

Limited functions of Hox genes in the larval development of the ascidian *Ciona intestinalis*

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SUMMARY

In animals, region specific morphological characters along the anteroposterior axis are controlled by a number of developmental genes, including Hox genes encoding homeodomain transcription factors. Although Hox genes have been regarded to play a key role in the evolution of morphological diversity, as well as in the establishment of the body plan, little is known about the function of Hox genes in invertebrates, except for in insects and nematodes. The present study addresses the role of Hox genes in body patterning during the larval development of the ascidian *Ciona intestinalis* conducting knockdown experiments of the seven Hox genes expressed during embryogenesis. Experimental results have demonstrated that *Ci-Hox12* plays an important role in tail development through the maintenance of expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* in the tail tip epidermis. Additionally, it has been shown that *Ci-Hox10* is involved in the development of GABAergic neurons in the dorsal visceral ganglion. Surprisingly, knockdown of *Ci-Hox1*, *Ci-Hox2*, *Ci-Hox3*, *Ci-Hox4* and *Ci-Hox5* did not give rise to any consistent morphological defects in the larvae. Furthermore, expression of neuronal marker genes was not affected in larvae injected with MOs against *Ci-Hox1*, *Ci-Hox3* or *Ci-Hox5*. In conclusion, we suggest that the contribution of Hox genes to the larval development of the ascidian *C. intestinalis* might be limited, despite the fact that *Ci-Hox10* and *Ci-Hox12* play important roles in neuronal and tail development.

KEY WORDS: Hox, Ascidian, Larval development, *Ciona intestinalis*

INTRODUCTION

Morphological diversification of body regions depends on the formation of characteristic structures along the various body axes. It has long been noted that the system specifying positional identity along the anteroposterior (AP) axis is based on an evolutionary conserved set of regulators, the Hox genes (Carroll, 1995; McGinnis and Krumlauf, 1992). Hox genes are known for the notable coordination, referred to as ‘colinearity’, between their expression patterns along the AP axis and the position within their clustered arrangement on a chromosome. This leads to the unique combination of Hox genes expressed at the different AP axial levels during development, which is also referred to as the ‘Hox code’ (Kessel and Gruss, 1991). Despite the fact that Hox genes have been regarded to play a key role in the evolution of morphological diversity, as well as in the establishment of the body plan, little is known about the function of Hox genes in invertebrates, except for in insects and nematodes. Therefore, it is considered necessary to understand the developmental role of Hox genes in a wide variety of invertebrates, especially in animal species occupying key positions in the phylogeny.

Ascidians belong to the subphylum Urochordata, a branch within the phylum Chordata. Their eggs develop into tadpole larvae, which share a prototypical morphogenesis and chordate body plan, characterized by the presence of a hollow dorsal neural tube, a notochord, paraxial mesoderm and a postanal tail (Satoh, 1994; Satoh, 2008). However, there are some crucial differences in the development between ascidians and vertebrates. In vertebrates, body

patterning along the AP axis proceeds from anterior to posterior, generating elaborate segmental vertebra, spinal cord and gut, for example. In this process, Hox genes play a crucial role; loss of the function of one Hox gene frequently leads to a drastic morphological defect, the homeotic transformation, in which the character of one body segment transforms into that of the flanking segment (Favier and Dolle, 1997). By contrast, ascidians exhibit extremely rapid and predominantly mosaic development, generating a simple tadpole-like body consisting of approximately 2600 cells without conspicuous segmental structures. Furthermore, they subsequently metamorphose into sessile adults with a unique body structure.

In the ascidian species *Ciona intestinalis*, nine Hox genes have been identified (Dehal et al., 2002). Previously we demonstrated that *Ciona* Hox genes are dispersed on two chromosomes and that eight Hox genes, *Ci-Hox1*, 2, 3, 4, 5, 10, 12 and 13 are expressed up to the juvenile stage. *Ci-Hox1*, 3, 5, 10 and 12 exhibit limited spatially coordinated expression in the larval neural tube, as do *Ci-Hox10*, 12 and 13 in post-larval gut development (Ikuta et al., 2004). Based on these observations, a scenario for the evolution of the ascidian body plan has been proposed, in which ascidians must have acquired their simple body plan and rapid embryogenesis together with extensive genomic rearrangement and gene loss, including disintegration of the Hox gene cluster and loss of some Hox gene members (Ikuta and Saiga, 2005). To fully evaluate the scenario, an in-depth functional analysis of the ascidian Hox genes needs to be performed.

To investigate the biological functions of the seven Hox genes of *C. intestinalis*, *Ci-Hox1*, 2, 3, 4, 5, 10 and 12, of which expression has been detected by whole-mount in situ hybridization (WISH) during embryogenesis, translational inhibition experiments using antisense morpholino oligonucleotides (MOs) directed against them were conducted. We have found that *Ci-Hox12* plays an important role in tail development by maintaining the expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* after the early tailbud stage. Additionally, *Ci-Hox10* is involved in the development of GABAergic neurons in

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the dorsal visceral ganglion. Conversely, for *Ci-Hox 1, 2, 3, 4* and *5*, no morphological defects were detected in the knockdown experiments. Furthermore, the expression of GABAergic and cholinergic neuronal marker genes was unaffected in larvae injected with MOs against *Ci-Hox1, 3* and *5*, despite the fact that *Ci-Hox1, 3, 5, 10* and *12* are coordinately expressed in the larval CNS (Ikuta and Saiga, 2005; Ikuta and Saiga, 2007; Ikuta et al., 2004). These results suggest that the roles of Hox genes are limited in ascidian larval development.

MATERIALS AND METHODS

Ascidians

C. intestinalis were cultivated at the Maizuru Fisheries Research Station of Kyoto University or at the International Coastal Research Centre, Ocean Research Institute, the University of Tokyo. Eggs and sperm were obtained surgically from the gonoducts.

Antisense morpholino oligonucleotides (MOs) and synthetic mRNAs

MOs (Gene Tools) used in the present study were as follows:

*Hox1*MO, equimolar mixture of 5'-AATCATCTTACACCTTCTATTAA-3' and 5'-CAGATCCCTCGTGTGCTGCTAC-3' (see below);

*Hox2*MO, 5'-TCCTGGCTCACATCTTTCCTAACTT-3';

*Hox3*MO, 5'-ATGCACTTTGTCGATCAATCTGTGA;

*Hox4*MO, 5'-TTGTCAGTAGAGATAATGAATTACC-3';

*Hox5*MO, 5'-AGCATTGTTCAAATCCATTATATCG-3';

*Hox10*MO1, 5'-TCGAAGTTTGAGGTTGAATATCCAT-3';

*Hox10*MO2, 5'-TGAGGTTGAATATCCATTGTTTTAA-3';

*Hox12*MO1, 5'-CATAGGGATTCAACAGACTAAATAT-3';

*Hox12*MO2, 5'-CGCCGCTAAAAACCTTTGCTGTCAT-3';

*Wnt5*MO, 5'-CATATTTCCGGCAACGATTCAAAC-3';

*Fgf8/17/18*MO, TACTCGCAATGCATTAAATCCGAAT (Imai et al., 2009).

Because both *Hox12*MO1 and *Hox12*MO2 produced the same morphological defects, only *Hox12*MO1 was used in the following experiments. In *Ci-Hox10* and *Ci-Hox12* knockdown experiments, mutated *Hox10*MO1 and *Hox12*MO1 were used as controls, respectively, with five base-pair mismatches: *Hox10*muMO, 5'-TCGCAGTTTCAGGTCAATATACAT-3'; and *Hox12*muMO, 5'-CATCGGGATACAACACACTAACTAT-3'. Additionally, a fluorescein-labelled standard control MO, FcMO (5'-CCTCTTACCTCAGTTACAATTTATA-3'), was used as the cell-lineage tracer.

The ability of *Hox1*MO, *Hox3*MO, *Hox4*MO, *Hox5*MO, *Hox10*MO1 and *Hox12*MO1 to inhibit translation was checked by examining the effect of the MOs on the expression of a *lacZ* reporter construct prepared by inserting a genomic DNA fragment of 3.5 kbp upstream of each Hox gene with the MO target sequence into the pPD46.21 vector (Fire et al., 1990). When eggs were injected with the construct alone, over 90% of the injected individuals exhibited positive X-gal staining at the tailbud or larva stage. By contrast, when eggs were co-injected with the reporter construct and the MO, none of the injected individuals showed positive X-gal staining for all the constructs, except that co-injection of two different MOs (see above) was required to inhibit *lacZ* expression of the *Ci-Hox1* reporter construct (data not shown). Accordingly, for knockdown experiments of *Ci-Hox1*, 10 fmole each of the two MOs were simultaneously introduced.

Additionally, MO target sequence in the genomic DNA of the MO-injected individuals was confirmed by direct nucleotide sequence determination of the DNA fragment amplified by PCR using genomic DNAs from ten MO-injected larvae as a template; for all MOs tested, no differences were observed in the target sequence between the MO and the genomic DNAs of the MO-injected individuals.

For the rescue experiments, synthetic *Ci-Hox12* mRNA lacking the MO target sequence was designed. For in vitro RNA synthesis, a cDNA fragment containing the full coding sequence of *Ci-Hox12* was subcloned into the pBluescriptRN3 vector (Lemaire et al., 1995) and used as a template for RNA synthesis with mMessage mMachine (Ambion).

Microinjection and sample preparation

For preparation of samples at the tailbud stage, unfertilized eggs were dechorionated and microinjected with 10–20 fmole of MO and/or 200 fg of reporter construct DNA, which were dissolved in 0.1×TE containing 0.5 mg/ml of Fast Green (Wako). For the knockdown of *Ci-Wnt5*, 2.5 fmole of *Wnt5*MO was co-injected with 1.25 fmole of FcMO into both b/h4.2 cells at the eight-cell stage. In rescue experiments, 10 fmole of MO and 20 pg of synthetic mRNA were introduced into an egg. Injected eggs or embryos were reared at 16–20°C in Millipore-filtered sea water (MFSW), or artificial sea water (Rohtomarine; Rohto, Japan). For preparation of samples of larvae, fertilized eggs with intact chorion were microinjected.

Histochemical staining and WISH

lacZ reporter gene expression was visualized by histochemical staining for β-galactosidase as described previously (Hikosaka et al., 1994). Phalloidin staining was carried out as described previously (Christiaen et al., 2005). Images were captured using a CCD camera on a Keyence BZ-8000 microscope, a Nikon E600 microscope equipped with Nikon EZC1 confocal scanning system, or a Zeiss LSM5 Exciter confocal scanning system. To measure the length of the notochord, AP dimensions of all the notochord cells were taken sequentially in a confocal sagittal section through the midline with ZEN LSM micro-imaging software (Zeiss) and added up for each specimen. For each measurement, twelve individuals were used.

WISH was carried out as described previously (Ikuta and Saiga, 2007; Ikuta et al., 2004; Katsuyama et al., 1995). Antisense RNA probes for *Ci-MRLC2*, *Ci-Wnt5*, *Ci-Otx*, *Ci-En*, *Ci-Sox1*, *Ci-Lhx3*, *Ci-Eph3*, *CiGC25h02* and *CiGC16g22* were synthesized using EST clones as templates, obtained from *Ciona intestinalis* Gene Collection release 1 (<http://ghost.zool.kyoto-u.ac.jp/indexr1.html>). Templates for RNA probe synthesis of *Ci-Hox1, 3, 5, 10* and *12*, *Ci-vAChTP*, *Ci-vGAT*, *Ci-Pax2/5/8* and *Ci-Fgf8/17/18* were as described previously (Ikuta and Saiga, 2007; Ikuta et al., 2004). For the simultaneous detection of three-marker gene expression, hybridization was performed in the presence of fluorescein-, digoxigenin-, and DNP-labelled probes. DNP was detected by anti-DNP-HRP and stained with cyanine-5 tyramide (Perkin Elmer). Imaging was as described previously (Ikuta and Saiga, 2007).

Treatment with Fgf signalling pathway inhibitor

U0126 (Promega), a MAPK kinase (MEK) inhibitor, or SU5402 (Calbiochem), an Fgf receptor inhibitor, was dissolved in dimethylsulfoxide (DMSO) to 10 mM and stored as stock solution at –80°C. Embryos at the neurula or early tailbud stages were transferred to freshly prepared 2, 10 or 20 μM U0126 or SU5402 in sea water, or to 0.02, 0.1 or 0.2% DMSO in sea water, reared until the mid-tailbud stage and fixed for phalloidin staining or WISH.

RESULTS

Translational inhibition of *Ci-Hox12* causes a morphological defect in the tail of tailbud embryos

To address the role of the seven Hox genes expressed during the larval development of *Ciona intestinalis*, translational inhibition experiments using MOs were carried out. In tailbud embryos and larvae injected with MOs against *Ci-Hox1, 2, 3, 4, 5* or *10*, no morphological defects were consistently detected (data not shown; see Fig. S1 in the supplementary material), despite the positive results of confirmatory experiments for the effectiveness of the MOs used (see Materials and methods). Furthermore, expression of the genes that share the expression domain with the Hox genes at tailbud or larval stage was unaffected in the tailbud embryos or larvae injected with MOs against *Ci-Hox1, 2, 3, 4* and *5* (see Fig. S1 in the supplementary material).

By contrast, embryos injected with a MO against *Ci-Hox12* had a round-ended tail, which was slightly shorter in length (9.9% on average, $n=12$, $P<0.001$) than that of control embryos (Fig. 1A,B;

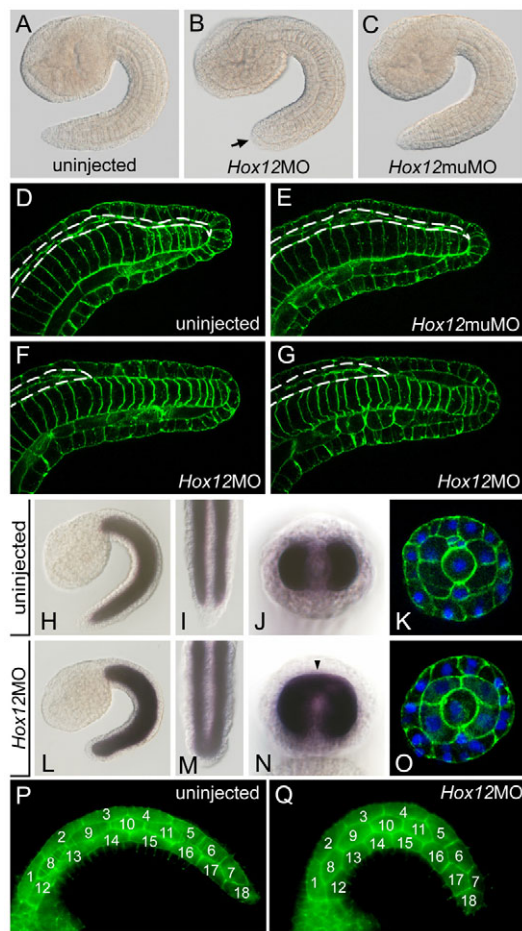


Fig. 1. Morphological defects in embryos injected with *Hox12*MOs. (A-C) Knockdown of *Ci-Hox12* gives rise to tailbud embryos with a shortened and round-ended tail (arrow, B). Anterior is to the left. (D-G) Confocal sections of mid-tailbud embryos indicated with phalloidin staining. Dotted lines indicate the range of the nerve cord; posterior is to the right, dorsal to the top. (H-O) Disposition of the muscle cells revealed by WISH for *Ci-MRLC2*, the myosin regulatory light chain 2 gene of *C. intestinalis* (H-J, L-N), and by phalloidin staining (K, O). (H, L) Lateral views, anterior is to the left. (I, M) Dorsal views of the tail. Anterior is to the top. (J, K, N, O) Optical (J, N) and confocal (K, O) sections at the level close to the tail end. In *Hox12*MO-injected embryos, muscle cell rows join posterior-dorsally (arrowhead, N). (P, Q) Phalloidin staining of uninjected control (P) and *Hox12*MO-injected (Q) embryos at mid-tailbud stage. In both panels, numbers are arbitrarily assigned to cells. Lateral views are shown; dorsal is to the top.

Fig. 4I, J). Eggs injected with the control mutated MO (*Hox12muMO*) developed into normal tailbud embryos with a tapered tail end (Fig. 1C).

In normal larval development, *Ci-Hox12* is expressed from neurula stage onwards in posterior ectodermal cells, and at the tailbud stage in the end of the posterior nerve cord and epidermis (Ikuta et al., 2004). To see the effect of *Ci-Hox12* knockdown, the morphology of *Hox12*MO-injected embryos was examined in detail by confocal microscopy. First, in embryos injected with *Hox12*MO, the epidermal cells at the tail tip were cuboidal, whereas they are columnar in control embryos (Fig. 1D-G). Second, the nerve cord did not reach the tail end and, in the region posterior to the nerve

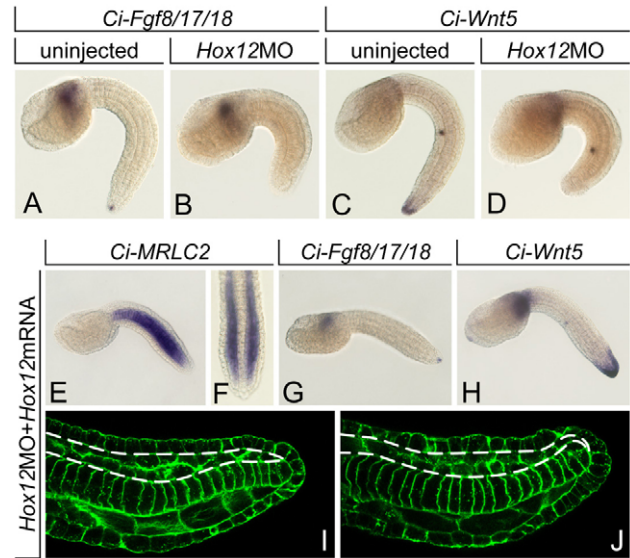


Fig. 2. Knockdown of *Ci-Hox12* specifically leads to loss of expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* at the tail tip and morphological defects. (A-D) WISH specimens at mid-tailbud stage. Genes examined for expression are indicated at the top. Lateral views are shown, anterior is to the left. (E-J) Co-injection of synthetic *Ci-Hox12* mRNA rescued the defects caused by the *Hox12*MO. (E-H) WISH specimens showing the muscle (E, F), and expression of *Ci-Fgf8/17/18* (G) and *Ci-Wnt5* (H). Lateral views are shown, anterior is to the left except for in F, which shows a dorsal view of the tail of the specimen shown in E. (I, J) Confocal sections of phalloidin-stained mid-tailbud embryos co-injected with *Hox12*MOs and *Ci-Hox12* mRNA. Anterior is to the left, dorsal to the top.

cord end, epidermal cells, or even more frequently one or two large cells, were located on the dorsal side of the notochord (Fig. 1F, G). The number of notochord cells was unchanged between *Hox12*MO-injected and control embryos (data not shown). Confocal transverse sections close to the tail end revealed that the muscle cells met on the dorsal side of the notochord in *Hox12*MO-injected embryos, whereas the nerve cord on the dorsal side of the notochord separated muscle cells laterally in control embryos (Fig. 1K, O). This was confirmed by visualizing the expression of a muscle marker gene *Ci-MRLC2*, the *Ciona* homologue of the myosin regulatory light chain gene (Fig. 1H-J, L, M). Furthermore, the number of muscle cells in *Hox12*MO-injected embryos was eighteen on each side of the tail, similar to the control embryos (Fig. 1P, Q); therefore, the abnormal localization of muscle cells is most likely to be indirectly produced by the defect of nerve cord elongation.

These observations indicate that *Ci-Hox12* plays an important role in tail development by controlling elongation of both the tail nerve cord and the tail itself, and by controlling the morphology of the tail end and of the epidermal cells at the tail tip.

Translational inhibition of *Ci-Hox12* affects signal transduction gene expression

To further understand the function of *Ci-Hox12* in the tail development, the effects of *Ci-Hox12* knockdown on gene expression at the tail end of tailbud stage embryos were examined. We identified *Ci-Fgf8/17/18* and *Ci-Wnt5* as the genes expressed in the tail tip epidermis in normal mid-tailbud embryos (our unpublished results; Fig. 2A, C; see also <http://hoya.zool.kyoto->

u.ac.jp/otherfr.html). Expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* in the tail tip epidermis was extensively reduced in *Hox12*MO-injected embryos, whereas expression outside of the tail tip was unaffected (Fig. 2A-D). Closer examination of the expression of these genes in embryos injected with *Hox12*muMO revealed that their expression was normal (data not shown).

In an attempt to demonstrate specificity of the action of *Hox12*MO, a rescue experiment was carried out in which embryos were co-injected with *Hox12*MO and *Ci-Hox12* synthetic mRNA. The positioning of the muscle cells was normal and the expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* at the tail tip was recovered (Fig. 2E,H). Furthermore, the nerve cord reached the tail end (Fig. 2I) and in some embryos epidermal cells at the tail tip recovered, exhibiting columnar morphology (Fig. 2J). Thus, the phenotypes observed in embryos injected with *Hox12*MO originated from the specific knockdown of *Ci-Hox12* gene function.

***Ci-Hox12* controls tail length through the maintenance of *Ci-Fgf8/17/18* expression**

To explore further the role of *Ci-Hox12* in controlling tail morphology, the roles of the signalling genes regulated by *Ci-Hox12* at the tail tip, *Ci-Fgf8/17/18* and *Ci-Wnt5*, were examined. At first, the effect of blocking Fgf signalling on tail morphogenesis was evaluated. Embryos were treated with SU5402, an Fgf receptor inhibitor, and U0126, an MEK inhibitor, from neurula stage, when the expression of *Ci-Fgf8/17/18* at the posterior end becomes detectable by WISH, and morphology was examined at the mid-tailbud stage. Embryos treated with 10 μ M SU5402 exhibited morphological defects over the whole body, tail elongation was severely inhibited and was accompanied by a slight intercalation defect of notochord cells; embryos treated with 2 μ M SU5402 did not show any defects (Fig. 3G,H). In embryos treated with U0126, a similar tail elongation inhibition was observed and was dose dependent (Fig. 3D-F). In this case, however, some columnar cells were observed on the dorsal side close to the tail tip (Fig. 3D,F, insets). This was in contrast to what was observed in embryos treated with SU5402, in which the columnar cells were located at the tail tip, similar to in normal embryos (Fig. 3H, inset). This difference might reflect a wider inhibitory effect of U0126 on activities downstream of various signalling molecules, including Fgfs. Furthermore, embryos injected with *Fgf8/17/18*MO had a shortened tail with columnar epidermal cells at the tip (Fig. 3I).

In normal development, *Ci-Fgf8/17/18* transcripts are detectable by WISH at the posterior end of the embryo from the neurula stage onwards, which is the same timing as the onset of *Ci-Hox12* expression. Therefore, it is likely that *Ci-Hox12* is involved in the maintenance but not the onset of *Ci-Fgf8/17/18* transcription. This is supported by the experiment examining *Ci-Fgf8/17/18* expression in *Hox12*MO-injected embryos at neurula and early tailbud stages. As shown in Fig. 4A-D, the frequency of *Ci-Fgf8/17/18*-positive embryos, which were indistinguishable from normal embryos, was 100% at the neurula stage and about 50% at the early tailbud stage. This is in contrast with the mid-tailbud stage, at which downregulation of *Ci-Fgf8/17/18*, as well as shortening of the tail, was observed in over 90% of *Hox12*MO-injected embryos. These observations suggest that the function of *Ci-Hox12* in maintaining *Ci-Fgf8/17/18* expression at the tail tip occurs from around the early tailbud stage onwards. Next, embryos were treated with SU5402 from the early tailbud but not neurula stage onwards and the length of the notochord was measured to see whether the treatment reproduces the tail shortening effect. The extent of tail shortening was 14.6% ($n=12$, $P<0.001$) in SU5402-treated embryos (Fig.

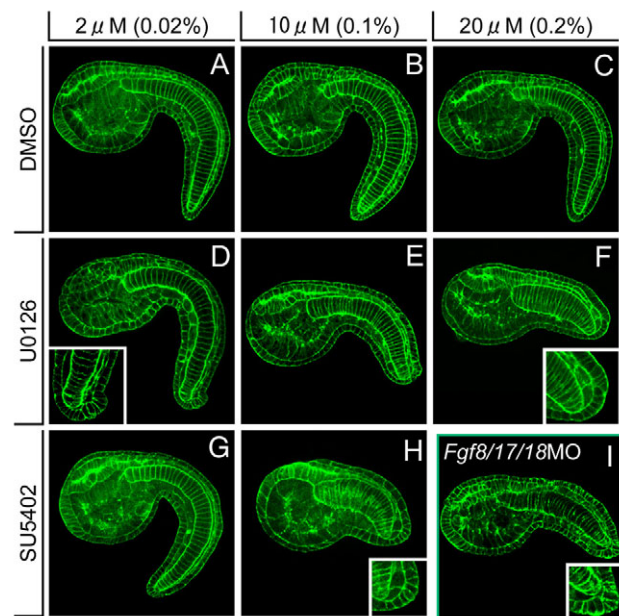


Fig. 3. Blocking of Fgf signalling inhibits elongation of the tail.

(A-H) Confocal sections of phalloidin staining of control embryos treated with DMSO (A-C) and embryos treated with U0126 (D-F) or SU5402 (G,H) from the neurula stage onwards and fixed at the time when control embryos reached the mid-tailbud stage. Concentrations of DMSO (0.02–0.2%), U0126 and SU5402 (2–20 μ M) are indicated at the top. (I) Confocal section of a mid-tailbud embryo injected with the *Fgf8/17/18*MO. Note that the embryo possesses a shortened tail with columnar epidermal cells at the tip. Insets show higher magnification of the tail end. Anterior is to the left, dorsal to the top.

4K,L), which was comparable to the 9.9% ($n=12$, $P<0.001$) observed in *Hox12*MO-treated embryos (Fig. 4I,J). It should be noted that the shortening of the tail was the only conspicuous morphological defect observed in the Fgf receptor inhibitor-treated embryos.

These observations suggest that *Ci-Hox12* plays an important role in maintaining the expression of *Ci-Fgf8/17/18* at the tail tip, which in turn controls the length of the tail, but not the cell morphology, after the early tailbud stage.

***Ci-Hox12* controls epidermal cell shape at the tail end through the maintenance of *Ci-Wnt5* expression**

Next, the involvement of *Ci-Wnt5* in tail morphogenesis was examined. For this, the b4.2 cells of the eight-cell stage embryo, the precursors of the posterior epidermal cells (Nishida, 1987), were co-injected with the MO against *Ci-Wnt5* and the tracer fluorescein-labelled standard control MO (FcMO). The morphology at mid-tailbud stage was examined after phalloidin staining. Control embryos injected with FcMO did not exhibit any defects. By contrast, embryos co-injected with *Wnt5*MO had cuboidal but not columnar cells at the tail tip, and a rounded rather than a tapered tail end (Fig. 5A,B). The length of the tail did not seem to be affected (data not shown). This observation suggested that *Ci-Wnt5* might be involved in the formation of columnar cells at the tail tip under the control of *Ci-Hox12*. As it was noted in the previous section that columnar cells were present in the embryos in which Fgf signalling was inhibited, the embryos treated with Fgf signalling inhibitors

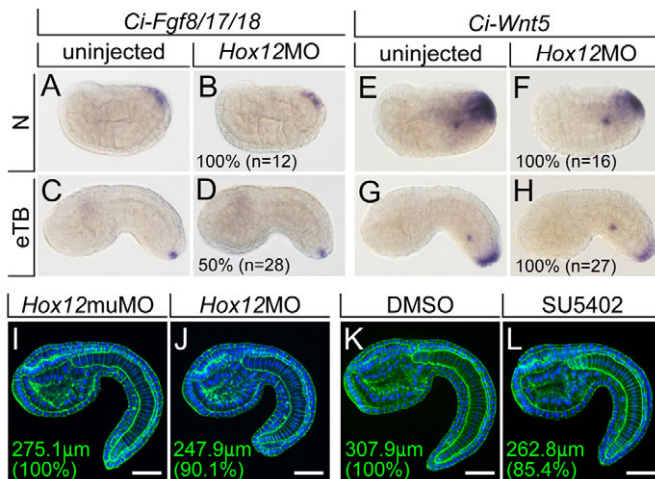


Fig. 4. *Ci-Hox12* regulates the expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* at the tail tip epidermis during tailbud stages.

(A-H) Expression of *Ci-Fgf8/17/18* (A-D) and *Ci-Wnt5* (E-H) in control embryos or *Hox12*MO-injected embryos at the neurula (A,B,E,F) and early tailbud (C,D,G,H) stages. Gene names are indicated at the top; stages investigated are indicated on the left. N, neurula; eTB, early tailbud. (I-L) Comparison of AP length of the notochord between control embryos and *Ci-Hox12* knockdown embryos (I,J), or between DMSO-treated control embryos and SU5402-treated embryos (K,L), using confocal sections. (K,L) Treatment with DMSO (0.1%) or SU5402 (10 µM) was done from the early tailbud stage onwards. Representative specimens from each experimental set ($n=12$ for each) are shown. Average length of the notochord and its percentage against that of the control are shown in green in each panel. For all panels, lateral views are shown, anterior is to the left. Scale bars: 50 µm.

were examined for *Ci-Wnt5* expression. As shown in Fig. 6, expression of *Ci-Wnt5* was detected in the tail end where the columnar epidermal cells were located (Fig. 6D-H). Furthermore, expression of *Ci-Wnt5* was also detected in the tapered tail end of the embryos injected with the *Fgf8/17/18*MO (Fig. 6I). Conversely *Ci-Fgf8/17/18* expression in the tail tip epidermis was unaffected in embryos injected into the epidermal lineage with *Wnt5*MO (Fig. 5C), which suggests that the function of *Ci-Wnt5* in the tail epidermis is independent of *Ci-Fgf8/17/18*.

Because the expression of *Ci-Wnt5* in the posterior epidermis becomes detectable by WISH at the gastrula stage, before the onset of *Ci-Hox12* expression, *Ci-Hox12* does not seem to be involved in the onset of *Ci-Wnt5* expression. Thus, the expression of *Ci-Wnt5* in *Hox12*MO-injected embryos at neurula and early tailbud stage was investigated. Expression of *Ci-Wnt5* was detected in the tail tip epidermis in 100% of the *Hox12*MO-injected embryos at both stages (Fig. 4E-H). This is in strong contrast to the mid-tailbud stage, in which over 90% of the *Hox12*MO-injected embryos showed a downregulation of *Ci-Wnt5*. These observations suggest that *Ci-Wnt5*, the expression of which at the tail tip is maintained by *Ci-Hox12* after early tailbud stage, plays a role in the formation of columnar cells at the tail tip and in the formation of tapered tail end morphology.

Functional analysis of *Ci-Hox1*, 3, 5 and 10 in larval CNS development

As described above, morphological defects were not detected in the tailbud embryos and larvae injected with MOs against *Ci-Hox1*, 2, 3, 4, 5 and 10. However, because they are coordinately expressed in

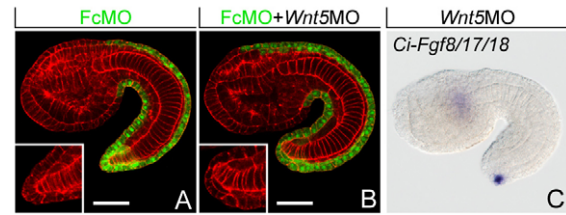


Fig. 5. *Ci-Wnt5* controls the morphology of epidermal cells at the tail end. (A,B) Confocal sections of embryos injected with FcMO (A) or co-injected with FcMO and *Wnt5*MO (B) in the b/b cells at the eight-cell stage. Fluorescein signal (green) indicates injected cell descendants. Embryos are counterstained with Alexa Fluor-546-phalloidin (red). Insets show higher magnification of the tail end. (C) Expression of *Ci-Fgf8/17/18* in embryos injected with *Wnt5*MO in the b/b cells. Anterior is to the left, dorsal to the top. Scale bars: 50 µm.

the neural tube during *C. intestinalis* embryogenesis, it has been envisaged that *Ci-Hox1*, 3, 5 and 10 might be involved in regionalization of the larval CNS (Ikuta and Saiga, 2007; Ikuta et al., 2004). Therefore, we examined whether a function of *Ci-Hox1*, 3, 5 or 10 in CNS development was detectable.

Ci-Hox1, 3, 5 and 10 have been reported to be expressed in a region corresponding to the prospective visceral ganglion and the anterior nerve cord (Ikuta and Saiga, 2007). Within the region, it has been shown that GABAergic neurons are present in the visceral ganglion and the anterior nerve cord, and that cholinergic neurons are located in the visceral ganglion. Both types of neuron are involved in controlling the locomotor activity of *Ciona* larvae (Brown et al., 2005; Takamura et al., 2002; Yoshida et al., 2004). To investigate the function of *Ciona* Hox genes in neuronal development, the expression of *Ci-vAChTP*, a cholinergic neuron marker, and of *Ci-vGAT*, a GABAergic neuron marker, was examined in larvae injected with MOs against *Ci-Hox1*, 3, 5 or 10. In uninjected control larvae, expression of *Ci-vAChTP* was detected in the sensory vesicle and the ventral visceral ganglion, and expression of *Ci-vGAT* was detected in the sensory vesicle, the dorsal visceral ganglion and the anterior nerve cord (Fig. 7A). Injection with MOs against *Ci-Hox1*, 3 and 5 did not affect the expression of either marker gene (Fig. 7B-D). Next, double knockdown experiments were conducted, as Hox genes have been described to function redundantly in a variety of animals (Akin and Nazarali, 2005; Favier and Dolle, 1997; Maconochie et al., 1996; Schilling and Knight, 2001). At the tailbud stage, *Ci-Hox1* and *Ci-Hox3* are co-expressed in a part of the presumptive visceral ganglion, and *Ci-Hox1* and *Ci-Hox5* are co-expressed in the anterior nerve cord (Ikuta and Saiga, 2007). Therefore, MOs against *Ci-Hox1* and *Ci-Hox3*, or *Ci-Hox1* and *Ci-Hox5*, were simultaneously introduced into fertilized eggs. In these cases too, no effects of the introduced MOs on neuronal marker gene expression or on larval morphology (Fig. 7G,H) were observed. By contrast, when either *Hox10*MO1 or *Hox10*MO2 was injected, the expression of *Ci-vGAT* in the dorsal visceral ganglion was downregulated in the larvae (Fig. 7E,F), whereas larvae injected with *Hox10*muMO exhibited normal expression of *Ci-vGAT* (data not shown). During normal development, *Ci-Hox10* is expressed in a small subset of dorsal cells of the anterior nerve cord at the tailbud stage, but expression becomes undetectable by the larval stage (Ikuta and Saiga, 2007; Ikuta et al., 2004); the developmental fate of the *Ci-Hox10* positive cells in relation to the larval CNS is not known. To assess the

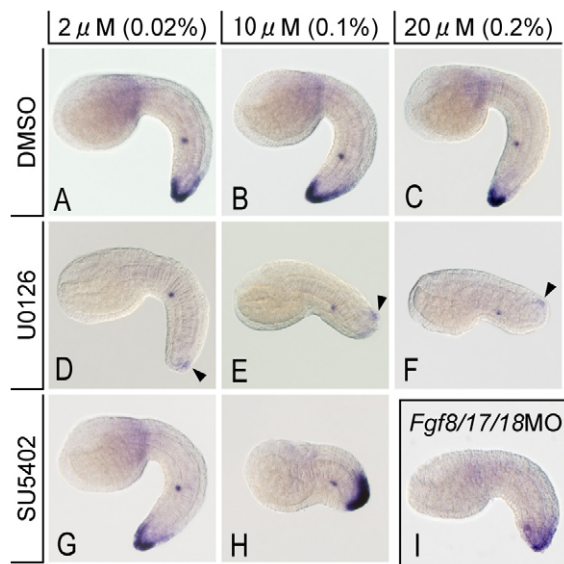


Fig. 6. Effects of blocking of Fgf signalling or knockdown of *Ci-Fgf8/17/18* on the expression of *Ci-Wnt5*. (A–H) Expression of *Ci-Wnt5* in embryos treated with DMSO (A–C), U0126 (D–F) or SU5402 (G,H). Note that expression of *Ci-Wnt5* in the tail tip epidermis was observed on the dorsal side close to the tail tip of embryos treated with U0126 (arrowheads) and at the tail end of embryos treated with SU5402. Concentrations of DMSO (0.02–0.2%), U0126 and SU5402 (2–20 μ M) are indicated at the top. (I) Expression of *Ci-Wnt5* in mid-tailbud embryos injected with *Fgf8/17/18*MO. For all panels, lateral views are shown; anterior is to the left.

developmental fate, a *lacZ* reporter construct harbouring 5 kb of DNA from upstream of *Ci-Hox10* was introduced into eggs. The construct recapitulated the endogenous expression of *Ci-Hox10* in the anterior dorsal nerve cord with a frequency of 28% ($n=21$) for X-gal staining and 33% ($n=32$) for WISH at the late tailbud stage (Fig. 7L,K). At the larval stage, X-gal staining was detected in the dorsal visceral ganglion in 24% ($n=21$) of the injected specimens (Fig. 7J); however, the transcriptional signal of *lacZ* gene was not detected at this stage ($n=21$; Fig. 7L), and thus this is likely to represent carry-over of the β -galactosidase protein from the tailbud stage. These results suggest that the cells expressing *Ci-Hox10* in the anterior dorsal nerve cord at tailbud stage contribute to GABAergic neurons in the dorsal visceral ganglion in the larva, and that the function of *Ci-Hox10* might be required for the development of these neurons.

DISCUSSION

The present study outlines the functional analysis of the Hox genes expressed during larval development of the ascidian *Ciona intestinalis* in order to understand their roles in 'chordate-type' larval development. We have demonstrated that *Ci-Hox12* and *Ci-Hox10* play roles in tail and neuronal development, respectively. Furthermore, and rather surprisingly, knockdown of *Ci-Hox 1, 2, 3, 4* and *5* did not cause any consistent morphological defects.

Involvement of *Ci-Hox12* in tail morphology

Upon the loss of *Ci-Hox12* functions, the distinctive morphology of the tail – a tapered end with several columnar epidermal cells at the tip – is lost and the length of the tail is also shortened. We have demonstrated that under the regulation of *Ci-Hox12*, *Ci-Wnt5*

contributes to the formation of the columnar cells and the tapered tail shape, and *Ci-Fgf8/17/18* contributes to the proper elongation of the tail. Although treatment with Fgf inhibitors could block the activity of any Fgf, *Ci-Fgf8/17/18* is most likely involved in this process. This was supported by the observation that embryos injected with *Fgf8/17/18*MO had a shortened tail with tapered end (Fig. 3I, Fig. 6I). Recently, it has been reported that *Ci-Fgf3/7/10/22* emanating from floor cells of the tail nerve cord directs notochord intercalation and, in turn, controls tail elongation through non-MAPK signalling (Shi et al., 2009). Our treatment with relatively high concentrations of U0126, and of SU5402, resulted in tail shortening with intercalation defects (Fig. 3). Because *Ci-Fgf8/17/18* is expressed only in a small number of cells in the tail tip epidermis and is expected to exert its effect in the close proximity of the tail tip, it is unlikely that *Ci-Fgf8/17/18* affects notochord intercalation over the length of the tail during tail elongation. We speculate that *Ci-Fgf8/17/18* might regulate tail elongation through the control of cell number or cell length along the AP axis of the posterior tail epidermis: if the function of *Ci-Fgf8/17/18* were inhibited, the tail epidermis would not elongate accordingly. The notochord intercalation defect observed upon the administration of a high dose of Fgf signalling inhibitors in the present study (Fig. 3) could be attributed to a secondary effect due to insufficient space in the tail for the notochord cells to intercalate properly.

For the impaired elongation of the nerve cord, a possible candidate downstream of *Ci-Hox12* that is involved in proper tail nerve cord elongation is *Ci-EphrinA-d*, which is expressed in the posterior half of the tail nerve cord and is downregulated upon knockdown of *Ci-Hox12* (data not shown). Ephrin/Eph signalling has been noted to regulate cell shape, movement and attachment (Himanen et al., 2007). Loss of *Ci-EphrinA-d* expression in the posterior nerve cord could induce a change in cell adhesion characters, which in turn might result in the loss of proper cellular contact between the posterior nerve cord and the tail end epidermis. Unfortunately, knockdown of *Ci-EphrinA-d* by MOs gave rise to such severe phenotypes of the embryo from early developmental stages onwards that the knockdown effect on nerve cord elongation could not be assayed (data not shown).

Gene regulatory network of Hox, Fgf, Wnt and Cdx in the posterior body might be different in *Ciona* to in vertebrates

In vertebrates, Fgf has been implicated in posterior body patterning via interactions with Wnt and retinoic acid, and in regulating the expression of Cdx, which in turn induces the expression of posterior Hox genes (Deschamps and van Nes, 2005; Keenan et al., 2006; Lohnes, 2003; Pilon et al., 2006; Shimizu et al., 2005). Regulation of Fgf and Wnt by Hox genes has never been reported in vertebrates. Gene regulatory relationships among Fgf, Wnt, Cdx and Hox in *C. intestinalis* seem to be different from those observed in vertebrates. In the present study, *Ci-Hox12* is required for the maintenance of expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* in the tail tip epidermis. As the expression of *Ci-Wnt5* at the tail tip was unaffected by treatment with an Fgf receptor inhibitor or injection of the *Fgf8/17/18*MO, and because expression of *Ci-Fgf8/17/18* at the tail tip was detectable in *Ci-Wnt5* knockdown embryos, the transcriptional regulation of these signalling molecules in the tail must be independent. Additionally, expression of *Ci-Hox12* was not affected by treatment with SU5402, injection of the *Fgf8/17/18*MO or the knockdown of *Ci-Wnt5* (see Fig. S2 in the supplementary material), which suggests that in *C. intestinalis* expression of the posterior Hox gene might not be regulated by Fgf or Wnt.

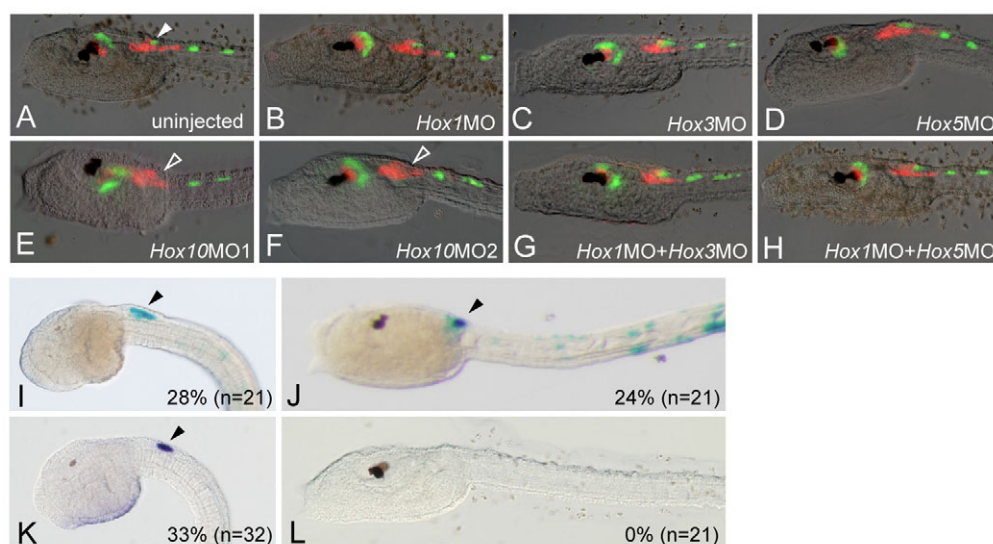


Fig. 7. Analysis of the effect of injection of MOs against *Ci-Hox1*, *3*, *5* and *10* on neuronal development at the larva stage. (A-H) Two-colour fluorescence WISH in larva injected with MO(s) or in uninjected control larva for the expression of *Ci-vAChTP* (red) and *Ci-vGAT* (green), which are markers of cholinergic and GABAergic neurons, respectively, in the visceral ganglion and the anterior tail. Note that expression of *Ci-vGAT* in the dorsal visceral ganglion was downregulated in larvae injected with either *Hox10MO1* or *Hox10MO2* (open arrowhead in E and F, which should be compared with the positive signal in control sample indicated by the white arrowhead in A). For all panels, bright-field and two-colour fluorescence images are merged. **(I-L)** Expression of a *lacZ* reporter gene driven by a putative *Ci-Hox10* enhancer shown by X-gal staining (I, J) and WISH (K, L) at the late tailbud stage (I, K) and the larval stage (J, L). Percentages of samples with positive reporter signals in the anterior nerve cord (black arrows in I, K) and dorsal visceral ganglion (black arrow in J) are shown at the bottom right. For all panels, lateral views of the trunk to the anterior tail are shown. Anterior is to the left.

Alternatively Fgf and Wnt might redundantly regulate the expression of *Ci-Hox12*. It is also possible that *Ci-Hox12* is downstream of the MAPK pathway and does not interact with Fgf, as expression of *Ci-Hox12* was not affected by treatment with SU5402 but was downregulated by treatment with U0126 (see Fig. S2 in the supplementary material). Additionally, the expression domain of *Ci-Hox12* is unlikely to overlap with that of *Ci-Cdx* (see Fig. S3 in the supplementary material), as the knockdown of *Ci-Hox12* or blocking of Fgf signalling did not affect the expression of *Ci-Cdx* (see Fig. S3 in the supplementary material). It should be noted that the expression of Fgf, Wnt and Hox genes in the ascidian tail is similar to the expression of the gene counterparts in the tailbud of vertebrate embryos, although the tail end of the ascidian embryo is not such a pool of undifferentiated cells as the vertebrate tailbud. In any case, the findings of this study point to novel interactions among the genes essential for chordate tail development.

Roles of *Ci-Hox1*, *2*, *3*, *4*, *5* and *10* in *Ciona* development

The neural tube in ascidian larva, in which *Ci-Hox1*, *3*, *5* and *10* are expressed, is an organ exhibiting regionalized structure, consisting of four regions along the AP axis: the sensory vesicle, the neck, the visceral ganglion and the tail nerve cord. As proposed previously, the neck, visceral ganglion and tail nerve cord could be homologous to the rhombospinal region of vertebrates (Ikuta and Saiga, 2007). In the vertebrate rhombomere, Hox genes have been reported to be key regulators for the differentiation of motoneurons. In many cases, knockdown or knockout of a Hox gene leads to striking defects in the character and distribution of motoneurons (Chandrasekhar, 2004; Guthrie, 2007). In the visceral ganglion and the anterior tail nerve cord of *Ciona* larva, GABAergic and cholinergic neurons have

been suggested to be regulators of locomotor activity (Brown et al., 2005; Takamura et al., 2002; Yoshida et al., 2004). Although we presumed that the identity or location of these neurons could have been affected by the knockdown of Hox genes, expression patterns of *Ci-vAChTP* and *Ci-vGAT*, which mark cholinergic and GABAergic neurons, respectively, were unaffected in larvae injected with MOs against *Ci-Hox1*, *3* and *5*, including after double knockdown of the co-expressed genes. Additionally, in the *Ci-Hox1* null mutant line (kindly provided by Y. Sasakura, University of Tsukuba, Japan), expression of *Ci-vAChTP* and *Ci-vGAT* in the larval visceral ganglion and anterior nerve cord was not affected (data not shown). Furthermore, expression of the regional marker genes of the CNS along AP axis was unaffected in mid- or late-tailbud embryos injected with MOs against *Ci-Hox1*, *3* and *5* (see Fig. S1 in the supplementary material). It should be noted, however, that the developmental fates of the cells expressing *Ci-Hox1*, *3* and *5* in the CNS are currently unknown. There should be cells other than cholinergic and GABAergic neurons in the CNS around the junction of the trunk and the tail, but their character and location still remain to be described. Moreover, the innervation patterns of individual neurons in ascidian larva are still unclear. Therefore, the possibility cannot be excluded that functions of *Ci-Hox1*, *3* and *5* in the CNS might be uncovered by investigating the differentiation and/or innervation of individual cells expressing Hox genes.

By contrast, our present data suggest that cells expressing *Ci-Hox10* in the anterior nerve cord at tailbud stages contribute to GABAergic neurons in the dorsal visceral ganglion of larva, and that *Ci-Hox10* is involved in the development of these neurons. Currently it is not clear whether the downregulation of *Ci-vGAT* in the dorsal visceral ganglion upon knockdown of *Ci-Hox10* results from cell death or a change in neuronal identity. Interestingly, it has

been previously reported that mouse Hox10 paralogous genes coordinately regulate lumbar motoneuron patterning (Lin and Carpenter, 2003; Wu et al., 2008). For further study, detailed anatomical data in combination with molecular data of the larval ascidian CNS are required.

Lastly, morphological defects in regions other than neural tissues were not detected in the embryos and larvae injected with MOs against *Ci-Hox 1, 2, 3, 4, 5* and *10*. Most of these genes have been reported to be expressed also in the mesoderm or endoderm during embryogenesis: *Ci-Hox1* in larval endoderm; *Ci-Hox4* in trunk lateral cells and the mesenchyme; *Ci-Hox5* in trunk lateral cells; *Ci-Hox10* in the posterior endoderm and the endodermal strand; and *Ci-Hox2* in the larval ectodermal atrial primordia [in a previous study, it was described in trunk lateral cells (Ikuta et al., 2004)]. As the cells in these regions remain undifferentiated in embryogenesis and later contribute to the adult body (Hirano and Nishida, 1997; Hirano and Nishida, 2000), it is possible that the roles of Hox genes in these tissues become evident during or after metamorphosis. In spite of this possibility, Hox genes seem to have very limited roles, if any, in the patterning of mesodermal and endodermal tissues during larval development.

Conclusions

Taken together, the findings of this study suggest that, apart from *Ci-Hox10* and *12*, *Ciona* Hox genes do not play crucial roles in the morphogenesis of tadpole larva. It has been proposed that Hox genes were organized into a cluster in the ancestor of deuterostomes and that the Hox gene cluster became disorganized in echinoderm and urochordate lineages (Cameron et al., 2006; Ikuta and Saiga, 2005; Lemons and McGinnis, 2006; Seo et al., 2004). It is an indisputable fact that ascidian Hox genes are no longer all clustered and that the colinearity is residual. The present study suggests that their functional roles are also limited as far as larval development is concerned. Coordinated functions of Hox genes to regionalize the segmental structure along the AP axis have been mainly described in relatively slow-developing animals; thus, a correlation is present between the limited roles of Hox genes in ascidian embryogenesis and the rapid embryogenesis that forms a tadpole larva with a simple body plan lacking obvious segmental structures.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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