dendrodoa grossularia (Mackie and Bone, 1976). This difference in innervation could be due to the difference in size between the larger Halocynthia and Dendrodoa larvae relative to Ciona. In Halocynthia, the middle band-innervating neuron is termed ‘Moto-b’, but it is not known whether ‘Moto-b’ corresponds to A11.117 or A11.118. It is conceivable that the frondose endplates of A11.118 in Ciona could represent the vestiges of the more ventral axon trajectories seen in Halocynthia and Dendrodoa.

**Ectopic Dmbx or Vsx expression abolishes A11.118-specific motor endplates**

Our observations on the morphological diversity of the visceral ganglion (summarized in Fig. 5) raised the possibility that the unique TF expression profile of each VG precursor might be functionally related to their particular identity. To investigate the potential role of these TFs in regulating morphology in the VG, we misexpressed Dmbx, Vsx, Islet and Nk6 in all differentiating VG neurons using regulatory DNA from the Coe gene. Coe (Collier/Olf/EBf) transcription factors are involved in myriad cell fate decisions in metazoans, particularly in neurogenesis (Dubois and Vincent, 2001). A 2.6 kb genomic DNA segment located upstream of the Coe gene directs expression in all of the differentiating VG neural precursors (see Fig. S3 in the supplementary material).

The visualization of individual neurons was sometimes compromised upon misexpression of certain TFs (see Fig. S4 in the supplementary material). For example, cross-repressive interactions between Dmbx, Vsx, and Islet were revealed by in situ hybridization assays (Fig. 6A-C). Dmbx and Vsx strongly repress each other (Fig. 6A,B), while Dmbx and Islet have seemingly little effect on each other’s expression (Fig. 6A,C). Vsx can actually induce ectopic Islet expression (Fig. 6C), while Islet can repress Vsx in A13.474, but not in A11.117 (Fig. 6B), hinting at a more complicated interaction between these two genes.

This modulation in reporter construct expression did not allow us to fully characterize the morphology of individual cells under these conditions. There were no obvious morphological defects under conditions without cross-repression (e.g. Islet>Dmbx, data not shown). Nonetheless, using Fgf8/17/18>GFP to label all neurons in the A9.30 lineage we were able to visualize their axons in swimming larvae. Using this reporter, we observed multiple axons and axon growth cones originating from the VG in larvae electroporated with Coe>Dmbx (Fig. 6A,D,H; see Fig. S5A in the supplementary material). These axons were all thin and did not form frondose endplates like those seen in control larvae electroporated with Coe>lacZ (Fig. 6H; see Fig. S5B in the supplementary material). Electroporation of Coe>Vsx also mimicked this phenotype (Fig. 6E,H), suggesting that exclusion of Dmbx and/or Vsx from A11.118 might be important for its specification. By contrast, electroporation of Coe>Nk6 or Coe>Islet did not have a visible effect on motor endplate formation (Fig. 6F-H). This observation is not surprising as Nk6 and Islet are transiently expressed in A11.118 in wild-type larvae.

**Conversion of visceral ganglion precursors into ectopic A11.117-like neurons**

The preceding results are consistent with the idea that a transcription factor code is required for the specification of morphologically distinct neuron subtypes. However, the cross-repressive interactions not only interfered with our ability to distinguish individual cell bodies and axons, they also meant that certain driver>transgene combinations (e.g. Vsx>Dmbx) could not work owing to strong auto-repression.

In an effort to circumvent this problem, we manipulated candidate upstream cell signaling events. The goal was to change the identity of some cells without losing expression of the reporter constructs used for visualizing morphology and axon trajectories.

FGF signaling has been shown to be a major mechanism of cell fate specification in the Ciona neurogenic ectoderm (Bertrand et al., 2003; Hudson et al., 2007). We asked whether FGF signaling is involved in specifying the identity of the various neuron subtypes in the VG. The Fgf8/17/18 enhancer was used to drive expression of a truncated, dominant-negative FGF receptor [dnFGFR (Davidson et al., 2006)] in A9.30. Lineage-specific perturbation of signaling downstream of FGFR resulted in ectopic A11.117-like cells. In 90% (n=90) of electroporated embryos, all A9.30 descendants express Vsx>GFP (Fig. 7B). By contrast, ectopic Vsx>GFP expression (in cells other than A11.117 and A13.474) is seen in only 11% (n=75) of control embryos co-electroporated with Fgf8/17/18>lacZ. These ectopic A11.117-like cells all project axons down the tail but do not form endplates (Fig. 7C). These neurons also express Pitx>YFP, albeit more weakly, indicating perhaps some later requirement for FGF signaling in Pitx activation or an inhibitory effect of excess Vsx (see Fig. S6D in the supplementary material). Dmbx>GFP reporter gene expression was completely abolished (see Fig. S6C in the supplementary material).

These results are consistent with a conversion of the entire lineage to an A11.117-like identity (summarized in Fig. 7H). Ectopic dendrites were not seen. This could be due to non-cell
autonomous effects of having multiple A11.117 cells in contact with each other, or could be related to lower levels of Pitx expression. However, the lack of endplates, the ipsilateral axon trajectory, and cell shape and size indicate an acquisition of the A11.117 fate. Thus, there is a correlation between transcriptional state (Vsx+, Pitx+) and neuronal morphology in the VG.

Conversion of visceral ganglion precursors into ectopic A12.239-like neurons

We next investigated whether other signaling pathways might be acting to specify the other neurons in the VG. We perturbed Notch signaling in the A9.30 lineage, using the Fgf8/17/18 enhancer to express a mutant form of Su(H) incapable of binding DNA [Su(H)DBM] (Hudson and Yasuo, 2006). Upon Fgf8/17/18> Su(H)DBM electroporation, both A12.237 and A12.239 (the posterior daughter cells of A11.119 and A11.120, respectively) express Dmbx. As a result, a striking ‘off-on-off-on’ GFP pattern is seen in 66% (n=100) of embryos electroporated with Fgf8/17/18> Su(H)DBM and Dmbx>GFP (Fig. 7E). An alternating pattern of endogenous Dmbx expression is also seen by in situ hybridization (Fig. 7F). In 100% (n=100) of wild-type embryos, A12.239 is the only cell that expresses Dmbx (Fig. 7D). This ‘off-on-off-on’ phenotype suggests the specification of an ectopic A12.239-like neuron is not due to a simple breakdown in lateral

Fig. 6. Effects of ectopic expression of Dmbx, Vsx, Isl and Nk6. (A) Percent of electroporated embryos showing Dmbx expression upon electroporation with Coe>mCherry (control condition), Coe>Vsx or Coe>Islet. Only Fgf8/17/18>lacZ+ embryo hemispheres (left/ right) were assayed for colocalization with Dmbx transcript (by immunofluorescence staining for β-gal coupled to fluorescent in situ hybridization). Green shading indicates the fraction of β-gal+ embryo hemispheres showing Dmbx expression. Grey shading indicates the fraction of β-gal- hemispheres that do not show expression of Dmbx. Paucity of colocalization relative to control indicates that the transcription factor being overexpressed probably represses Dmbx. Panels to the right of the graph are representative embryos from each condition, showing A9.30 lineage in red and Dmbx expression in green. Dotted line represents the embryo midline. In second and third panels, both hemispheres are visible but only the left side has been electroporated. In Coe>Vsx condition, Dmbx expression is seen mainly in unelectroporated hemispheres, indicating that Vsx represses Dmbx. Left-right mosaic incorporation of plasmid thus provides us with an internal control. In Coe>Islet, Dmbx expression is just as likely to be seen in electroporated hemispheres as in unelectroporated hemispheres. This indicates relative lack of repression of Dmbx by Islet. Arrow indicates A12.239. (B) As in A, but instead looking at effects of Dmbx and Islet overexpression on Vsx. Open arrowhead indicates A13.474 cell; closed arrowhead indicates A11.117. Dmbx appears to repress Vsx in both cells, while Islet appears to repress Vsx in A11.117 but not in A13.474. Thus, although in our quantitative assay Coe>Islet is indistinguishable from the control, there is a qualitative difference between the two (A11.117-specific loss of Vsx). (C) Same as in A and B, but instead looking at effects of Dmbx and Vsx overexpression on Islet (Isl). Islet is expressed in A10.57 (open arrows), therefore we scored for ‘adjacent’ Fgf8/17/18>lacZ and neuronal Islet expression, instead of colocalization. Islet expression is also seen in the notochord, just ventral to the nerve cord. This notochord expression was not assayed. Double arrowhead indicates ectopic Islet expression in A12.239 caused by overexpression of Vsx. All in situ hybridizations carried out on a mixture of embryos at stages 23-25, from at least three independent electroporations per condition. (D) Larva electroporated with Coe>Dmbx and Fgf8/17/18>GFP. Axons from A9.30 descendants in the VG project down the tail but do not form motor endplates (inset). (E) Larva electroporated with Coe>Vsx and Fgf8/17/18>GFP, showing lack of endplates as in D. (F) Larva electroporated with Coe>Islet and Fgf8/17/18, showing endplates typical of control larvae. (G) Same as in F, but in a larva electroporated with Coe>Nk6 instead. (H) Quantification of endplate formation under conditions represented in D-G, plus Coe>lacZ control. Larvae were assayed for frondose endplates visible by GFP fluorescence, from Fgf8/17/18>GFP expression in A9.30 lineage. Bars represent percentage of larvae showing endplates, averaged over three replicates (±s.e.m.). Embryos in A-C were from a batch of mixed stages, from E65-E80.
Inhibition of neurogenesis. Rather, it suggests a conversion of A11.119 into an A11.120-like progenitor cell upon inhibition of Notch signaling (summarized in Fig. 7). By contrast, perturbation of Notch signaling in the A9.30 lineage does not affect the specification of A11.117 and A11.118, based on the lack of ectopic Dmbx>GFP expression in these cells, and normal Vsx>GFP expression in A11.117 (see Fig. S6B in the supplementary material).

Moreover, in 25% (n=300) of Fgf8/17/18>Su(H)DBM-electroporated embryos, ectopic Dmbx+ neurons grew axons that crossed the midline (Fig. 7G; see Fig. S6A in the supplementary material). Thus, ectopic Dmbx+ neurons project contralaterally, a feature unique to the Dmbx+ A12.239 pair within the VG. This observation suggests that the contralateral projection of A12.239 correlates with a unique transcriptional state as assayed by Dmbx>GFP expression; duplicating a transcriptional state results in a duplicate neuron with the same axon trajectory.

DISCUSSION
We have shown enhancers associated with TFs expressed in specific VG neuronal precursors to visualize the neurons controlling the swimming behavior of the Ciona tadpole. We have shown that neuronal subtypes in the Ciona VG arise in a stereotyped manner from cells expressing distinct combinations of TFs, which correlate with specific morphological features such as contralaterally projecting axons and frondose motor endplates (summarized in Fig. 5). These qualitative traits were largely invariant, though gross errors in axon outgrowth and targeting were sporadically seen in embryos displaying other non-specific developmental defects attributed to the electroporation protocol. Our observations on cell-specific morphological attributes such as cell shape and axon trajectory are consistent with previous studies that distinguished each neuron based on their position within the VG (Imai and Meinertzhagen, 2007; Takamura et al., 2010).

These neuron-specific reporter constructs should be useful for the visualization and manipulation of individual neurons in vivo. In fact, with a combination of three enhancers and three different fluorescent reporter genes, we were able to distinguish five pairs of neurons in a single tadpole through co-electroporation and multi-plexed fluorescent imaging (Fig. 5). This Ciona ‘brainbow’ (Livet et al., 2007) demonstrates the potential usefulness of these constructs as a tool for future studies on the ascidian CNS.

We have begun to document the exact signaling events involved in setting up the transcriptional and subsequent morphological diversity of the ascidian VG. These signaling events are not necessarily shared with vertebrates. For example, the Ciona neural tube is comprised of cells arranged in just four rows: one ventral, one dorsal and two lateral rows. All VG neurons arise from cells within the lateral rows. It is hard to reconcile this simple layout with dorsoventral patterning by long-range BMP and Shh signals in the vertebrate neural tube (Dessaud et al., 2008). The short-range patterning of the Ciona neural tube might instead depend on ascidian-specific processes. We have already shown that FGF and Notch are two signaling pathways involved in VG neuronal subtype specification. Signaling events used for a given cell fate decision can vary even between different ascidian species (Hudson and Yasuo, 2008), indicating flexibility of signaling pathway deployment in evolution. However, we believe the transcriptional networks operating downstream of cell fate choice may prove to be more conserved, and thus more interesting from a comparative standpoint.
It has been assumed that all five pairs of neurons in the VG are motoneurons, based on their expression of cholinergic markers (Horie et al., 2010). However, only A11.118 forms the large lateral motor endplates, whereas A10.57 forms smaller endplates. In vertebrates and Drosophila, motoneurons and interneurons are specified by a ‘motoneuron code’. The combinational activity of Islet and Lhx3 specifies primary motoneurons, while interneurons are specified in the absence of Islet, through action of Chx10/Vsx2 (Lee et al., 2008; Thaler et al., 2002; Thor et al., 1999). Our study suggests this regulatory code might also apply to the VG of Ciona. For example, Islet and Lhx3 are transiently co-expressed in A10.57, A11.117 and A11.118 (Katsuyama et al., 2005; Imai et al., 2009). The putative interneuron A11.117 goes on to express Vsx, consistent with conservation of a motoneuron code. The inhibition of motor endplate formation by misexpression of Vsx can be interpreted as the specification of ectopic interneurons at the expense of motoneurons.

We have also found a correlation between Dmbx expression and contralateral projections in the VG. Ectopic Dmbx+ neurons project contralaterally. In mice, Dmbx1 is expressed initially in the prospective midbrain, and later in hindbrain and spinal cord neurons (Gogoi et al., 2002). Dmbx1 knockout mice show early neonatal lethality owing to diminished milk intake, probably related to its function in the hindbrain (Fujimoto et al., 2007; Ohtoshi and Behringer, 2004). In zebrafish, Dmbx1 paralogs in zebrafish have been shown to regulate cell-cycle exit of neuronal progenitors in the retina and optic tectum (Kawahara et al., 2002; Wong et al., 2010). It is possible that Dmbx genes are involved in a regulatory code for decussating interneuron subtypes in vertebrates, such as the aforementioned Mauthner cells of fish and amphibians.

Future work will be required to determine the causal link between TFs and morphology in the VG. A gene network analysis might reveal the regulation of rate-limiting cellular effectors responsible for some of the distinctive properties of VG neurons. It is also possible that some TFs might regulate transient cellular processes, such as morphogenetic movements, rather than subtype identity. However, some possibly represent ‘selector genes’ that directly regulate the terminal differentiation genes responsible for subtype-specific morphological and physiological properties (Hobert et al., 2010). Future work on how these distinct neurons interconnect to control swimming will bring us closer to understanding how gene regulatory networks create behavior in a chordate.

Acknowledgements
We thank Justin Bosch, Phillip Cleves and Andy Cooc for assistance; the members of the Levine Lab for insightful discussion and suggestions; and Lionel Christiaen for critical reading of the manuscript. Supported by NSF grant 108 0445470 to M.L. and an ARCS fellowship to A.S. for critical reading of the manuscript. Supported by NSF grant.

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