*Hox*-mediated endodermal identity patterns the pharyngeal muscle formation in
the chordate pharynx

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Summary statement

Hox1 establishes the anterior-posterior identity in the pharyngeal endoderm of chordate ascidian. This regional identity of endoderm is essential for proper formation of pharyngeal muscles.

Abstract

The pharynx, possessing gill slits and the endostyle, is a characteristic of chordates that is a complex of multiple tissues well organized along the anterior-posterior (AP) axis. Although Hox genes show AP coordinated expression in the pharyngeal endoderm, tissue specific roles of these factors for establishing the regional identities within this tissue is largely unknown. Here, we show that Hox1 is essential for the establishment of AP axial identity of the endostyle, a major structure of the pharyngeal endoderm, in the ascidian Ciona intestinalis. We found that Hox1 knockout causes posterior to anterior transformation of the endostyle identity, and Hox1 represses Otx expression and anterior identity, and vice versa. Furthermore, alteration of the regional identity of the endostyle disrupts the formation of body wall muscles, suggesting that the endodermal axial identity is essential for the coordinated pharyngeal development. Our results reveal an essential role of Hox genes for establishment of the AP regional identity in the pharyngeal endoderm and crosstalk between endoderm and mesoderm for the development of chordate pharynx.
Introduction

The pharynx, with gill slits and the endostyle (or thyroid gland), is a defining chordate feature, while existence of gill slits can be extended to deuterostomes. The chordate pharynx is comprised of multiple tissues such as nerves, muscles and endodermal epithelia that are well-organized along the embryonic anterior-posterior (AP) axis. *Hox* genes are thought to play important roles to establish the regional identity of the pharynx along the AP axis (Couly et al., 1998; Gendron-Maguire et al., 1993; Hunt et al., 1991; Rijli et al., 1993). In the pharyngeal endoderm, *Hox* genes also exhibit coordinated expression during the vertebrate development (Shone et al., 2016). In mice, loss of *Hoxa1* and *Hoxb1* functions causes the absence of the third pharyngeal pouch derivatives, such as the thymus and parathyroid (Rossel and Capecchi, 1999). However, tissue-specific role of *Hox* genes in the pharyngeal endoderm is not well understood, except for *Hoxa3* which is involved in the development of the third pouch-derived organs (Chojnowski et al., 2014). A reason for the scarce understanding of the tissue-specific role of *Hox* genes is related with the multiplicity of their functions; *Hox* genes are expressed and function in various organs including pharyngeal endoderm. Therefore, little is known about how the regional identities are established within this tissue by *Hox* genes and how the patterning is coordinated with those in other tissues.

We have previously found that *Hox1* is expressed in the posterior part of the endostyle in the ascidian, *Ciona intestinalis* (Sasakura et al., 2012). The endostyle is a mucus-producing organ formed on the ventral midline of pharyngeal endoderm in non-vertebrate chordates and in larval lampreys, and is thought to be the precursor of the vertebrate thyroid gland (Salvatore, 1969). The ascidian endostyle is a representative structure of the pharynx and extends from the anterior to posterior ends of the pharynx.
(see Fig. 1K). Although $Hox1$ expression in the posterior endostyle suggests involvement of this gene in the AP patterning of the pharynx, its function is unclear. Here, we report an essential role of $Hox1$ for establishing the AP axial identity of the endostyle in $Ciona$. We also find that the AP axial identity of endostyle is necessary for the patterning of muscles in the pharynx, indicating that the endodermal patterning is the key event for establishing organized pharynx in chordates.

**Results and Discussion**

**$Hox1$ and $Otx$ establish the regional identity of the endostyle**

To explore the role of $Hox1$ in the endoderm, we carried out tissue-specific knockout of $Hox1$ using transcription activator-like effector nucleases (TALENs) (Treen et al., 2014). A TALEN pair designed to target $Hox1$ locus was expressed in the endodermal tissues under the control of $Tifl$ regulatory sequence that expresses downstream genes in the endoderm and small subset of neural cells (Ristoratore et al., 1999; Sasakura et al., 2012). The endoderm restrictive disruption of $Hox1$ allowed embryos to develop to juveniles with normal atrial siphons and gill slits, both of which are lost in the $Hox1$ mutant animals (Sasakura et al., 2012). We first examined the expression of $Otx$ and $Hox1$, which respectively mark the anterior and posterior endostyle as reported in another tunicate species (Cañestro et al., 2008) (Fig. 1A, E). Because TALENs generally introduce small deletions and/or insertions, expression of target genes usually remains in knockout animals, thereby allowing us to examine whether target genes are necessary for their own expression. Expression of $Hox1$ in the posterior endostyle was not detected when $Hox1$-
TALENs were expressed in the endodermal tissues (Fig. 1B). By contrast, Otx was ectopically expressed in the posterior endostyle in addition to the anterior endostyle (Fig. 1F). These results suggest that the identity of the posterior endostyle is transformed into the anterior one in the Hox1-knockout juveniles, and Hox1 is required to repress the anterior identity in the posterior endostyle. Then we tested whether Hox1 is capable of suppressing the anterior identity. Overexpression of Hox1 in the endodermal tissues was carried out using the Titf1 cis-element (Titf1>Hox1). Expression of Otx was not detected in the anterior endostyle of Titf1>Hox1 introduced animals (Fig. 1I), confirming that Hox1 represses the anterior identity in the endostyle.

It has been reported that Hox1 is expressed in the posterior trunk endoderm giving rise to the caudal pharynx in larval stages (Ikuta et al., 2004). To distinguish the role of Hox1 in the endostyle from the larval endoderm, we expressed Hox1-TALENs using an enhancer that drives gene expression in the endostyle after metamorphosis (Awazu et al., 2004). The stage-limited knockout of Hox1 recapitulated the ectopic expression of Otx in the posterior endostyle (Fig. S1). We also found that knockout of Hox1 in whole endoderm does not affect the expression of Otx at the larval stage (Fig. S1). These results indicate that the alteration of the posterior endostyle identity observed in Hox1-TALEN introduced juveniles is not a secondary effect of its disruption in the larval endoderm.

Next, we examined the role of Otx for establishing anterior identity in the endostyle. Knockout of Otx in the endoderm resulted in the loss of Otx expression in the anterior endostyle (Fig. 1G). On the other hand, Hox1 expression was expanded throughout the endostyle of juveniles introduced with Otx-TALENs (Fig. 1C). These results indicate that Otx is required for establishing the anterior identity of the endostyle
and repressing the posterior one. Then we carried out overexpression of Otx in the endoderm. Expression of Hox1 in the posterior endostyle was not detected when the Titf1>Otx was introduced (Fig. 1J). Taken together these results suggest that Hox1 and Otx play an opposing role for organize AP identity in the pharyngeal endoderm: Hox1 establishes the posterior identity of the endostyle and represses Otx expression and subsequent anterior identity, and vice versa.

Because Otx is expressed in the endodermal cells of cleavage stage embryos (Hudson and Lemaire, 2001), we examined whether the phenotypes seen in Otx-knockout juveniles are the results of the disruption of this gene in early embryogenesis. First, we did not detect mutations in the Otx locus of early gastrula embryos introduced with Otx-TALENs, while mutations were detectable at the larval stage (Fig. S1), suggesting that function of Otx in early development was not disrupted by TALENs expressed by Titf1 driver. We also found that expression of Hox1 is not altered in the larvae introduced with Otx-TALENs (Fig. S1). These results support the notion that expression of Otx in the anterior endostyle is required for establishing the anterior identity of endostyle.

We also found that morphology of the endostyle depends on the molecular identity. The anterior tip of endostyle is comprised of large columnar cells and shows protruded morphology, whereas the posterior tip has no such structure. In the Hox1-TALEN introduced juveniles, the posterior tip of endostyle showed the anterior-like morphology as well as expressing Otx (Fig. 1B, F and Fig. S1). On the other hand, the anterior tip became the posterior-like shape upon the knockout of Otx (Fig. 1C, G and Fig. S1). These results indicate that morphology of the endostyle is determined by Hox1 and Otx.
We next carried out simultaneous knockout of *Hox1* and *Otx*. In the double-knockout animals, *Hox1* expression was not detected whereas *Otx* was expressed in both the anterior and posterior endostyle (Fig. 1D, H). This result suggests that *Hox1* function is indispensable for *Hox1* expression itself, and the default identity of the posterior endostyle is the same as the anterior one expressing *Otx*.

**A retinoic acid-** *Hox1* **feedback loop maintains the posterior identity**

The default of anterior identity in the endostyle suggests that posterior identity with *Hox1* expression might require induction from other tissues. We investigated the mechanism that induces *Hox1* expression in the posterior endostyle. Retinoic acid (RA) is a well-known regulator of *Hox1* in *Ciona* (Kanda et al., 2009; Nagatomo and Fujiwara, 2003) as well as in vertebrates (Marshall et al., 1996). In *Ciona*, RA is involved in patterning of the central nervous system and posterior epidermis (Imai et al., 2009; Pasini et al., 2012). RA is also known to regulate endodermal patterning in tunicates (Hinman and Degnan, 1998, 2000). We carried out RA administration assay using the transgenic line EJ[MiTSAdTPOG]124 (hereafter referred to as EJ124) which is the enhancer trap line that expresses GFP under the control of *Hox1* enhancer (Sasakura et al., 2012). GFP expression was detected throughout the endostyle of EJ124 juveniles cultured with RA after metamorphosis, suggesting that *Hox1* expression in the endostyle is activated by RA signaling (Fig. 2A, B). To determine whether RA signaling is necessary to induce *Hox1* expression in the posterior endostyle, we carried out endoderm-specific knockout of the retinoic acid receptor, RAR (Nagatomo et al., 2003). Expression of GFP in the posterior endostyle became detectable from the 2 days post-fertilization (dpf) in control EJ124 animals (Fig. 2D). By contrast, GFP expression in the posterior endostyle was
undetectable in EJ124 animals introduced with TALENs for RAR (Fig. 2C). This suggests that RAR is indispensable to induce Hox1 expression in the posterior endostyle. We next analyzed expression of Raldh2 that encodes the RA synthesizing enzyme (Zhao et al., 1996). Raldh2 is expressed in a subset of the tail muscle cells during embryogenesis (Nagatomo and Fujiwara, 2003). In addition we found that Raldh2 is expressed in the posterior trunk endoderm of swimming larvae (Fig. S2). To test whether RA synthesized in the larval tissues is required to induce Hox1 expression in the endostyle, we carried out knockout of Raldh2 in muscle and endodermal tissues simultaneously. Expression of Hox1 in the posterior endostyle was diminished in animals introduced with Raldh2-TALENs (Fig. S2), suggesting that RA signaling from larval muscle and endoderm induces Hox1 expression in the endostyle.

To examine whether maintenance of the Hox1 expression in the posterior endostyle depends on RA, we analyzed expression of Raldh2 in juveniles. Raldh2 was expressed in the posterior end of the endostyle in juveniles (Fig. 2I). This localized expression became evident after tail absorption (Fig. S2). Then we tested whether this localized Raldh2 expression depends on the posterior identity of the endostyle. Expression of Raldh2 was undetectable both in the Hox1 knockout and in the Otx overexpressed animals (Fig. 2J, K). On the other hand, overexpression of Hox1 or knockout of Otx resulted in the duplicated expression of Raldh2 in both the anterior and posterior endostyle (Fig. 2L, M). These results suggest that Raldh2 is expressed in the posterior endostyle depending on Hox1-mediated posterior identity.

In order to test if Raldh2 in the endostyle is required for maintenance of Hox1 expression, we carried out an endoderm-specific knockout of Raldh2 using EJ124 animals. GFP expression in the posterior endostyle was observed in Raldh2-TALEN introduced
animals as well as in controls at 2 dpf (Fig. 2E, F), suggesting that Raldh2 function in the endoderm is not necessary for initiation of Hox1 expression in the endostyle. However, at 6 dpf, GFP expression in the posterior endostyle became hardly detectable in the Raldh2 knockout animals (Fig. 2G, H). This indicates that RA synthesis in the posterior endostyle is required to maintain Hox1 expression in the posterior endostyle. Taken together, these findings suggest that the RA-Hox1 positive feedback loop is necessary to establish the posterior identity of the endostyle: RA signaling from surrounding tail muscles and the posterior trunk endoderm can initiate Hox1 expression in the posterior endostyle region; in turn Hox1 up-regulates the Raldh2 expression in the endostyle, and RA synthesized in the posterior endostyle maintains Hox1 expression by the positive feedback (Fig. 2N). These data also suggest that broader expression of Hox1 throughout the endostyle observed in Otx knockout juveniles (Fig. 1C) is likely due to excess production of RA in both the anterior and posterior endostyle (Fig. 2M). We examined expression of Raldh2 in larvae introduced with TALENs targeting Hox1, Otx or RAR. Expression of Raldh2 in the posterior trunk endoderm was unaffected by knockout of these genes (Fig. S2). This suggests that RA-Hox1 and Otx genetic network establishes the AP identity of pharyngeal endoderm during post-larval development.

**Endodermal identity is essential for the directional elongation of body wall muscles**

In order to address the significance of the regional identity of the endostyle for the development of the pharynx, we analyzed the formation of the body wall muscles (BWMs), which develop along the AP axis of the pharynx. Ascidian BWMs, as well as atrial siphon muscles, are specified through a shared genetic program with vertebrate pharyngeal muscles (Diogo et al., 2015; Stolfi et al., 2010). BWM precursor cells are
located in the atrial siphon primordial before metamorphosis and BWMs elongate posteriorly towards the endostyle during metamorphosis (Stolfi et al., 2010) (Fig. 3A). Formation of BWMs was observed by expression of *myosin heavy chain 3 (MHC3)* gene, which marks the oral and atrial siphon muscles and BWMs (Stolfi et al., 2010). We found that BWMs failed to elongate toward the posterior endostyle when *Hox1* was knocked out in the endoderm. In *Hox1* knockout juveniles, approximately 45% of observed BWMs displayed misdirected elongation (Fig. 3B) and 29% of them did not show elongation (Fig. 3C). These results suggest that the posterior identity of the endostyle is required for the directional elongation of BWMs toward the posterior endostyle. Because the endostyle and the atrial siphon where precursors of BWMs situate are not adjacent to each other, there should be a signaling factor that is downstream of *Hox1* and promotes the directional elongation of BWMs. RA is a feasible candidate factor to promote the posterior elongation of BWMs. To test this possibility, we examined the BWM formation in the animals treated with a RA synthesis inhibitor, citral (Kanda et al., 2009; Marsh-Armstrong et al., 1994). In the citral treated condition, BWM seemed to start the posterior elongation but failed to complete it (Fig. 3D, E). Then we observed BWM formation in juveniles introduced with *Raldh2* TALENs. Knockout of *Raldh2* in the endodermal tissue disrupted the elongation of BWMs (Fig. 3F, G), suggesting that RA synthesized in the posterior endostyle is required for posterior elongation of BWMs. Next we treated juveniles with RA and analyzed BWM formation. In RA treated animals, elongated BWMs was not observed, suggesting that ubiquitous RA input inhibits the elongation or differentiation of BWMs (Fig. S3). In order to clarify whether BWMs are not formed or failed to elongate by RA treatment, we carried out live-imaging of BWM formation by expressing Kaede fluorescent protein in the BWMs with a *cis*-regulatory region of *MHC3*. This imaging
analysis revealed that BWMs made ectopic protrusions toward various directions but the muscles failed to posteriorly directed elongation upon the RA administration (Fig. 3H, I). These results further support the idea that RA synthesized in the posterior endostyle is necessary for directional elongation of the BWM towards the posterior endostyle. It is also possible that there is another factor that regulates directional elongation of BWMs, because citral treatment or Raldh2 knockout disrupted posterior elongation of BWMs but did not recapitulate the misdirected elongation observed in Hox1 knockout animals. To fully understand the mechanism underlining the directional elongation of BWMs, identification of the factor(s) that is downstream of Hox1 and/or Otx is necessary.

Conclusions

This study shows that in C. intestinalis, the anterior and posterior ends of the endostyle, a representative structure of pharyngeal endoderm, have distinct identities established by Otx and Hox1 transcription factors, respectively. In chordates, these factors are expressed in the pharyngeal endoderm during embryogenesis. In mice development, Otx2 is expressed in the first arch endoderm (Ang et al., 1994), and Hoxa1 and Hoxb1 are expressed in the caudal pharynx in a RA and Raldh2 dependent manner (Niederreither et al., 2003; Wendling et al., 2000). In the cephalochordate amphioxus, Hox1 is expressed in the endoderm under the control of RA and represses Otx expression, which determines the posterior limit of the pharynx (Schubert et al., 2005). Therefore, the AP patterning mechanism of Ciona endostyle, involving Otx and RA-Hox1, may have utilized the shared genetic mechanism that patterns the AP axis of the early pharyngeal endoderm in chordate development.
In vertebrate pharyngeal development, patterning and differentiation of pharyngeal muscles are regulated by cranial neural crest cells (NCC), which give rise to skeletal elements and tendons associated with muscles (Noden and Trainor, 2005; Rinon et al., 2007). Our present study demonstrates that proper formation of the BWM, a musculature surrounding the pharynx, is dependent on the regional identity of the endodermal organ. This suggests that in *Ciona*, which has no definitive NCC, the endoderm primarily plays an important role for organizing the pharynx along the AP axis.

**Materials and Methods**

**Animals**

Wild type *Ciona intestinalis* was cultivated at Maizuru (Kyoto), Misaki (Kanagawa), Mukaishima (Hiroshima) and Usa (Kochi). Transgenic line of *Hox1* enhancer trap, EJ[MiTSAdTPOG]124, was maintained by an inland culture system (Joly et al., 2007).

**Constructs and electroporation**

TALENs were assembled by 4-module golden gate method (Sakuma et al., 2013). The activity of the constructed TALENs were estimated by previously described method (Fig. S4) (Treen et al., 2014). Details of construction of expression vectors and electroporation procedures are described in supplementary Materials and Methods.

**In situ hybridization, imaging and retinoic acid administration**

Whole-mount *in situ* hybridization (WISH) was done basically according to the previous study (Yoshida and Sasakura, 2012). Details of WISH, imaging and RA administration
assay are described in supplementary Materials and Methods.

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Competing interests

The authors declare no competing interests.

Author contributions


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References


Fig. 1. *Hox1* and *Otx* establish the axial identity of the endostyle. (A-D, J) Whole-mount *in situ* hybridization (WISH) for *Hox1* in 6 days post fertilization (dpf) juveniles. (A) A control juvenile. (B) A *Hox1*-TALEN introduced juvenile. (C) A *Otx*-TALEN introduced juvenile. (D) A *Hox1*+*Otx*-TALEN introduced juvenile.
introduced juvenile. (D) A Hox1 and Otx double knockout juvenile. (J) Otx-overexpressed juvenile. (E-H, I) WISH for Otx in 6 dpf juveniles. (E) A control juvenile. (F) A Hox1-TALEN introduced juvenile. (G) A Otx-TALEN introduced juvenile. (H) A Hox1 and Otx double knockout juvenile. (I) A Hox1-overexpressed juvenile. Black, red and white arrows indicate the normal, ectopic and loss of expression of analyzed gene, respectively. (K) A schematic illustration of Ciona juvenile. AS, atrial siphon; BWM, body wall muscle; En, endostyle; Es, esophagus; Gi, gill; In, intestine; OS, oral siphon; St, stomach. In all panels anterior is to the top and ventral is to the left. Numbers on the top right of panels indicate the proportion of juveniles showing the phenotype represented by the panel. Scale bar: 50 \( \mu m \).
Development • Advance article

retinoic acid vs DMSO

TALEN introduced vs Control

RAR

Tfl-TALENs

Raldh2

RAR

wild type

Hox1 TALEN

Tfl-Otx

Otx TALEN

Otx 

Hox1

RA

(larval muscle and endoderm)

RA

(endostyle)

Anterior

Posterior
**Fig. 2. Retinoic acid-Hox1 feedback establishes the posterior identity.** (A-H) Transgenic line of GFP enhancer trap for Hox1. In each panel GFP fluorescence image (pseudocolored) is on the left and merged image of GFP and differential interference contrast is on the right. (A) A retinoic acid treated 3 days post fertilization (dpf) juvenile. (B) A control juvenile. (C) An RAR-TALEN introduced 2 dpf juvenile. GFP expression was not observed in the posterior endostyle (dotted circle), while its expression in the epidermis (e) and autofluorescence of absorbed tail muscle (t) were detected. (D) A control juvenile. (E) A Raldh2-TALEN introduced 2 dpf animal. (F) A control 2 dpf animal. (G) A Raldh2-TALEN introduced 6 dpf juvenile. (H) A control 6 dpf juvenile. White arrowheads in D, E, F and H indicate GFP expression in the posterior endostyle. (I-M) Whole-mount *in situ* hybridization for Raldh2 in 6 dpf juveniles. (I) A control juvenile. (J) A Hox1-TALEN introduced juvenile. (K) A Otx-overexpressed juvenile. (L) A Hox1-overexpressed juvenile. (M) A Otx-TALEN introduced juvenile. Black, red and white arrowheads indicate the normal, ectopic and loss of Raldh2 expression, respectively. (N) Model for the establishment of anterior-posterior identities in the endostyle. In all panels anterior is to the top and ventral is to the left. Numbers on the top right of panels indicate the proportion of animals showing the phenotype represented by the panel. Scale bars: 50 μm.
Fig. 3. Endodermal identity is essential for the directional elongation of BWMs. (A-G) Whole-mount in situ hybridization for MHC3 in 3 days post fertilization juveniles. (A) A control juvenile. (B, C) Hox1-TALEN introduced juveniles. BWMs showed misdirected elongation (B) or no elongation (C). (D) A citral treated juvenile. (E) A control EtOH. (F) A Raldh2-TALEN introduced juvenile. (G) A control juvenile. Numbers on the top right of panels indicate the proportion of BWMs showing the
phenotype represented by the panel. (H, I) Time-lapse analysis of the BWM formation using MHC3> Kaede. The 3D-reconstructed fluorescent images of the BWM and atrial siphon muscles are shown. Approximate developmental times are indicated on the bottom. Arrows indicate ectopic protrusions of BWMs. A, anterior; hpf, hours post fertilization; P, posterior. In all panels anterior is to the top and ventral is to the left. Scale bars: 50 μm.
Figure S1. Functions of Hox1 and Otx in the endostyle are required for establishing the identity of the posterior endostyle. (A, B) Whole-mount in situ hybridization (WISH) for Otx in 6 days post fertilization juveniles. (A) A Musashi>Hox1-TALEN introduced juvenile. (B) A control juvenile. One side of TALEN pair targeting Hox1 was electroporated. When Hox1 TALENs were expressed in the endostyle of juveniles, an ectopic expression of Otx in the posterior endostyle (red arrow) was detected in addition to the normal one in the anterior endostyle (black arrow). Anterior is to the top and ventral is to the left. (C, D) WISH for Otx in swimming larvae. (C) A Titf1>Hox1-TALEN introduced larva. (D) A control larva. Expression of Otx was detected only in the sensory vesicle in Hox1-TALEN introduced larvae as well as in control larvae. Anterior is to the left and dorsal is to the top. (E) Detection of mutations in the Otx locus of animals introduced with Titf1>Otx-TALENs. The PCR fragments containing the target site of Otx-TALEN were analyzed by heteroduplex mobility shift assay. The presence of shifted bands (brackets) indicates the formation of heteroduplexes with mismatched nucleotides, indicating the presence of mutations. The arrowhead indicates the position of PCR bands without mismatches. Mutations in the Otx locus were only detectable in TALEN-introduced larvae. eG: early gastrula. (F, G) WISH for Hox1 in swimming larvae. (F) A Titf1>Otx-TALEN introduced larva. (G) A control larva. Expression of Hox1 in the endoderm marks presumptive posterior pharynx (white arrows). This expression pattern was not affected in Otx-TALEN introduced larvae. Anterior is to the left and dorsal is to the top. (H-K) Magnified images of the anterior (H, I) and posterior tips (J, K) of the endostyle in control (H, J), Titf1>Hox1- (K) and Titf1>Otx-TALEN (I) introduced animals. Numbers on the top right of panels indicate the proportion of larvae showing the phenotype represented by the panel. Scale bars: 50 µm.
Figure S2. Retinoic acid synthesis in the larva is required for expression of Hox1 in the posterior endostyle. (A-D) Whole-mount in situ hybridization (WISH) for Raldh2 in swimming larvae. (A) A Titf1>Hox1-TALEN introduced larva. (B) A Titf1>Otx-TALEN introduced larva. (C) A Titf1>RAR-TALEN introduced larva. (D) A control larva. Expression of Raldh2 was detected in the posterior trunk endoderm and anterior tail muscle cells. This expression pattern was not affected by knockout of Hox1, Otx or RAR. (E, F) WISH for Raldh2 in tail-absorbed animals (30 hpf). (E) A Titf1>Hox1-TALEN introduced animal. Expression of Raldh2 in the posterior endostyle was not observed (dotted circle). (F) A control animal. Expression of Raldh2 was detected in the posterior endostyle (arrow head). Anterior is to the left and dorsal is to the top. (G, H) WISH for Hox1 in 3 days post fertilization (dpf) juveniles. (G) A TALEN pair designed to target Raldh2 were expressed in both muscle and endoderm. (H) A control juvenile. In Raldh2-TALEN introduced animal, expression of Hox1 in the posterior endostyle was absent (white arrow), while this expression was detectable in the control animal (black arrow). Numbers on the top right of panels indicate the proportion of juveniles showing the phenotype represented by the panel. Scale bars: 50 µm.
Figure S3. Retinoic acid disrupts posterior elongation of BWMs. Whole-mount in situ hybridization for MHC3 in 3 days post fertilization juveniles treated with retinoic acid or dimethylsulfoxide (DMSO). Numbers on the top right of panels indicate the proportion of BWMs showing the phenotype represented by the panel. Scale bar: 50 µm.
Hox1 TALEN  TTCACTACAAACAGCTTACCGAGCTTGAAGAAAAGGTTTCACTTTCAATA
TTCACTACAAACAGCT---------AAAAGAGGTTTCACTTTCAATA 1x
TTCACTACAAACAGCTTACCGAGCT-------AAAAGAGGTTTCACTTTCAATA 1x
TTCACTACAAACAGCTTACCGAGCTTACCGAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCACTACAAACAGCTTACCGAGCTTACCGAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
Mutation frequency 87.5% (n=8)

Otx TALEN  TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
TCGGAAAGACAAAGATATCCCGAGCTTGAAAAAGAGTTTCACTTCAATA 1x
Mutation frequency 83.3% (n=6)

RAR TALEN  TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
TTCTTTTGGACTCAGCTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA 1x
Mutation frequency 87.5% (n=8)

Raldh2 TALEN  TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
TCCGCTGACTACAGACGTACCAGGAGCAGCTCTCAATATCTCCGAGGTTGAA 2x
Mutation frequency 100% (n=10)

Figure S4. Activity of TALENs. Examples of the sequenced mutations found in animals introduced with a TALEN pair targeting Hox1, Otx, RAR or Raldh2. PCR fragments that include the binding sites of each TALEN pair were sequenced. Sequence of wild type genome is shown on the top. TALEN binding regions are highlighted in blue. "-" represents deletion of a nucleotide. The nucleotides that were not seen in the normal sequence are shown in red. The number at the right side indicate the frequency of the appearance.
Supplementary Materials and Methods

Constructs
TALENs were assembled by 4-module golden gate method (Sakuma et al., 2013). The previously described TALEN structure (Treen et al., 2014) was simplified by putting the TALEN and mCherry on a single ORF separated by a 2A peptide sequence (GSGEGRGSLLTCGDVEENPGP) (Szymczak et al., 2004) by amplifying the backbone TALEN and 2A::mCherry insert by PCR with 15bp overlapping regions and recombining them using an In-Fusion HD cloning kit (Clontech). The activity of the constructed TALENs were estimated by expressing under the control of the EF1α promoter according to the previous method (Treen et al., 2014) (Figure S6). The EF1α promoter was replaced with the promoter of Titf1 (Sasakura et al., 2012) for endoderm-specific expressions using the In-Fusion HD cloning kit. An enhancer element of Musashi gene (designated as fragment 3) fused with the TPO promoter (Awazu et al., 2004) was used to drive TALEN expression in the endostyle of juveniles. For driving TALEN expression in the muscle lineage, the EF1α promoter was replaced with the promoter of Tul (Davidson and Levine, 2003). The Titf1>HOX1 construct was described previously (Sasakura et al., 2012). The promoter of Titf1 and cDNA of Otx (Ciinte.CG.KH2012.C4.84) was amplified by polymerase chain reaction (PCR) using following primer pairs (F: 5’- CGACTCTAGAGGATCCTAGTTAGCAGATGAC-3’; R: 5’- GGCCGCAAGGGATCCTCAGCAAGTG-3’) and (F: 5’- GATCCCCCTGCGGCCCATGATCTTTCCAAATCTCCC-3’; R: 5’- CCTGATCCTGCGCGCCAAGACTTGAATTTCC-3’), respectively. PCR fragments of Titf1 promoter and Otx cDNA are fused with 2A::mCherry using the In-Fusion HD cloning kit to create Titf1>Otx. Genomic upstream region of MHC3 (Ciinte.CG.KH2012.C3.774) was isolated by PCR from Ciona genomic DNA using following primers (F: 5’- AATCTGACAGAAAACGTCGTCTTTCCGAAC-3’; R: 5’- TTTTCTAGATTTCACCACGCCATCCAC-3’) and inserted into the Pst I (5’) and Xba I (3’) sites of pSP-Kaeede (Hozumi et al., 2010) to generate the MHC3>Kaeede construct. The official names of the vectors and transgenic lines according to the nomenclature rule of tunicates (Stolfi et al., 2015) were as follows: Titf1>TALENs, pCiinte.REG.KH2012.C10.3638397-3636215[Titf1>TALENs::2A::mCherry, Musashi>TALENs, pCiinte.REG.KH2012.C10.4438567-4440059], Musashi>TALENs, pCiinte.REG.KH2012.L3.178445-177583[Tpo>TALEN::2A::mCherry; TnI>TALENs, pCiinte.REG.KH2012.C11.1684372-1685258[TnI>TALENs::2A::mCherry].
Electroporation

Plasmid DNAs were electroporated to 1-cell embryos according to the previous reports (Corbo et al., 1997; Treen et al., 2014). Dechorionated eggs of wild type animals were inseminated with sperm isolated from wild type or EJ[MiTSAAdTPOG]124 (EJ124) (Sasakura et al., 2012) animals. In knockout experiments, 20 μg (Hox1- and Otx-TALENs) or 30 μg (RAR- and Raldh2-TALENs) of expression vectors of L and R TALENs were electroporated for each electroporation. For control of knockout experiments, twofold amount of expression vectors of only one side of each TALEN pair (L or R) were electroporated. In the other experiments, 30 μg of DNA was electroporated. Animals with strong RFP (in Titf1>TALENs or Titf1>Otx electroporation) or CFP (in Titf1>Hox1 electroporation) fluorescence in the endoderm were selected at the tailbud stage for further culturing. Individuals expressing GFP were selected among animals developed from eggs inseminated with EJ124 sperm at 2 days post fertilization for further experiment.

Detection of mutations

Genomic DNAs were isolated from the early gastrula embryos and swimming larvae developed from eggs, into which the Titf1>Otx-TALEN pair or the left side of Otx-TALEN (control) was electroporated, using the Wizard genomic DNA purification kit (Promega), and the genomic region including the target site of the Otx-TALEN pair was amplified by PCR. The PCR bands were analyzed by heteroduplex mobility shift assay with polyacrylamide gel electrophoresis (Ota et al., 2013) to examine their heterogeneity of the sequence that reflects the presence of mutated gene.

Retinoic acid and citral administration

Preparation of stock solutions of retinoic acid (RA) and citral was done as described previously (Kanda et al., 2009). Metamorphosing larvae were treated with 1 μM all-trans RA, 0.1%
dimethylsulfoxide, 20 μM Citral or 0.1% ethanol from 45 hours post fertilization (hpf), prior to the timing that the BWM elongation starts, to 62 hpf, after the posterior elongation of BWMs competed.

**In situ hybridization and imaging**

Whole-mount *in situ* hybridization (WISH) was done basically according to the previous study (Ikuta et al., 2010; Yoshida and Sasakura, 2012) with some modifications described below: juveniles of appropriate stages were relaxed with L-menthol and fixed with 4% formaldehyde in seawater for at least three days at 4°C; and after incubation with proteinase K, specimens were washed four times with PBST and then tunics were removed manually using tungsten needles. The signals were visualized with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphosphate substrates or with TSA Plus Fluorescein Kit (Perkin Elmer). Digoxigenin-labeled RNA probes for *Otx* (Ciinte.CG.KH2012.C4.84), *Raldh2* (Ciinte.CG.KH2012.C4.697) and *MHC3* (Ciinte.CG.KH2012.C3.774) were synthesized using Gateway ORF clones (Roure et al., 2007) as templates. RNA probes for *Hox1* (Ciinte.CG.KH2012.L171.16) was described previously (Sasakura et al., 2012). The WISH images are acquired by Axio Observer.Z1 and AxioCam MRm (Carl Zeiss). Fluorescent images were taken with a Zeiss fluorescent microscope AxioImager.Z1 and AxioCam MRm. For time-lapse imaging of BWM formation, embryos were electroporated with *MHC3>Kaede* at the 1-cell stage. At 48-50 hours post fertilization, individuals were mounted on a glass-based dish (Iwaki) and time-lapse 3D imaging was performed using LSM700 confocal microscope (Carl Zeiss). The recording interval was 10 min. Three-dimensional images were reconstructed from z-stack images using ZEN2010 software (Carl Zeiss).
Supplementary Reference


