

1 **Possible co-option of *engrailed* during brachiopod and mollusc shell**

2 **development**

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17

18 **Abstract**

19 In molluscs, two homeobox genes, *engrailed* (*en*) and *distal-less* (*dlx*), are transcription factors that  
20 are expressed in correlation with shell development. They are expressed in the regions between  
21 shell-forming and non-shell-forming cells, likely defining the boundaries of shell-forming fields.  
22 Here we investigate the expression of two transcription factors in the brachiopod *Lingula anatina*.  
23 We find that *en* is expressed in larval mantle lobes, whereas *dlx* is expressed in larval tentacles. We  
24 also demonstrate that the embryonic shell marker, mantle peroxidase (*mpox*), is specifically  
25 expressed in mantle lobes. Our results suggest that *en* and *mpox* are possibly involved in brachiopod  
26 embryonic shell development. We discuss the evolutionary developmental origin of  
27 lophotrochozoan biomineralization through independent gene co-option.

28

29 **Keywords:** brachiopods, shell formation, *engrailed*, *distal-less*, evolution

## 30 **Introduction**

31 Brachiopods and molluscs are the major lophotrochozoans that are capable of biomineralization,  
32 possessing shells comprised of calcium phosphate or calcium carbonate. Shelled invertebrates first  
33 appeared in the early Cambrian, more than 540 million years ago, and have accumulated an ample  
34 fossil record. Although brachiopod and mollusc shells are secreted from outer mantle epithelial  
35 cells derived from ectoderm [1,2], the possible homology of their mantle tissues and cell types  
36 remains unresolved.

37           Delimitation of the shell forming region and specification of shell-secreting cells are the  
38 initial developmental events of shell formation. In gastropods, embryonic and/or larval shells are  
39 formed by the shell gland, which originates from ectodermal cells in early trochophore larvae [2].  
40 Also, developmental genes including *dpp/bmp*, *hox1* and *hox4* are expressed around the shell gland  
41 [3-4]. During mollusc shell development, the homeobox-containing transcription factor genes,  
42 *engrailed (en)*, is expressed at boundaries defining the larval shell-forming region [3, 5-9]. On the  
43 other hand, *distal-less (dlx)* is also expressed in a similar manner in a gastropod [10]. However, *en*  
44 and *dlx* have diverse functions in animals, suggesting that shell formation is probably not the  
45 ancestral role of these genes. For example, in annelids, *en* and *dlx* may have roles in segmentation  
46 and neurogenesis, respectively, other than shell formation [11,12] (supplementary table S3). In  
47 arthropods, *en* and *dlx* are required for segmentation and limb formation [11,13]. Furthermore, in

48 echinoderms, vertebrates, and arthropod, *en* also functions in neurogenesis [11,14-16]. Thus,  
49 co-option of *en* and *dlx* for shell formation probably only occurred in the lineage leading to  
50 molluscs, because such shells are not present in most other taxa.

51           Recent studies have advanced our understanding of brachiopod embryogenesis to  
52 molecular levels [17-22] by applying molecular analyses to traditional morphological and  
53 experimental embryology [1,23-25], yet the molecular basis of shell development in brachiopods  
54 remains unclear. In the lingulid *Lingula anatina* and the rhynchonellid *Terebratalia transversa*,  
55 embryonic shells (i.e., protogula) are formed by the mantle lobes derived from ectodermal cells [1,  
56 26]. In *T. transversa* and the craniid *Novocrania anomala*, Hox genes, such as *scr* and *antp*, are  
57 expressed in the shell-forming epithelium, but unlike molluscs, *hox1* and *hox4* are not expressed  
58 [20]. Interestingly, in *N. anomala*, *en* is expressed in the shell primordium [21]. However, details of  
59 *en* expression in relation to shell formation have not been reported. Moreover, expression of *dlx*,  
60 which occurs in the gastropod larval shell region [10], has not been observed in brachiopods. Here,  
61 we found that in *L. anatina*, *en* is expressed in the mantle lobes, possibly participating in shell  
62 formation. In contrast, *dlx* is expressed in tentacles, but not in shell-forming regions. Given its  
63 different developmental modes in brachiopods and molluscs, we argue that *en* and its gene  
64 regulatory circuit but not *dlx* may be involved in brachiopod and mollusc shell development, and  
65 that shell formation in the two lineages may have evolved by independent co-option of *en*. Future

66 studies on the developmental function of *en* in brachiopods and molluscs will be needed to test this  
67 hypothesis.

## 68 **Methods**

69 Specimens of *L. anatina* were collected at Amami Island, Kagoshima, Japan. Methods of egg  
70 collection and culturing of the embryos followed those reported previously [19]. Late gastrulae (9  
71 hour-post-fertilization, hpf) and larvae (29 hpf) were fixed with 4% paraformaldehyde (with 10 mM  
72 EPPS in filtered seawater) at 4°C overnight. We performed whole mount *in situ* hybridization using  
73 the protocol from the previous study on the brachiopod *T. transversa* [17] with slight modifications.  
74 Detailed methodology is provided in the supplementary material.

## 75 **Results and Discussion**

### 76 *Expression of en, mpox, and dlx in L. anatina embryos*

77 We first annotated *en*, *mpox*, and *dlx* in the *L. anatina* genome (using v1.0 gene models) [19]. We  
78 identified three *en* homologs (20932, 20933, and 29453) on two different scaffolds (609 and 1364);  
79 *en* homologs 20932 and 20933 probably resulted from tandem duplication (supplementary figure  
80 S1). Of these three homologs, 20932 is not expressed during early development, whereas 20933 and  
81 29453 are alleles that both correspond to the same transcript, which begins to be expressed during  
82 late gastrula stage (supplementary figure S1). *En* is expressed at the boundary of embryonic shells  
83 in molluscan larvae, and it has been associated with shell formation [3,5-9]. Interestingly, in the

84 brachiopods *N. anomala* and *T. transversa*, *en* is expressed at boundaries between the apical region  
85 and the mantle lobe region in larvae [21]. At the gastrula stage, *en* is expressed at the blastopore and  
86 dorsal sides in *T. transversa* and *N. anomala*, respectively [21] (figure 1g). However, in *L. anatina*,  
87 we found that *en* is expressed in the ectoderm at the gastrula stage in two domains: one at the  
88 animal pole and the other near the blastopore (figure 1a). In larval stage, *L. anatina en* is expressed  
89 in both the dorsal and ventral mantle lobes (figure 1d). This finding is similar to the observation that  
90 in the lingulid brachiopod, *Glottidia*, animal pole ectoderm develops into the dorsal mantle lobe  
91 [24]. In the larvae of *N. anomala* and *T. transversa*, *en* is expressed at the apical-mantle boundary  
92 (figure 1g) [21]. In *N. anomala* bilobed larvae, *en* is also expressed in the shell rudiment that is  
93 located on the dorsal side, and a corresponding domain also occurs on the dorsal surface of *T.*  
94 *transversa* trilobed larvae (figure 1g) [21]. However, unlike *L. anatina* and *N. anomala*, *T.*  
95 *transversa* larvae do not develop a shell. They start to secrete shells during the juvenile stage, just  
96 after metamorphosis [26].

97           The *mpox* gene, originally annotated as peroxidasin, has been reported as a shell matrix  
98 enzyme that is specifically expressed in the adult mantle of *L. anatina* [19]. After a detailed  
99 phylogenetic analysis and orthology assignment, it was renamed *mpox* (peroxidase expressed in the  
100 mantle) (supplementary figure S2). *Mpox* has an animal heme peroxidase domain, but contains none  
101 of the other domains found in a *bona fide* peroxidasin (supplementary figure S2). We showed that

102 *mpox* is not expressed in late gastrulae, but is expressed in larval mantle lobes (figure 1*b,e*). This is  
103 consistent with the fact that MpoX is an embryonic shell matrix protein, indicating that *mpox* can be  
104 used as an embryonic shell marker, and that it may reside downstream of *en*. While functional  
105 experiments have yet to be conducted, these expression patterns in *L. anatina* suggest that *en* likely  
106 participates in determination of larval shell-forming regions to regulate expression of shell-related  
107 genes such as *mpox*.

108           We found a single *dlx* gene in the *L. anatine* genome, which begins to be expressed at the  
109 cleavage stage (supplementary figure S1). *Dlx* is a transcription factor gene with well-known  
110 functions in limb and nervous system development in arthropods and vertebrates [13]. In gastropod,  
111 *dlx* could be involved in shell formation, because it is expressed in boundary regions of developing  
112 shells [10]. However, we found that *dlx* is not expressed in the shell-forming region in *L. anatina*.  
113 Instead, it is expressed in the anterior domain and the tentacle domain in gastrulae and larvae,  
114 respectively (figure 1*c,f*). Thus, *dl* has likely acquired a novel function in initiating shell formation  
115 only in molluscs, among animals hitherto examined.

### 116 ***Evolution of shell formation involving en***

117 Some developmental processes of homoplastic organs involve homologous transcription factors, a  
118 condition which is defined as ‘deep homology’ (e.g. appendage formation in arthropods and  
119 chordates) [27]. Based upon the earliest fossils of mineralized tissues and molecular phylogeny,

120 major bilaterian lineages with biominerals—e.g. arthropods, annelids, molluscs, brachiopods,  
121 bryozoans, echinoderms, and chordates—probably evolved their mineralized tissues independently  
122 during the early Cambrian. However, it is still unknown whether homologous genes are used to  
123 develop those biominerals. Accordingly, we focused on mollusc and brachiopod shell development.  
124 Previous studies took particular note of the end products of shell formation, that is, shell matrix  
125 proteins (SMPs), and compared them with other invertebrate biomineralization-related proteins.  
126 Most brachiopod SMPs do not have homologs in other invertebrates, although some contain  
127 domains that are also found in the SMPs of molluscs (e.g., epidermal growth factor and Von  
128 Willebrand factor, type C) [19,28,29]. Interestingly, we found that *en* is expressed in larval mantle  
129 lobes of *L. anatina* in the region responsible for biomineralization, rather than at the border of the  
130 shell-forming region, as seen in molluscs (figures 1 and 2) [3,5-9].

131 To explore possible conservation of the *en* gene at the genomic level, we compared the  
132 amino acid sequences and the upstream genomic region of *en* (~10 kb), as well as microsynteny  
133 around *en* among lophotrochozoans, including brachiopods, annelids, oysters, and limpets. We  
134 found that there is no conserved non-coding sequences and genomic organization of brachiopod and  
135 mollusc *en* genes (supplementary figure S3–6). Accordingly, *en* probably has a complex  
136 evolutionary history among lophotrochozoans. For instance, tandem gene duplication probably  
137 occurred independently in brachiopods and molluscs (e.g. g20932 and g20933 of *L. anatina*;

138 219968 and 219971 of *L. gigantea*) (supplementary figure S5). Previous studies regarding  
139 diversification of *En* functions among bilaterians indicated that ancestrally it was probably  
140 associated with neurogenesis and head-trunk boundary formation, and that its function has  
141 diversified in lophotrochozoans in various ways [15,21] (supplementary table S3). While functional  
142 analyses of *en* in brachiopods and molluscs are needed, their expression patterns suggest that *en* is  
143 involved in initial shell development in both brachiopods and molluscs. However, given differences  
144 in genomic structure and expression pattern, our findings suggest that the involvement of *en* in shell  
145 formation has evolved by independent gene co-option. Thus, brachiopods and molluscs in relation  
146 to the shell-forming cells probably do not share common ancestry. Although efforts in taxonomic  
147 sampling and genetic analysis are far from complete, our results provide a new perspective on the  
148 evolution and development of shell formation in brachiopods.

149

## 150 **Ethics**

151 All animal collection and experiments followed the Collecting and Experimental Ethics Policy of  
152 the Japan Agency for Marine-Earth Science and Technology.

153

## 154 **Data accessibility**

155 Raw sequence data (*engrailed*, *distal-less*, and *mantle peroxidase*) were deposited into GenBank  
156 under accession LC143447–LC143449.

157

### 158 **Competing interests**

159 The authors have no competing interests.

160

### 161 **Authors' contributions**

162 KS, KE and NS designed the project. KS and Y-JL collected *L. anatina* embryos. KS performed  
163 RNA extractions, cDNA syntheses, gene cloning, *in situ* hybridization, and drafted the manuscript.  
164 Y-JL performed phylogenetic and gene expression analyses. KE, Y-JL, and NS helped to draft the  
165 manuscript, and all authors approved the final version of manuscript.

166

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174

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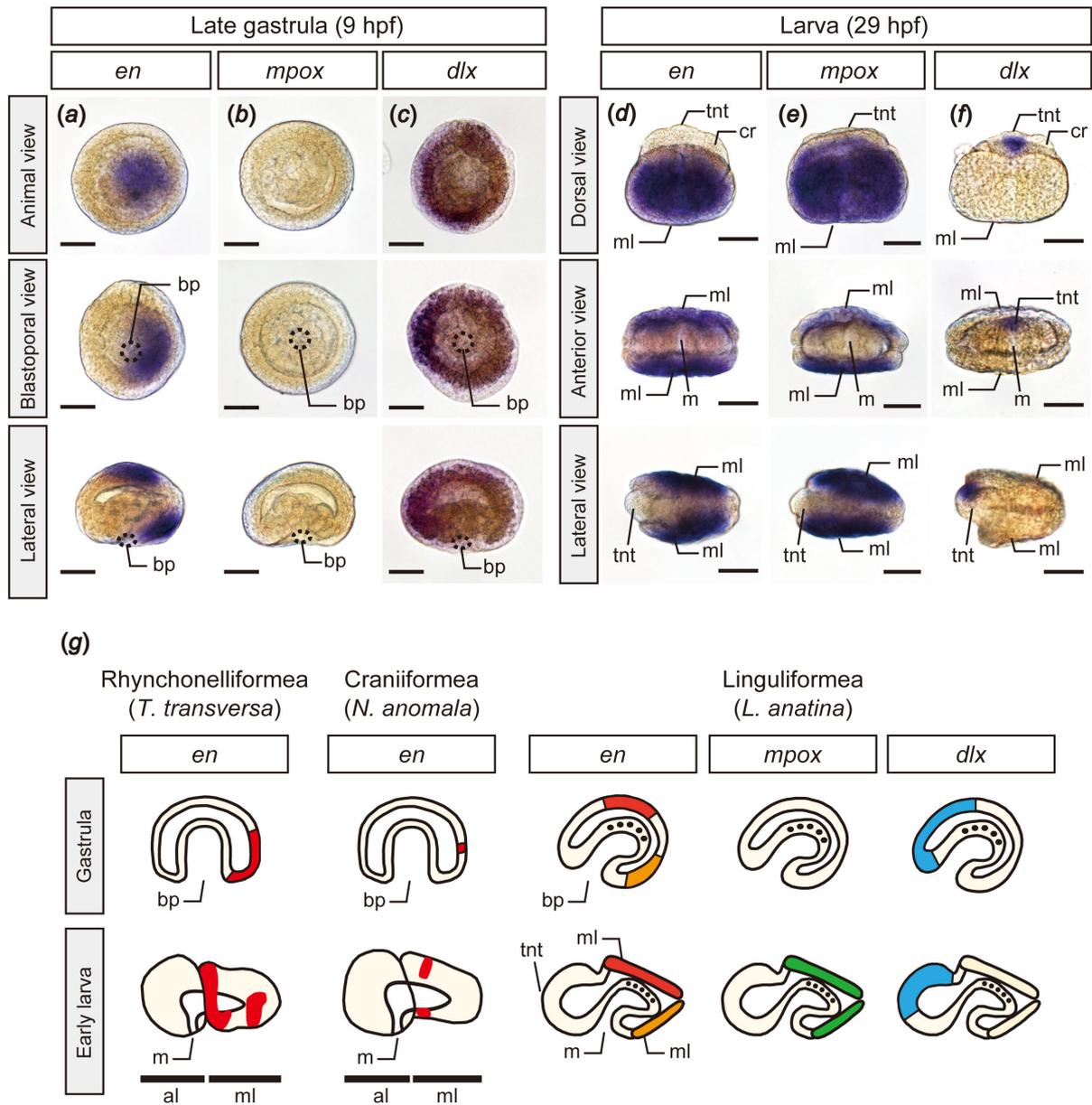
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261

## 262 **Figure Legends**

263

Figure 1



264

265 **Figure 1.** Expression of *en* (a, d), *mpox* (b, e), and *dlx* (c, f) in late gastrula and larval stages of *L.*

266 *anatina*. (g) Schematic illustration of *en*, *mpox* and *dlx* expression in three brachiopods *T.*

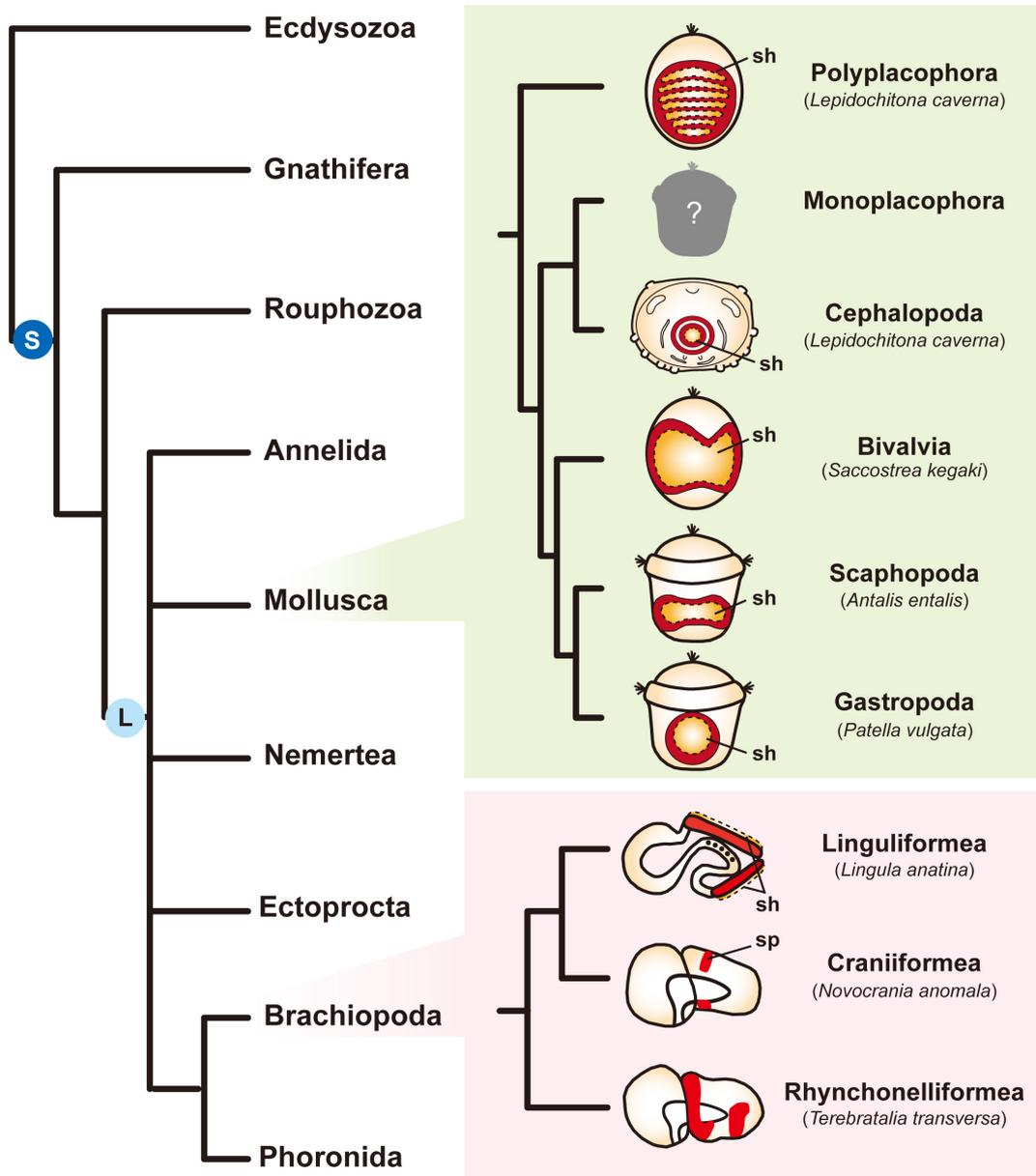
267 *transversa*, *N. anomala*, and *L. anatina*. Larval drawings are adapted from Vellutini and Hejnol [21].

268 Al, apical lobe; bp, blastopore; cr, cirri; g, gut; ml, mantle lobes; m, mouth; tnt, tentacle. Scale bars,

269 20  $\mu$ m.

270

**Figure 2**



271

272 **Figure 2.** Expression patterns of *engrailed* (red) in mollusc and brachiopod larvae. Larval drawings

273 are adapted from refs. [3,6-9,21]. The phylogeny is based on the study of Laumer et al. [30].

274 However, we noticed that there is no consensus among phylogenomic data, except for the close

275 relationship of Brachiopoda and Phoronida. Dashed lines indicate the shell-forming region. L,

276 Lophotrochozoa; ml, mantle lobe; S, Spiralia; sh, shell; sp, shell primordium.