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Bicaudal-C is required for the formation of anterior neurogenic ectoderm in the sea urchin embryo

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bicaudal-C (bicC) mRNA encodes a protein containing RNA-binding domains that is reported to be maternally present with deflection in the oocytes/eggs of some species. The translated protein plays a critical role in the regulation of cell fate specification along the body axis during early embryogenesis in flies and frogs. However, it is unclear how it functions in eggs in which bicC mRNA is uniformly distributed, for instance, sea urchin eggs. Here, we show the function of BicC in the formation of neurogenic ectoderm of the sea urchin embryo. Loss-of-function experiments reveal that BicC is required for serotonergic neurogenesis and for expression of ankAT-1 gene, which is essential for the formation of apical tuft cilia in the neurogenic ectoderm of the sea urchin embryo. In contrast, the expression of FoxQ2, the neurogenic ectoderm specification transcription factor, is invariant in BicC morphants. Because FoxQ2 is an upstream factor of serotonergic neurogenesis and ankAT-1 expression, these data indicate that BicC functions in regulating the events that are coordinated by FoxQ2 during sea urchin embryogenesis.

The concentration and localization of maternal factors in oocytes/eggs influence the development of most organisms. In some animals, mRNA specific for the formation of one of the tissues or organs accumulates at the small region of the egg during oogenesis, and the protein translated from the localized message specifies the cell fate at the precise time. This type of localized message has been reported in animals that undergo mosaic development. For instance, macho-1 mRNA is the muscle-cell fate determinant in ascidians and is localized mostly at the vegetal hemisphere in unfertilized eggs[1]. Because macho-1 mRNA tightly associates with the egg cortex endoplasmic reticulum[2], the message localization is packed at the vegetal pole when the egg is fertilized and shifts to the posterior end to the centrosome-attracting body, CAB[3], during ooplasmic segregation before 1st cleavage. Blastomeres containing macho-1 are specified as muscle by tadpole larvae. In ascidian embryos, a number of genes coding for this type of posterior-localized message are named postplasmic/PEM-like genes[4]. In *C. elegans*, P-granule-associated proteins are well reported as factors that localize to the posterior end and maintain germ line cell fate[5]. In contrast to those organisms, in mammals that perform regulative development such as critically localized messages or proteins are not well reported. Blastomeres during early cleavage stages may develop into most of the body cells. For example, identical human twins derive from separated blastomeres from a single embryo after cleavage, indicating that no essential polarization is present in either the egg or very early cleavage stage embryo of mammals. Thus, how asymmetrical cell fate is determined in these non-mosaic organisms has yet to be clearly shown.

Bicaudal-C (BicC) is a polarized factor in *Drosophila* eggs. During oogenesis, *bicC* mRNA accumulates in the pre-oocyte and is transported to the anterior end of the oocyte[6]. As the message is translated to protein, it begins to exert its function, and one of the main functions of this maternal BicC is regulating poly(A) tail length of target mRNAs, including its own mRNA[7]. BicC directly interacts with NOT3/5, a component of CCR4 core deadenylase complex, and therefore controls deadenylation[7]. BicC protein contains two specific domains, SAM (Sterile Alpha Motif) and KH (hnRNP K homology) domains[6]. Both have RNA-binding activity, but the direct binding-target is rarely identified. One example is *Xenopus* Cripto-1 mRNA, the BicC targeting site of which was reported recently[8,9]. Osk accumulation is premature in the absence of BicC in *Drosophila* oocytes[10], but the direct interaction is unclear. The localized expression of *bicC* mRNA or protein is required for development of asymmetry in the body plan. However, the maternal or very early expression of *bicC* is not always polarized in some animals, especially in mammals. Thus, the analysis of BicC function in animals in which the mRNA or protein is uniformly present in the oocytes/eggs is essential in order to understand the properties of this protein.

In sea urchin embryos, there are not many maternally polarized factors. However, the forth cleavage can produce an asymmetry, and cytoplasmic transplantation or dissection of an egg into animal and vegetal halves is significant experimental evidence for the presence of a maternally polarized factor[11]. The protein of Dishevelled is maternally localized at the vegetal egg cortex, and it functions in the initiation of Wnt signaling at the vegetal hemisphere during embryogenesis[12]. This localization is revealed by microinjection of mRNA encoding fluorescent protein-fused Dishevelled but is not shown by *in situ* hybridization, indicating that its mRNA is not tightly localized in the sea urchin eggs. Additionally, it has been reported that ubiquitously localized factors can produce asymmetric cell fate specification in sea urchin embryogenesis. For example, *soxB1* mRNA localizes everywhere in the eggs, and the translated protein is detected in every blastomere in the early cleavage stages. However, the distribution of protein progressively reveals an anterior-posterior gradient, and based on this gradient, it functions in cell-fate specification in a concentration-dependent manner[13]. The gradient from the ubiquitous expression is produced by a site-specific turnover system through a combination of protein degradation and mRNA expression[14]. Because of the presence of these site-specific turnover systems for maternal/zygotic factors, there may be upstream mechanisms that promote polarized formation of eggs/early cleavage. To understand these mechanisms, it is necessary to accumulate knowledge of the functions of each maternally expressed factor. Here, we show the function of sea urchin BicC, whose message is not polarized in eggs/early cleavages, in the formation of anterior neurogenic ectoderm and add to the understanding of polarization of the animal body during sea urchin embryogenesis.

Results

bicC mRNA is expressed at multiple areas during early development of sea urchin embryos.

To investigate the *bicC* spatial pattern during embryogenesis, we employed *in situ* hybridization using *bicC* RNA-probe. As previously reported[15], *bicC* mRNA is maternally expressed and ubiquitously detected until the cleavage stage and is almost absent at the blastula stages (Fig. 1A–F). Fluorescent *in situ* hybridization using a tyramide amplification system[16] under the same microscopic conditions shows that the gene is ubiquitously expressed during the early stages and is almost completely absent at the blastula stage (Fig. 1C–E). When gastrulation is initiated, the vegetal plate cells begin to express *bicC* mRNA (Fig. 1G, H). The vegetal plate expression is maintained (Fig. 1I, J), but expression is not detected in the invaginated gut cells until the late gastrula stage. The expression pattern becomes complicated in the prism stage. The vegetal plate expression is diminished as foregut expression begins. Moreover, in the ciliary band, a few cells express the gene at the lateral and ventral regions (Fig. 1K, L). These complex and dynamic patterns of *bicC* mRNA localization suggest that the gene expression is regulated by multiple inputs during sea urchin development.

BicC is required for the formation of the anterior neurogenic ectoderm.

To investigate the function of BicC during sea urchin embryogenesis, we injected morpholino oligonucleotide (BicC-MO) against BicC mRNA, by which we inhibited its translation, and observed embryonic development. Initially, we tried to microinject BicC-MO into the fertilized eggs, and the injected embryos showed delayed development but still had normal morphology as prism larvae with invaginated gut, whereas the control reached to early pluteus stage at 52 hours (see Supplementary Fig. S1 online). It is possible that the morpholino is not sufficient for interfering with the translation of the maternal message[1]. To efficiently inhibit the translation of maternal bicC mRNA, we microinjected BicC-MO into unfertilized eggs, stored the injected eggs at 4°C overnight and inseminated the next day. BicC morphants developed more slowly than control embryos, and the cleavage timing was delayed by almost 1 mitosis cycle during the early stages (data not shown). The blastocoel of morphants forms but is narrower than that of control embryos (Fig. 2). Gastrulation is never observed in the morphants (Fig. 2I, 3E).

The spatial expression pattern of *foxQ2*, the earliest zygotic gene expressing at the anterior neuroectoderm[17], is almost normal, indicating that Wnt signals that restrict the anterior neuroectoderm fate function normally (Fig. 2). However, the message level in the morphants, especially in BicC-MO2 embryos, is lower than that of control embryos during very early blastula stages (Fig. 2A–D). These results suggest that initially spatial control of the anterior neuroectoderm formation[18–20] is likely normal but that BicC function affects on the quantity control of *foxQ2* expression. Although the morphology of the control and BicC morphants is very different, the expressing region of the *foxQ2* in morphants is still restricted at the anterior end (Fig. 2E–J). This is confirmed by the result, in which we detected FoxQ2 protein (Fig. 2G, H). To investigate whether the development of this anterior neuroectoderm is normal, we tried to detect the expression of *ankAT-1* gene and serotonergic neurons. AnkAT-1 is responsible for elongation of the immotile apical tuft cilia of non-neural cells in the anterior neuroectoderm[21,22]. Surprisingly, although the expression of FoxQ2 appears to be almost normal and *ankAT-1* is one of the strongest target genes of FoxQ2 function during embryogenesis of the sea urchin[21], *ankAT-1* is not expressed in the morphants (Fig. 3A, F). In addition, the serotonergic neurons in the anterior neuroectoderm are not differentiated in the morphants at 48 h (Fig. 3B–E, G–J). Because *ankAT-1* expression and serotonergic neurogenesis are downstream of FoxQ2 function[19,21], these results
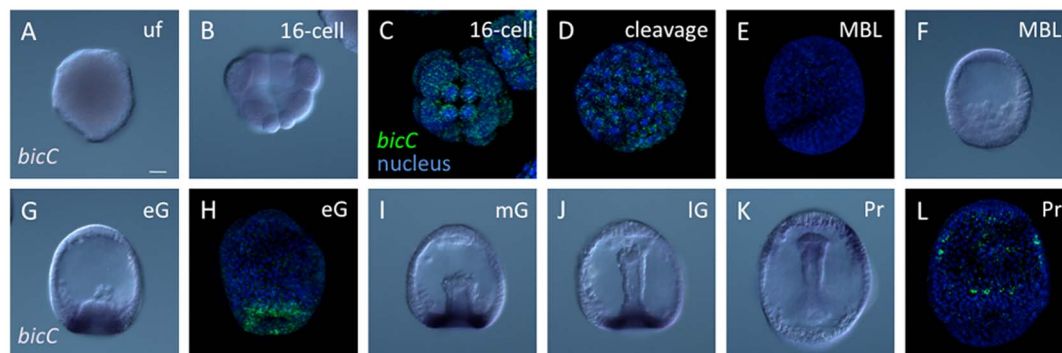
Figure 1 | The expression pattern of *bicC* during sea urchin embryogenesis. Maternal *bicC* mRNA was detected at the unfertilized egg (A), 16-cell (B, C) and cleavage stages (D). Fluorescent *in situ* hybridization with DAPI staining was performed on a 16-cell embryo (C), cleavage stage (D), mesenchyme blastula (MBL; E), and early gastrula (eG; H). The signal was missing in MBL (E, F). *bicC* was expressed at the vegetal plate of eG (G, H), mid-gastrula (mG; I) and late gastrula (lG; J). In prism larva, *bicC* was expressed at the ciliary band and the tip of gut (K, L). The bar in (A) is 20 μm.
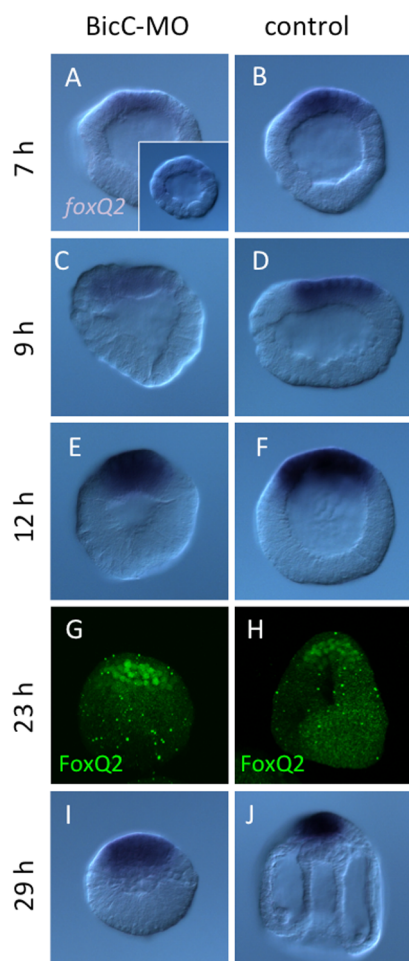
Figure 2 | foxQ2 expression is spatially invariant without BicC. *foxQ2* expression in BicC morphants (A, C, E, I) and glycerol-injected control embryos (B, D, F, J). The signals were detected at 7 hours (h; A, B), 9 h (C, D), 12 h (E, F), and 29 h (I, J). FoxQ2 protein was also detected in the BicC morphant (G) and in control (H) at 23 h. The inset of (A) shows a morphant injected with BicC-MO2 to confirm the specificity of the morpholinos. The expression of *foxQ2* was weaker than that of the BicC-MO1 injected embryo, but the spatial pattern was invariant.

indicate that FoxQ2 does not play a normal role despite the fact that its message and protein are expressed at the correct location without BicC protein.

To confirm the lack of FoxQ2 function in BicC morphants, we examined the relationship between the animal plate and oral ectoderm. It has been suggested that FoxQ2 inhibits the specification of oral ectoderm by Nodal and oral ectoderm genes are never expressed in the *foxQ2*-positive animal plate region after early blastula stages[19]. We tried to detect *lefty* mRNA, whose expression is controlled by Nodal activity[23], in morphants and control embryos. In control embryos, *lefty* is restricted at the oral ectoderm region, and the region never overlaps with the animal plate, which expresses *foxQ2* (Fig. 3O–R). In contrast, in BicC morphants, *lefty* mRNA is expressed at almost the entire ectoderm region, including the animal pole (Fig. 3K–N). This indicates that without BicC function, the ectoderm is covered with oral ectoderm, and as a result, the animal plate, ciliary band and aboral ectoderm are diminished. Additionally, this *lefty* expression pattern confirms that FoxQ2 does not function normally in BicC morphants, indicating that the anterior neurogenic ectoderm formation is dependent on BicC function.

BicC is required for the formation of endoderm. At 48 hr, BicC morphants have dispersed cells at the vegetal pole but no gut formation (Fig. 3E). These dispersed cells have P4 antigen (Fig. 3B–E, G–J), indicating that they are differentiated primary mesenchyme cells (PMC)[24]. As expected, *pmar1*, an essential gene for PMC specification, is unchanged in BicC morphants compared to control embryos (Fig. 4A, B). These results indicate that the specification and differentiation of PMC, i.e., skeletogenic mesenchyme cells, are normal in BicC morphants. In contrast, *endo16*, an early endomesoderm and late endoderm marker, is not expressed during embryogenesis, at least, until 26 hr, without BicC (*cf.* Fig. 4C,E with D, F). However, *foxA* is expressed at the vegetal region (Fig. 4G–L), but the expression pattern is invariant until 29 hr because the endoderm never invaginates. These data suggest that some but not all aspects of endomesoderm specification occurred in BicC morphants. Although the ectoderm region is covered with oral ectoderm in the BicC morphant (Fig. 3), *foxA* expression is not detected around the mouth region (Fig. 4K, L). Because a mouth stomodeum is not observed even in 48 hr morphants (Fig. 3E), the morphants do not form a mouth structure without proper BicC function.

Based on these data, BicC morphants lose the differentiated anterior neurogenic ectoderm and endomesoderm. Next, we explored what happens on the lateral ectoderm (ectoderm except for anterior neurogenic ectoderm). We employed *in situ* hybridization to detect *univin*, which is initially expressed in the entire region and lateral ectoderm marker after cleavage stages[25]. Intriguingly, the expression pattern of *univin* in BicC morphants is invariant to that of control embryos (Fig. 5A–H). The size of the *univin*-absent area at the anterior end is almost identical between morphants and controls (Fig. 5A, E). This finding indicates that some molecular mechanisms suppressing *univin* expression are maintained without BicC function. Additionally, the mechanisms might be the same or related to those inducing *foxQ2* expression at the anterior end of the embryo.

To explore the effect of BicC mis-expression, we microinjected mRNA encoding a full length of BicC and detected *foxQ2*, *lefty* and *foxA*. As expected, because *bicC* is ubiquitously expressed maternally, the excess mRNA did not affect the expression pattern of those marker genes or morphology (Fig. 6A–F). These data suggest that BicC expression is not spatially controlled during early stages, but the function likely depends on modification of its protein and/or the localization of its partners or targets.

Discussion

The data presented here show that BicC is required for the formation of anterior neurogenic ectoderm and endoderm of sea urchin embryos. Because the majority of the previous studies on the formation of anterior neuroectoderm have focused on the function of transcription factors exclusively expressed at the region, this work suggests that maternally and ubiquitously expressed factors are also essential for the development of anterior neuroectoderm in sea urchin embryos.

During the genome-sequencing project of *Strongylocentrotus purpuratus*, BicC was initially annotated by two gene numbers, SPU_17139 and SPU_18221[26]. However, in a current version of sea urchin genome assembly (Spur3.1), bicC is encoded in one scaffold_1503 (http://Echinobase.org), indicating that these two annotated genes are the same. In fact, in genomic and transcriptomic data of *Hemicentrotus pulcherrimus* (which will be published elsewhere by Yaguchi et al.), bicC is encoded in one scaffold, similar in the *S. purpuratus* genome. This likely indicates that there is only one gene that encodes BicC in the sea urchin genome. However, it is possible that alternative splicing produces the variant types of mRNA, as is predicted in human genome (NM_001080512, XM_005270166-70).

Based on the analysis of spatial gene expression patterns, *bicC* mRNA is maternally and ubiquitously expressed until the end of
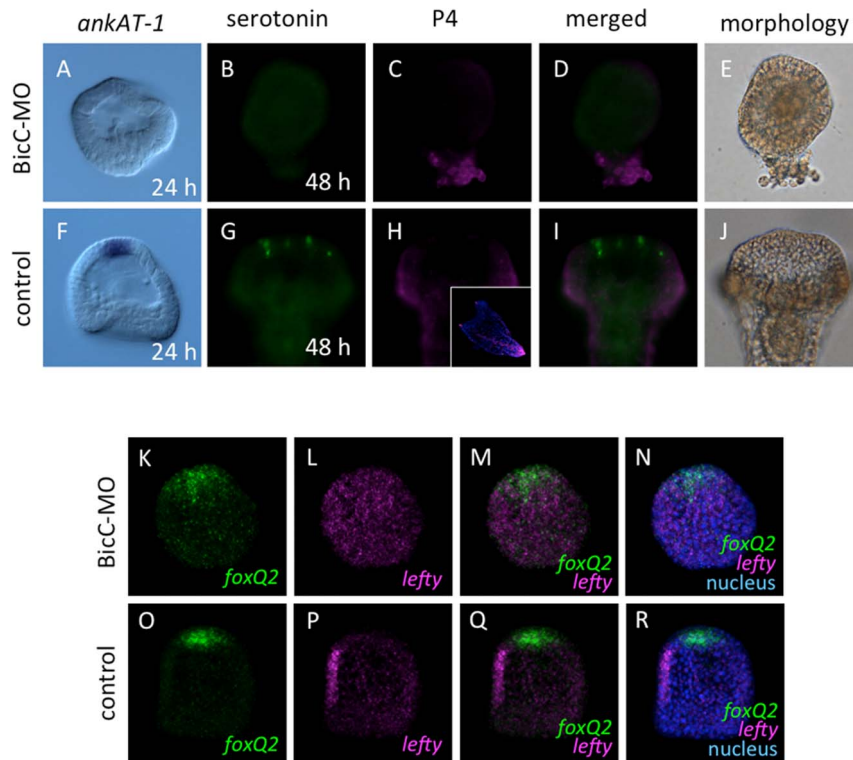
Figure 3 | BicC is required for FoxQ2 function. The *ankAT-1* gene and serotonin were not detected in BicC morphants (A, B) but were detected in the control (F, G). Inset in (A) showed no *ankAT-1* gene expression in BicC-MO2 morphant to ensure the specificity of the morpholinos. (C) and (H) show a P4 antigen pattern detecting differentiated spicule cell lineage. The inset of (H) shows a P4 signal with DAPI staining in the body rod of a 48 h embryo because the P4 signal in the oral lobe at this stage is almost missing. (D) is a merged image of (B) and (C). (I) is a merged image of (G) and (H). (E) and (J) show the morphology of a BicC morphant and control, respectively, at 48 h. Double fluorescent *in situ* hybridization staining with *foxQ2* (K, O) and *lefty* (L, P) in a BicC morphant (K–N) and control (O–R). (M) Merged image of (K) and (L). (Q) Merged image of (O) and (P). (N, R) DAPI staining shows the morphology with a merged image of (M) and (Q), respectively.

cleavage stages (Fig. 1). Maternally accumulated mRNA of *bicC* is well reported in other organisms, for instance, in *Drosophila* and in *Xenopus*[7,10,27]. *bicC* mRNA in *Drosophila* is localized to the anterior margin of the stage 8–9 oocyte[6], and in *Xenopus* it localizes mainly to the vegetal half[27]. The uniform localization of maternal mRNA such as *bicC* is frequently observed in sea urchin eggs. In other organisms,
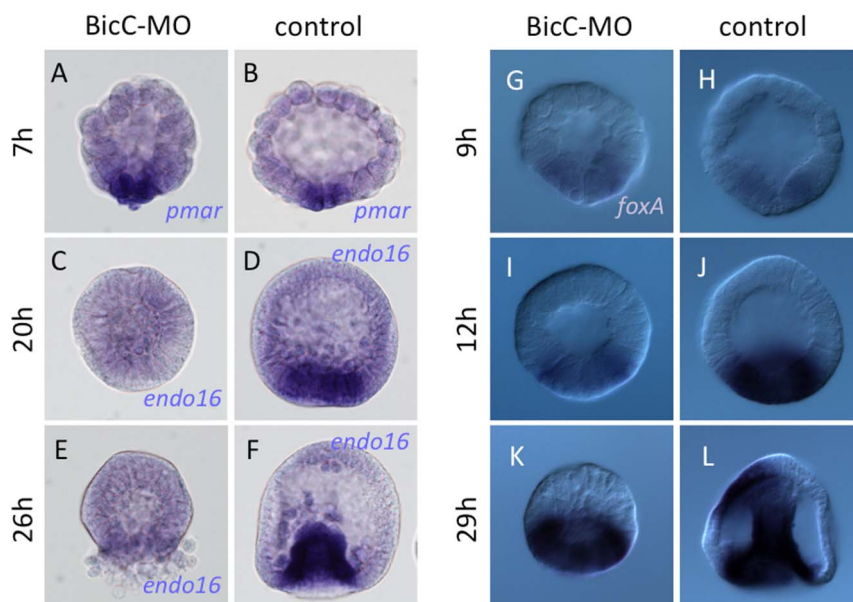
Figure 4 | BicC is required for endoderm formation. The *pmar1* gene was expressed in the BicC morphant (A) as it was in the control (B), but *endo16* was not detected in BicC morphants (C, E) at the time that it was expressed in control embryos (D, F). In contrast, spatial control of *foxA* expression was almost invariant in the BicC morphant (G, I, K) and in the control (H, J, L).
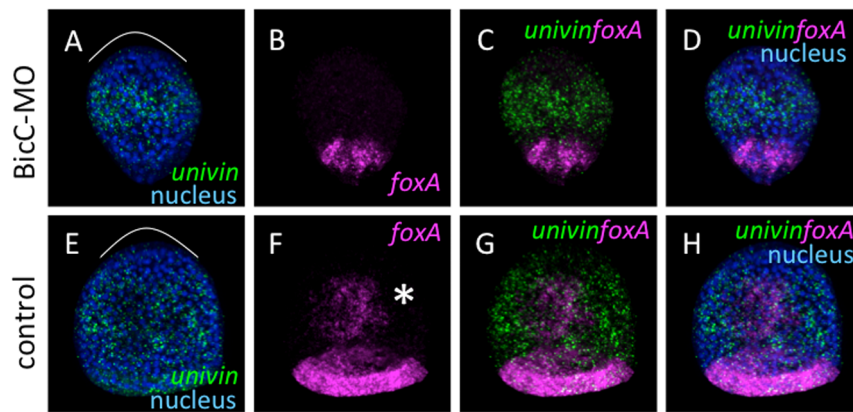
Figure 5 | *univin* expression is independent of BicC function. Double fluorescent *in situ* hybridization staining with *univin* (A, E) and *foxA* (B, F) in a BicC morphant (A–D) and control (E–H). (C, D) Merged image of (A) and (B). (G, H) Merged image of (E) and (F).

the same genes are localized to specific regions of the oocytes/eggs. *vg1* mRNA is localized to the vegetal pole in *Xenopus* eggs[28], whereas its related gene *univin* is maternally and ubiquitously present in sea urchin eggs[25,29]. Although Sox3, a member of SoxB1 family, is localized to the animal hemisphere of frog eggs[30], *soxB1* mRNA and its protein are localized ubiquitously in sea urchin eggs[13,31]. This might be because the sea urchin undergoes highly regulative development during early embryogenesis, similar to mammalian embryos. In fact, the *bicC* message in mice is ubiquitously localized in their growing oocytes[32]. Because of the ubiquitous expression of sea urchin *bicC* in early stages, it is suggested that its function depends on its post-transcriptional regulation and/or the localization of its target molecules.

Based on the diminishment of mRNA during blastula stages, it is likely that zygotic expression of *bicC* does not occur until gastrula stage. This is suggested by the result that exogenous mRNAs do not last longer than 24 hours in the sea urchin embryo (Yaguchi et al., unpublished data). However, we cannot rule out the possibility of zygotic expression or a specific degradation system of *bicC* mRNA. The stability of mRNA is frequently dependent on its polyadenylation state[33], and it has been reported that one of the functions of BicC is controlling deadenylation of mRNA[7]. It remains unclear how the transcription or maintenance of endogenous *bicC* message is regulated in the sea urchin embryo. The dynamic nature of the *bicC* expression pattern from the vegetal plate in gastrula stages to the ciliary band in the foregut region in the prism stage suggests that the

regulation of zygotic *bicC* mRNA expression and stability is precisely controlled by multiple mechanisms (Fig. 1G–L).

The data shown here indicate that BicC is required for the formation of anterior neurogenic ectoderm in the sea urchin embryo. One of the earliest zygotic genes expressing at the anterior neurogenic ectoderm is FoxQ2[17,34], and its message is weaker than that of control embryos but still present near the normal location in BicC morphants (Fig. 2). This suggests that the mechanisms that control the *foxQ2* spatial expression pattern do not depend on BicC function. However, intriguingly, FoxQ2-dependent events such as *ankAT-1* expression and serotonergic neurogenesis do not occur in the morphants, indicating that BicC is required for FoxQ2 function. Because BicC is an RNA-binding protein, there are two hypotheses that may explain BicC function in the regulation of FoxQ2 activity: 1) direct binding to *foxQ2* mRNA or 2) indirect effects through binding to genes that are related to FoxQ2 function. In *Drosophila*, it is suggested that BicC functions in the regulation of *oskar* mRNA in oocytes to interfere with immature translation of the gene, although the direct binding of BicC and *oskar* is not reported yet[10]. *oskar* mRNA gradually accumulates at the posterior end during oogenesis and translation starts at a precise time after localization, but BicC does not function normally, OSK protein will be mis-localized. BicC plays a role in regulating its own mRNA in *Drosophila* and negatively regulates the length of poly(A) tails[7]. This causes decreased stability of *bicC* mRNA. These reports indicate that BicC regulates the desired protein localization by controlling the stability of target mRNAs. In our data, knockdown of BicC inhibits FoxQ2 function, suggesting that BicC does not directly bind *foxQ2* mRNA in the sea urchin embryo. In fact, the presence or absence of BicC does not affect the translation of FoxQ2 (Fig. 2G, H). Thus, BicC likely functions in the regulation of other genes related to the formation of neurogenic ectoderm. We could not find any candidates with which BicC directly associates during sea urchin embryogenesis, but based on these results, in which FoxQ2 protein does not function normally in BicC morphants, it is possible that the function of co-factor(s) of FoxQ2 is inhibited. In vertebrate embryos, it has been reported that BicC regulates a micro-RNA, *miR-17*, to negatively control its binding efficiency to the target RNA, *polycystin 2*, resulting in precise control of translation of the protein[35]. It is likely that this type of regulation might act in the control of FoxQ2 function.

Despite lacking FoxQ2 function, *foxQ2* mRNA continues to be expressed at the anterior region of BicC morphants (Fig. 2). This indicates that mechanisms controlling *foxQ2* expression are independent of BicC function. Because FoxQ2 is one of the earliest zygotic factors reported so far in the sea urchin ectoderm, it is suggested that maternal factors regulate its initial induction, and the combination of maternal and the consequent zygotic factors maintain the
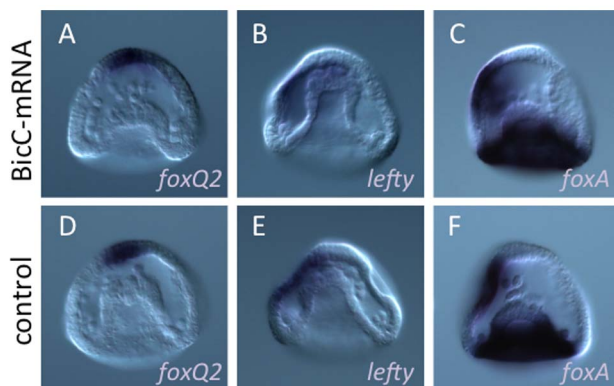
Figure 6 | BicC mRNA injection does not affect the development of sea urchin embryos. The expression patterns of *foxQ2*, an animal plate marker (A, D), *lefty*, an oral ectoderm marker (B, E), and *foxA*, an endoderm and a mouth stomodeum marker at this stage (C, F) were observed in control (D–F) and BicC mRNA-injected embryos (A–C).

expression. This putative mechanism is one of the candidates that essentially specify the anterior end of the embryos. The presence of this system is supported by our results, in which *univin* is not expressed at the anterior end region in BicC morphants as it is in normal embryos (Fig. 5). Because *univin* is maternally expressed at the entire region of the early embryos and disappeared from anterior and posterior ends in later stages[25], the suppressing mechanisms of *univin* expression functions at both ends in BicC morphants, suggesting that there is at least one anterior end characteristic still present in the morphants.

It is indicated that Nodal protein diffuses to the whole area of BicC morphants because *lefty* mRNA, a downstream gene of Nodal signaling, is expressed everywhere in ectoderm, including the anterior end (Fig. 3K–N). Normally, Nodal protein is restricted to the oral ectoderm region by Lefty due to a reaction-diffusion model[23]. In addition, it is suggested that FoxQ2 interferes with Nodal function including its own positive-autoregulation at the anterior end, with the result that Nodal cannot induce itself or oral ectodermal features at the neurogenic ectoderm even though Lefty function is inhibited[19]. Thus, *lefty* expression occurs only when the functions of FoxQ2 and Lefty, which both negatively regulate Nodal function, are doubly knocked down[19]. Therefore, our data (Fig. 3) suggest that FoxQ2 does not function without BicC even when the protein is present at the anterior end of the morphants. It is still unclear how the function of Lefty is inhibited in the morphants.

The involvement of BicC in endomesoderm formation is more complicated than in ectoderm. Based on the data showing that *pmar1* expression during the cleavage stage (Fig. 4) and P4 expression (Fig. 3) are normal in BicC morphants, the specification and the differentiation of large micromere descendants are independent of BicC. However, because the morphants lack spicule structures, BicC affects other factors that are essential for the formation of spicules in normal conditions. A lack of *endo16* expression throughout early embryogenesis indicates that some aspects of the specification of endoderm and non-skeletogenic mesoderm depend on BicC function (Fig. 4). In contrast, *foxA* expression at the endomesoderm seems to be normal in BicC morphants during early stages (Fig. 4). Thus, other aspects of endomesoderm formation are dependent on the function of BicC in the sea urchin embryo. Additionally, the result of microinjection into the fertilized eggs indicated that the deficient endomesoderm formation is likely dependent on maternal *bicC* (see Supplementary Fig. S1 online). The delayed but normal gastrulation in those morphants suggested that the function of regulatory and constitutive genes was almost normal even though the functional timing might be later than those in control embryos. Although we could detect the exclusive expression of *bicC* at the vegetal plate during gastrulation, it is possible that the zygotic BicC might have no specific function at least until 52 hours when we observed. Alternatively, the zygotic BicC might be required to control the precise timing of gastrulation. To further elucidate the detailed function of BicC on endomesoderm formation, further analysis is required.

Taking our results together, it becomes clear that the formation of all three embryonic germ layers depends on the function of BicC. The evidence obtained in this study suggests that the control of FoxQ2 function by BicC is essential for the development of the anterior end of the sea urchin embryos, but more research is required to understand whole mechanisms of formation in the anterior neurogenic ectoderm.

Methods

Animals and embryo culture. Adult *Hemicentrotus pulcherrimus* were collected at the Shimoda Marine Research Center, University of Tsukuba. The gametes were collected by intrabrastocoelar injection of 0.5 M KCl, and the embryos were cultured in 3.5 cm plastic dishes with filtered natural seawater (FSW) at 15°C.

Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization (WISH) was designed based on a previous report[36] with some modifications. The eggs/embryos were fixed with 3.7% formaldehyde-FSW overnight at 4°C. After the samples were washed with MOPS buffer (0.1 M MOPS, pH 7.0, 0.5 M NaCl, 0.1% Tween-20) 5 times, pre-hybridization was performed in the hybridization buffer (70% formamide, 0.1 M MOPS, pH 7.0, 0.5 M NaCl, 1.0 mg/ml bovine serum albumin [BSA], 0.1% Tween-20) at 50°C for 1 hour. Hybridization with 0.5–1.0 ng/µl digoxygenin (DIG: Roche Diagnostics GmbH, Mannheim, Germany)-labeled RNA-probe was performed at 50°C for 7 days. After being washed with MOPS buffer 5 times (7 min each) at room temperature (RT), 3 times (45 min each) at 50°C, and 2 times (7 min each) at RT, the sample was blocked in blocking buffer (MOPS buffer containing 5% lamb serum) for 1 hour at RT. Diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) in blocking buffer (1 : 1,000) was used for overnight incubation to detect the probe. After several washes in MOPS buffer, NBT/BCIP system (Promega Corporation, Madison, WI, USA) was used to detect the signal. To perform two-color *in situ* hybridization, we added DIG-labeled probe and FITC-labeled probe simultaneously and peroxidase conjugated antibodies and used Cy3- and FITC-tyramide signal amplification system (PerkinElmer, Waltham, MA, USA) to detect DIG and FITC probes, respectively.

Whole-mount immunohistochemistry. Immunohistochemistry for detecting serotonin (Sigma-Aldrich, St. Louis, MO, USA), synaptotagminB (synB)[37], FoxQ2[38], and P4[24] was performed as described previously[18,38]. The primary antibodies (dilutions; serotonin 1 : 2,000, synB 1 : 100, FoxQ2 1 : 100, and P4 1 : 100) were detected with the secondary antibodies conjugated with Alexa Fluor 488 or 568 (Life Technologies, Carlsbad, CA, USA, 1 : 2,000 dilution). The specimens were observed with a Zeiss AxioImager.Z1, and optical sections were stacked and analyzed with ImageJ and Adobe Photoshop software.

Microinjection of morpholino antisense oligonucleotides (MO) and mRNA. We used the following morpholinos (Gene Tools, Philomath, OR, USA) designed based on *Hemicentrotus pulcherrimus* BicC mRNA sequence in GenBank (accession number is AB968521) in 24% glycerol in injection needles:

BicC-MO1: 5′- GAGGGTCATAACTAAAGGAGTTCAT -3′ and
BicC-MO2: 5′- AAACTTGAGGTAGTGGTAAAGTTCC -3′.

BicC-MO1 (3.8 mM in needle) and -MO2 (2.0 mM in needle) are non-overlapped morpholinos that block the translation of BicC. BicC-MO2 was used to confirm the specificity of morpholinos, and the additional results to prove the unfertilized-egg injection of BicC-MO were shown in Supplementary Figure S1. BicC mRNA was synthesized with mMessage mMachine kit (Life Technologies) and injected at 2.2 µg/µl in 24% glycerol. The control experiments were performed with an injection of 24% glycerol alone. In each experiment and/or observation we used more than 200 embryos and data was collected from three independent batches.

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Author contributions

S.Y. designed the study, performed the experiments, prepared the figures, and wrote the manuscript. J.Y. and K.I. performed the experiments.

Additional information

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