Functional evolution of *Ets* in echinoderms with focus on the evolution of echinoderm larval skeletons

Hiroyuki Koga[1], Mioko Matsubara[1, 2], Haruka Fujitani[1], Norio Miyamoto[1], Miéko Komatsu[3], Masato Kiyomoto[4], Koji Akasaka[5], Hiroshi Wada[1]

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

[2] Seto Marine Biological Laboratory, FSERC, Kyoto University, 459 Shirahama, 649-2211, Japan

[3] Department of Biology, Faculty of Science, Toyama University, 3190 Gofuku, Toyama 930-8555, Japan

[4] Tateyama Marine Laboratory, Marine Coastal Research Center, Ochanomizu University, Kou-yatsu 11, Tateyama 294-0301, Japan

[5] Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Koajiro 1024, Misaki, Miura 238-0225, Japan

Author for correspondense: Hiroshi Wada

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572 JAPAN

Tel & Fax: +81-29-853-4671

E-mail: hwada@biol.tsukuba.ac.jp

Abstract

Convergent evolution of echinoderm pluteus larva was examined from the standpoint of functional evolution of a transcription factor *Ets1/2*. In sea urchins, *Ets1/2* plays a central role in the differentiation of larval skeletogenic mesenchyme cells. In addition, *Ets1/2* is suggested to be involved in adult skeletogenesis. Conversely, in starfish, although no skeletogenic cells differentiate during larval development, *Ets1/2* is also expressed in the larval mesoderm. Here, we confirmed that the starfish *Ets1/2* is indispensable for the differentiation of the larval mesoderm. This result led us to assume that, in the common ancestors of echinoderms, *Ets1/2* activates the transcription of distinct gene sets, one for the differentiation of the larval mesoderm, and the other for the development of the adult skeleton. Thus, the acquisition of the larval skeleton involved target switching of *Ets1/2*. Specifically, in the sea urchin lineage, *Ets1/2* activated a downstream target gene set for skeletogenesis during larval development in addition to a mesoderm target. We examined whether this heterochronic activation of the skeletogenic target set was achieved by the molecular evolution of the *Ets1/2* transcription factor itself. We tested whether starfish *Ets1/2* induced skeletogenesis when injected into sea urchin eggs. We found that, in addition to ectopic induction of mesenchyme cells, starfish *Ets1/2* can activate some parts of the skeletogenic pathway in these mesenchyme cells. Thus, we suggest that the nature of the transcription factor *Ets1/2* did not change, but rather that some unidentified co-factor(s) for *Ets1/2* may distinguish between targets for the larval mesoderm and for skeletogenesis. Identification of the co-factor(s) will be key to understanding the molecular evolution underlying the evolution of the pluteus larvae.

Introduction

Echinoderm larvae can be classified into two types: pluteus and auricularia. The former is seen in sea urchins and brittle stars, whereas the latter is observed in crinoids, sea cucumbers, and starfish. The evolution of pluteus larvae is a classic example of convergent evolution. Classical phylogeny based on adult morphology and paleontological data agree with molecular phylogenetic analyses suggesting that sea urchins and brittle stars are not closely related(Paul and Smith 1984; Wada and Satoh 1994; Littlewood et al. 1997; Janies 2001). Specifically, studies from these two camps support the phylogeny that crinoids represent the most primitive status of echinoderms and diverged first among these groups. In the remaining four classes, a close relationship between sea urchins and sea cucumbers is well supported, whereas whether brittle stars are closely related to starfish or the sea urchin–sea cucumber clade remains unclear. Given this phylogeny, the pluteus type larvae are thought to have evolved independently in both the lineage to brittle stars and that to sea urchins.

The most obvious difference between pluteus larvae and auricularia-type larvae is the presence of a well-developed skeleton in the former. While the auricularia of some sea cucumber species possess a tiny spine in the posterior part of the body, they never develop into arms. The spine was observed to retain up to juvenile stage in *Holothuria leucospilota* (Y. Hiratsuka, personal communication). This contrasts with the pluteus larval skeleton, which does not contribute to the adult skeleton(Yajima 2007). Thus, the convergent evolution of pluteus was achieved by independent acquisition of a larval skeleton in sea urchins and brittle stars.

The genetic mechanisms of skeletogenesis are well studied in sea urchins. During sea urchin embryogenesis, skeletogenic mesenchyme cells are derived from small micromeres (reviewed by Ettensohn 2009). Specification of the skeletogenic mesenchymal cells commences from nuclear deposition of β-catenin. β-catenin in turn activates *Pmar/micro1* transcription, which

inactivates transcription of the ubiquitous inhibitor of endomesoderm differentiation pathway, *Hes*, and allows the activation of transcription of *Ets1/2*, *deadringer* (*Dri*), *Tbx*, *Alx1*, leading to the activation of the skeletogenic gene regulatory network (reviewed by Ettensohn 2009). A recent study demonstrated the existence of a distinct pathway for the activation of *Alx1*, which occurs independently from *Pmar/micro1* but is dependent on unequal cleavage(Sharma and Ettensohn 2010). Some transcription factors in the skeletogenic gene regulatory network directly activate the effector genes of skeletogensis. *Ets1/2* was shown to directly activate transcription of *SM50* and *cyclophilin1* (Kurokawa et al. 1999; Amore and Davidson 2006). *Dri* has also been shown to be involved in the transcription of *cyclophilin1*(Amore and Davidson 2006). These two transcription factors show additional functions during larval development; for example, *Dri* is also involved in the differentiation of the aboral ectoderm(Amore et al. 2003), and *Ets1/2* also shows expression in secondary mesenchymal cells(Röttinger et al. 2003).

Recently, Gao and Davidson (2008) showed that most transcription factors in the larval skeletogenic network are also expressed in cells producing adult skeleton. Therefore, they proposed that a heterochronic shift of the genome regulatory cassette utilized for adult skeletogenesis led to the evolution of the larval skeleton of sea urchins. This observation led us to question what type of molecular evolution is responsible for the heterochronic shift. Thus, we started to examine the mesoderm development of starfish, which do not produce a larval skeleton. Published studies together with our unpublished data revealed that most of the transcription factors, such as *Ets1/2*, *Tbr* and *Dri*, are expressed in mesodermal cells of starfish embryo(Shoguchi et al. 2000; Hinman and Davidson 2007; Hinman et al. 2009), and these observations led McCauley et al. (2010) to suggest that the heterochronic shift did not require a dramatic change in the presence of particular transcription factors, but rather that it was due to more subtle changes in transcription factors. Here, we examined the possibility that functional evolution of the

transcription factor *Ets1/2* may be involved in the heterochronic activation of skeletogenesis. We focused on this transcription factor because it is proven to directly activate the effector genes for skeletogenesis, such as *SM50* and *cyclophilin1* (Kurokawa et al. 1999; Amore and Davidson 2006) . Therefore, this gene is involved in skeletogenesis directly, not by the subsequent result of a function in mesoderm development. We examined the detailed time course of the gene's expression in starfish, sea cucumber, and brittle stars. We then examined *Ets1/2* function during starfish early development. Finally, we examined whether starfish *Ets1/2* can substitute for the function of *Ets1/2* in the skeletogenesis of sea urchin larva.

Materials and Methods

Fertilization and embryo rearing

Adult starfish (*Asterina pectinifera*) were collected from Tateyama (Chiba Prefecture), Kashima (Ibaraki Prefecture), and Asamushi (Aomori Prefecture). Mature eggs were obtained by dissection and treated with 1 mM 1-methyladenine (Sigma). Eggs were fertilized with dissected sperm and cultured in artificial seawater (artificial seawater for invertebrates, Senju Seiyaku, Osaka) at 22°C.

Adult brittle stars (*Amphipholis kochii*) were collected from Abuta (Hokkaido Prefecture) and Himi (Toyama Prefecture). Spawning of gametes was induced by 1–2 hrs of cold shock at 4˚C (Yamashita 1985). Eggs were fertilized with sperm and cultured in artificial seawater at 23°C.

Adult sea cucumbers (*Holothuria leucospilota*) were collected from Shirahama (Wakayama Prefecture). Mature eggs were obtained by dissection and treated with 1 mM Dithiothreitol (Maruyama 1980). Eggs were fertilized with dissected sperm and cultured in artificial seawater at 28°C.

Adult sea urchins (*Hemicentrotus pulcherrimus*) were collected from Kominato (Chiba Prefacture). Gametes were obtained by coelomic injection of 1mM Acetylcholine, and fertilized eggs were cultured in artificial seawater at 16°C.

Gene isolation and molecular phylogenetic analyses

Ets1/2 orthologues were isolated by PCR using the following the degenerate primer set (F1: 5’-TGGACNGGNGAYGGNTGGGA-3’, F2: 5’-GGNCCNATHCARYTNTGGCARTT-3’ and R: 5’- TTRTGDATDATRTTYTTRTC -3’). Template cDNA was reverse-transcribed from embryonic RNA using ReverTra Ace (Toyobo). The amplified DNA fragments were cloned into pGEM-T Easy vector and sequenced. Longer cDNA fragments were obtained either by screening a cDNA

library made from mRNA of gastrula embryos (*ApEts1/2*, Library screening by Alphos Direct, Amersham), or by RACE (*AkEts1/2* and *HlEts1/2*, by BD SMART RACE cDNA amplification kit, Clontech). The full sequence of each homolog was deposited in the DDBJ data bank (accession numbers, *ApEts1/2*: AB569245; *AkEts1/2*: AB569246; *HlEts1/2*: AB569247).

Molecular phylogenetic analyses were performed by PhyML ver. 3.0 (Guindon and Gascuel 2003). Amino acid evolutionary models were selected using Modelgenerator (Keane et al. 2006).

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were synthesized *in vitro* from the cDNA clones using the DIG RNA Labeling Kit (Roche). The embryos were fixed in a solution containing 4% paraformaldehyde, 0.1 M MOPS (pH 7.5), and 0.5 M NaCl and stored in 80% ethanol at -20°C. *In situ* hybridization was performed following the protocol for ascidian embryos (Yasuo and Satoh 1994), except that the RNase treatment was omitted during the washing process. Briefly, after rehydration, the embryos were treated with 2 μg/ml Proteinase K at 37°C for 20 min and then post-fixed in 4% paraformaldehyde. After prehybridization, the embryos were hybridized with digoxigenin-labeled probes at 55°C (hybridization buffer: 50% formamide, 6× SSC, 5× Denhart's solution, 100 μg/ml yeast RNA, and 0.1% Tween 20). Excess probes were removed by washing the embryos twice in 50% formamide, 4× SSC, and 0.1% Tween 20, twice in 50% formamide, 2× SSC, and 0.1% Tween 20, and twice in 50% formamide, 1× SSC, and 0.1% Tween 20. The embryos were then incubated with 0.5% blocking reagent in PBT for 30 minutes at room temperature. After blocking, embryos were incubated with alkaline phosphate-conjugated anti-digoxigenin antibodies, and positive immunoreactions were visualized using Nitro blue tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution (Roche).

Morpholino oligo injection

Antisense morpholino Oligonucleotide (MO) was designed for blocking translation of Ap*Ets 1/2* (5'- ATGATCCTCCGACGCCTCAGCCATG -3', designed and produced by GeneTools). Commercially available standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') was utilized for the control experiment. A 14-mM aliquot of MO was injected into dissected immature eggs using the equipments described by Sweet et al. (2004). After injection, eggs were treated with 1 mM 1-methyladenine and fertilized with dissected sperm. Reared embryos were fixed 36 hrs after fertilization in 4% paraformaldehyde in PBT and stained by NBT/BCIP for detection of alkaline phosphatase.

Overexpression of ApEts in sea urchin eggs

The full coding region of *ApEts 1/2* or sea urchin *Ets 1/2* (Hp*Ets*; (Kurokawa et al. 1999) was inserted into pRN3, which possesses a 5' and 3' untranslated region of *Xenopus* cytoplasmic actin (Lemaire et al. 1995). mRNA was *in vitro* synthesized using mMASSAGE mMACHINE T3 Kit(Ambion), and then purified by using MegaClear Kit (Ambion), and 1mg/ml mRNA was injected into sea urchin fertilized eggs . mRNA for *lac-Z* was injected as a control. Larva were examined for morphology and further stained with anti-SM50 antibody BG2 (Kitajima and Urakami 2000).

QRT-PCR

RNA was extracted from five larvae in three distinct batches for each experiment. Quantitative real-time PCR (QRT-PCR) was performed using Power SYBR Green Cells-to-Ct Kit (Applied Biosystems), and the ABI PRISM 7900 HT (Applied Biosystems). We examined the

expression of SM50 using the following primers: (F: 5'-CTACGTCCGCAGTCAATCC-3', R: 5'-CTGGTCCATTTCCACAAGGT-3'). The expression levels were normalized using that of EF-1α amplified with the following primers: F: 5'-ATCGACCACTACCGGTCATC-3', R: 5'-AACCCAGGCATACTTGAACG-3'.

Results

Expression of Ets1/2 in starfish, sea cucumber, and brittle stars

The molecular phylogenetic analyses (Fig. 1) clearly indicated the orthology of the isolated *Ets1/2* from starfish (*A. pectinifera*), sea cucumber (*H. leucospilota*), and brittle star (*A. kochii*). The expression pattern of starfish *Ets1/2* in the mesodermal cells of the blastula has been previously reported, albeit fragmentally (Hinman and Davidson 2007; McCauley et al. 2010). We found that the earliest expression of *ApEts1/2* was detected in the vegetal plate of hatching blastula (Fig. 2A). During gastrulation, the expression was maintained in mesoderm cells at the tip of the archenteron (Fig. 2B). Expression was also observed in mesenchyme cells delaminated from the tip of the archenteron. In the late gastrula stage, the *ApEts1/2* expression was no longer detected in the archenteron, and expression was only observed in the mesenchyme cells (Fig. 2C), whereas in bipinnaria larvae, expression was still observed in the mesenchyme cells delaminating from the coeloms (Fig. 2D).

The expression of sea cucumber *Ets1/2* also commenced in the mesoderm cells of early gastrula (Fig. 3A). In the mid-gastrula stage, expression was detected in scattered mesenchyme cells (Fig. 3B). In the late gastrula, although most of the mesenchyme cells shut down *Ets1/2* expression, we observed strong expression in a few cells located close to the blastopore (Fig. 3C). Judging from its position, the *Ets1/2*-positive cell may be a skeletogenic cell for the first spicule (Fig. 3D). This expression is consistent with *Ets1/2* expression seen in the adult skeletogenic cells of sea urchin and starfish (Gao and Davidson, 2008).

We analyzed the expression of *Ets1/2* in the brittle star, which also possess a larval skeleton. The earliest expression of *AkEts1/2* was detected in the vegetal region of hatching blastula (Fig. 4A). Expression was maintained in primary mesenchyme cells and the vegetal region of the mesenchymal blastula stage (Fig. 4B). In the early gastrula, expression was detected in the

archenteron as well as mesenchyme cells (Fig. 4C). At the mid-gastrula stage, strong expression of *AkEts1/2* was maintained in mesenchyme cells located near the tip of the archenteron, whereas mesenchyme cells in the lateral part of the body showed relatively weak signals. The mesenchyme cells with strong *AkEts1/2* signals at this stage may represent secondary mesenchyme cells that were just delaminated from the tip of the archenteron, whereas mesenchyme cells in the lateral part with weaker *AkEts1/2* expression may be derivatives of cells that delaminate before gastrulation (primary mesenchyme cells; Fig. 4D). In the early pluteus stage, expression of *AkEts1/2* was detected in mesenchyme cells underneath the arm as well as at the tip of the archenteron. The expression in mesenchyme cells underneath the arms may suggest that *AkEts1/2* is also involved in skeletogenesis of pluteus larva (Fig. 4E).

Function of Ets is required for mesoderm cell differentiation in starfish

To investigate the function of the *ApEts1/2* in starfish embryogenesis, we performed a morpholino oligonucleotide (MO) knockdown experiment. Approximately 10% of both control and *ApEts1/2*-MO embryos died before hatching due to damage induced by injection (Table 1). Approximately 16% (12/74) of the hatched larvae failed to form an archenteron when injected with *ApEts1/2*-MO (Fig. 5C), whereas all the hatched larvae developed normally up to the bipinnaria larval stage (29/29). At the early gastrula stage (24 hrs after fertilization), control-MO injected larvae showed an archenteron subdivided into an alkaline phosphatase (AP) positive endoderm region, and an AP negative mesodermal region (Fig. 5A, B). However, even at 36 hrs after fertilization, approximately 70% (51/74) of the larvae lacked an AP negative mesoderm region and showed an archenteron mostly positive for alkaline phosphatase (Fig. 5D, E). Almost no mesenchyme cell was observed in *ApEts1/2*-MO injected larvae. This indicated that mesoderm cell differentiation was suppressed when injected with *ApEts1/2*-MO.

Overexpression of the starfish Ets1/2 in the sea urchin embryos

This study provided evidence that starfish *Ets1/2* plays a critical role in the differentiation of the embryonic mesoderm. Together with the observations that *Ets1/2* orthologues are commonly expressed in larval mesoderm regions of four echinoderm classes, our data support that *Ets1/2* maintains its original function in early mesoderm differentiation in the common ancestors of echinoderms. In the linage to echinoids (and perhaps to brittle stars as well), *Ets1/2* activated skeletogenic target genes, which were originally activated only in adult stages. We asked whether the molecular evolution of *Ets1/2* itself was responsible for the activation of skeletogenic target genes in early embryonic stages. We analyzed whether starfish *Ets1/2*, when over-expressed in embryogenesis, can produce excess skeletogenic cells similar to the sea urchin orthologue(Kurokawa et al. 1999).

When *ApEts1/2* mRNA was injected into sea urchin eggs, over-production of mesenchyme cells was observed (221/326; Fig. 6E, Table2), similar to the result when sea urchin *Ets1/2* (*HpEts*) was introduced (112/184; Fig. 6C, Table 2; Kurokawa et al. 1999). This phenotype is consistent with the hypothesis that *Ets1/2* has a conserved role in mesoderm differentiation, particularly in the epithelium-to-mesenchyme transition (EMT) (Röttinger et al. 2003; Smith and Davidson 2008). Given the over-production of mesenchyme cells, the injected larvae do not develop to the stage when calcite spicules develop. However, ectopic SM50 protein was detected in the mesenchyme cell mass where *ApEts1/2* was over-expressed (37/48; Fig. 6F, Table 2). The activation of *SM50* gene expression was confirmed by QRT-PCR measurement of mRNA (Fig. 6G, Table 2). Therefore, we concluded that the starfish *Ets1/2* is able to activate, at least, some parts of the skeletogenic pathway.

Discussion

Dual function of Ets1/2 in echinoderm development

We examined the expression and function of *Ets1/2* in echinoderm embryogenesis. In starfish, *Ets1/2* is expressed in the early phases of the mesoderm development, and the function of *Ets1/2* was shown to be indispensable for mesoderm development. Starfish *Ets1/2* is expressed in adult skeletogenic cells (Gao and Davidson, 2008), and we also observed the expression of *Ets1/2* in the putative adult skeletogenic cells of sea cucumber. Although involvement of *Ets1/2* in skeletogenesis of holothuroids and ophiuloids need to be examined by further experiments, based on these expression and functional data, we suggest that in the common ancestors of echinoderms, *Ets1/2* has at least two distinct functions: one for the early development of mesoderm cells, and the other for skeletogenesis (Kurokawa et al. 1999; Smith and Davidson 2008). If the larval skeleton evolved secondarily in sea urchins and brittle stars, the latter function would likely be restricted to the adult stage. Thus, in the common ancestors of the echinoderms, *Ets1/2* has two distinct targets; in early larval phases, it activates genes required for mesoderm cell differentiation and EMT, and in the adult stages, it activates a distinct gene set required for skeletogenesis. *SM50* and *cyclophilin1* are probably descendants from genes included in the latter set.

Molecular evolution of the heterochronic shift of skeletogenesis

The dual functions of *Ets1/2* probably underlie the heterochronic shift of skeletogenesis into the larval stage. For the *Ets1/2* to be involved in the evolution of the larval skeleton, it was not necessary for its *cis*-regulation to change because *Ets1/2* was likely expressed in early mesoderm cells of echinoderm ancestors. On the other hand, for the evolution of the larval skeleton, *Ets1/2* must have switched downstream target genes. That is, in the embryonic stage, *Ets1/2* was able to activate, in addition to the target gene set for the larval mesoderm, the skeletogenic downstream

target gene set, which was originally activated only during adult skeletogenesis. Thus, we asked whether the molecular evolution of *Ets1/2* itself is involved in the evolutionary change of the target. Several studies documented the evolution of the transcription factors leading to changes in the nature of transcriptional regulation and to subsequent phenotype evolution (e. g. Galant and Carroll 2002; Ronshaugen et al. 2002; Lynch et al. 2008).

However, in the case of echinoderm *Ets1/2*, the transcription factor itself may not have experienced drastic changes because starfish *Ets1/2* can activate larval skeletogenesis when introduced to sea urchin eggs. This suggests the existence of unidentified transcription co-factor(s) with which *Ets1/2* can distinguish between embryonic mesoderm development and skeletogenesis. Lack of the unknown co-factor(s) may explain the inactivation of the skeletogenic gene regulatory network during starfish embryogenesis. Alternatively, *cis*-regulatory change in the skeletogenic downstream genes may have occurred. However, we think the latter case less likely, because in that case we must assume that all the skeletogenic downsream genes changed their *cis*-regulation independently. Thus, we think identification of the co-factor is key to understanding the molecular evolution responsible for the evolution of the sea urchin larval skeleton.

Insight to the convergent evolution of larval skeleton

Evolution of echinoderm pluteus larvae is a classical example of convergent evolution. We present evidence that *Ets1/2* is expressed in skeletogenic mesenchyme cells in brittle star larvae. However, the present observation of gene expression is not sufficient to determine whether *AkEts1/2* activates the skeletogenic target set. Even by functional assay, whereby *Ets1/2*-MO is injected into eggs, it is not possible to tell whether *AkEts1/2* is only involved in mesoderm development or in both larval mesoderm differentiation and skeletogenesis because when the former function is suppressed, the subsequent skeletogenesis is also disrupted. In either case, whether the same molecular evolutionary

history underlies the convergent evolution of echinoderm larval skeleton in sea urchin and brittle stars remains an open question.

From the perspective of convergent evolution, it is worth noting that many transcription factors involved in the larval skeleton are expressed in the embryonic mesoderm of starfish (Shoguchi et al. 2000; Hinman and Davidson 2007; Hinman et al. 2009; McCauley et al. 2010). This similarity implies that the architect ure of the gene regulatory network for embryonic mesoderm differentiation and adult skeletogenesis was quite similar in the echinoderm ancestors. In other words, very little evolutionary change may have led to the evolution of larval skeleton (McCauley et al. 2010). This may account for the independent evolution of the larval skeleton. Convergent evolution of the pluteus, however, must be considered from another perspective, namely the shape of larval skeleton. Echinopluteus and ophiopluteus are similar not only in the presence of the larval skeleton, but also in the shape of the skeleton. The shape of the skeleton is achieved by interaction with the epidermis (Kinoshita and Okazaki 1984; Armstrong et al. 1993; Di Bernardo et al. 1999; Duloquin et al. 2007; Röttinger et al. 2008). Some common feature may exist in the larval epidermis of echinoderms that led to a similar interaction with the skeletogenic mesenchyme cells.

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Figure Legends

Figure 1. Molecular phylogenetic tree of the echinoderm *Ets1/2* genes.

The tree was made using 102 amino acid sites of the Ets domain. An amino acid substitution model was selected by Modelgenerator (Keane et al. 2006), and the tree was calculated by the maximum likelihood method using PhyML 3.0 (Guindon and Gascuel 2003) under JTT+G model. The numbers on the nodes indicate 1000 bootstrap supporting values for each node (values higher than 500 are shown). SpEts1: *Strongylocentrotus purpuratus* Ets1/2 (L19541), HpEts, *Hemicentrotus pulcherrimus* Ets1/2 (AB008365), MmEts1, 2: *Mus musculus* Ets1, 2 (P27577, P15037), HsEts1, 2: *Homo sapiens* Ets1, 2 (P14921, P15036), SpERG: *S. purpuratus* ERG (Q6R7X7), HsERG: *H. sapiens* ERG (P11308), MmELK: *M. musculus* ELK (P41969), HsELK: *H. sapiens* ELK (P19419), SpELK: *S. purpuratus* ELK (AY049979).

Figure 2. Expression pattern of the starfish *Ets1/2*, Ap*Ets1/2*.

(A) During hatching blastula stage, expression was observed in the vegetal plate cells. (B) In gastrula, expression was seen in the mesoderm cells at the tip of the archenteron and in mesenchyme cells delaminated from the tip of the archenteron. (C) During the late gastrula stage, expression was only observed in the mesenchyme cells. (D) In bipinnaria larvae, expression was still observed in the mesenchymal cells. Scale bars: 50 μm in (A, B), and 100 μm in (C, D).

Figure 3. Expression pattern of the sea cucumber *Ets*, *HlEts*.

(A) Expression was detected in the archenteron of early gastrula. (B) During the mid-gastrula stage, expression was detected in scattered mesenchymal cells. (C). In the late gastrula, although most of the mesenchyme cells shut down *Ets1/2* expression, strong expression was observed in a cell

located close to the blastopore. (D) Image of auricularia larva with a tyny spicuke in the posterior part of the body (arrohead) Scale bars: 50 μm.

Figure 4. Expression pattern of the brittle star *Ets1/2*, *AkEts1/2*

(A) In hatching blastula, expression was detected in the vegetal region. (B) During the mesenchymal blastula stage, expression was observed in primary mesenchymal cells and the vegetal region. (C) In the early gastrula, expression was detected in the archenteron and mesenchyme cells. (D) During the mid-gastrula stage, strong expression was maintained in mesenchyme cells located near the tip of the archenteron, whereas mesenchyme cells in the lateral part of the body showed relatively weaker signals (arrows). (E) During the early pluteus stage, expression of *AkEts1/2* was detected in mesenchymal cells underneath the arm (arrows) as well as at the tip of the archenteron (arrowheads). The expression in mesenchymal cells underneath the arms strongly suggests that *AkEts1/2* is also involved in skeletogenesis of pluteus larva. Scale bars: 20 μm.

Figure 5. Morpholino knockdown experiment of the starfish *Ets1/2*, *ApEts1/2*.

(A, B) Control MO injected larvae at 24 hr (A) and 36 hr (B) stained for alkaline phosphatase (AP) activity. At the tip of the archenteron, an AP-negative mesoderm region is clearly visible. (C–E) *ApEts1/2*-MO injected larvae at 36 hr. Some larvae failed to form archenteron (C). Even in larvae from the archenteron, the AP negative region was lost or severely reduced in the *ApEts1/2* knockdown larvae. Scale bars: 50 μm.

Figure 6. Over-expression of the starfish *Ets1/2* in sea urchin embryos

(A, B) When control *lac-Z* mRNA was injected, sea urchin embryos developed normally. (C, D) When sea urchin *HpEts* mRNA was injected, ectopic mesenchyme cells were induced (C), and ectopic expression of SM50 was observed (D). (E, F) Starfish *Ets1/2* mRNA also induced ectopic mesenchyme cells (E) and ectopic expression of SM50 (F). (G) QRT-PCR measurement of the *SM50* mRNA in five larvae at 24 hrs. Data were shown as ΔCt relative to untreated larvae. The expression level was normalized by the EF-1α gene.

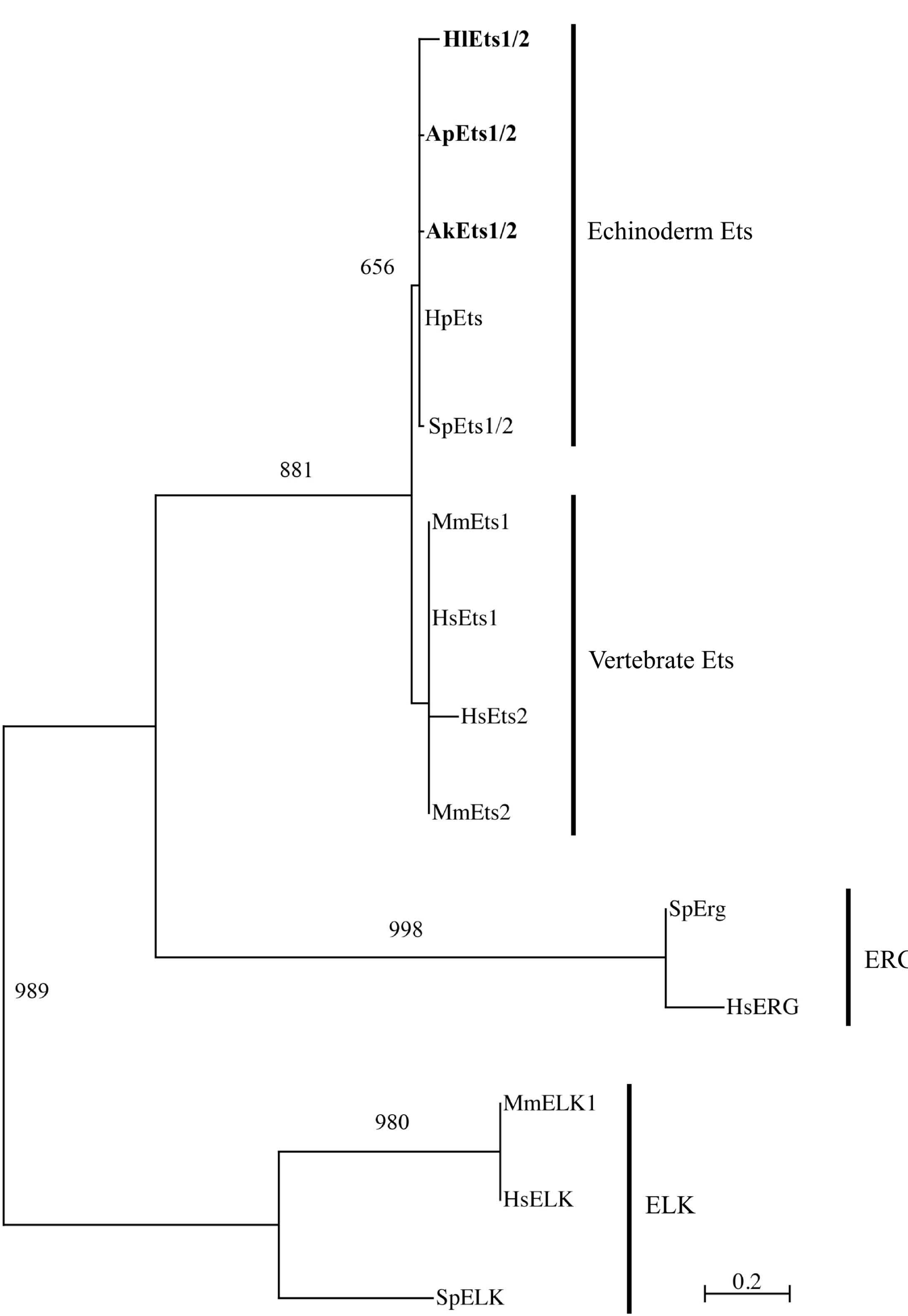
Table 1 Effect of *ApEts1/2*-MO on gastrulation and differentiation of mesoderm

	hatched/injected (%)	Normal (AP negative mesoderm present)	Archenteron formed, but AP negative mesoderm absent or drastically reduced	Gastrulation failed
Control-MO	29/37 (78)	29 (100)	0 (0)	0 (0)
ApEts-MO	74/88 (84)	11 (15)	51 (69)	12 (16)

Numbers in the brackets indicate either percentage of hatched larvae from injected, or percentage of the larvae showing the phenotypes from total number of larvae hatched.

Table 2. Effect of ectopic expression Ets mRNAs

	Number of embryos that show ectopic mesenchyme cells (%)	Total injected	Number of embryos that show ectopic SM50 expression (%)	Total injected and SM50 expression examined
LacZ	0 (0%)	87	–	–
HpEts	112 (60.9%)	184	35 (83.3%)	42
ApEts1/2	221 (67.8%)	326	37 (77.0%)	48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ApEts1/2
AkEts1/2
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HpEts
SpEts1/2
Echinoderm Ets
881
MmEts1
HsEts1
HsEts2
MmEts2
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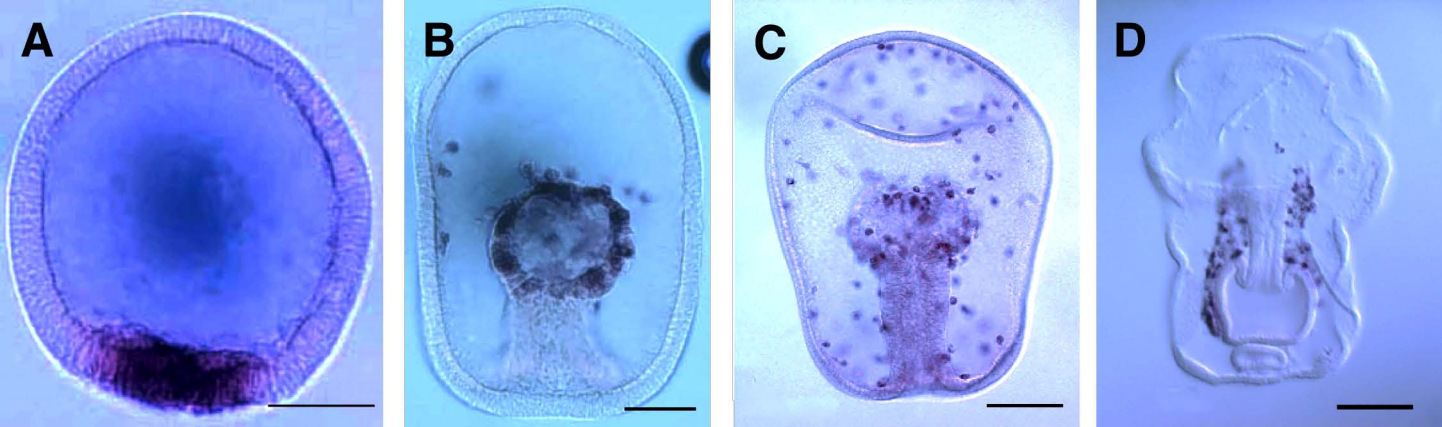
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B
C
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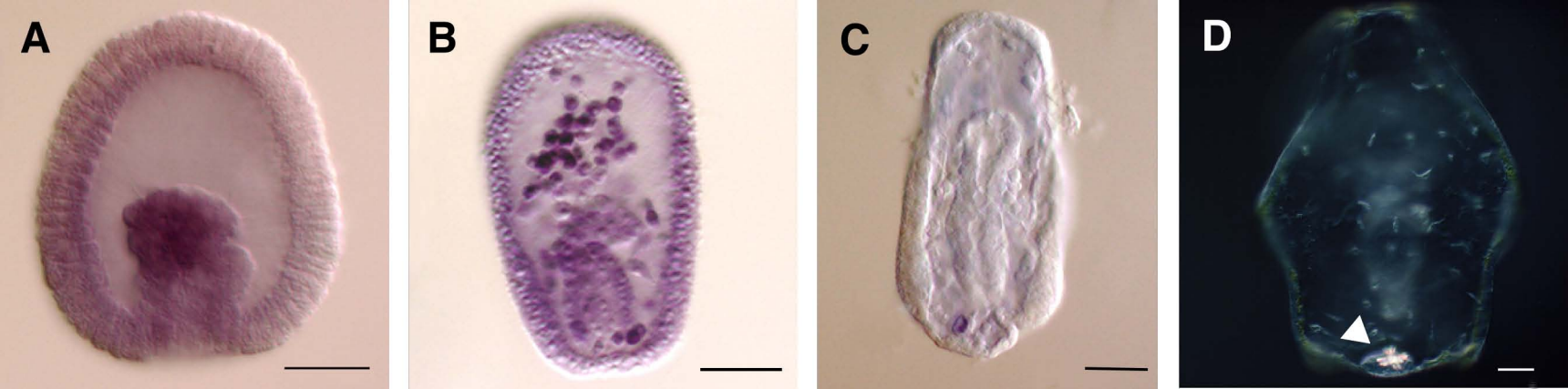
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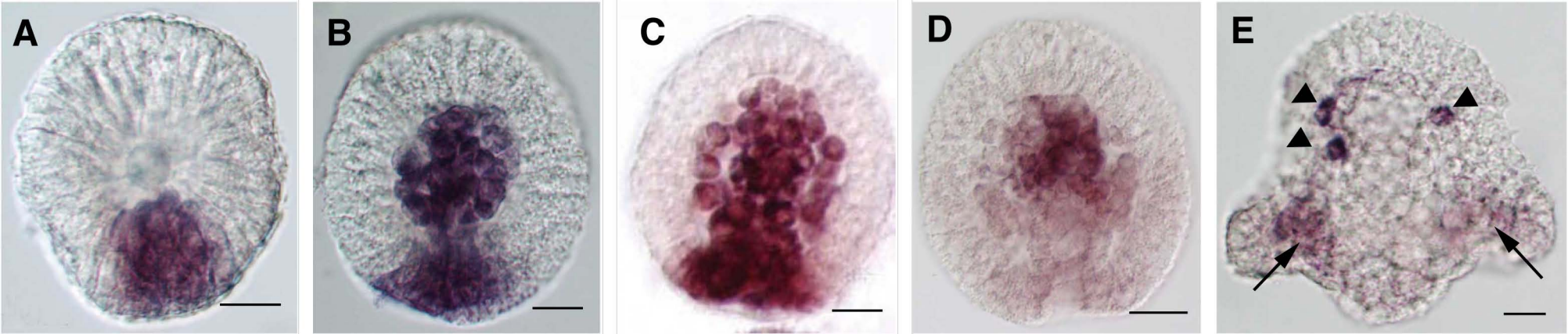
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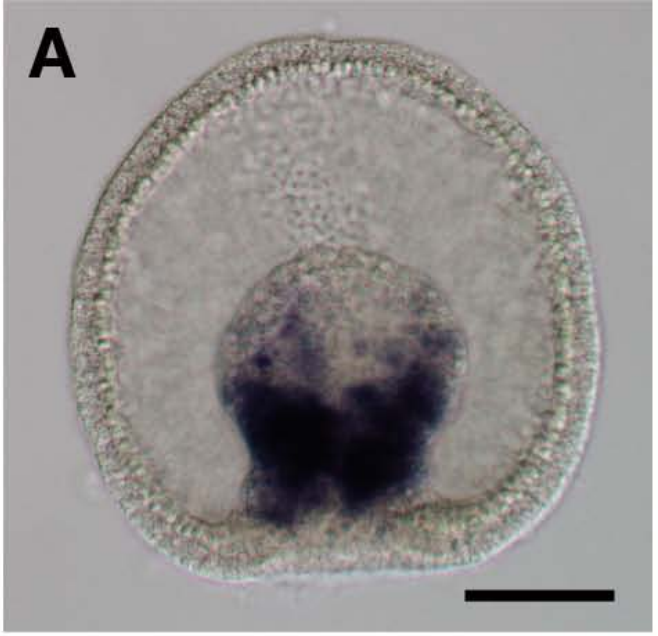
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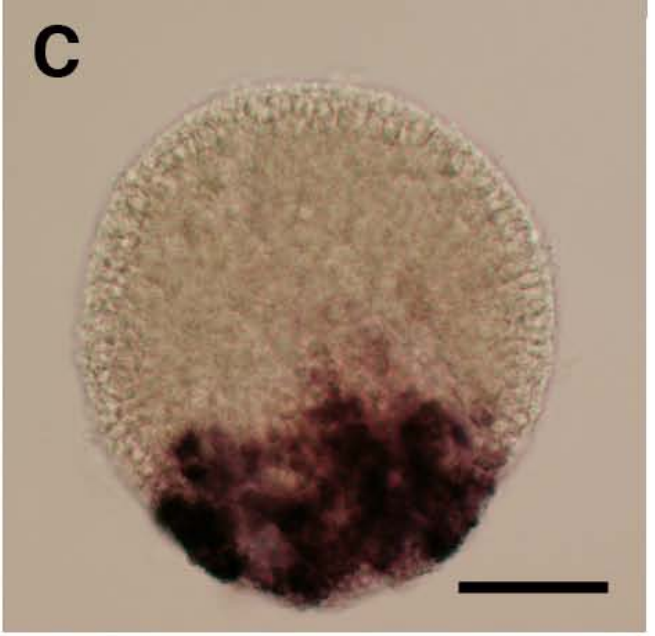
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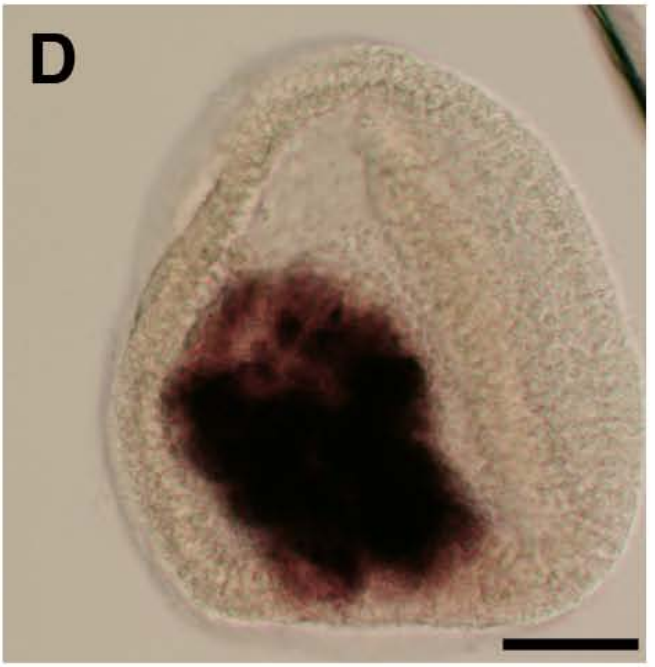
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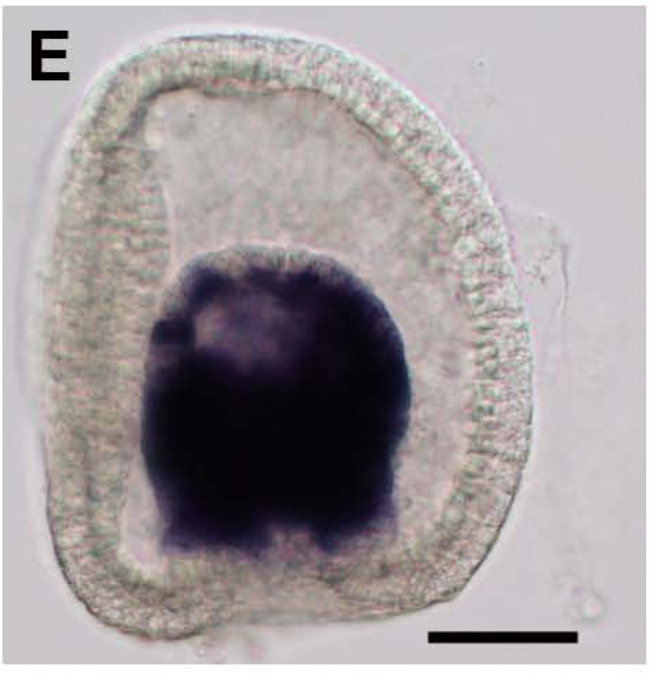
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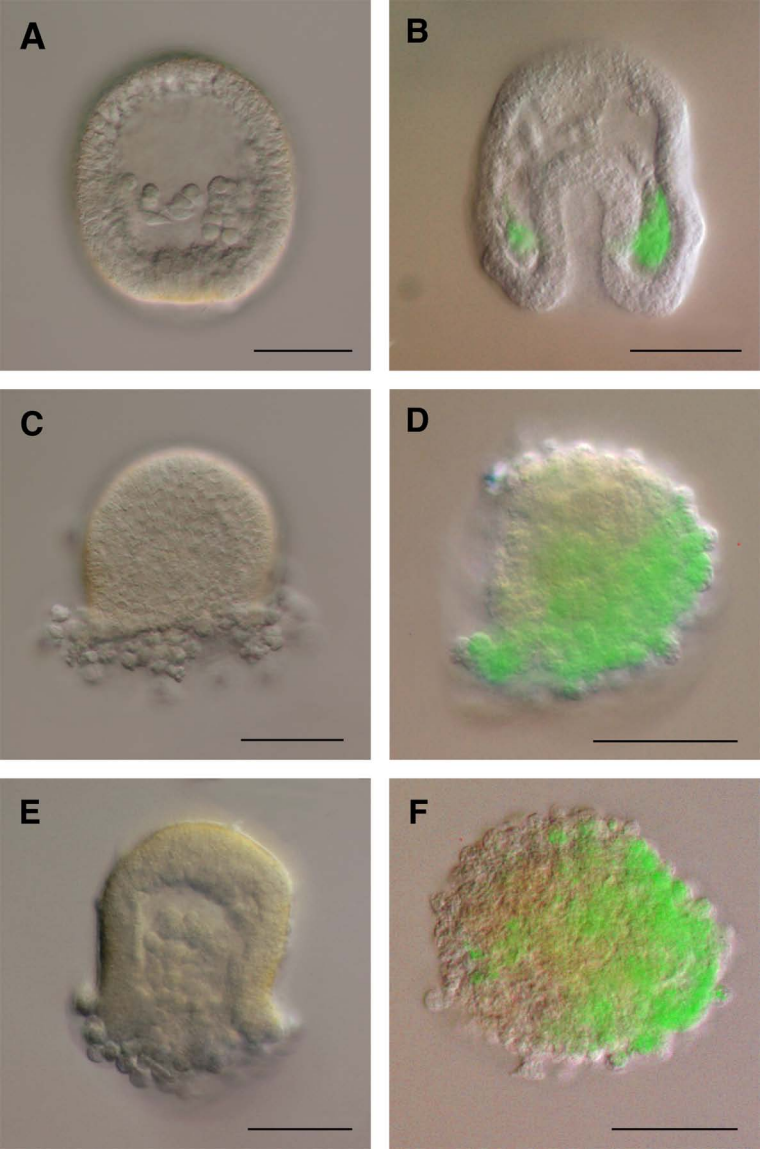
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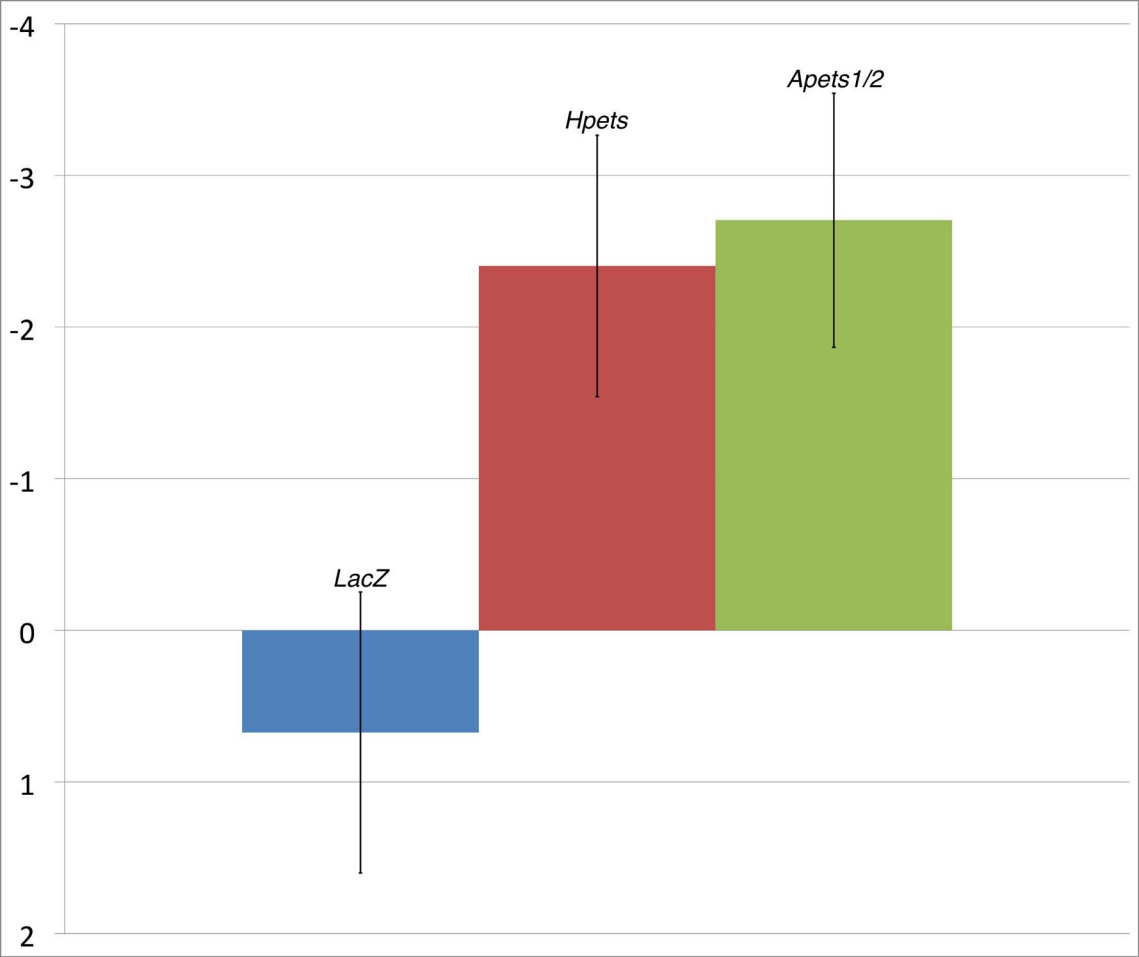
-4
-3
-2
-1
0
1
2
LacZ
Hpets
Apets1/2