



## I-SceI Meganuclease-mediated transgenesis in the acorn worm, *Saccoglossus kowalevskii*



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### ABSTRACT

Hemichordates are a phylum of marine invertebrate deuterostomes that are closely related to chordates, and represent one of the most promising models to provide insights into early deuterostome evolution. The genome of the hemichordate, *Saccoglossus kowalevskii*, reveals an extensive set of non-coding elements conserved across all three deuterostome phyla. Functional characterization and cross-phyla comparisons of these putative regulatory elements will enable a better understanding of enhancer evolution, and subsequently how changes in gene regulation give rise to morphological innovation. Here, we describe an efficient method of transgenesis for the characterization of non-coding elements in *S. kowalevskii*. We first test the capacity of an I-SceI transgenesis system to drive ubiquitous or regionalized gene expression, and to label specific cell types. Finally, we identified a minimal promoter that can be used to test the capacity of putative enhancers in *S. kowalevskii*. This work demonstrates that this I-SceI transgenesis technique, when coupled with an understanding of chromatin accessibility, can be a powerful tool for studying how evolutionary changes in gene regulatory mechanisms contributed to the diversification of body plans in deuterostomes.

### 1. Introduction

Transcriptional enhancers direct the spatiotemporal expression of genes, thus playing a crucial role in development. Advances in genome-wide analysis of chromatin state and transcription factor binding have predicted hundreds of thousands of potential regulatory elements in the human genome (Neph et al., 2012; Rada-Iglesias et al., 2011), and comparative genomic studies have suggested that components of this *cis*-regulatory landscape may be broadly conserved across animal phyla (Simakov et al., 2015). However, performing comparative functional studies of *cis*-regulatory elements remains challenging due to a general lack of transgenesis techniques in taxonomically important non-model systems. A goal of evolutionary developmental biology is to differentiate between *cis*-regulatory elements with ancient origins versus those with more recent origins that are associated with the emergence of morphological novelties. To explore such questions of gene regulatory evolution, efficient transgenesis techniques need to be developed in organisms in key phylogenetic positions that are informative about phenotypic innovation.

The hemichordate *S. kowalevskii* is a promising model in which to interrogate the correspondence between the evolution of *cis*-regulatory elements and deuterostome morphological innovation, due to: (1) its key phylogenetic position as a close outgroup to chordates (Cannon et al., 2014; Simakov et al., 2015); and (2) the compelling similarities in axial patterning mechanisms it shares with vertebrates that are involved in patterning key aspects of the vertebrate body plan despite strong morphological differences between the two groups (Lowe et al., 2015, 2006, 2003; Yao et al., 2016). Compared to vertebrates, *S. kowalevskii* has a modest body plan consisting of three major domains (proboscis, collar, and trunk), and a relatively simple nervous system composed of a diffuse basiepithelial nerve net, and dorsal and ventral nerve cords (Kaul and Stach, 2010; Kaul-Strehlow et al., 2015; Knight-Jones, 1952). Despite this simple anatomy, recent work has revealed close similarities in the gene regulatory network regulating early vertebrate brain regionalization and the anterior ectoderm of *S. kowalevskii* (Darras et al., 2018; Lowe et al., 2003; Pani et al., 2012). Furthermore, the genome of *S. kowalevskii* displays extensive synteny with the genome of the basal chordate amphioxus, and

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contains more than 6500 conserved non-coding elements found in all deuterostomes (Simakov et al., 2015). This remarkable level of genomic conservation, in addition to the common axial patterning mechanisms found between hemichordates and chordates despite the striking differences in their body plans, makes *S. kowalevskii* a valuable system for exploring the role of *cis*-regulatory elements in the evolution of these disparate morphologies. For this study, we sought to develop an efficient transgenesis technique that could be used to functionally characterize non-coding regulatory elements found within the *S. kowalevskii* genome.

Here, we present methods for I-SceI meganuclease-mediated transgenesis in *S. kowalevskii* embryos. I-SceI is an intron-encoded restriction endonuclease originally isolated from baker's yeast, *Saccharomyces cerevisiae* (Jacquier and Dujon, 1985). Previous work has shown I-SceI enables integrative transgenesis in Medaka, zebrafish, frogs, sea urchins, newts, lamprey, and the starlet sea anemone, *Nematostella vectensis* (Casco-Robles et al., 2010; Grabher et al., 2004; Ochiai et al., 2008; Ogino et al., 2006; Parker et al., 2014b; Renfer and Technau, 2017; Thermes et al., 2002). In this technique, I-SceI protein is co-injected into the fertilized zygote with a plasmid carrying a transgene flanked by 18-bp I-SceI restriction sites, which enable the enzyme to integrate the transgene into pseudo-sites within the genome (Thermes et al., 2002). To test this transgenesis method in *S. kowalevskii*, we demonstrate that meganuclease-mediated integration of promoter:GFP fusion cassettes can be used to: (1) drive ubiquitous expression; (2) efficiently replicate expression patterns for genes with restricted spatiotemporal profiles; and (3) label specific cell types. Further, we show that for some genes, a minimal chromosome immunoprecipitation sequencing (ChIP-seq) dataset was sufficient for the identification of putative enhancer and promoter elements, and describe a minimal promoter from the *S. kowalevskii* Gbx gene that can be used to characterize putative native enhancers found in *S. kowalevskii*, as well as to test exogenous enhancers from other animals. This work adds to the toolkit of experimental techniques available in *S. kowalevskii*, and demonstrates that it is now a model capable of providing insights into deuterostome evolution and chordate origins through the functional testing of *cis*-regulatory elements.

## 2. Materials and methods

### 2.1. Embryonic cultures

Gravid *S. kowalevskii* adults were collected from the intertidal in Waquoit Bay, MA and maintained in flow-through seawater tables at the Marine Biological Laboratory, Woods Hole, MA. Spawning and animal maintenance were carried out as previously described (Lowe et al., 2004).

### 2.2. Hemichordate transgenesis

I-SceI meganuclease-mediated transgenesis was adapted from previously established protocols from other organisms (Ogino et al., 2006; Parker et al., 2014b). Putative *S. kowalevskii* promoters and enhancers were identified by eye using a H3K27ac ChIP-seq dataset (see below) by selecting regions strongly enriched for H3K27ac signal, and these regions plus approximately 100–200 bp of flanking sequence were cloned into a reporter plasmid containing I-SceI meganuclease restriction sites (Parker et al., 2014a) upstream of an eGFP coding sequence and a SV40 late polyadenylation signal sequence using Gibson assembly (Gibson et al., 2009). In cases where no obvious H3K27ac enrichment was observed, 5–10 kb of genomic sequence immediately upstream of the predicted start codon was used for cloning instead (as for Synapsin, Supplemental Fig. 1D). All primer sequences used in this study can be found in Supplemental Table 1. To prepare injection mixtures, 10  $\mu$ l restriction digests containing 5 units of I-SceI enzyme (NEB), 1  $\mu$ l of CutSmart buffer, and 130 ng of reporter

plasmid (final concentration 13 ng/ $\mu$ l), were incubated at 37 °C for 40 min. Upon completion, 0.5  $\mu$ l of 50 mg/ml rhodamine was added to the restriction digest as a tracer and this mix was injected into *S. kowalevskii* embryos as previously described (Darras et al., 2011; Lowe et al., 2006).

### 2.3. Live imaging

Injected animals were screened for fluorescence using a Zeiss Axio Zoom.V16 or Zeiss SteREO Discovery.V20 stereo microscope. Photographs and movies of fluorescent embryos were captured with Zeiss AxioCam MRC5 or MRm cameras using the ZEN software package.

### 2.4. ChIP-seq library preparation and sequencing

The chromatin immunoprecipitation protocol was adapted from previous reports (Buecker et al., 2014). Briefly, 80–100  $\mu$ l of 48 hpf and 72 hpf old embryos were crosslinked with 1% formaldehyde for 10 min at RT, quenched with glycine at a final concentration of 0.125 M, and flash frozen and stored at –80 °C. Chromatin was sheared by sonication while emerged in an ice bath to 100–500 bp fragments. Sonicated chromatin was incubated with 5–10  $\mu$ g antibody and incubated overnight at 4 °C. Antibody was bound to Dynabeads by incubating 1 mL of antibody bound chromatin with 100  $\mu$ l Dynabead solution at 4 °C for 2–4 h. ChIP DNA were subject to end repair, A-tailing, adaptor ligation and cleavage with USER enzyme, followed by size selection to 250–500 bp and amplification with NEBNext sequencing primers (Cat#E7335S). Libraries were purified, quantified, multiplexed (with NEBNext Multiplex Oligos for Illumina kit, E7335S). Samples were sequenced with 40-bp single-end read (~150 million reads) on an Illumina HiSeq. 2000-seq (BioMicroCenter). Data were processed using the Galaxy sequence analysis platform and aligned to the *S. kowalevskii* genome using BowTie2 (Goecks et al., 2010; Langmead and Salzberg, 2012; Simakov et al., 2015). The antibody used for ChIP-seq was H3K27ac from Active Motif (#39133, 39134).

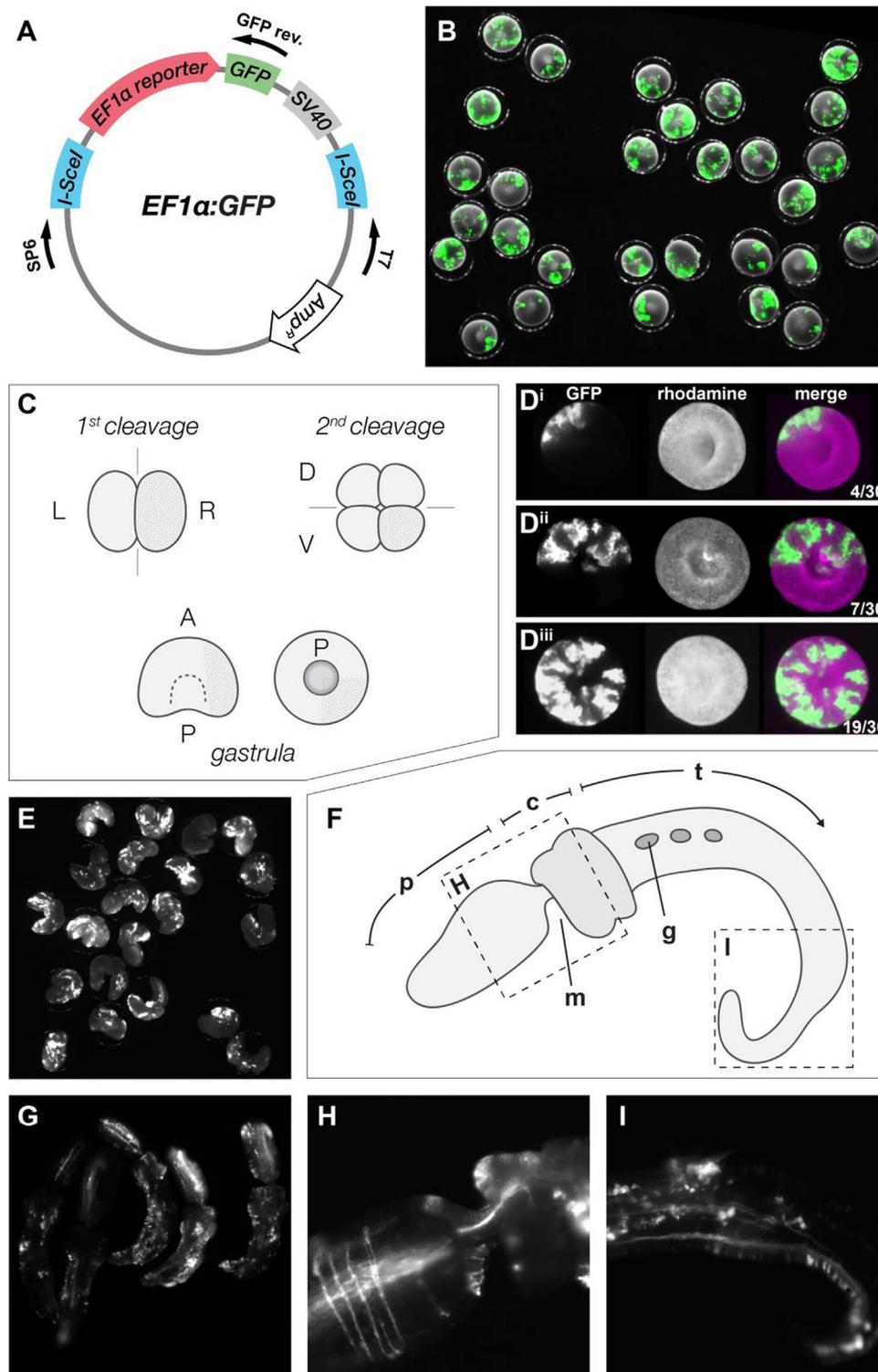
### 2.5. Fixation, antibody staining, and confocal microscopy

72-h embryos were fixed and stained as described previously (Gillis et al., 2012; Lowe et al., 2004). Briefly, embryos were fixed for 30 min at room temperature in fixation buffer (3.7% formaldehyde, 0.1 M MOPS pH 7.5, 0.5 M NaCl, 2 mM EGTA pH 8.0, 1 mM MgCl<sub>2</sub>, 1X PBS), and subsequently stored in ethanol at –20 °C. For antibody staining, embryos were rehydrated into 1x PBS +0.1% Triton X-100 (PBSTr), rinsed 3  $\times$  10 min in PBSTr, and placed into a blocking solution of PBSTr +5% goat serum for 2 h at room temperature. Embryos were incubated with anti-GFP antibody (Rabbit anti-GFP serum; Life Technologies #A-6455) at a 1:500 dilution overnight at 4 °C. After primary antibody incubation, embryos were washed 4  $\times$  30 min in PBSTr, and then incubated for 4 h at room temperature with secondary antibody (Alexa-Fluor 555 goat anti-mouse IgG, ThermoFisher #A28180) diluted 1:500 in blocking solution. Samples were then washed 4  $\times$  30 min in PBSTr and transitioned into 100% glycerol. Images were captured on a LSM 700 confocal microscope with a 10 $\times$  objective using the Zen software package (Carl Zeiss).

## 3. Results

### 3.1. I-SceI meganuclease is an efficient means of transgenesis in *S. kowalevskii*

To test the efficiency and mosaicism of I-SceI-mediated transgenesis in *S. kowalevskii*, we sought to create a transgene capable of driving ubiquitous expression in the developing embryo. Translation elongation factor 1- $\alpha$  (EF1 $\alpha$ ) is an essential component of the ribosome-



**Fig. 1.** Expression of EF1α in *S. kowalevskii*. (A) Schematic of the EF1α transgenesis plasmid. Approximately 1200 bp upstream of the *S. kowalevskii* EF1α gene was amplified from genomic DNA and cloned into the I-SceI plasmid. (B) A group of mid-gastrulae screened for positive GFP expression. (C) Early cleavage patterns of *S. kowalevskii* can be used to determine the timing of plasmid integration. The first cleavage defines the left and right axis of the embryo and the second defines the dorsal and ventral axis. Integration of the EF1α plasmid at the one, two, or four-cell stage should drive GFP expression ubiquitously, in half, or in one quadrant, of the developing embryo, respectively. (D) Expression of EF1α in mid-gastrulae. The left images in D<sup>i</sup>, D<sup>ii</sup>, and D<sup>iii</sup> show GFP in individual embryos expressed in one quadrant, one half, and throughout the embryo, respectively. Rhodamine was used as a tracer to follow successfully injected embryos. Merged GFP and rhodamine images are shown on the left along with the number of GFP expressing embryos that can be placed into each category. Integration of the EF1α construct occurs at the one-cell stage in the majority of GFP positive embryos. (E) Expression of EF1α in kink stage embryos. Strong GFP expression is seen throughout development of *S. kowalevskii*. (F) Schematic illustrating the tripartite body plan of the 3 gill slit stage *S. kowalevskii*. p, proboscis; c, collar; t, trunk; m, mouth; and g, gill slit. (G) GFP expression is maintained post hatching. (H) GFP expression shown in the proboscis and collar. (I) GFP expression in the trunk and post-anal tail of a hatched animal.

associated translational machinery in all eukaryotic cells, and its promoter element has been used to drive ubiquitous transgene expression in diverse organisms ranging from plants to vertebrates

(Curie et al., 1993; Krieg et al., 1989). We identified an EF1α ortholog in the *S. kowalevskii* genome. To aid in the identification of candidate promoters and enhancers, we performed a ChIP-seq experiment using

**Table 1**  
I-SceI Plasmids mediate transgenesis in *S. kowalevskii*.

I-SceI	Construct	Enhancer Element	Promoter	GFP Expression	Total Injected	Figure
+	pGPS2 NotI-linearized Control Plasmid	–	–	0 (0%)	25	Not shown
+	EF1 $\alpha$ :GFP	<i>ef-1 alpha</i> endogenous upstream region	<i>ef-1 alpha</i>	23 (92%)	25	Fig. 1; Supp. Movies 1–2
–	linearized EF1 $\alpha$ :GFP	<i>ef-1 alpha</i> endogenous upstream region	<i>ef-1 alpha</i>	5 (17%)	30	Not shown
–	PCR-amplified EF1 $\alpha$ :GFP	<i>ef-1 alpha</i> endogenous upstream region	<i>ef-1 alpha</i>	1 (4%)	27	Not shown
+	EF1 $\alpha$ :GFP without preincubation	<i>ef-1 alpha</i> endogenous upstream region	<i>ef-1 alpha</i>	1 (3%)	30	Not shown
+	Actin1:GFP	<i>actin1</i>	<i>actin1</i>	10 (67%)	15	Supp. Fig. 2
+	Engrailed:GFP	<i>engrailed</i> endogenous upstream region	<i>engrailed</i>	16 (52%)	31	Fig. 2; Supp. Movies 3–4
+	Gbx:GFP	–	<i>gbx</i>	0 (0%)	60	Fig. 4E
+	Hox11/13c:GFP	<i>hox11/13c</i> endogenous upstream region	<i>hox11/13c</i>	18 (62%)	29	Figs. 2, 4F; Supp. Movies 5–6
–	Hox11/13c:GFP	<i>hox11/13c</i> endogenous upstream region	<i>hox11/13c</i>	0 (0%)	25	Not shown
+	–2.7Hox11/13c-Pax6:GFP	–2.7 kb <i>hox11/13c</i> enhancer	<i>pax6</i>	0 (0%)	20	Not shown
+	–2.7Hox11/13c-Gbx:GFP	–2.7 kb <i>hox11/13c</i> enhancer	<i>gbx</i>	19 (42%)	45	Fig. 4G; Supp Movie 7
+	–38.6skSBE1-Gbx:mNeonGreen	+38.6 kb S.k. hh SBE1 enhancer	<i>gbx</i>	6 (10%)	60	Yao et al. (2016)
+	–7.5mmSBE1-Gbx:mNeonGreen	+7.5 M.m. shh SBE1 enhancer	<i>gbx</i>	5 (9%)	55	
+	–1111mmSBE5-Gbx:mNeonGreen	–1111 M.m. shh SBE5 enhancer	<i>gbx</i>	5 (33%)	15	

Plasmids generated and microinjected into *S. kowalevskii*. The columns from left to right reflect the presence or absence of the I-SceI meganuclease enzyme, name of the construct generated, enhancer element present, native or ectopic promoter used, penetrance of GFP expression, the number of viable embryos successfully injected, and the corresponding figure number for each construct.

an antibody to detect Histone 3 Lysine 27 acetylation (H3K27ac), a conserved marker of active chromatin (Mundade et al., 2014; Schwaiger et al., 2014). From this dataset we identified a 1.2-kb fragment upstream of its translational start site enriched for active chromatin marks during embryonic stages (Supplemental Fig. 1A). This region was amplified from genomic DNA and cloned into the I-SceI plasmid (Fig. 1A).

When microinjected into fertilized eggs, the EF1 $\alpha$ :GFP construct produced transgenic embryos with high efficiency (92%; Fig. 1B, Table 1, Supplemental Movie 1). No embryos with completely ubiquitous GFP expression were observed, but rather all embryos displayed a range of mosaic expression patterns. The early cleavage patterns of *S. kowalevskii* can be used to determine the timing of plasmid integration (Fig. 1C). The first division defines the left-right axis whereas the second defines the dorsal-ventral axis; therefore, integration of the plasmid at the one, two, or four-cell stage should lead to expression in the whole, one half, or one quadrant of the embryo, respectively. As predicted, the expression patterns of EF1 $\alpha$ :GFP could largely be sorted into three categories based on quadrants of GFP expression: mosaic expression restricted to one quadrant of the embryo (Fig. 1Di), restricted to one half (Fig. 1Dii), or throughout the whole embryo (Fig. 1Diii). GFP expression remained persistent through development, and strong GFP expression was maintained past hatching into juvenile stages (Fig. 1E–I). At 7 days post-fertilization, mosaic GFP expression was observed to continue in a number of adult tissues, including musculature in the proboscis (Fig. 1H, Supplemental Movie 2) and the ventral nerve cord (Fig. 1I). GFP expression was sustained for the duration of experimentation and observation, up to 21 days post-fertilization.

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In some transgenesis systems, such as Bacterial Artificial Chromosome (BAC)-mediated transgenesis (Gong et al., 2002; Jeong et al., 2006), injection of linearized DNA fragments is sufficient to drive genomic incorporation of transgenes. To test whether the inclusion of I-SceI protein in the injection mixture is necessary for transgenesis, the EF1 $\alpha$ :GFP construct was linearized, purified, and microinjected into fertilized eggs without supplemental enzyme. The linearized construct alone was capable of driving GFP expression, but the efficiency of transgenesis was much lower and the level of mosaicism was higher compared to injections containing I-SceI protein, thus illustrating the efficiency and efficacy of the enzyme in creating transgenic *S. kowalevskii* embryos (17%; Table 1). We next tested the PCR-amplified reporter fragment containing EF1 $\alpha$ :GFP and found that it was capable of driving expression, but with much lower efficiency and higher

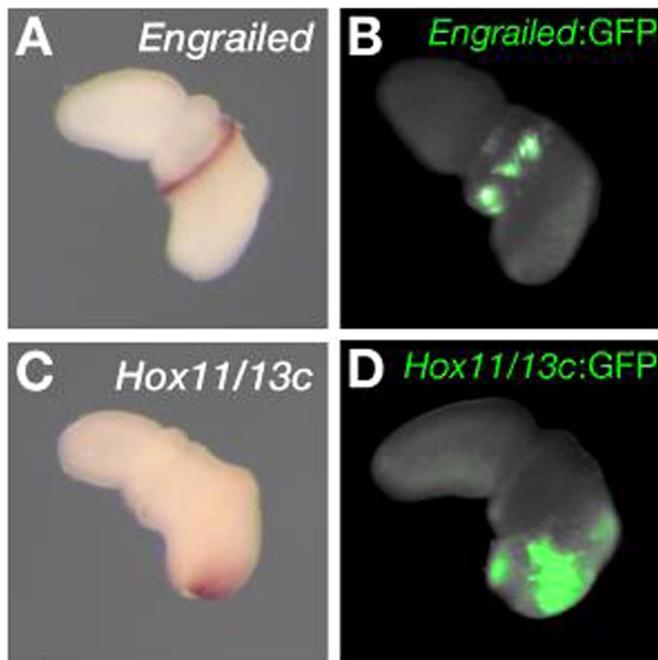
mosaicism (4%; Table 1). Finally, we sought to test the necessity of preincubating the I-SceI reaction for 40 min at 37 °C prior to injection. Injection of the EF1 $\alpha$ :GFP plasmid and I-SceI without preincubation again resulted in a much lower efficiency and high mosaicism (3%; Table 1). Together these results show the necessity of preincubating the I-SceI reaction prior to injection in order to efficiently drive GFP expression in *S. kowalevskii* embryos. A second ubiquitous expression construct using the *S. kowalevskii* Actin-1 gene was also tested, but was observed to be less efficient and more mosaic than EF1 $\alpha$ , and therefore was not further characterized (Table 1, Supp. Fig. 1B).

To further assess the utility of I-SceI transgenesis in *S. kowalevskii*, we sought to test whether the system could be used to drive spatiotemporally restricted gene expression without excessive ectopic expression. To this end, we selected two well-characterized transcription factor genes with highly regionalized expression patterns along the anteroposterior axis, Engrailed and Hox11/13c (Lowe et al., 2003). Upstream regions of these genes containing one or more putative enhancers and the native promoter were identified using the ChIP-seq dataset, and cloned into the I-SceI vector. Both the Engrailed:GFP and Hox11/13c:GFP constructs drove GFP expression with high efficiency (52% and 62%, respectively; Table 1), and successfully recapitulated the expression patterns detectable by *in situ* hybridization (Fig. 2, Supplemental Movies 3–6). The GFP expression observed with the Hox11/13c:GFP construct (Fig. 2D) extended slightly more anteriorly than the endogenous expression pattern detected (Fig. 2C), but was generally in the appropriate position along the anteroposterior axis (far posterior). Further, although mosaic, little ectopic GFP expression for both the Engrailed:GFP and Hox11/13c:GFP constructs was detected outside of the expected domains, thus showing I-SceI provides a simple and effective tool to produce accurate gene expression in *S. kowalevskii*.

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### 3.2. I-SceI can be used to label individual cell types

We next tested the efficacy of I-SceI-mediated transgenesis for labeling specific cell types. A cell type of interest in *S. kowalevskii* are neurons, due to longstanding questions about the similarities between hemichordate and chordate nervous systems (Lowe et al., 2015). The ability to label individual neurons or specific neuronal subtypes in *S. kowalevskii* would increase our capacity to study the organization of the hemichordate nervous system. As a general proof-of-concept experiment, we chose a gene that could likely serve as a general neuronal marker, the structural neural gene Synapsin. Synapsins are a



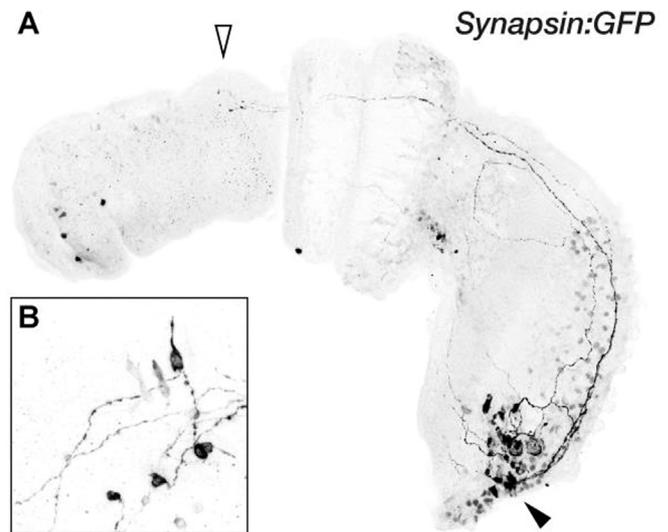
**Fig. 2.** Reporter constructs for genes with spatiotemporally restricted expression patterns recapitulate *in situ* hybridization results. (A) *In situ* hybridizations for Engrailed at the kink stage, 72 hpf. (B) GFP expression in *Engrailed:GFP*-injected embryos at the same stage as in (A) recapitulating the *in situ* hybridization expression pattern. (C) *In situ* hybridizations for Hox11/13c at the kink stage, 72 hpf. (D) *Hox11/13c:GFP* expression at the same stage.

family of neuronal phosphoproteins that function in synapse formation and plasticity and play a role in the regulation of synaptic transmissions (Cesca et al., 2010). Synapsins are expressed in both the central and peripheral nervous system and are generally absent or expressed at very low levels in non-neuronal cells (De Camilli et al., 1983a, 1983b, 1979). To this end, we cloned an 8 kb region upstream of the *S. kowalevskii* Synapsin ortholog into the I-SceI plasmid to test the hypothesis that the regulatory information within this upstream region should drive GFP expression specifically in neurons.

After the onset of neurogenesis, the Synapsin:GFP construct produced strong GFP expression in neuronal cell bodies and axonal projections in developing embryos at 72 h (Fig. 3). The high level of GFP expression, in combination with the inherent mosaicism of I-SceI transgenesis, was sufficient to allow for the observation of individual neurons and their processes, which was previously not possible using either *in situ* hybridization or immunofluorescence with common neurotransmitter antibodies. Using this technique, we were able to trace axonal projections of individual neurons projecting between distant body regions, such as from cell bodies in the posterior tip of the trunk across the collar and into the proboscis (Fig. 3A; arrows). Further, it was possible to observe the detailed morphology of individual neural cell bodies, which will allow for characterization of neuronal cell types (Fig. 3B).

### 3.3. A minimal promoter from the *S. kowalevskii* Gbx can be used to characterize putative enhancer elements

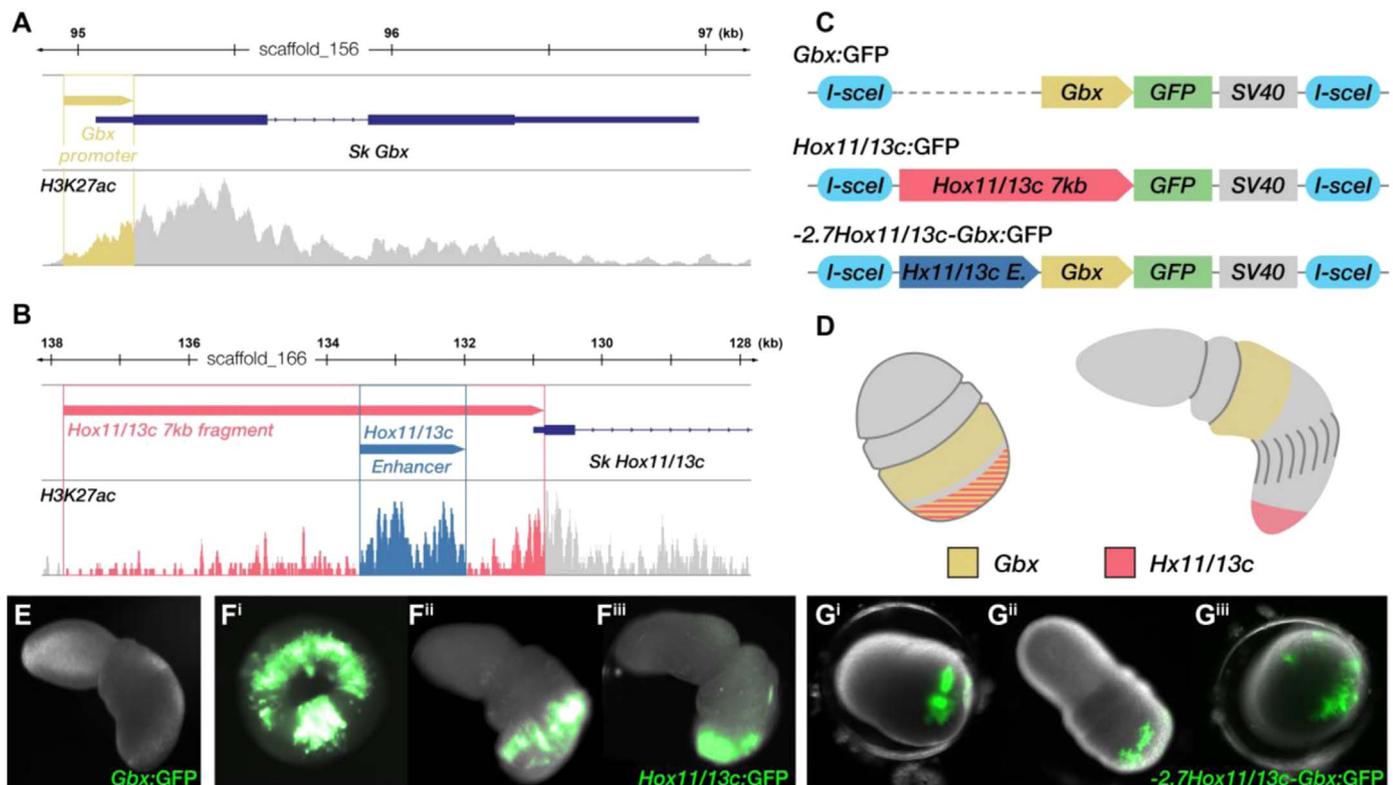
We next wanted to determine if the I-SceI transgenesis method could be used to test the activity of enhancer elements in *S. kowalevskii*. To this end, it was necessary to identify a minimal promoter with little to no background expression that could potentially interfere with the functional characterization of enhancers. We first tried common vertebrate minimal promoters - the mouse *c-Fos* and zebrafish *hsp70* promoters have proven to be effective for enhancer studies in lamprey and zebrafish; however, both were unsuccessful in driving



**Fig. 3.** Expression of Synapsin:GFP in the juvenile illustrates the possibility of labeling specific cell types with I-SceI meganuclease in *S. kowalevskii*. (A) Synapsin:GFP expression in the juvenile worm. Expression is seen in neuronal cell bodies in the far posterior of the worm, shown by the black arrowhead, with axon trajectories stretching to the proboscis, shown by the white arrowhead. This plasmid confirms that it is possible to label individual cell types in *S. kowalevskii*, in this case neurons. The mosaicism of the I-SceI method makes it easier to track individual neurons in the animal. (B) 40 $\times$  magnification of neuronal cell bodies and axon projections found in the proboscis.

GFP expression in *S. kowalevskii* (data not shown; (Parker et al., 2014a, 2011, 2014b)). We then reasoned that a native *S. kowalevskii* minimal promoter would likely be more effective at driving expression than an exogenous vertebrate promoter.

Utilizing the ChIP-seq dataset, we identified putative minimal promoters from several *S. kowalevskii* genes based on enriched H3K27ac signal proximal to the transcriptional start site, including Gbx, Pax6, and Engrailed (Fig. 4A). We also identified a 1.5 kb region enriched for H3K27ac located 2.7 kb upstream of *hox11/13c*, and within the 7 kb region in our validated *Hox11/13c:GFP* construct, as a potential enhancer of interest for this study (Fig. 4B). In order to test this region for enhancer activity, we used three constructs: (1) a negative control containing only the Gbx minimal promoter cloned into the I-SceI plasmid directly upstream of GFP; (2) the complete *Hox11/13c:GFP* construct with 7 kb of upstream sequence as a positive control; and (3) the 1.5 kb putative enhancer region cloned upstream of the Gbx minimal promoter (-2.7Hox11/13c-Gbx:GFP) (Fig. 4C). The expression patterns of Gbx and *Hox11/13c* have been well-characterized throughout *S. kowalevskii* development, and are expressed in the anterior and far posterior trunk, respectively, in spatially distinct domains, which we reasoned would allow for unambiguous interpretation of GFP expression resulting from the enhancer-promoter fusion (Fig. 2D; (Aronowicz and Lowe, 2006; Freeman et al., 2012; Lowe et al., 2003)). When injected into fertilized eggs, the minimal promoter control construct, Gbx:GFP, produces no expression (Fig. 4E). As previously observed, the *Hox11/13c:GFP* construct produced far-posterior expression beginning in the gastrula stage and persisting past hatching in a pattern recapitulating the expression observed in previous *in situ* studies (Fig. 4F). The enhancer construct, -2.7Hox11/13c-Gbx:GFP, produced GFP expression closely matching the pattern observed from the full 7-kb fragment containing the endogenous *Hox11/13c* promoter (Fig. 4G, Supplemental Movie 7). With the success of testing the *hox11/13c* and hedgehog enhancer elements from *S. kowalevskii* as well as sonic hedgehog enhancers from the mouse (Yao et al., 2016) with the same Gbx promoter, it is likely that this minimal promoter is general enough to be used in future enhancer tests in *S. kowalevskii*. An initial screen utilizing similar enhancer:-promoter fusions between the -2.7Hox11/13c element and the mini-



**Fig. 4.** The Gbx minimal promoter can be used to test enhancer activity in *S. kowalevskii*. (A) The Gbx minimal promoter, as identified from ChIP-seq data, is a 222 bp fragment located directly 5' of the Gbx start site. (B) The ChIP-seq dataset for the 7 kb region directly upstream of the Hox11/13c coding sequence. A putative Hox11/13c enhancer is found approximately 1 kb upstream of the start site. (C) The cloning strategy to test the efficacy of the putative Gbx minimal promoter and Hox11/13c enhancer element. Three different plasmids were created: (1) the putative Gbx minimal promoter cloned immediately upstream of the GFP start site, (2) the entire 7 kb region directly upstream of Hox11/13c cloned immediately upstream of the GFP start site, and (3) the putative Hox11/13c enhancer and cloned upstream of the Gbx minimal promoter. (D) Schematics of Gbx and Hox11/13c expression from *in situ* data during *S. kowalevskii* development. (E) Injection of the Gbx:GFP plasmid yields no expression. (F) Three different embryos (shown at mid-gastula 24 hpf (4F<sup>i</sup>) and kink stage 72 hpf (4F<sup>ii-iii</sup>)) that have been injected with the Hox11/13c:GFP plasmid. GFP expression recapitulates *in situ* data. Expression is only seen in the far posterior of the developing embryo. (G) Expression patterns of the -2.7Hox11/13c-Gbx:GFP plasmid in the far posterior of the embryo throughout development (shown at gumedo 36 hpf (G<sup>i</sup>), double-groove 48 hpf (G<sup>ii</sup>), and a posterior view of a double-groove embryo at 48 hpf (G<sup>iii</sup>)).

mal promoters identified upstream of Pax6 or Engrailed fusion constructs failed to produce any GFP expression, and therefore these promoters were not characterized further (data not shown).

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#### 4. Discussion

Here we report an I-SceI meganuclease approach to create transgenic *S. kowalevskii* embryos. The high efficiency of this protocol enables, for the first time in hemichordates, the functional characterization of putative non-coding regulatory elements in the genome. We demonstrate the feasibility of following gene expression in live embryos by cloning large upstream regions of genomic DNA containing regulatory information and the native promoter into a I-SceI plasmid. This protocol also enables the labeling of individual cell types, such as neurons, which will allow for a better understanding of the organization and diversity of neuronal subtypes that make up the *S. kowalevskii* nervous system. As additional cell-specific expression and single cell sequencing data becomes available, this technique will greatly improve our ability to explore cell type evolution within deuterostomes. Furthermore, the Gbx minimal promoter can be used to test enhancer activity in *S. kowalevskii* using both endogenous elements and enhancers from other species, making it a powerful tool for studying enhancer evolution.

The observation that GFP expression is seen predominantly to be either ubiquitous, or to be restricted to halves or quadrants of embryos developing from EF1 $\alpha$ :GFP-injected zygotes provides insight as to

when the I-SceI construct is integrating into the genome. This range of expression is likely explained by how the first two cell divisions establish the body plan of *S. kowalevskii*: the first cell division defines the left-right axis of the embryo, whereas the second defines the dorsoventral axis (Fig. 1C, after (Colwin and Colwin, 1953)). Therefore, plasmid integration at the one-cell stage would lead to expression throughout the embryo, two-cell stage in half of the embryo, and four-cell stage in one quadrant of the embryo. By screening EF1 $\alpha$ :GFP transgenic embryos, we found that the majority of I-SceI-mediated transgene integration likely occurs before first cleavage (Fig. 1D).

Despite the apparent early integration, all transgenic vectors showed variable signs of mosaicism, which has also been seen in I-SceI-mediated transgenesis in other systems (Ogino et al., 2006; Parker et al., 2014b; Renfer and Technau, 2017). In certain scenarios, such as labeling individual neurons, mosaicism may be advantageous: observing the complete morphology of a subset of cells is more informative than observing an entire population of labeled cells in which sub-structures and features of individual cells are obscured by one another. However, mosaicism can also give an incomplete picture of the expression domain of a gene, or of the full activity of an enhancer. Mosaic transgene expression likely results from integration at a later developmental stage. Further work will be necessary to more accurately determine the timing and genomic location of transgene integration using the I-SceI system. However, this work demonstrates that integration occurs early enough in development for this technique to serve as an effective means of transgenesis in *S. kowalevskii*.

Beyond mosaicism, some constructs also produced slight ectopic expression, or expression that did not completely mimic the endogen-

ous gene expression pattern observed by *in situ* hybridization. A challenge with the I-SceI system is the inability to control the genomic location of transgene integration. The genomic environment surrounding the site of integration greatly influences transgene expression, possibly due to activity of peripheral endogenous regulatory elements (Liu, 2013). Furthermore, in cases where only a short upstream fragment of genomic DNA was used in a reporter construct, it is possible that not all regulatory elements necessary to drive the complete expression pattern were included. A potential solution to address this concern is to apply the I-SceI system to BACs: a BAC containing the entire genomic locus surrounding a gene of interest is more likely to possess all of the regulatory information necessary to accurately recapitulate the endogenous expression pattern. Also, due to their large size, BACs may shield the transgene from unwanted positional effects at the site of integration, such as adjacent transcriptional activators or repressors. Due to the low efficiency of transgenesis seen when injecting linearized plasmids in *S. kowalevskii* without I-SceI enzyme, it may be necessary to incorporate I-SceI sites into the BAC vector to increase the likelihood of integration into the genome.

Finally, the choice of minimal promoter used when screening putative enhancers may be an important consideration for achieving accurate and robust reporter expression. When testing the  $-2.7\text{Hox11}/13\text{c}$  element using the Gbx minimal promoter, we observed more mosaicism than was seen with the complete 7 kb Hox11/13c:GFP construct, which may suggest that the Gbx minimal promoter is not as efficient as the native Hox11/13c promoter. However, we observed no ectopic expression outside of the expected posterior domain in these experiments, which suggests that this minimal promoter may not be a source of 'leaky' ectopic expression. Thus, the Gbx minimal promoter is an excellent candidate for future studies of enhancer activity.

Previous work has shown that *S. kowalevskii* is a promising model for understanding deuterostome evolution and chordate origins (Aronowicz and Lowe, 2006; Darras et al., 2018; Fritzenwanker et al., 2014; Gillis et al., 2012; Green et al., 2013; Lowe et al., 2006, 2003; Pani et al., 2012). However, studies of gene expression in this species were previously limited to *in situ* hybridization, with no means of assessing the contribution of non-coding elements to gene regulation. Initial studies of *cis*-regulation in *S. kowalevskii* have shown that an enhancer that is necessary for expression of Sonic Hedgehog during vertebrate brain development predates the origin of chordates (Table 1; (Epstein et al., 1999; Kiecker and Lumsden, 2004; Vieira and Martinez, 2006; Yao et al., 2016)), demonstrating that there is much that can be learned about enhancer evolution from cross-phyla comparisons between hemichordates and other deuterostomes. The continued development of I-SceI transgenesis will increase the ease and feasibility of further studies of *cis*-regulatory evolution in *S. kowalevskii*, and will greatly expand the range of experimental possibilities using this emerging model system.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.10.022.

## References

- Aronowicz, J., Lowe, C.J., 2006. Hox gene expression in the hemichordate *Saccoglossus kowalevskii* and the evolution of deuterostome nervous systems. *Integr. Comp. Biol.* 46, 890–901.
- Buecker, C., Srinivasan, R., Wu, Z., Calo, E., Acampora, D., Faial, T., Simeone, A., Tan, M., Swigut, T., Wysocka, J., 2014. Reorganization of enhancer patterns in transition from naive to primed pluripotency. *Cell Stem Cell* 14, 838–853.
- Cannon, J.T., Kocot, K.M., Waits, D.S., Weese, D.A., Swalla, B.J., Santos, S.R., Halanych, K.M., 2014. Phylogenomic resolution of the hemichordate and echinoderm clade. *Curr. Biol.* 24, 2827–2832.
- Casco-Robles, M.M., Yamada, S., Miura, T., Chiba, C., 2010. Simple and efficient transgenesis with I-SceI meganuclease in the newt, *Cynops pyrrhogaster*. *Dev. Dyn.* 239, 3275–3284.
- Cesca, F., Baldelli, P., Valtorta, F., Benfenati, F., 2010. The synapsins: key actors of synapse function and plasticity. *Prog. Neurobiol.* 91, 313–348.
- Colwin, A.L., Colwin, L.H., 1953. The normal embryology of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morphol.* 92, 401–453.
- Curie, C., Axelos, M., Bardet, C., Atanassova, R., Chaubet, N., Lescure, B., 1993. Modular organization and development activity of an Arabidopsis thaliana EF-1 alpha gene promoter. *Mol. Gen. Genet.* 238, 428–436.
- Darras, S., Fritzenwanker, J.H., Uhlinger, K.R., Farrelly, E., Pani, A.M., Hurley, I.A., Norris, R.P., Osowitz, M., Terasaki, M., Wu, M., et al., 2018. Anteroposterior axis patterning by early canonical Wnt signaling during hemichordate development. *PLoS Biol.* 16, e2003698.
- Darras, S., Gerhart, J., Terasaki, M., Kirschner, M., Lowe, C.J., 2011. beta-catenin specifies the endomesoderm and defines the posterior organizer of the hemichordate *Saccoglossus kowalevskii*. *Development* 138, 959–970.
- De Camilli, P., Cameron, R., Greengard, P., 1983a. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* 96, 1337–1354.
- De Camilli, P., Harris, S., Huttner, W., Greengard, P., 1983b. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. II. its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96, 1355–1373.
- De Camilli, P., Ueda, T., Bloom, F., Battenberg, E., Greengard, P., 1979. Widespread distribution of protein I in the central and peripheral nervous systems. *Proc. Natl. Acad. Sci. USA* 76, 5977–5981.
- Epstein, D.J., McMahon, A.P., Joyner, A.L., 1999. Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. *Development* 126, 281–292.
- Freeman, R., Ikuta, T., Wu, M., Koyanagi, R., Kawashima, T., Tagawa, K., Humphreys, T., Fang, G.C., Fujiyama, A., Saiga, H., et al., 2012. Identical genomic organization of two hemichordate hox clusters. *Curr. Biol.* 22, 2053–2058.
- Fritzenwanker, J.H., Gerhart, J., Freeman, R.M., Jr., Lowe, C.J., 2014. The Fox/Forkhead transcription factor family of the hemichordate *Saccoglossus kowalevskii*. *EvoDevo* 5, 17.
- Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., 3rd, Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.
- Gillis, J.A., Fritzenwanker, J.H., Lowe, C.J., 2012. A stem-deuterostome origin of the vertebrate pharyngeal transcriptional network. *Proc. Biol. Sci.* 279, 237–246.
- Goecks, J., Nekrutenko, A., Taylor, J., Galaxy, T., 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* 11, R86.
- Gong, S., Yang, X.W., Li, C., Heintz, N., 2002. Highly efficient modification of bacterial artificial chromosomes (BACs) using novel shuttle vectors containing the R6Kgamma origin of replication. *Genome Res.* 12, 1992–1998.
- Grabher, C., Joly, J.S., Wittbrodt, J., 2004. Highly efficient zebrafish transgenesis mediated by the meganuclease I-SceI. *Methods Cell Biol.* 77, 381–401.
- Green, S.A., Norris, R.P., Terasaki, M., Lowe, C.J., 2013. FGF signaling induces mesoderm in the hemichordate *Saccoglossus kowalevskii*. *Development* 140, 1024–1033.
- Jacquier, A., Dujon, B., 1985. An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell* 41, 383–394.
- Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M., Epstein, D.J., 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. *Development* 133, 761–772.
- Kaul, S., Stach, T., 2010. Ontogeny of the collar cord: neurulation in the hemichordate *Saccoglossus kowalevskii*. *J. Morphol.* 271, 1240–1259.

- Kaul-Strehlow, S., Urata, M., Minokawa, T., Stach, T., Wanninger, A., 2015. Neurogenesis in directly and indirectly developing enteropneusts: of nets and cords. *Org. Divers. Evol.* 15, 405–422.
- Kiecker, C., Lumsden, A., 2004. Hedgehog signaling from the ZLI regulates diencephalic regional identity. *Nat. Neurosci.* 7, 1242–1249.
- Knight-Jones, E., 1952. On the nervous system of *Saccoglossus cambrensis* (Enteropneusta). *Philos. Trans. R. Soc. Lond. B* 236, 315–354.
- Krieg, P.A., Varnum, S.M., Wormington, W.M., Melton, D.A., 1989. The mRNA encoding elongation factor 1-alpha (EF-1 alpha) is a major transcript at the midblastula transition in *Xenopus*. *Dev. Biol.* 133, 93–100.
- Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359.
- Liu, C., 2013. Strategies for designing transgenic DNA constructs. *Methods Mol. Biol.* 1027, 183–201.
- Lowe, C.J., Clarke, D.N., Medeiros, D.M., Rokhsar, D.S., Gerhart, J., 2015. The deuterostome context of chordate origins. *Nature* 520, 456–465.
- Lowe, C.J., Tagawa, K., Humphreys, T., Kirschner, M., Gerhart, J., 2004. Hemichordate embryos: procurement, culture, and basic methods. *Methods Cell Biol.* 74, 171–194.
- Lowe, C.J., Terasaki, M., Wu, M., Freeman, R.M., Jr., Runft, L., Kwan, K., Haigo, S., Aronowicz, J., Lander, E., Gruber, C., et al., 2006. Dorsoroventral patterning in hemichordates: insights into early chordate evolution. *PLoS Biol.* 4, e291.
- Lowe, C.J., Wu, M., Salic, A., Evans, L., Lander, E., Stange-Thomann, N., Gruber, C.E., Gerhart, J., Kirschner, M., 2003. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. *Cell* 113, 853–865.
- Mundade, R., Ozer, H.G., Wei, H., Prabhu, L., Lu, T., 2014. Role of ChIP-seq in the discovery of transcription factor binding sites, differential gene regulation mechanism, epigenetic marks and beyond. *Cell Cycle* 13, 2847–2852.
- Neph, S., Vierstra, J., Stergachis, A.B., Reynolds, A.P., Haugen, E., Vernot, B., Thurman, R.E., John, S., Sandstrom, R., Johnson, A.K., et al., 2012. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature* 489, 83–90.
- Ochiai, H., Sakamoto, N., Suzuki, K., Akasaka, K., Yamamoto, T., 2008. The *Ars* insulator facilitates I-SceI meganuclease-mediated transgenesis in the sea urchin embryo. *Dev. Dyn.* 237, 2475–2482.
- Ogino, H., McConnell, W.B., Grainger, R.M., 2006. High-throughput transgenesis in *Xenopus* using I-SceI meganuclease. *Nat. Protoc.* 1, 1703–1710.
- Pani, A.M., Mullarkey, E.E., Aronowicz, J., Assimacopoulos, S., Grove, E.A., Lowe, C.J., 2012. Ancient deuterostome origins of vertebrate brain signalling centres. *Nature* 483, 289–294.
- Parker, H.J., Bronner, M.E., Krumlauf, R., 2014a. A Hox regulatory network of hindbrain segmentation is conserved to the base of vertebrates. *Nature* 514, 490–493.
- Parker, H.J., Piccinelli, P., Sauka-Spengler, T., Bronner, M., Elgar, G., 2011. Ancient Pbx-Hox signatures define hundreds of vertebrate developmental enhancers. *BMC Genom.* 12, 637.
- Parker, H.J., Sauka-Spengler, T., Bronner, M., Elgar, G., 2014b. A reporter assay in lamprey embryos reveals both functional conservation and elaboration of vertebrate enhancers. *PLOS One* 9, e85492.
- Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., Wysocka, J., 2011. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* 470, 279–283.
- Renfer, E., Technau, U., 2017. Meganuclease-assisted generation of stable transgenics in the sea anemone *Nematostella vectensis*. *Nat. Protoc.* 12, 1844–1854.
- Schwaiger, M., Schonauer, A., Rendeiro, A.F., Pribitzer, C., Schauer, A., Gilles, A.F., Schinko, J.B., Renfer, E., Fredman, D., Technau, U., 2014. Evolutionary conservation of the eumetazoan gene regulatory landscape. *Genome Res.* 24, 639–650.
- Simakov, O., Kawashima, T., Marletaz, F., Jenkins, J., Koyanagi, R., Mitros, T., Hisata, K., Bredeson, J., Shoguchi, E., Gyoja, F., et al., 2015. Hemichordate genomes and deuterostome origins. *Nature* 527, 459–465.
- Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J., Joly, J.S., 2002. I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mech. Dev.* 118, 91–98.
- Vieira, C., Martinez, S., 2006. Sonic hedgehog from the basal plate and the zona limitans intrathalamica exhibits differential activity on diencephalic molecular regionalization and nuclear structure. *Neuroscience* 143, 129–140.
- Yao, Y., Minor, P.J., Zhao, Y.T., Jeong, Y., Pani, A.M., King, A.N., Symmons, O., Gan, L., Cardoso, W.V., Spitz, F., et al., 2016. Cis-regulatory architecture of a brain signaling center predates the origin of chordates. *Nat. Genet.* 48, 575–580.