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Developmental role of *dpp* in the gastropod shell plate and co-option of the *dpp*
signaling pathway in the evolution of the operculum

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Abstract

The operculum is a novel structure in gastropod molluscs. Because the operculum shows notable similarities to the shell plate, we asked whether there were an evolutionary link between these two secretory organs. We found that some of the genes involved in shell-field development are expressed in the operculum, such as *dpp* and *grainyhead*, whereas *engrailed* and *Hox1* are not. Specific knockdown of *dpp* by injection of double-stranded RNA (dsRNA) resulted in malformation of the shell plate. The shell plate was smaller due to failure of activation of cell proliferation in the shell-field margin. The expressions of *grainyhead* and *chitin synthase 1* in the shell field margin were suppressed by *dpp*-dsRNA. However, matrix secretion was not completely abolished, and the expressions of *ferritin*, *engrailed* or *Hox1* were not affected by *dpp*-dsRNA, indicating that *dpp* is partly involved in the developmental pathway for shell matrix secretion. We also present evidence that *dpp* performs a key role in operculum development. Indeed, *dpp*-dsRNA impaired matrix secretion in the operculum as well as expression of *grainyhead*. Based on these observations that *dpp* is important for development of both the shell plate and operculum, we conclude that co-option of *dpp* to the posterior part of the foot contributed to the innovation of the operculum in gastropods.

Key word: *dpp*, *grainyhead*, gastropod, mollusc, shell plate, operculum

Introduction

Mollusca is one of the most divergent phyla of lophotrochozoans; its members are characterized by a calcitic shell. The hard shell is an effective apparatus to protect the body from predators; however, the shell plate does not cover the whole body, allowing uptake of food/oxygen and the excretion of waste. To better protect the body against predators, gastropods and bivalves developed distinct strategies. On the one hand, bivalves' shells separated into two distinct plates, and by developing a ligament and a novel adductor muscle to control opening of these separated shell plates, they can protect their soft tissues. On the other hand, gastropods developed a distinct apparatus, the operculum, in the posterior part of the foot.

In the present study, we examined the evolution of the gastropod operculum using the limpet, *Nipponacmea fuscoviridis*. Although adult limpets lack an operculum, it is present in the posterior part of the foot in larvae. The operculum is a proteinaceous and sometimes calcified structure that is secreted by specific gland cells on the posterior part of the foot (Voltzow, 1994). Additionally, just as the shell coils in many gastropod species, the operculum also shows a spiral growth pattern. Thus, there was an old argument stressing that the operculum is homologous with the shell, and that shell and operculum are together indicative of an original bivalve condition (Gray,

1850; Fleischmann, 1932). Here, including a test of the above hypothesis, we examined the evolutionary link between the shell plate and the operculum. In support of the link, several genes are expressed in both the shell field and the operculum, such as *ubfm* and *ferritin* (Jackson et al., 2007).

In all molluscan groups, the shell plate develops on the dorsal ectoderm (Kniprath, 1981). In gastropods, the first sign of shell plate morphogenesis is observed as shell-field invagination, which occurs at the gastrula stage as an invagination of dorsal epidermal cells (Kniprath, 1981; Nederbragt et al., 2002). Accompanied by shell matrix secretion into the invaginated extracellular space, shell-field cells evaginate, and subsequently, shell plate covers a wide area of the larval body (Kniprath, 1981). After the evagination of the shell field, cells along the margin of shell field are responsible for further secretion of the shell plate matrix. Several genes have been identified to be involved in the development of shell-field cells. *Engrailed* is expressed in cells responsible for shell matrix secretion in chitons, scaphopods, and bivalves as well as in gastropods (Jacobs et al., 2000; Wanninger and Haszprunar, 2001; Nederbragt et al., 2002; Kin et al., 2009). *Hox1* has also been shown to be expressed in the shell-field margin of gastropods (Hinman et al., 2002; Samadi and Steiner, 2009). In gastropods, *dpp* is expressed in cells surrounding the *engrailed*-positive cells

(Nederbragt et al., 2002; Iijima et al., 2008). However, none of these genes has been examined in terms of function, such as through RNAi knockdown experiments.

To assess whether there is an evolutionary link between the shell and operculum, we first observed the developmental process of the operculum in *N. fuscoviridis* and examined the expression patterns of *engrailed*, *dpp*, *Hox1*, and *grainyhead*. We found that *dpp* and *grainyhead* were expressed in the operculum as well as in the shell-field margin, but *engrailed* and *Hox1* were not. Double-stranded RNA-based inhibition of *dpp* function resulted in failure of both the shell plate and operculum to develop. We propose that co-option of *dpp* function in the operculum partially explains the innovation of the gastropod operculum.

Materials and Methods

Animals and in vitro fertilization

Sexually mature individuals of *Nipponacmea fuscoviridis* were collected in Yoshidahama Harbor, Miyagi Prefecture, Japan, during the breeding season (April to June and September to November).

In vitro fertilization was performed following the methods described by Deguchi (2007). Embryos were cultured in filtered seawater (FSW) at 22°C.

Histology

Specimens were observed under Nomarsky optics using Nikon E-800. Matrix of shell plate and operculum were observed as refringent matrix under Nomarsky optics.

Embryos were fixed in a solution containing 4% paraformaldehyde, 0.1 M MOPS (pH 7.5), 2 mM EGTA, and 0.5 M NaCl. Fixed embryos were embedded in 2% agar.

They were dehydrated through a graded ethanol series, which was then replaced by a graded ethanol-*n*-butanol series. Then, the agar blocks were embedded in paraffin.

Sections (3 µm thick) were stained with Mayer's hematoxylin and eosin.

Cloning of genes and in situ hybridization

Using the primers shown in Sup. Table 1, *dpp*, *engrailed*, *Hox1*, *grainyhead*, *chitin synthase 1(CS1)*,

and *ferritin* were amplified with PCR. The primers were designed with reference to sequences from another species of limpet, *Lottia gigantea* (<http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html>). GenBank/EMBL/DDBJ accession numbers are as follows: AB612238 for *dpp*, AB639757 for *engrailed*, AB639756 for *Hox1*, AB639758 for *grainyhead*, AB646432 for *CSI*, and AB639755 for *ferritin*. *In situ* hybridization was performed as described by Kin *et al.* (2009).

RNAi

Microinjection was performed using micromanipulators (Narishige) and an injection apparatus (Femtojet; Eppendorf). After injection, embryos were cultured in FSW (22°C) until fixed at 12, 16, or 20 hours post-fertilization (hpf). Template cDNAs of each gene for double-stranded RNA (947 bp for *Nfdpp* and 667 bp for the control *hedgehog* gene from the Japanese purple mussel, *Septifer virgatus*) were amplified using the primers shown in Sup. Table 1. Double-stranded RNA was generated following Clemens *et al.* (2000). dsRNA dissolved in water was injected into fertilized eggs following Sweet *et al.* (2004)

Cell proliferation was analyzed using 5-bromo-2-deoxyuridine (BrdU; Roche Applied Science). dsRNA-injected larvae (12 hpf) were transferred to FSW containing 1 μ M BrdU until fixation at 15 hpf. Fixed larvae were processed for immunochemical staining with an anti-BrdU antibody (Roche Applied Science) and anti-mouse secondary antibody conjugated to Alexa 488 (Molecular

Probes). After visualizing the actin filaments underlying plasma membrane using phalloidin, specimens were observed under a confocal laser-scanning microscope, as described previously (Kurita et al., 2009; Kurita and Wada, 2011). By this mean, the edge of shell field was unambiguously identified (Supplementary Fig. 1). Statistical analyses of cell proliferation were performed using the Mann–Whitney *U*-test.

Results

Development of the operculum

First, we observed the developmental time-course of the shell plate and the operculum in *N. fuscoviridis*. At 8 hpf, the shell field was observed as a small invagination on the dorsal side (Fig. 1A, B). The ventral part was flat, and we did not observe any sign of foot development or the operculum at this stage. At 10 hpf in the trochophore larva, when the shell-field invagination became more prominent, development of the foot began to be observed as a small protrusion in the ventral part (Fig. 1C, D). At 14 hpf, the shell field evaginated and expanded (Fig. 1E, F). Due to the expansion of the shell field, the telotroch moved upward and the mantle cavity began to form. At this stage, in the posterior part of the foot, we recognized cells of a distinct shape, specifically, long and columnar, oriented along the apical–basal axis (Fig. 1F). Note that the shell-field cells at 10 hpf showed a similar morphology. At 18 hpf in veliger larvae, the shell matrix covered a wide part of the body, and the mantle cavity became prominent (Fig. 1G, H). In the posterior part of the foot, we could recognize secretion of the operculum matrix, which was underlain by tall columnar cells (Fig. 1G, H).

Expression patterns of engrailed, dpp, grainyhead, and Hox1

As reported in several species of gastropods (Nederbragt et al., 2002; Iijima et al., 2008), *dpp* expression was detected in cells surrounding the shell field at 10 hpf as well as in anterior ectoderm cells (Fig. 2A; the signal of anterior ectoderm is out of focus in this panel). Subsequently, from 14 hpf, we detected new expression of *dpp* in the ventral epidermis of the foot (black arrowhead in Fig. 2B). Expression in the shell-field margin was no longer detected at this stage. Expression in the posterior foot was detected at 18 hpf, when operculum matrix secretion was also observed. The cells underlying the operculum were marked by *dpp* expression (Fig. 2C, posterior view in Supplementary Fig. 2).

As reported by Nederbragt et al. (2002) and Iijima et al. (2008), expression of *engrailed* was detected in the shell-field margin and anterior ectoderm cells at the trochophore stage (10 hpf; Fig. 2D). At 14 hpf, as operculum development proceeded, expression was newly detected in the foot (white arrowhead in Fig. 2E). The signal persisted until 18 hpf, with the expression clear inside the foot and not in the epidermal layer (Fig. 2F). Thus, this *engrailed* expression did not mark cells involved in the matrix secretion of the operculum. FMRF-positive nerve cells were detected in a similar part of the foot at 22 hpf (Supplementary Fig. 3), when *engrailed* expression was no longer detected. This suggests that this later expression of *engrailed* may be

involved in neurogenesis in the foot ganglion.

A transcription factor, *grainyhead* has been proposed to have a conserved role in the differentiation of exocrine cells (Yamaguchi et al., 2006). Because the shell field and operculum are both secretory organs, we reasoned that *grainyhead* may be involved in the development of both. Indeed, we detected expression of *grainyhead* in the shell field and operculum. At 10 hpf, expression was detected in cells adjacent to the shell field and in the most anterior region of shell field (Fig. 2G). At 14 hpf, expression was detected in the posterior part of the foot, and stronger expression was detected in a more limited part of the foot epithelium as the foot region grew bigger (Fig. 2H). At 18 hpf, expression was observed in cells underlying the operculum, but the expression was more restricted compared with *dpp* (Fig. 2I; compare with Fig. 2C, posterior view in Supplementary Fig. 2).

As reported by Hinman et al. (2002), the 10 hpf trochophore larvae show a half circle of *Hox1*-positive cells in the shell field (Fig. 2J). At the veliger stage (14–18 hpf), *Hox1* expression remained at the edge of the mantle, corresponding to the position of the shell glands, but we did not detect *Hox1* expression in the foot region (Fig. 2K, L).

We also examined developmental expression of two shell plate effector genes, ferritin and *chitin synthase 1 (CS1)*. At 10 hpf, both *ferritin* and *CS1* was expressed in

cells surrounding the shell plate (Fig. 2M, P). Ferritin expression was also detected in a pair of anterior cells, whose nature was unknown. At 14-18 hpf, expression of both genes were detected in the shell field margin, while only *ferritin* expression was detected in the operculum (Fig. 2N, O, Q, R, posterior view of ferritin expression in operculum shown in Supplementary Fig. 2).

Function of dpp in the shell field and operculum

Because *dpp* is expressed in the operculum as well as in the shell-field margin, we examined the function of *dpp* in these organs. We found that inhibition of *dpp* by RNAi resulted in the failure of shell-field development, which was not observed with a control RNAi using bivalve *hedgehog* (Fig. 3A, B, Table 1). We confirmed that 0.5 µg/µl of dsRNA was sufficient to degrade endogenous *dpp* (Fig. 4A, B, Table 2), and we performed further analyses injecting this concentration of dsRNA. At this concentration, after injecting any dsRNAs, approximately 80% of larvae survived and kept swimming up to 20 hpf (Table 1). Among the survivors, the development of 10–20% larvae was apparently arrested at the trochophore stage (comparable to 10 hpf in normal development) and they failed to form a mantle cavity, although they continued to swim. When injected with *dpp*-dsRNA, more than half of the larvae

(62/94: 66%, arrested larvae excluded) showed abnormal and smaller shell plates, whereas no such effect was observed in control dsRNA-injected larvae (Fig. 3A, B). However, in *dpp*-dsRNA-injected larvae, some matrix was still observed (Fig. 3B), and thus shell development was not completely abolished. *CSI* expression was impaired in more than half of the injected larvae examined (14/23: Fig. 4G, H). Thus, *CSI* expression is likely to be under the control of *dpp* signaling. Expression of *grainyhead* in the shell-field margin was also severely affected in *dpp*-dsRNA-injected larvae; in most of the injected larvae (35/42), expression was not detected (Fig. 4M, N). On the other hand, expression of the other shell effector gene, *ferritin*, was unaffected (0/37: Fig. 4I, J). No effect was observed in the expression of *engrailed* or *Hox1* (Fig. 4C-F).

Because the *dpp*-dsRNA-injected larvae showed notably smaller shell plates, we examined the effect of *dpp* on the proliferation of shell-field cells. Cell-proliferation activity was assessed by BrdU incorporation during shell-field evagination and the early expansion period (from 12 to 15 hpf). Whereas control larvae showed high cell-proliferation activity, especially in the shell-field margin, *dpp*-dsRNA larvae showed significantly reduced cell-proliferation activity in the shell field (Fig. 3C-F, G). We did not detect a difference in cell proliferation in the head region (anterior to the protroch) between *dpp*-dsRNA larvae and control dsRNA larvae (Fig. 3C-F, H),

indicating that the effect was specific to shell-field cells. Thus, *dpp* appears to function in the activation of cell proliferation at the edge of the shell field as well as in matrix secretion, where *CSI* is involved. However, secretion of some other matrix component in which *ferritin* is involved is not dependent on *dpp* signaling.

Operculum development was also impaired by *dpp*-dsRNA. In larvae surviving up to 20 hpf after being injected with 0.5 $\mu\text{g}/\mu\text{L}$ *dpp*-dsRNA, approximately 40% of the larvae (47/117) showed no matrix secretion in the operculum, whereas the shell plate developed to some degree (Fig. 3A, B). No control dsRNA-injected larvae showed such a phenotype without an operculum. Additionally, *grainyhead* expression was almost abolished in *dpp*-dsRNA larvae when examined at 16 hpf (24/29; Fig. 4O, P). However, expression of *ferritin* in the operculum was not affected (Fig. 4K, L), while morphology of the larvae was deformed due to the effect on shell field expansion. In addition, we observed tall columnar cells in the posterior part of the foot even when *dpp* function of inhibited (Fig. 3D). Thus, *dpp* has certain roles for the matrix secretion in the operculum, but cell differentiation was not completely abolished by *dpp*-dsRNA.

Discussion

Developmental role of dpp in shell-field cell development

In the present study, we provide evidence that *dpp* plays an important role in shell-field development. We found that cell proliferation at the shell-field margin was significantly suppressed by *dpp*-dsRNA (Fig. 3). Cell proliferation of the shell-field margin is important for normal morphogenesis of gastropods to cover and protect the posterior body mass (Kniprath, 1981). We also found that, although shell matrix secretion was not completely abolished by *dpp*-dsRNA, expression of one of the shell matrix effectors, *CS1*, was impaired (Fig. 4G, H). Thus, *dpp* signaling performs an important, but limited, role in shell matrix secretion. That is, shell matrix secretion is likely controlled in a complex and hierarchal manner. It is likely that *dpp* signaling is involved in certain aspect of matrix secretion, such as chitin synthesis. Shimizu et al. (2011) indicated that chemical inhibition of *dpp* signaling resulted in failure of calcification of the shell plate in pond snail. On the other hand, other aspects of shell development processes are not dependent on *dpp* signaling, such as the expression of *ferritin* (Fig. 4I, J). Several transcription factors are also shown to be expressed in the shell field, including *engrailed* and *Hox1*. Indeed, *engrailed* shows conserved expression in the shell field of several molluscs (Jacobs et al., 2000; Wanninger and

Haszprunar, 2001; Nederbragt et al., 2002; Kin et al., 2009); thus, it may perform a key role in matrix secretion. However, unfortunately, our attempt to inhibit *engrailed* by means of dsRNA injection was not successful; dsRNA did not lead to degradation of the *engrailed* mRNA (data not shown).

Development and evolution of the operculum

The operculum is a novel structure in gastropods. Because the operculum shows notable similarities with the shell, we explored the idea that co-option of the shell-field developmental process may account for the evolution of the operculum by comparing developmental mechanisms of these two tissues. Development of the operculum begins at about 14 hpf with differentiation of thick cells in the posterior part of the foot (Fig. 1). In addition to the similarity in morphology of the thick columnar cells, we observed co-expression of *dpp*, *grainyhead*, and *ferritin* in both the operculum and the shell-field margin (Fig. 2). Inhibition of *dpp* signaling impaired matrix secretion in the operculum (Fig. 3A, B). Because *dpp* signaling is involved in multiple contexts in animal development, shared involvement of *dpp* cannot be a strong evidence for an evolutionary link. However, because the expression of *grainyhead* is dependent on *dpp* signaling in both the shell field and the operculum (Fig. 4M-P), it is probably safe to

propose an evolutionary link in the developmental processes between the shell plate and the operculum. Thus, we suggest that co-option of the developmental process of the shell plate occurred during the evolution of the operculum.

Our results may also be consistent with the idea that operculum originated from one of the bivalve shell plates, supposing that *dpp-grainyhead* is also involved in bivalve shell development. However, we did not detect expression patterns such that *dpp*-positive cells migrate from the shell plate and form operculum cells. Rather the expression of *dpp* and *grainyhead* in operculum cells commences notably later than that in the shell plate (Fig. 2). Furthermore, lack of gene expression of *engrailed* or *Hox1* in the operculum does not support the origin of the operculum from one of the bivalve shells. Thus, our data are more consistent with co-option of the developmental process of the shell plate to the operculum.

The co-option of *dpp-grainyhead* pathway may have contributed to providing a novel function, namely as matrix secretory cells, to the cells in the posterior part of foot. However, perhaps the co-option of the *dpp-grainyhead* pathway was insufficient, because *ferritin* expression is not under the control of *dpp* signaling in either the shell field or the operculum. Thus, additional evolutionary events may have been required for the evolution of the operculum. Alternatively, co-option of a regulatory molecule

further upstream of *dpp* might have occurred. In either case, co-option of the genetic cascade of *dpp-grainyhead* has provided a unique cellular nature as matrix secretors, and was an essential step for operculum evolution. Such a phenomenon of shuffling the cellular nature within a body may be one of the major driving forces for the evolution of novel structures as expressed by Gould (1977) when he stated; “permutation of the old within complex systems can do wonders.” The innovation of the molluscan operculum is a typical example of a novel structure due to permutation of the old (shell plate).

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Figure legends

Figure 1. Outline of development of the shell plate and operculum in *N. fuscoviridis*

(A, B) At 8 hpf, the shell field is observed as a small invagination on the dorsal side. However, no sign of foot development is observed. Lateral view (A, dorsal to the left) and dorsal view (B). Shell field is encircled by white broken lines. (C, D) At 10 hpf, the shell-field invagination was more prominent, and foot development was observed as a small protrusion in the ventral part. Lateral view of whole-mount larvae (C) and sectioned image (D). Dorsal to the left. Shell field is encircled by white broken lines. (E, F) The shell plate matrix was first observed in 14-hpf early veliger larvae. Due to the expansion of the mantle epithelium, the foot moved upward, and a mantle fold emerged. Operculum cells were observed as long cells in the posterior part of the foot (encircled by black broken line). Lateral view of whole-mount larvae (E) and sectioned image (F). Dorsal to the left. (G, H) At 18 hpf, the shell plate developed with a dome-like shape

and surrounded a wide part of the larval body. The matrix of the operculum also emerged at this stage (black arrow). Lateral view of whole-mount larvae (G) and sectioned image (H). Dorsal to the left. Black arrowheads: foot, white arrows: mantle edge, black arrow: operculum, mc: mantle cavity. Scale bars: 20 μ m.

Figure 2. Expression pattern of *dpp*, *engrailed*, *grainyhead* and *Hox1*.

Expression patterns of *dpp* (A–C), *engrailed* (D–F), *grainyhead* (G–I), *Hox1* (J–L), *ferritin* (M–O) and *CSI* (P–R). Dorsal view of the 10-hpf trochophore larvae (A, D, G, J, M, P). Lateral views at 14 hpf (B, E, H, K, N, Q) and 18 hpf (C, F, I, L, O, R). Dorsal to the left. The expressions in the operculum are indicated by black arrowheads. Black arrows indicate expressions in the mantle edge. White arrowheads indicate the expression of *engrailed* in the internal cells of the foot. Asterisks indicate non-specific staining of shell plate.

Figure 3. Effect of *dpp*-dsRNA on larval morphology and cell proliferation in the shell

field. (A-D) Morphology of control dsRNA-injected larvae (A), and that of *dpp*-dsRNA larvae (B) at 20 hpf. pt: prototroch, f: foot. (C, D) Enlarged images of the foot region of the larvae shown in (A) and (B), respectively. Operculum region is indicated by

arrowheads. Clear operculum matrix is observed in control larvae (C), but no matrix was observed in *dpp*-dsRNA larvae, while tall columnar cells were still observed as indicated by arrowheads (D). (E, F) Lateral view of the control dsRNA-injected larva (E) and *dpp*-dsRNA larva (F). BrdU signals were detected as green signals. White broken lines indicate the edge of the shell field. (G, H) Comparison of BrdU-positive cell numbers at the shell field (G) and head region (H) between control dsRNA-injected larvae ($n = 8$) and *dpp*-dsRNA larvae ($n = 13$). Means and standard deviations are shown. Mann–Whitney *U*-test, n.s.: not significant. * $P < 0.01$. Scale bars: 50 μm .

Figure 4. Effect of *dpp*-dsRNA on gene expressions

Expression of *dpp* (A, B), *engrailed* (C, D), *Hox1* (E, F), *CS1* (G, H), *ferritin* (I-L) and *grainyhead* (M-P) in control dsRNA-injected larvae (A, C, E, G, I, K, M, O, Q) or *dpp*-dsRNA-injected larvae (B, D, F, H, J, L, N, P, R). Expression was examined at the 12-hpf trochophore stage (A-J, M, N: dorsal views) or the 16-hpf veliger stage (K, L, O, P: lateral views, dorsal to the left). Arrowheads indicate the position of the operculum cells.

Figure
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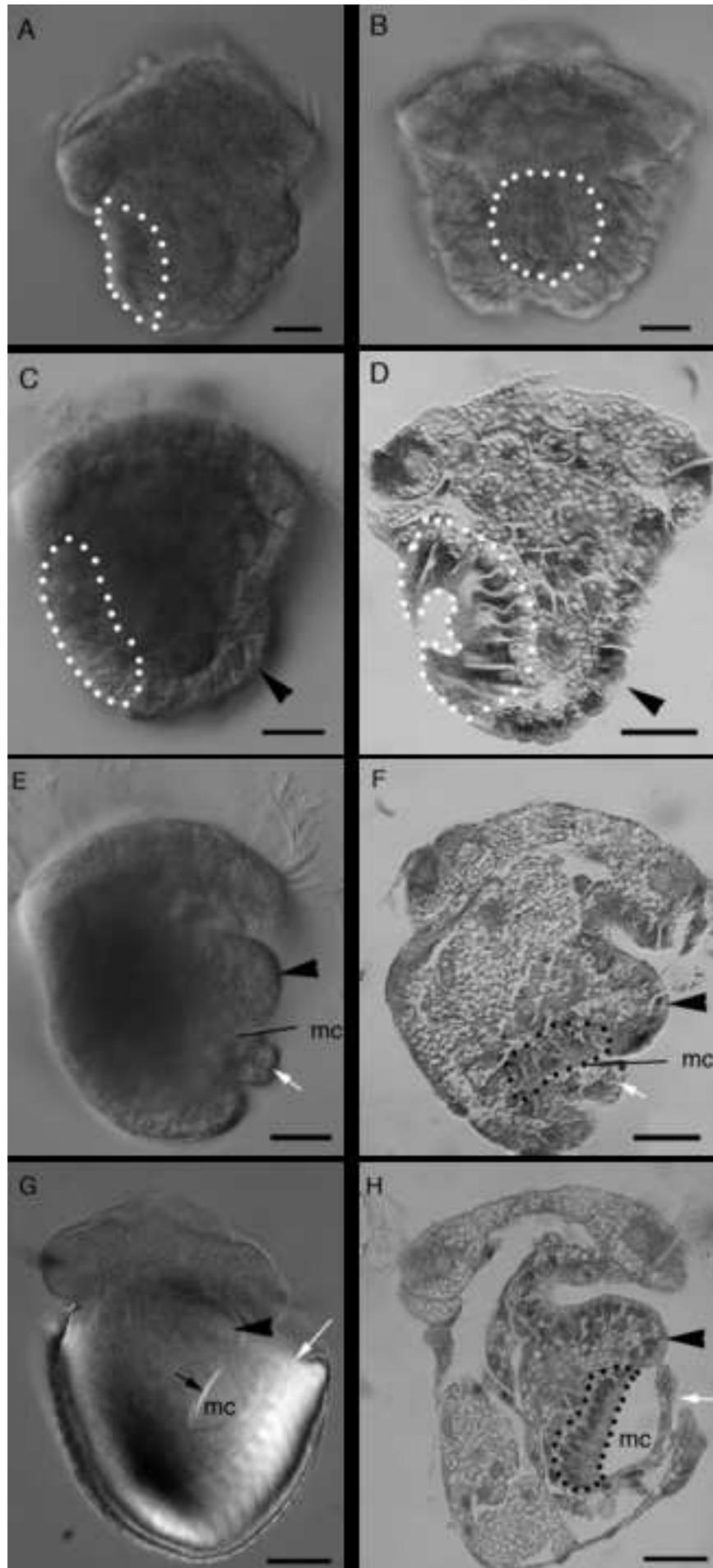


Figure2

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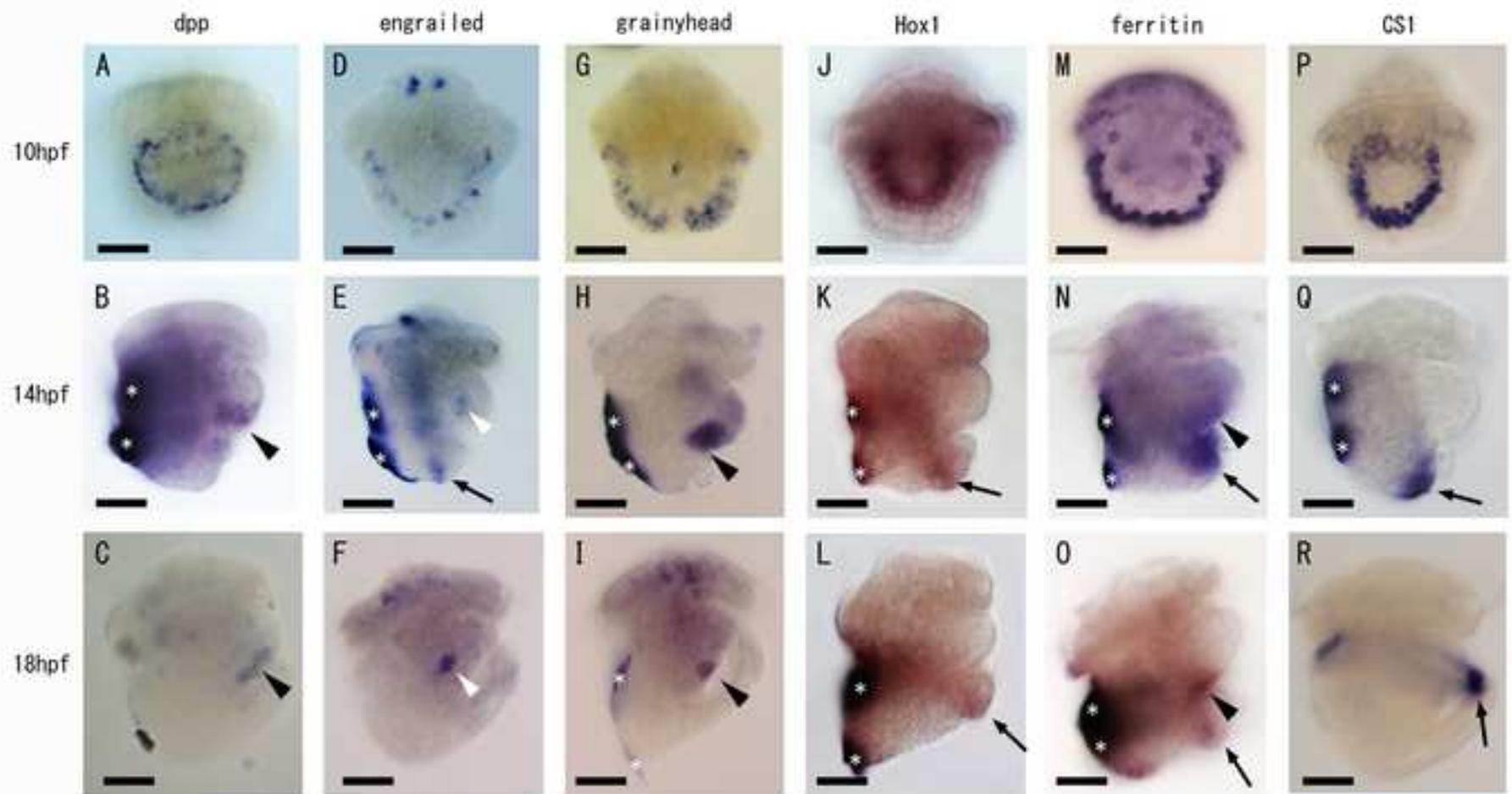


Figure3
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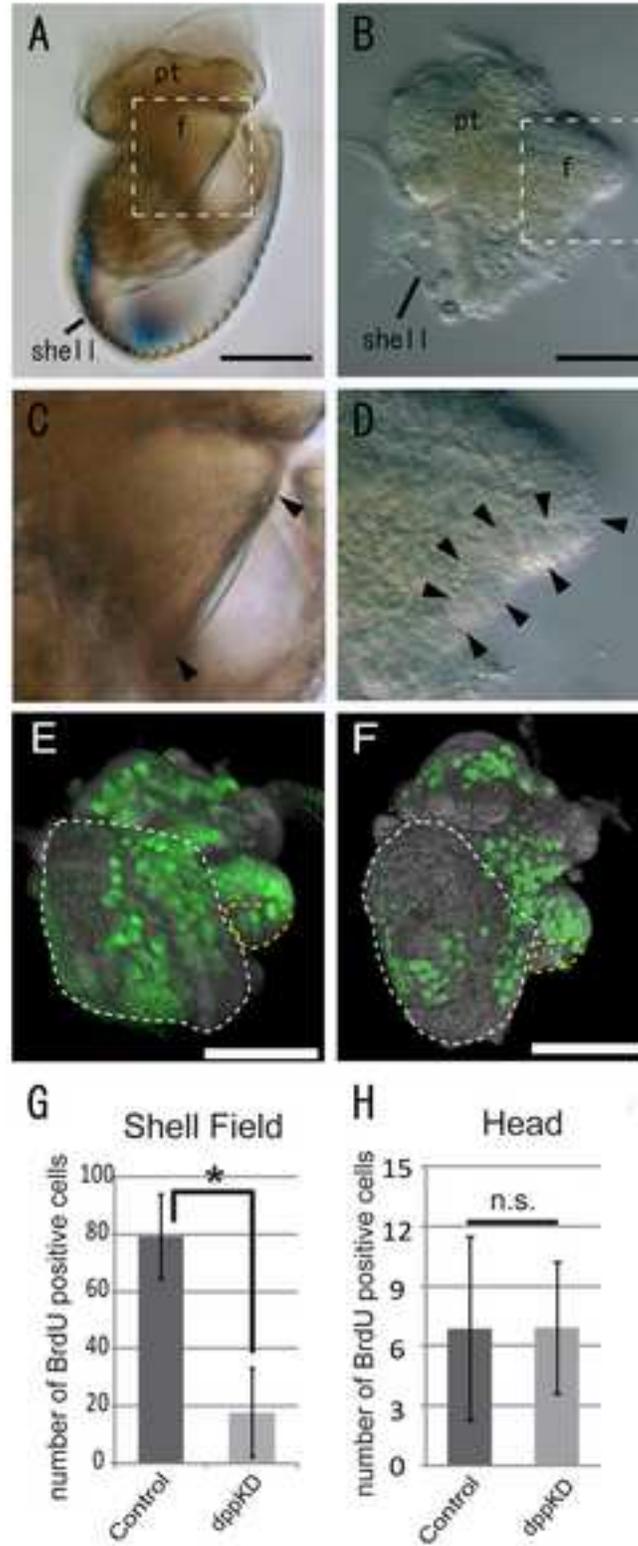


Figure4

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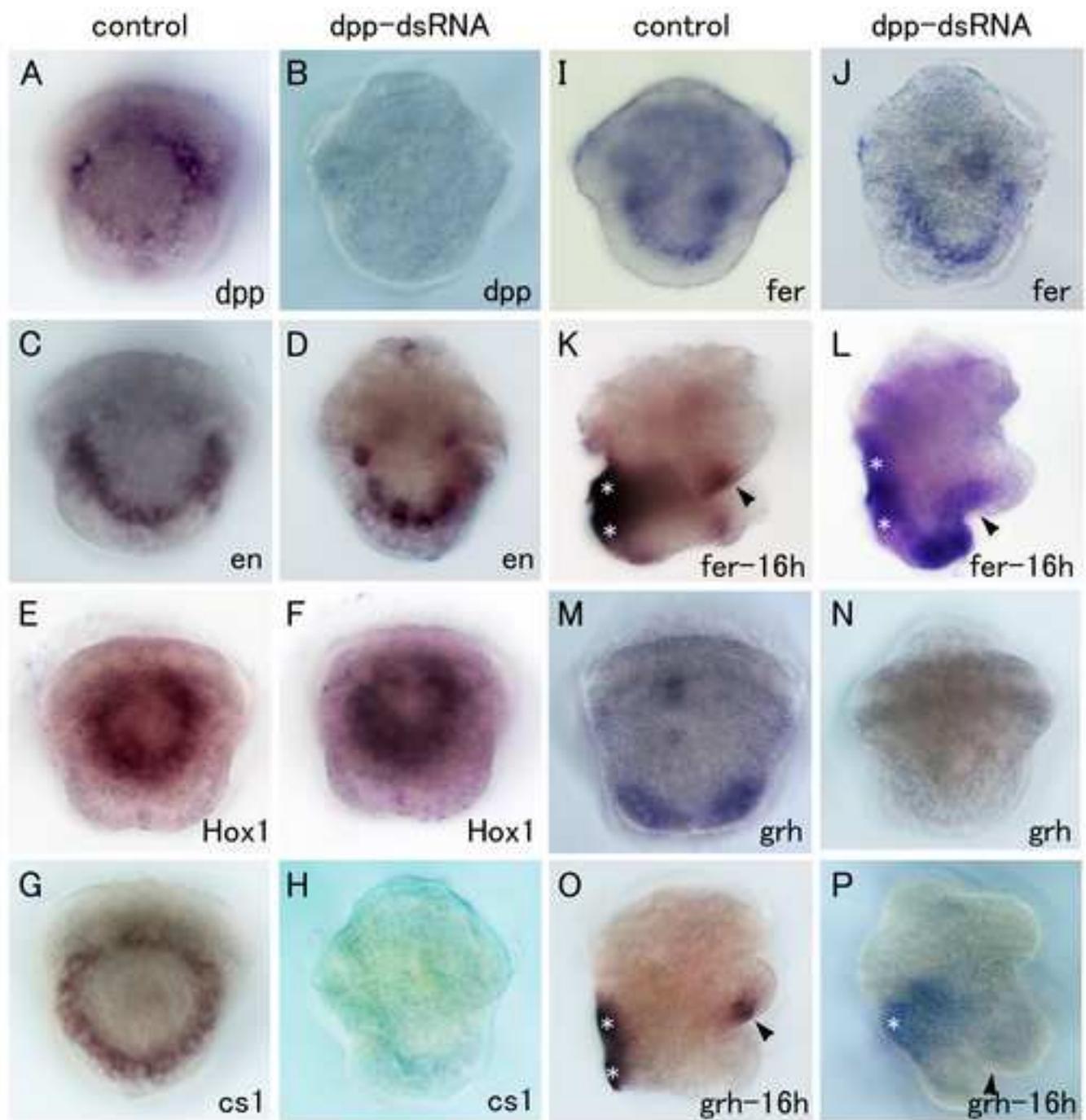


Table 1. Effect of dpp-RNAi on the larval morphology at 20hpf

	No. injected	Survived up to 20h	Normal	Shell ab. Op +	Shell ab. Op -	Shell -, Op-*
control dsRNA	96	84	73	0	0	11
dpp dsRNA	145	117	32	15	47	23

Shell ab.: abnormal shape of shell plate, Op +: operculum present, Op -: operculum absent

*Shell -, Op -: shell plate and operculum were absent due to arrested development

Table 2. Effect of dpp RNAi on gene expressions (no. of larvae in which expression was not detected / no. of larvae examined)

	dpp	en 12h (SF)	Hox1	cs1	ferritin		grainyhead	
					12h (SF)	16h (Op)	12h (SF)	16h (Op)
control dsRNA	2/25	0/35	0/32	0/63	0/22	0/21	0/42	0/41
dpp dsRNA	43/46	0/58	0/69	14/23	0/37	0/18	35/42	24/29

SF: shell field margin, Op: operculum