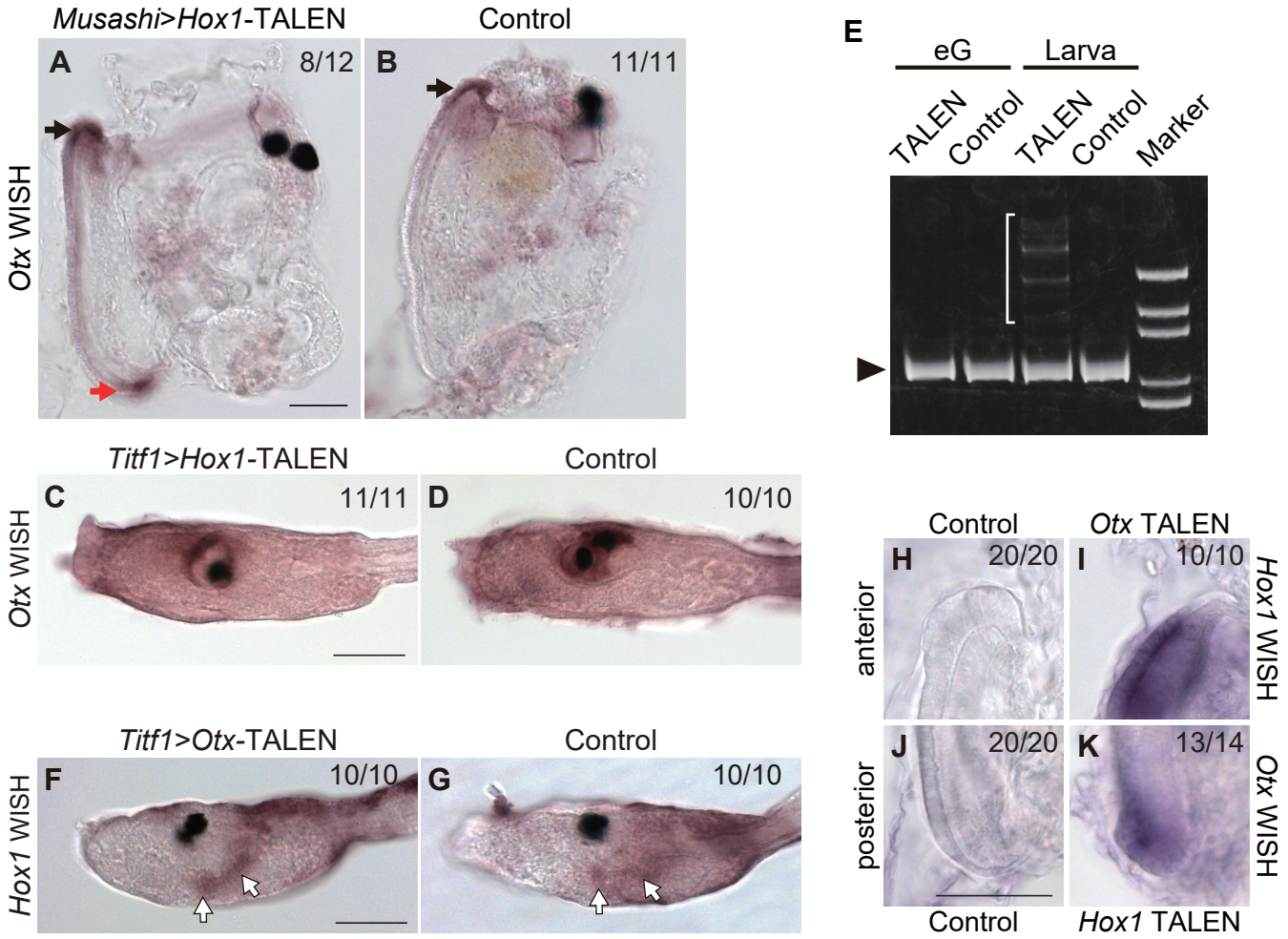

Figure S1. Functions of *Hox1* and *Otx* in the endostyle are required for establishing the identity of the posterior endostyle. (A, B) Whole-mount *in situ* hybridization (WISH) for *Otx* in 6 days post fertilization juveniles. (A) A *Musashi>Hox1*-TALEN introduced juvenile. (B) A control juvenile. 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 mismatchs. 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.

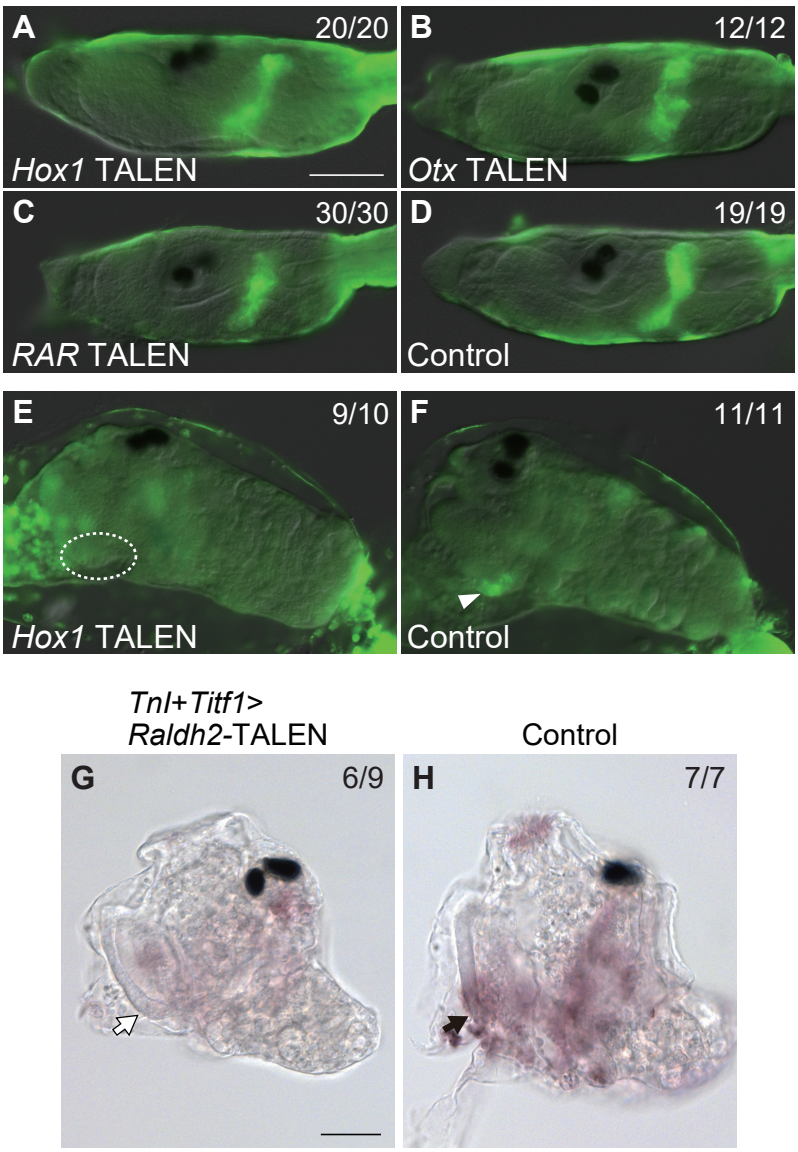

Figure S2. Retinoic acid synthesis in the larva is required for expression of *Hox1* in the posterior endostyle. (A-D) Whole-mount *in situ* hybridization (WISH) for *Raldh2* in swimming larvae. (A) A *Titf1>Hox1*-TALEN introduced larva. (B) A *Titf1>Otx*-TALEN introduced larva. (C) A *Titf1>RAR*-TALEN 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.

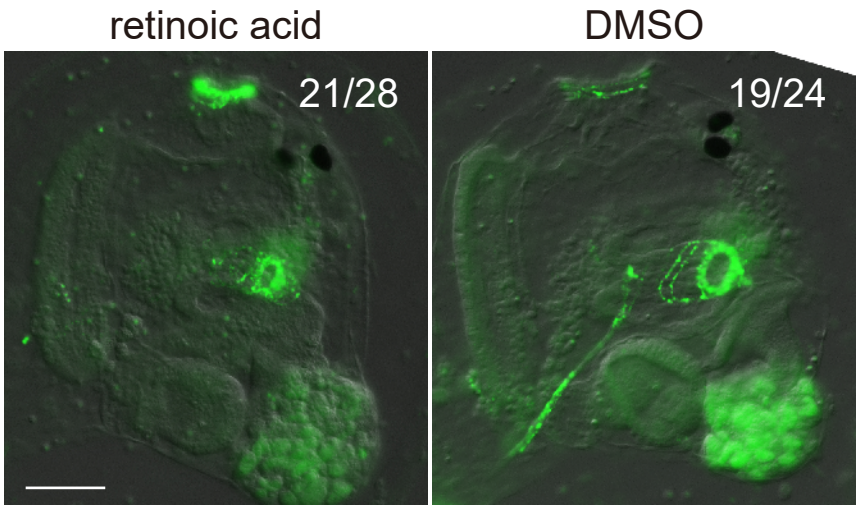

Figure S3. Retinoic acid disrupts posterior elongation of BWMs. Whole-mount in situ hybridization for *Mhc3* in 3 days post fertilization juveniles treated with retinoic acid or dimethylsulfoxide (DMSO). Numbers on the top right of panels indicate the proportion of BWMs showing the phenotype represented by the panel. Scale bar: 50 μm.

Hox1 TALEN

```
TTCACTACAAAACAGCTTACCGAGCTTGAAAAAGAGTTTCACTTCAATA

TTCACTACAAAACAGCT----------AAAAAGAGTTTCACTTCAATA  1x
TTCACTACAAAACAGCTT---------GAAAAAGAGTTTCACTTCAATA  1x
TTCAGTACAAAACAGCTTACCG----AAAAAAAGAGTTTCACTTCAATA  1x
TTCACTACAAAACAGCTTACCGA------AAAAGAGTTTCACTTCAATA  1x
TTCACTACAAAACAGCTTACCGAATTTCACTACAAAACAGCTTACCTTG
AAAAAGAGTTTCACTTCAATA                              1x
```

Mutation frequency 100% (n=5)

Otx TALEN

```
TCGGAAAGACAAGATATCCCGATATCTTTATGAGAGAAGAAGTTGCCCTA

TCGGAAAGACAAGATATCCCGA----------GAGAAGAAGTTGCCCTA  1x
TCGGAAAGACAAGATAT-------ATATTATGAGAGAAGAAGTTGCCCTA  1x
TCGGAAAGACAAGAT-----------TATGAGAGAAGAAGTTGCCCTA  1x
TCGGAAAGACAAGATAT----------TTATGAGAGAAGAAGTTGCCCTA  1x
TCGGAAAGACAAGATAT----------TATGAGAGAAGAAGTTGCCCTA  1x
TCGGAAAGACAAGATATCCCGATATCTTTATGAGAGAAGAAGTTGCCCTA  1x
```

Mutation frequency 83.3% (n=6)

RAR TALEN

```
TTCTTTCGACGTAGTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA

TTCTTTCGACGTAGTGTGCAGAA-----TGCAGTATACTTGTCATCGTAACA  1x
TTCTTTCGACGTAGTGTGCAGAA------GCAGTATACTTGTCATCGTAACA  1x
TTCTTTCGACGTAGTGTGCAGAA---------GTATACTTGTCATCGTAACA  1x
TTCTTTCGACGTAGTGTGCAGA----------ATACTTGTCATCGTAACA  2x
TTCTTTCGACGTAGTGTGCAG----------TATACTTGTCATCGTAACA  2x
TTCTTTCGACGTAGTGTGCAGAAGAACATGCAGTATACTTGTCATCGTAACA  1x
```

Mutation frequency 87.5% (n=8)

Raldh2 TALEN

```
TTCAGTCGTATCAGCAGTACCAGCAGCCTCTCAAATCCCCGAGGTGAACA

TTCAGTCGTATCAGCAGTACC------CTCTCAAATCCCCGAAGTGAACA  2x
TTCAGTCGTATCAGCAGTACC-------TCTCAAATCCCCGAAGTGAACA  2x
TTCAGTCGTATCAGCAGTACCAGC----TCTCAAATCCCCGAAGTGAGCA  4x
TTCAGTCGTATCAGCAGTACC---CCCCTCTCAAATCCCCGAAGTGAACA  1x
TTCAGTCGTATCAGCAGTACCAGTCAAATAAACATAAGCTTTATCAGCAG
TACCCGTATCAGCAGTACCAGTCCTCTCAAATCCCCGAAGTGAACA      1x
```

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 *TnI* (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'- CGACTCTAGAGGATCCTAGTTCATGGTTAGCAATGAC-3'; R: 5'-GGCCGCAAGGGGATCCTCACAGCAAAGTTTCCAGTG-3') and (F: 5'-GATCCCCTTGCGGCCATGTCGTATTTGAAATCTCCC-3'; R: 5'-CCTGATCCTGCGGCCGCCAAGACTTGGAATTTCC-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'-AATCTGCAGTAAAACGTCCGTTTCCGAAC-3'; R: 5'-TTTTCTAGATTTTCCCCACTTGAATCCAC-3') and inserted into the *Pst* I (5') and *Xba* I (3') sites of pSP-Kaede (Hozumi et al., 2010) to generate the *Mhc3>Kaede* 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*, p*Ciinte*.REG.KH2012.C10.3638397-3636215|*Titf1>TALEN*::*2A*::*mCherry*; *Musashi>TALENs*, p*Ciinte*.REG.KH2012.C10.4438567-4440059|*Musashi*:*Ciinte*.REG.KH2012.L3.178445-177583|*Tpo> TALEN*::*2A*::*mCherry*; *TnI>TALENs*, p*Ciinte*.REG.KH2012.C11.1684372-1685258|*TnI>TALEN*::*2A*::*mCherry*;

Titf1>Hox1, p*Ciinte*.REG.KH2012.C10.3638397-3636215|*Titf1>CFP*::*Hox1*; *Titfi>Otx*, p*Ciinte*.REG.KH2012.C10.3638397-3636215|*Titf1>Otx*::*2A*::*mCherry*; *Mhc3>Kaede*, p*Ciinte*.REG.JGIv2.chr03q.1986302-1989326|*Mhc3>Kaede*; EJ[MiTSAdTPOG]124, *Ciinte*.E[pMi-TSA-*Ciinte*.REG.KH2012.L3.178445-177583|*Tpo>GFP*]124.KH2012.L171.188604.

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[MiTSAdTPOG]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>TALEN*s 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.

***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).

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