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Supplementary Information for

BMP controls dorsoventral and neural patterning in indirect developing hemichordates providing insight into a possible origin of chordates

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Supplementary Materials and Methods

Phylogenetic analysis

Complete protein sequences were aligned using ClustalW with default settings on MEGA ver. 6.06. The alignments were analyzed using Bayesian inference analysis (MrBayes ver. 3.2.6, CIPRES Science Gateway). The Markov chain Monte Carlo analyses were specified at 100,000 or 1,000,000 generations, and the other parameters were defaults. FigTree ver. 1.4.3 was used to illustrate the trees. GenBank accession numbers of the genes used for the phylogenetic analyses are listed in Table S2.

Larval culture and feeding experiments

P. flava and *S. purpuratus* larvae were cultured in 200 ml of filtered seawater with constant gentle stirring. The culture medium was replaced every other day with fresh filtered seawater containing *Rhodomonas* (1). Equal amount of *Rhodomonas* were added into control and mBMP4-treated samples.

In situ hybridization of the amphioxus *onecut*

The amphioxus *onecut* was cloned by RACE PCR with two nested forward primers (forward primer F743: 5'-AACGTTCAACGGTTATGCCAACCACC-3'; forward primer F1308: 5'-AAGCGGAAAGAACAAGAAGCTGCG-3') and the 3' RACE primer provided in the FirstChoice RLM-RACE Kit (Thermo Fisher Scientific). *B. floridae* adults and embryos were cultured at room temperature and *in situ* hybridization was performed as described (2).

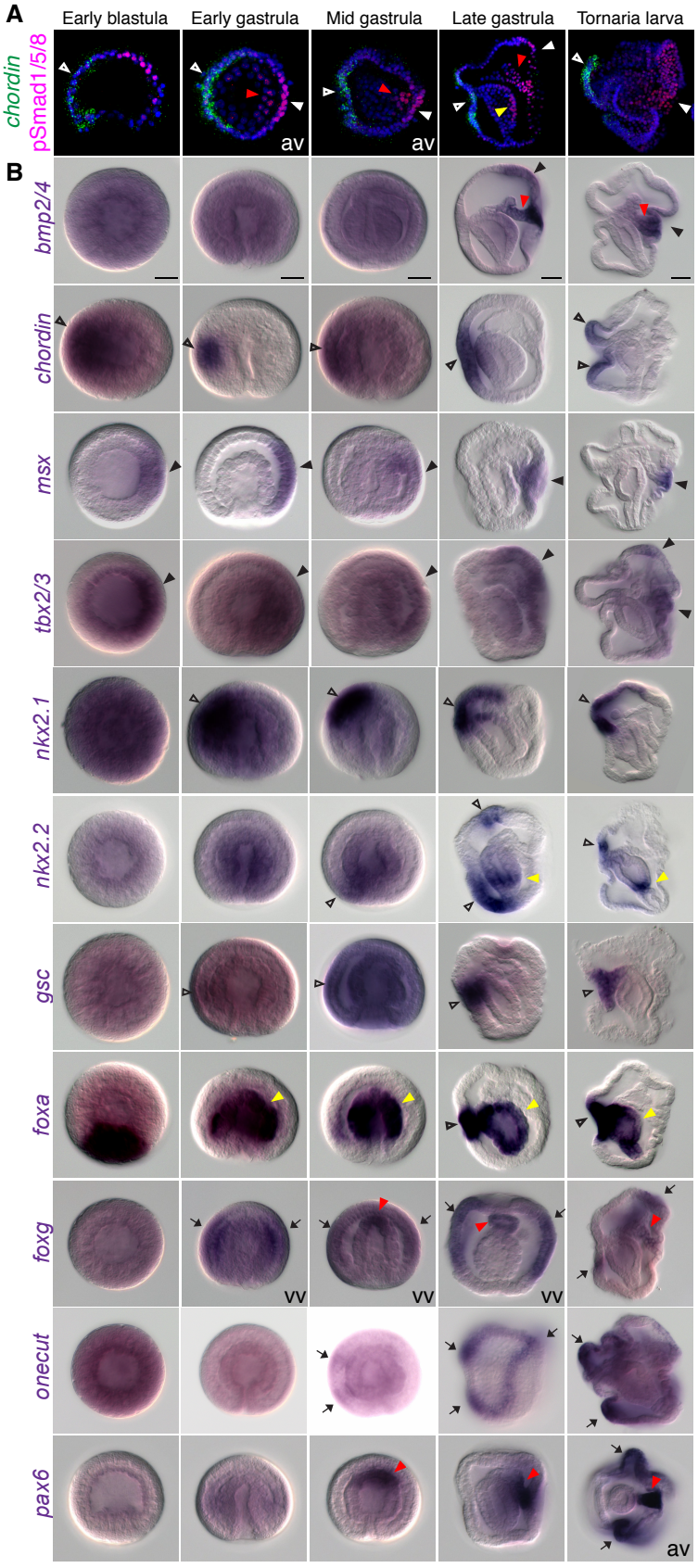
A
Early blastula
Early gastrula
Mid gastrula
Late gastrula
Tornaria larva
chordin
pSmad1/5/8
av
av
B
bmp2/4
chordin
msx
tbx2/3
nkx2.1
nkx2.2
gsc
foxa
foxg
vv
vv
vv
onecut
pax6
av

Fig. S1. Ectodermal gene expression patterns throughout embryogenesis of *P. flava*. (**A**) Double staining of *chordin* fluorescent *in situ* hybridization and immunostaining with an anti-pSmad1/5/8 antibody at various embryonic stages. (**B**) *In situ* hybridization of genes investigated in this study. Specific expression of *bmp2/4* on the dorsal side was initiated at the late gastrula stage. Expression of *chordin* in the presumptive ventral ectoderm at the blastula stage possibly acts on ubiquitously distributed, maternal BMP ligands. Genes encoding Msx and Tbx2/3 transcription factors were expressed in the presumptive dorsal ectoderm, beginning at the blastula stage. Genes that showed specific expression in subdomains of the ventral ectoderm during gastrulation include *nkx2.1* and *nkx2.2* (near the animal and vegetal poles, respectively), and *gsc* and *foxa* (in the stomodeal region). The *foxg*, *onecut*, and *pax6* genes were expressed in the lateral ectoderm, which marks the positions of future ciliary bands between the dorsal and ventral ectoderm. Except *pax6*, orthologs of these genes exhibit similar expression patterns in sea urchin embryos (3, 4), suggesting that the patterning mechanism and the deployment of the transcription factors for establishing the larval DV axis, including the ciliary band between the dorsal and ventral ectoderm, was present in the ambulacrarian ancestor. Scale bar is 30 μm. Empty arrowheads, black arrowheads, and arrows indicate the expression in the ventral ectoderm, dorsal ectoderm, and lateral ectoderm, respectively. Red and yellow arrowheads indicate the mesodermal and endodermal expression patterns, respectively. All images are side views unless otherwise indicated; av, apical view; vv, ventral view.

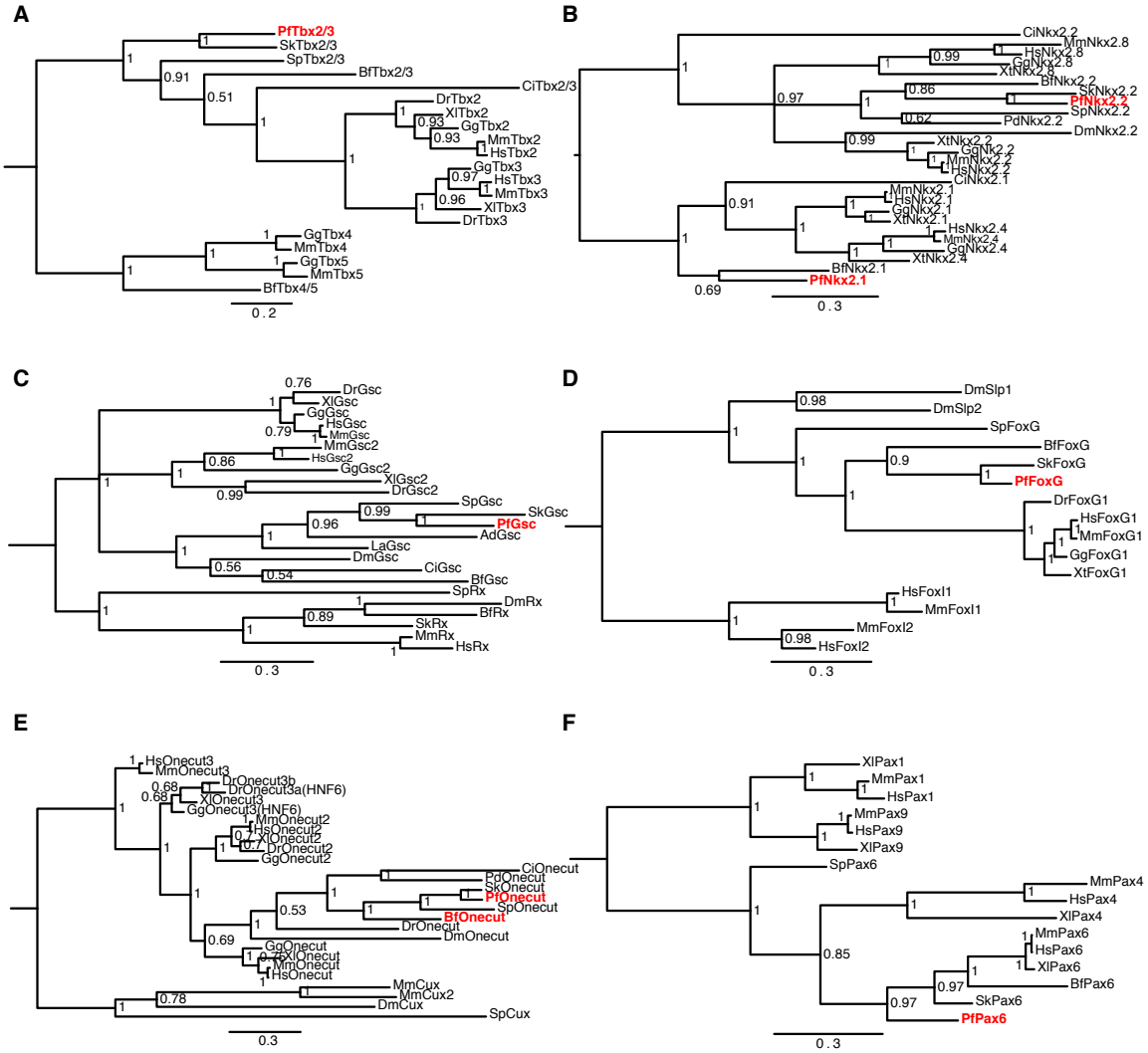

Fig. S2. Phylogenetic analyses of genes identified in this study. Complete protein sequences of (**A**) Tbx2/3, (**B**) Nk2.2, (**C**) Gsc, (**D**) FoxG, (**E**) Onecut, and (**F**) Pax6 from various species were aligned and analyzed using Bayesian inference analysis. The Markov chain Monte Carlo analysis was set to run for 100,000 (**a,c,f**) or 1,000,000 (**b,d,e**) generations. The outgroup sequences used in the analyses were (**A**) Tbx4/5, (**B**) Nk2.1/Nk2.4, (**C**) Rx, (**D**) FoxI, (**E**) Cux, and (**F**) Pax1/Pax9. The nodes indicate the posterior possibilities, and the scale bars are changes per site. GenBank accession numbers of the analyzed protein sequences are given in Table S2.

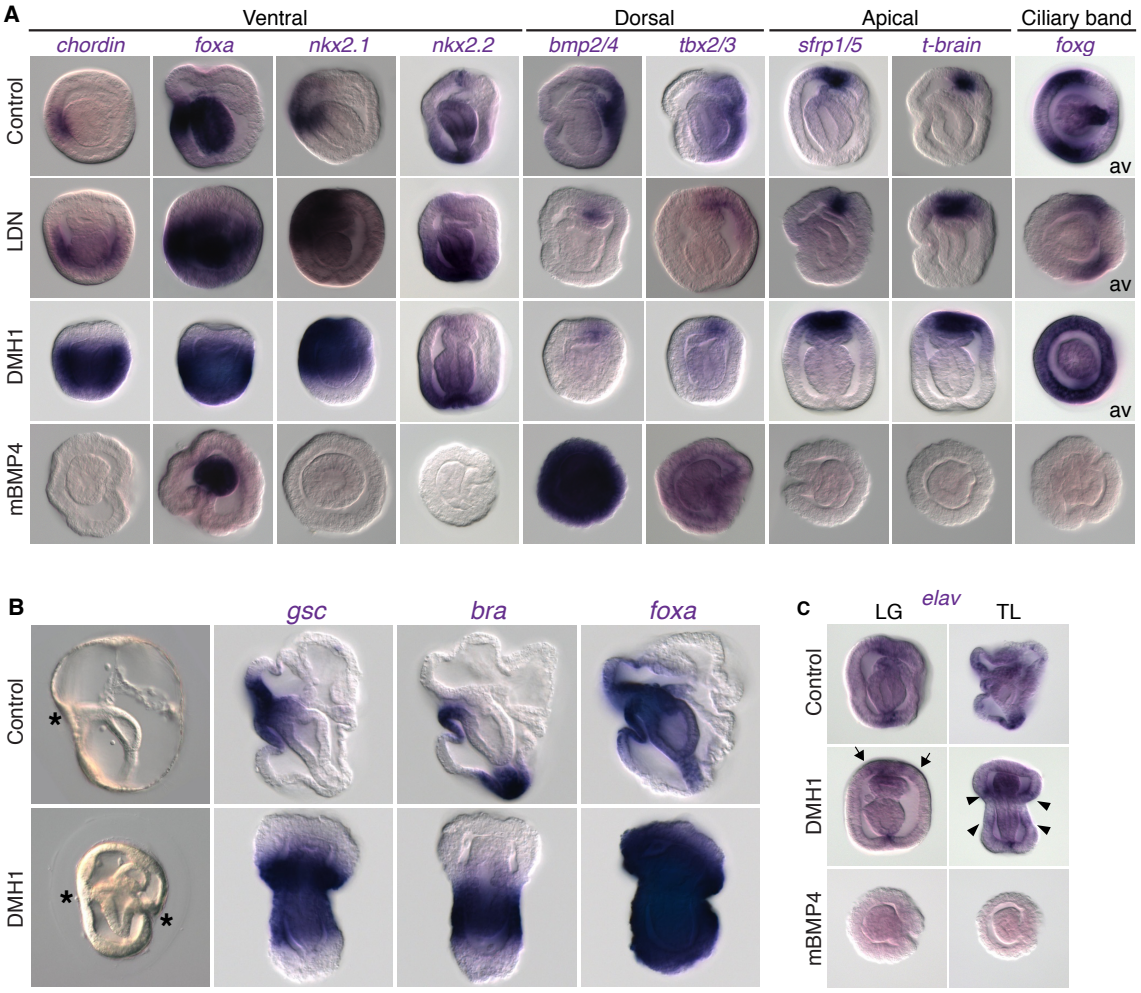

Fig. S3. BMP signaling controls DV patterning in *P. flava*. (**A**) Expression of ventral, dorsal, apical, and ciliary band marker genes in control gastrulae and embryos treated with 2 μM LDN, 2 μM DMH1, or 125 ng/ml mBMP4 after fertilization. (**B**) Expression of the stomodeal marker genes in control tornaria larvae and larvae treated with 2 μM DMH1. Asterisks indicate mouth openings. (**C**) Expression of *elav* was detected in several domains, including the apical region, the ciliary band, cells around the blastopore, and some mesodermal cells. In DMH1-treated embryos, *elav* expression in the apical region (arrows) and ciliary band expanded (arrowheads). Exogenous mBMP4 abolished its expression. All images are side views unless otherwise indicated. av, apical view. The data presented represent the phenotypes of most samples (>95%).

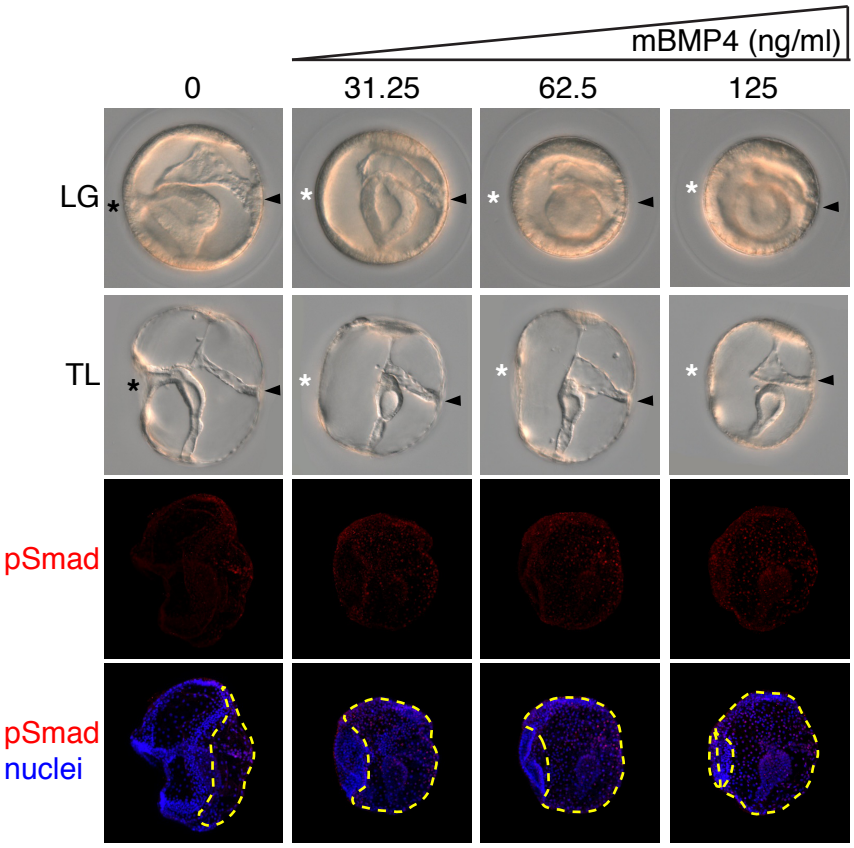

Fig. S4. To vary the level of the BMP signaling, embryos were treated with different concentrations of mBMP4 for 4 hrs during early gastrulation (from 24 to 28 hrs post fertilization). The phenotypes were observed at the late gastrula (LG) and the tornaria larval (TL) stages. To examine regions that receive BMP signaling, the TL-stage samples were stained with an anti-phospho-Smad1/5/8 (pSmad) antibody. When mBMP4 concentrations were increased, the pSmad-positive regions expanded (yellow dashed regions), while the pSmad-negative regions became smaller. The black asterisks and arrowheads indicate the mouth and the hydropore openings, respectively. The white asterisks denote the disappearance of the mouth. All images are side views (ventral to the left). The data presented represent the phenotypes of most samples (>95%).

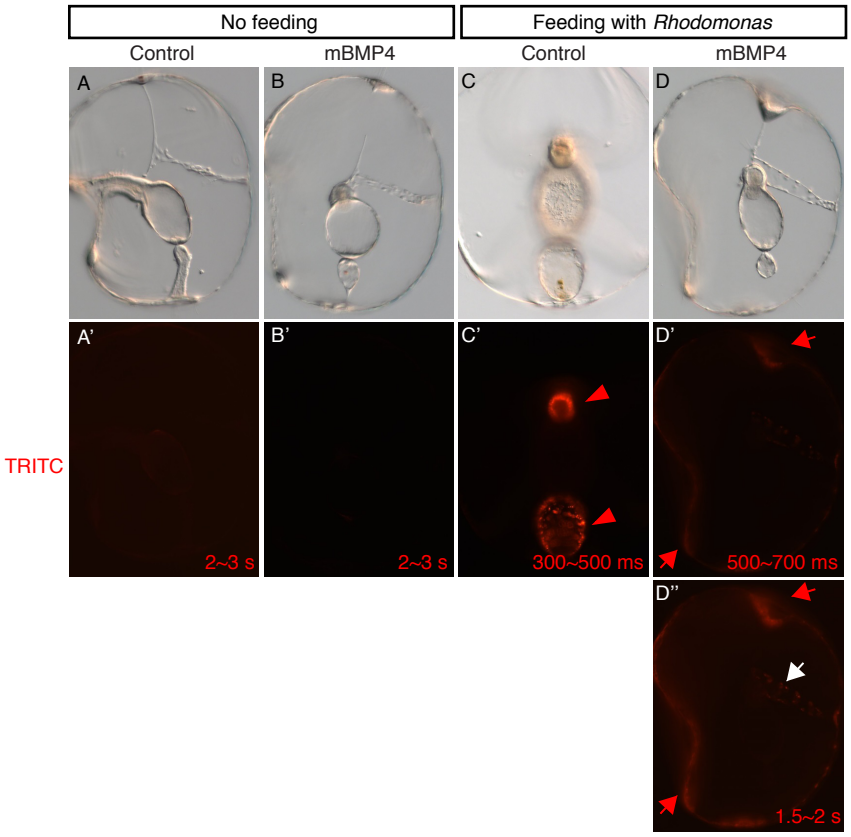

Fig. S5. Feeding experiments for control and mouthless tornaria larvae. (**A**-**B**) Eight-day-old control tornaria and mouthless larvae (mBMP4 treatment from 24 to 28 hpf) cultured in filtered seawater without feeding. (**C**-**D**) Eight-day-old control and mouthless larvae (mBMP4) fed with *Rhodomonas* (feeding started from 3 dpf). A TRITC filter was used to detect fluorescent signals emitted from *Rhodomonas*. The exposure time for the fluorescent images is indicated at the bottom-right corner. The arrowheads (**C'**) denote the presence of *Rhodomonas* in the digestive tract. The red and white arrows (**D'** and **D''**) indicate fluorescent signals in the ectoderm and mesoderm, respectively. Fluorescent signals were not detected in the mouthless larva without feeding (**B'**), suggesting that the mouthless larva may be able to take-up *Rhodomonas* or its debris from seawater. (**A**), (**B**), and (**D**) are side views (ventral to the left) and (**C**) is viewed from the ventral side.

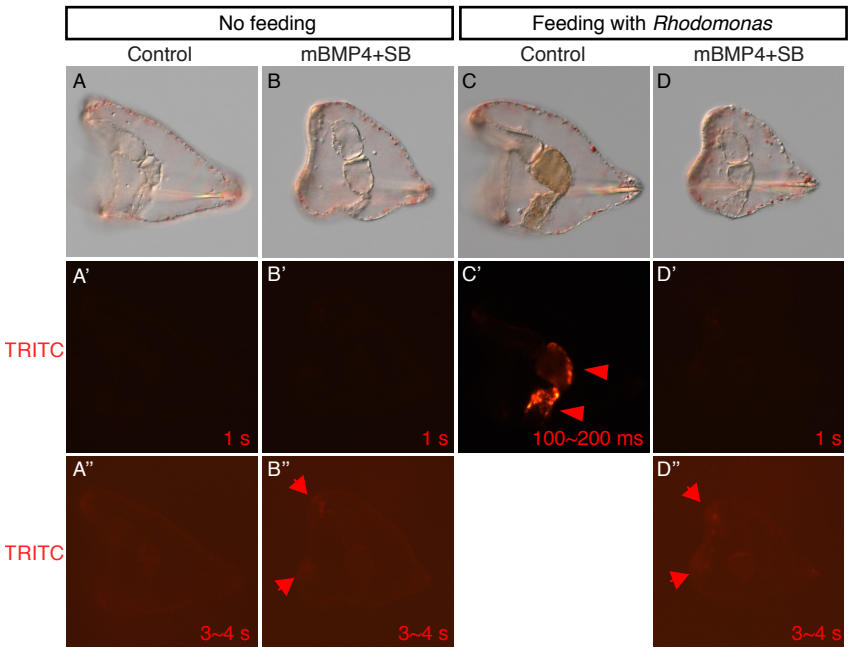

Fig. S6. Feeding experiments for control and mouthless sea urchin pluteus larvae. (**A**-**B**) Seven-day-old control and mouthless sea urchin larvae (mBMP4+SB treatment from 24 to 28 hpf) cultured in filtered seawater without feeding. (**C**-**D**) Seven-day-old control and mouthless larvae (mBMP4+SB) fed with *Rhodomonas* (feeding started from 3 dpf). A TRITC filter was used to detect *Rhodomonas*. The exposure time for the fluorescent images is indicated at the bottom-right corner. The arrowheads (**C'**) denote the presence of *Rhodomonas* in the digestive tract. The red arrows (**B''** and **D''**) indicate fluorescent signals in some mesodermal cells. The mesodermal fluorescent signals were detected in the mouthless larvae regardless of feeding, demonstrating that the fluorescence was not due to the presence of algae. All images are side views (ventral to the left).

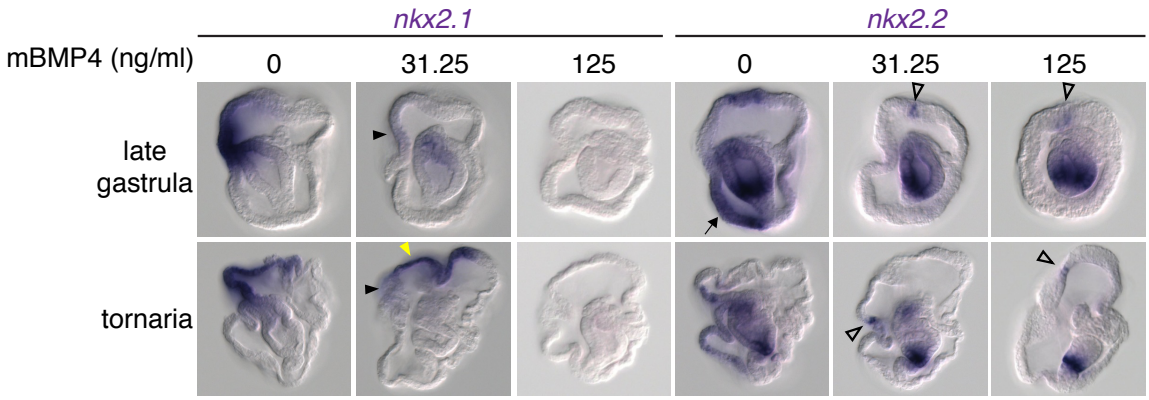

Fig. S7. Transient over-activation of BMP signals affects ectodermal expression of *nkx2.1* and *nkx2.2*. *In situ* hybridization of *nkx2.1* and *nkx2.2* was performed in control and mBMP4-treated (treatment from 24 to 28 hpf) *P. flava* embryos. At the late gastrula stage, the anterior-ventral ectodermal expression of *nkx2.1* (arrowhead) was decreased at a lower concentration of mBMP4 (31.25 ng/ml) and completely disappeared at a high level of mBMP4 (125 ng/ml). Its anterior ectodermal expression was detected at the tornaria stage (yellow arrowhead). Upon treatment, the posterior-ventral ectodermal expression of *nkx2.2* (arrow) disappeared, and only a few *nkx2.*2-positive cells remained in the ventral ectoderm (empty arrowheads). All images are side views (ventral to the left).

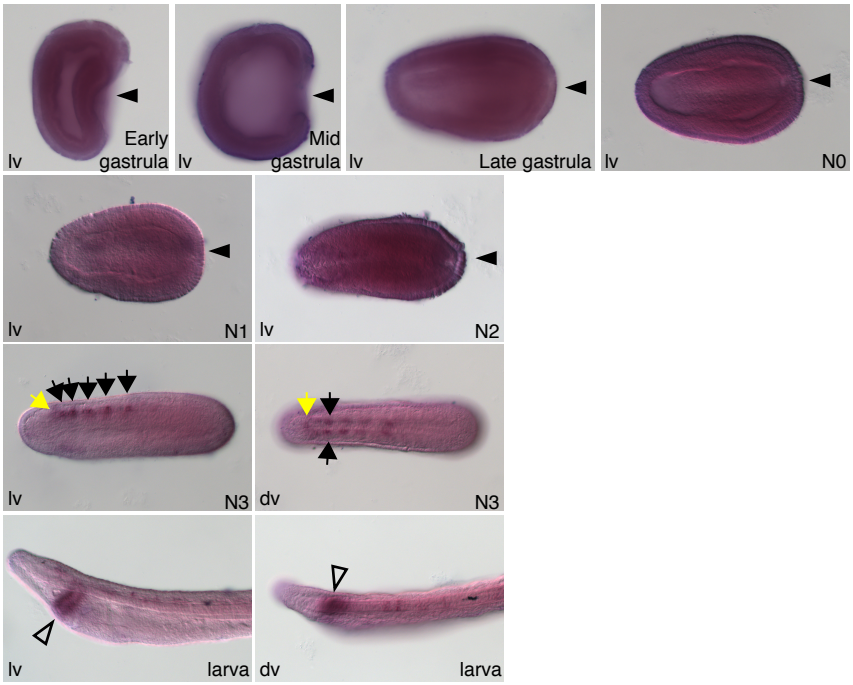

Fig. S8. Expression of the amphioxus *onecut* gene during embryogenesis. The *Branchiostoma floridae* homolog of *onecut* was cloned (Fig. S2) and *in situ* hybridization was performed at various developmental stages, beginning from early and mid-gastrula, extending to different neurula (N0-N3) stages, and finally to the larval stage. Developmental stages are indicated at the bottom-right corner. Views of the samples are indicated at the bottom-left corner (lv, lateral view; dv, dorsal view). Anterior is to the left and the arrowheads indicate the blastopores. No specific expression is detected before the N2 stage. At the N3 stage, *onecut* transcript is detected in the posterior part of the cerebral vesicle (yellow arrow) and in five pairs of neurons in the neural tube (black arrows; only one pair of neurons is indicated in the dorsal-view N3 embryo). At the larval stage (only the anterior part of the larva is shown), strong expression in the endostyle (empty arrowheads) is observed.

Table S1. Primers used for cloning *P. flava* genes

Gene Name	primer direction	Sequence
Goosecoid (*gsc*)	F	TCACGTCGCGTCACGTCAAAGTCAC
	R	CCCAACATTCGAGCAATCGTTCACTC
tbx2/3	F	ACAGCGTTAGACTGCCACCTGTGGTC
	R	CGCTAAGATGTGTGTTGAACGACTTC
foxg	F	ACACACACGCGCACATCCGTACATC
	R	CCGTTCGTAAGTCCTGCAAAAGTTGTC
pax6	F	CGCCTTCTGAACGGCCAGACAT
	R	TTGCGATGAGTGGGTGTCTGCC
onecut	F	GAGCAATGTGAGCGGTAGTTTTAC
	R	GCATGTGGCCAGTCGAATTATAAC

F: forward; R: reverse.

Table. S2. List of genes used in the phylogenetic analyses.

Gene	Species	Accession Number	Abbreviation in figures
Gsc	*Ciona intestinalis*	BAE06479.1	CiGsc
Gsc	*Danio rerio*	AAH81381.1	DrGsc
Gsc	*Branchiostoma floridae*	AAF97935.1	BfGsc
Gsc	*Strongylocentrotus purpuratus*	NP_999663.1	SpGsc
Gsc	*Gallus gallus*	NP_990662.1	GgGsc
Gsc	*Xenopus laevis*	XP_018087379.1	XlGsc
Gsc	*Homo sapiens*	EAW81594.1	HsGsc
Gsc	*Mus musculus*	EDL18791.1	MmGsc
Gsc	*Drosophila melanogaster*	AAB17948.1	DmGsc
Gsc	*Lingula anatina*	XP_013379052.1	LaGsc
Gsc	*Acropora digitifera*	BAQ19108.1	AdGsc
Gsc	*Saccoglossus kowalevskii*	NP_001161560.1	SkGsc
Gsc	*Ptychodera flava*	MH782152	PfGsc
Gsc2	*Mus musculus*	AAI25357.1	MmGsc2
Gsc2	*Xenopus laevis*	XP_018099595.1	XlGsc2
Gsc2	*Gallus gallus*	XP_025011442.1	GgGsc2
Gsc2	*Homo sapiens*	NP_005306.1	HsGsc2
Gsc2	*Danio rerio*	XP_021332017.1	DrGsc2
FoxG1	*Danio rerio*	AAH92710.1	DrFoxG1
FoxG1	*Homo sapiens*	AAH50072.1	HsFoxG1
FoxG1	*Xenopus tropicalis*	NP_001116933.1	XtFoxG1
FoxG	*Branchiostoma floridae*	AAC18392.1	BfFoxG
FoxG	*Saccoglossus kowalevskii*	XP_002735197.2	SkFoxG
FoxG	*Strongylocentrotus purpuratus*	NP_001123284.1	SpFoxG
FoxG	*Ptychodera flava*	MH782154	PfFoxG
Slp1	*Drosophila melanogaster*	NP_476730.1	DmSlp1
Slp2	*Drosophila melanogaster*	NP_476834.1	DmSlp2
FoxI1	*Homo sapiens*	AAH29778.2	HsFoxI1
FoxI1	*Mus musculus*	AAH07475.2	MmFoxI1
FoxI2	*Homo sapiens*	NP_997309.2	HsFoxI2
FoxI2	*Mus musculus*	AAH96623.1	MmFoxI2
FoxG1	*Mus musculus*	NP_001153584.1	MmFoxG1
FoxG1	*Gallus gallus*	NP_990524.1	GgFoxG1
Pax1	*Mus musculus*	AAK01146.1	MmPax1
Pax4	*Mus musculus*	BAA24517.1	MmPax4
Pax6	*Mus musculus*	AAH36957.1	MmPax6
Pax9	*Mus musculus*	NP_035171.1	MmPax9
Pax1	*Xenopus laevis*	ABG57077.1	XlPax1
Pax4	*Xenopus laevis*	XP_017948145.1	XlPax4

Pax6	*Xenopus laevis*	AAB36683.1	XlPax6
Pax9	*Xenopus laevis*	NP_001167485.1	XlPax9
Pax1	*Branchiostoma floridae*	XP_002210646.1	BfPax1
Pax6	*Branchiostoma floridae*	CAA11368.1	BfPax6
Pax1	*Strongylocentrotus purpuratus*	SPU_006683.1	SpPax1
Pax6	*Strongylocentrotus purpuratus*	SPU_006786.1	SpPax6
Pax1	*Ptychodera flava*	BAA78380.1	PfPax1
Pax6	*Ptychodera flava*	MH782156	PfPax6
Pax1	*Saccoglossus kowalevskii*	NP_001158408.1	SkPax1
Pax6	*Saccoglossus kowalevskii*	NP_001158383.1	SkPax6
Pax1	*Homo sapiens*	NP_006183.2	HsPax1
Pax4	*Homo sapiens*	NP_006184.2	HsPax4
Pax6	*Homo sapiens*	NP_001121084.1	HsPax6
Pax9	*Homo sapiens*	NP_006185.1	HsPax9
Nkx2.2	*Mus musculus*	AAI38160.1	MmNkx2.2
Nkx2.8	*Mus musculus*	NP_032727.2	MmNkx2.8
Nkx2.2	*Homo sapiens*	NP_002500.1	HsNkx2.2
Nkx2.8	*Homo sapiens*	NP_055175.2	HsNkx2.8
Nkx2.2	*Gallus gallus*	NP_001264647.1	GgNkx2.2
Nkx2.8	*Gallus gallus*	XP_003641408.3	GgNkx2.8
Nkx2.2	*Xenopus tropicalis*	XP_002939477.1	XtNkx2.2
Nkx2.8	*Xenopus tropicalis*	NP_988951.1	XtNkx2.8
Nkx2.2	*Branchiostoma floridae*	XP_002589199.1	BfNkx2.2
Nkx2.2	*Saccoglossus kowalevskii*	NP_001158404.1	SkNkx2.2
Nkx2.2	*Strongylocentrotus purpuratus*	AAS58444.1	SpNkx2.2
Nkx2.2	*Platynereis dumerilii*	ABO93209.1	PdNkx2.2
vnd	*Drosophila melanogaster*	CAA60619.1	DmNkx2.2(vnd)
Nkx2.2	*Ciona intestinalis*	NP_001071957.1	CiNkx2.2
Nkx2.1	*Ciona intestinalis*	NP_001027667.1	CiNkx2.1
Nkx2.1	*Mus musculus*	AAH57607.1	MmNkx2.1
Nkx2.1	*Homo sapiens*	NP_001073136.1	HsNkx2.1
Nkx2.1	*Gallus gallus*	CAA11493.1	GgNkx2.1
Nkx2.1	*Xenopus laevis*	AAG17405.1	XlNkx2.1
Nkx2.1	*Branchiostoma floridae*	AAC35350.1	BfNkx2.1
Nkx2.4	*Homo sapiens*	NP_149416.1	HsNkx2.4
Nkx2.4	*Gallus gallus*	XP_015138916.1	GgNkx2.4
Nkx2.4	*Mus musculus*	NP_075993.1	MmNkx2.4
Nkx2.4	*Xenopus laevis*	AAI69924.1	XlNkx2.4
Nkx2.1	*Ptychodera flava*	AAM93268.1	PfNkx2.1
Nkx2.2	*Ptychodera flava*	MH782155	PfNkx2.2
Onecut	*Mus musculus*	NP_032288.1	MmOnecut
Onecut	*Ciona intestinalis*	NP_001071994.1	CiOnecut

Onecut	*Saccoglossus kowalevskii*	NP_001161619.1	SkOnecut
Onecut	*Drosophila melanogaster*	NP_524842.2	DmOnecut
Onecut	*Platynereis dumerilii*	AHI16250.1	PdOnecut
Onecut	*Danio rerio*	AAL02365.1	DrOnecut
Onecut	*Strongylocentrotus purpuratus*	AAR28054.1	SpOnecut
Onecut	*Gallus gallus*	XP_015147374.1	GgOnecut
Onecut	*Xenopus tropicalis*	NP_001093730.1	XtOnecut
Onecut	*Homo sapiens*	NP_004489.1	HsOnecut
Onecut	*Ptychodera flava*	MH782157	PfOnecut
Onecut	*Branchiostoma floridae*	MK679618	BfOnecut
Onecut2	*Mus musculus*	AAI03669.1	MmOnecut2
Onecut2	*Homo sapiens*	NP_004843.2	HsOnecut2
Onecut2	*Xenopus tropicalis*	XP_004910425.1	XtOnecut2
Onecut2	*Danio rerio*	XP_001920308.3	DrOnecut2
Onecut2	*Gallus gallus*	XP_003642951.1	GgOnecut2
Cux	*Mus musculus*	CAA52922.1	MmCux
Cux2	*Mus musculus*	NP_001299837.1	MmCux2
Cux	*Drosophila melanogaster*	NP_524764.1	DmCux
Cux	*Strongylocentrotus purpuratus*	XP_011671723.1	SpCux
Hnf6	*Danio rerio*	XP_694209.1	DrOnecut3a(Hnf6)
Onecut3b	*Danio rerio*	XP_017207968.2	DrOnecut3b
Onecut3	*Xenopus tropicalis*	XP_002938867.1	XtOnecut3
Hnf6	*Gallus gallus*	XP_003642905.1	GgOnecut3(Hnf6)
Onecut3	*Homo sapiens*	NP_001073957.1	HsOnecut3
Onecut3	*Mus musculus*	AAL86921.1	MmOnecut3
Tbx2	*Danio rerio*	AAF59835.1	DrTbx2
Tbx2	*Gallus gallus*	XP_001235321.4	GgTbx2
Tbx2	*Xenopus laevis*	BAA93081.1	XlTbx2
Tbx2	*Homo sapiens*	AAA73861.1	HsTbx2
Tbx2	*Mus musculus*	AAC52697.1	MmTbx2
Tbx3	*Gallus gallus*	NP_001257807.1	GgTbx3
Tbx3	*Xenopus laevis*	AAH77254.1	XlTbx3
Tbx3	*Homo sapiens*	AAD50989.2	HsTbx3
Tbx3	*Mus musculus*	AAH96551.1	MmTbx3
Tbx3	*Danio rerio*	NP_001095140.2	DrTbx3
Tbx2/3	*Ciona intestinalis*	NP_001027620.1	CiTbx2/3
Tbx2/3	*Ptychodera flava*	MH782153	PfTbx2/3
Tbx2/3	*Branchiostoma floridae*	XP_002598922.1	BfTbx2/3
Tbx2/3	*Strongylocentrotus purpuratus*	NP_001123280.1	SpTbx2/3
Tbx2/3	*Saccoglossus kowalevskii*	NP_001158392.1	SkTbx2/3
Tbx4/5	*Branchiostoma floridae*	ABU50779.1	BfTbx4/5
Tbx4	*Gallus gallus*	NP_001025708.1	GgTbx4

Tbx5	*Gallus gallus*	NP_989504.1	GgTbx5
Tbx4	*Mus musculus*	NP_035666.2	MmTbx4
Tbx5	*Mus musculus*	NP_035667.1	MmTbx5
Rx	*Strongylocentrotus purpuratus*	XP_003726050.1	SpRx
Rx	*Drosophila melanogaster*	NP_726006.3	DmRx
Rx	*Mus musculus*	NP_038861	MmRx
Rx	*Branchiostoma floridae*	AFQ55891	BfRx
Rx	*Saccoglossus kowalevskii*	NP_001158375	SkRx
Rx	*Homo sapiens*	AAH51901	HsRx

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