The brine shrimp *Artemia* reproduces either ovoviviparously, producing free-swimming nauplii, or oviparously, producing encysted embryos (diapause cysts) able to cope with harsh and complex habitats. When the cysts enter diapause they are encased in a complex external shell that protects them from certain extreme environments. The genomic comparison of oviparous and ovoviviparous ovisacs has been described previously. We isolated three significantly up-regulated genes in oviparous oocytes and identified them as *Arp-CBP* (*Artemia parthenogenetica* chitin-binding protein) genes. Quantitative real-time PCR indicated that the expression of *Arp-CBP* genes gradually increases during diapause cyst formation and significant mRNA accumulation occurs during the ovisac stage of oviparous development. Moreover, in situ hybridization results demonstrated that *Arp-CBP* mRNAs are expressed in the embryo. Interestingly, the results of immune electron microscopy showed that all three *Arp-CBP* CBPs are distributed throughout the cellular ECL (embryonic cuticle layer) of the cyst shell. Furthermore, knockdown of *Arp-CBP* by RNA interference resulted in marked changes in the composition of the embryonic cuticular layer. The fibrous layer of the cyst shell adopted a loose conformation and the inner and outer cuticular membranes exhibited marked irregularities when *Arp-CBP* expression was suppressed. Finally, an in vitro recombinant protein-binding assay showed that all three *Arp-CBPs* have carbohydrate-binding activities. These findings provide significant insight into the mechanisms by which the ECL of *Artemia* cyst shell is formed, and demonstrate that *Arp-CBPs* are involved in construction of the fibrous lattice and are required for formation of the ECL of the cyst shell.

Key words: *Artemia*, chitin-binding protein, diapause cyst, embryonic cuticle layer.

INTRODUCTION

The brine shrimp *Artemia* is an ancient crustacean that escapes predation and competition by residing in high-salinity habitats, where they are frequently subjected to dry conditions, anoxia, food depletion and temperature fluctuations [1,2]. Females release either swimming larvae (nauplii) or encysted gastrula (cysts) that enter diapause, a physiological condition in which development stops, metabolism is greatly reduced and stress tolerance is high. Upon the onset of favourable changes in external conditions, the embryos receive and process appropriate environmental signals and proceed to resume development, and eventually emerge as nauplii.

A dormant *Artemia* encysted gastrula embryo is composed of approximately 4000 cells arrested at G2/M-phase of the cell cycle. Formation of a complex external shell, consisting of a non-cellular CL (chorion layer) and a cellular ECL (embryonic cuticle layer), protects the embryos from certain harsh environmental conditions for long periods. The CL is a hypochlorite-soluble substrate secreted by cells of the maternal shell gland and provides a mechanical barrier between the embryo and external environment. The embryonic cuticle is formed by blastodermic cells and is hypochlorite-resistant and impermeable to non-volatile solutes, and thus might play an important role as a biochemical barrier that maintains the osmotic properties of the embryo [3].

TEM (transmission electron microscopy) in previous work [3,4] revealed that the ECL is a tripartite structure consisting of a complex OCM (outer cuticular membrane), a loose fibrous membrane and a distinctive ICM (inner cuticular membrane). Ultrastructurally, the OCM is the most complex of the membranes within the cyst with the greatest number of distinct layers. The region between the OCM and ICM is finely fibrillar and consists predominantly of chitin and proteins, creating a fuzzy appearance in transverse view. In addition, some lamellar regions are delineated as polygonal plates adjacent to a middle area, often without lamellations. The ICM is the innermost layer and typically separates during prenauplius emergence [3,4].

Although it is known that the *Artemia* cyst shell contains chitin, proteins and some metal elements [5–9], the molecular formation of the cyst shell is complex and the details remain to be fully elucidated. In previous studies, three extracellular matrix peptides of the cyst shell, the shell gland-specific genes, *SGEG1*, *SGEG2a* and *SGEG2b*, were identified. They are specifically expressed in the cells of shell glands, distributed throughout the non-cellular CL of the cyst shell and are involved in protecting encysted embryos [6,7].

Until now, information on diapause-related proteins that are differentially expressed in oocytes from oviparous and ovoviviparous *Artemia* has been slow to emerge. In a previous study, we analysed the differential gene expression between oocytes from oviparous and ovoviviparous *Artemia* reared under different photoperiods. Three up-regulated chitin-binding protein genes were found to be specifically expressed in oviparous oocytes [10]. They have CBDs (chitin-binding
were reared in 4% seawater under 5 h light/19 h dark cycles. Ovoviviparous and ovoviviparous ovisacs [10]. The sequenced cDNAs were deposited in GenBank® (http://www.ncbi.nlm.nih.gov/blast). The nucleotide sequences obtained were used to examine the internal reference. All data are given as the means ± S.E.M.

In the present study, we focused on chitin-binding proteins that are key molecules in the ECL of Artemia cyst shells. The transcripts of Arp-CBP (Artemia parthenogenetica chitin-binding protein) genes, Arp-CBP-A, Arp-CBP-B and Arp-CBP-C, are expressed during the ovisac stage of oviparous embryos. The proteins are distributed throughout the ECL of the cyst shell. Gene knockdown by RNAi (RNA interference) and subsequent recombinant protein-binding assays indicated that Arp-CBPs are required for the formation of the encysted embryonic FL (fibrous layer).

MATERIALS AND METHODS

Culture of A. parthenogenetica

Oviparous A. parthenogenetica were reared in 8% (w/v) artificial seawater under 5 h light/19 h dark cycles. Ovoviviparous Artemia were reared in 4% (w/v) artificial seawater under a 16 h light/8 h dark cycle. The water temperature was kept at 28°C. The water was supplemented with Chlorella powder (Fuqing King Dharma Spirulina) as brine shrimp food.

Sequence validation and analysis

Three diapause-destined chitin-binding protein genes were identified from a suppression subtractive hybridization cDNA library of A. parthenogenetica oocytes created from oviparous and ovoviviparous ovisacs [10]. The sequenced cDNAs were edited and analysed using Lasergene® 7 (DNASTar), and the deduced amino acid sequences of the peptides were predicted using PredictProtein (http://www.ics.uci.edu/~baldig/scratch/) websites. BLAST searches were performed using the NCBI website (http://www.ncbi.nlm.nih.gov/blast). The nucleotide sequences for Arp-CBP-A, Arp-CBP-B and Arp-CBP-C can be found under GenBank®/DDBJ/EMBL accession numbers GH635256, GH635605 and GH635849 respectively.

Real-time RT (reverse transcription)-PCR

Both oviparous and ovoviviparous oocytes of A. parthenogenetica were dissected and prepared as described in [10]. Total RNAs were extracted individually using TRIzol® reagent (Invitrogen). After reverse transcription, qRT-PCRs (quantitative real-time PCRs) were performed on the Bio-Rad MiniOpticon™ Real-Time PCR System using SYBR® Premix Ex Taq™ (TaKaRa Bio) and Arp-CBP-specific primers (RT-F/RT-R and α-tubulin-F/α-tubulin-R in Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490285add.htm). The cycling parameters were: 40 cycles of 10 s at 95°C (30 s only for the first cycle), 10 s at 56°C and 10 s at 72°C (5 min only for the last cycle). Dissociation curves were analysed at the end of each run to determine the purity of the product and the specificity of the amplification. Relative transcript levels are presented as fold-changes calculated using the comparative Ct method with cDNA for α-tubulin as the internal reference. All data are given as the means ± S.E.M.

For gene expression characterization, Artemia used for ovisac isolation were placed in an ice bath for 1–2 min until lightly anaesthetized. The ovisac was then dissected, snap-frozen in liquid nitrogen and stored at −80°C until RNA preparation. Total RNAs were extracted from ovisacs of cyst-destined adults at different developmental stages. After RT, all qRT-PCRs were performed as described above.

ISH (in situ hybridization)

DIG (digoxigenin)-labelled sense and antisense RNA probes corresponding to nucleotides 1–818 of Arp-CBP-A cDNA, 167–706 of Arp-CBP-B cDNA and 112–619 of Arp-CBP-C cDNA were amplified (using ISH-F and ISH-R primers, as described in Supplementary Table S1) and cloned into the pSPT18 plasmid vector at the HindIII and KpnI sites. They were then transcribed in vitro from HindIII- and KpnI-linearized templates (DIG RNA Labelling kit SP6/T7).

For the preparation of tissue sections, Artemia were placed in an ice bath for 1–2 min until lightly anaesthetized, snap-frozen in liquid nitrogen and embedded in Tissue-Tek™ (Sakura Finetechanical). Frozen sections 8-μm-thick were then prepared using a frozen ultramicrotome (CM1950, Leica). The sections were air-dried, fixed with PFA (paraformaldehyde) 4% (w/v), digested with Proteinase K and hybridized at 42°C overnight. The sections were then washed at 52°C and blocked using blocking solution (Roche). They were then treated with AP (alkaline phosphatase)-conjugated anti-DIG antibody (1:500 dilution, Roche) and visualized using the colorimetric NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yI phosphate; Roche) according to the manufacturer’s instructions. Photographs of the stained sections were taken on an inverted microscope (ECLIPSE TE2000-S).

RNAi

The 180-bp Arp-CBP-A, 182-bp Arp-CBP-B, 192-bp Arp-CBP-C and 359-bp GFP (green fluorescent protein) cDNA fragments were amplified with sequence-specific primers (RNAi-F/RNAi-R and GFP-F/GFP-R in Supplementary Table S1) and subcloned into the plasmid pET-T7 at the XbaI and NcoI sites. The recombinant plasmids were transformed into Escherichia coli HT115 cells and ds (double-stranded) RNAs were produced and purified as described in [19]. A total of 200 individual Artemia per group were injected with 1 μg of dsRNA just before ovarian development. An UltraMicroPump II equipped with the Micro4™ MicroSyringe Pump Controller (World Precision Instruments) was used for the microinjection. RNAi-injected Artemia were cultured in 8% artificial seawater under a 5 h light/19 h dark cycle. The RNAi-induced ovisacs (lateral pouches filled with oocytes) and cysts were observed by light microscopy and collected. To examine the effect of RNAi-mediated gene suppression, total
RNA were extracted from the ovisacs (middle stage of cyst-destined adults; lateral pouches filled with oocytes) of Arp-CBP RNAi-treated and GFP RNAi-treated Artemia at 6 days after injection. After reverse transcription, qRT-PCRs were performed as described above.

**Western blot analysis**

The anti-Arp-CBP-A antibody was raised in rabbits (HuaAn Biotechnology) against a GST (glutathione transferase)-fusion protein expressed in E. coli strain BL21(DE3) cells with the pGEX4T-1 vector. The anti-Arp-CBP-B and anti-Arp-CBP-C antibodies were raised in rabbits (HuaAn Biotechnology) against peptides containing the partial amino acid sequences CEDGEGKIVT and CPNQRHFRRL respectively. Each sample was homogenized in 2× SDS-loading buffer (125 mM Tris/HCl, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.02–0.04% Bromophenol Blue, pH 6.8) and boiled for 10 min. After centrifugation at 12 000 g for 10 min at 4°C to remove insoluble shell fragments, supernatants were quantified using the direct UV method. A total of 50 μg of protein from each sample were separated by SDS/PAGE (12.5% gel) and transferred on to PVDF membranes (Millipore Corporation). The membranes were incubated with anti-Arp-CBP-A (1:2000 dilution), anti-Arp-CBP-B (1:100 dilution), or anti-Arp-CBP-C (1:500 dilution) antibody, and coated with 15 nm of gold nanoparticle-conjugated secondary antibody (1:30 dilution; Sigma–Aldrich). Cysts were viewed by TEM (JEM-1230, JEOL), and photographed at a voltage of 70 kV.

**Immunohistochemistry**

Cysts were soaked in ice-cold 2.0 M sucrose for 20 min to reduce the internal turgor pressure of the embryos and to prevent tissue extrusion and damage. Cysts were fixed and fixed for 12 h in 2.5% glutaraldehyde prepared in 3% NaCl. They were then washed and post-fixed in 1% osmium tetroxide in a 3% NaCl solution, dehydrated in a graded acetone series and embedded in Spurr resin. Sections of 70 nm were cut with a microtome (Leica EM UC6), stained with 2% uranyl acetate and Reynold’s solution (0.2% sodium citrate and 0.2% lead nitrate), then viewed by TEM (JEM-1230, JEOL), and photographed at a voltage of 70 kV.

**TEM analysis**

**Immunohistochemistry**

Cysts were soaked in ice-cold 2.0 M sucrose for 20 min and the cyst walls were then nicked. Cysts were fixed in 4% PFA and 0.2% glutaraldehyde overnight at 4°C. To obtain ultrathin sections, the cysts were embedded in 1% gelatin in 1 M PBS at 37°C for 30 min, and transferred to 4°C until solidification. Cysts in gelatin were then cut into nubs of approximately 0.1 cm×0.1 cm×0.1 cm. These nubs were sequentially immersed in 30, 50 and 70% (v/v) sucrose solution while sedimenting. Sections of 70 nm were cut using a microtome (Leica EM UC6), incubated with anti-Arp-CBP-A (1:1000 dilution), anti-Arp-CBP-B (1:100 dilution) or anti-Arp-CBP-C (1:500 dilution) antibody, and coated with 15 nm of gold nanoparticle-conjugated secondary antibody (1:30 dilution; Sigma–Aldrich). Cysts were viewed by TEM (JEM-1230) and photographed at a voltage of 80 kV.

**Prokaryotic expressed proteins and carbohydrate-binding analysis in vitro**

The ORFs (open reading frames) of Arp-CBP-A, Arp-CBP-B and Arp-CBP-C were amplified using the primers Exp-F and Exp-R respectively, and cloned into the pGEX4T-1 vector. GST-fusion proteins were expressed in E. coli BL21(DE3) and purified using glutathione beads. For carbohydrate-binding analysis in vitro, the proteins were bathed with chitin, cellulose, xylan or chitosan in binding buffer (500 mM NaCl, 10 mM Tris/HCl, 0.5% Triton X-100 and 1 mM PMSF, pH 7.4) at room temperature (25°C) for 4 h, centrifuged at 10 000 g for 1 min, washed five times with binding buffer and eluted twice with 2× SDS-loading buffer. Protein binding was then analysed by SDS/PAGE.

**RESULTS**

**Arp-CBPs encoded by oviparous oocytes of cyst-destined Artemia**

Three chitin-binding protein genes were identified from a full-length suppression subtractive hybridization cDNA library of A. parthenogenetica [10] and designated Arp-CBP-A, Arp-CBP-B and Arp-CBP-C. The results are shown in Figure 1.

**Arp-CBPs encoded by oviparous oocytes of cyst-destined Artemia**

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**Arp-CBP expression characteristics from oviparous and ovoviviparous oocytes**

Quantitative real-time RT–PCR was used to characterize the different expression patterns of Arp-CBP genes in oviparous and ovoviviparous oocytes. The results showed that Arp-CBPs are significantly up-regulated in Artemia oocytes that subsequently enter diapause. The mRNA level of Arp-CBP-A in oviparous stages increased more than 1738-fold over that of ovoviviparous oocytes (P=6.9×10⁻²; Figure 2A). The amount of Arp-CBP-B in oviparous oocytes was increased 81-fold over that in ovoviviparous oocytes (P=3.4×10⁻⁴; Figure 2A). Finally, the
mRNA level of Arp-CBP-C in oviparous oocytes was up-regulated 50-fold compared with that in ovoviviparous oocytes ($P = 6.57 \times 10^{-4}$; Figure 2A). These results demonstrate that Arp-CBP-A, Arp-CBP-B and Arp-CBP-C are diapause-specific genes up-regulated in oviparous oocytes. These results also showed that the mRNA levels of the three Arp-CBPs in oviparous oocytes were different (Figure 2B). Expression of Arp-CBP-A mRNA was the most abundant, Arp-CBP-B mRNA was approximately one-tenth of the amount of Arp-CBP-A and the mRNA level of Arp-CBP-C was the lowest in oviparous oocytes (Figure 2B).

**Characterization and localization of Arp-CBP mRNA expression**

To characterize the stage-specific expression patterns of Arp-CBP genes during cyst shell formation, quantitative real-time RT–PCR and Western blotting were used to analyse Arp-CBP expression in oviparous developmental stages. During the oviparous developmental cycle, oocytes emerge bilaterally in the abdomen and are then stored in lateral pouches until they are transferred to the ovisacs, where development occurs and the embryos remain throughout the reproductive cycle (for 5 days) until the encysted diapause embryos are released [1].

The results of quantitative real-time RT–PCR and Western blotting clearly showed an increase in Arp-CBP mRNA accumulation and protein expression during oocyte and embryo development. Significant accumulation of Arp-CBP-A mRNA was observed in late embryos in the abdomen of oviparous Artemia and in diapause cysts. By contrast, Arp-CBP-A expression in oviparous Artemia at other stages of the life cycle was relatively weak (Figure 2A). Thus Arp-CBP-A mRNA levels gradually increase during embryo development and reach peak expression in late embryos during the ovisac period. The results of Western blotting clearly revealed that Arp-CBP-A protein expression in late embryos and diapause cysts, whereas no signal was detected in oviparous Artemia at other time points (Figure 3A). These findings indicate that Arp-CBP-A plays an important role in the formation of diapause cysts.

In addition, mRNAs levels of both Arp-CBP-B and Arp-CBP-C were markedly increased in late embryos in the abdomen of oviparous Artemia, whereas only weak signals were observed in oviparous Artemia during other periods of the life cycle and in diapause cysts (Figure 3A). Western blotting showed that Arp-CBP-B and Arp-CBP-C protein expression was observed only in late embryos and no signal was detected in oviparous Artemia at
Chitin-binding proteins participate in formation of Artemia cyst shells

Figure 2 Characterization of Arp-CBP mRNA expression in oviparous and ovoviviparous oocytes

(A) Expression levels of Arp-CBP mRNA from oviparous and ovoviviparous oocytes. Results are means ± S.D.; ***P < 0.001. The level of α-tubulin mRNA was used as an internal control.

(B) Relative expression levels of Arp-CBP mRNA in oviparous oocytes.

other life cycle stages. These findings suggest that Arp-CBP-B and Arp-CBP-C participated mainly in the development of late embryos.

ISH was used to determine the cellular localization of Arp-CBP mRNAs in oviparous ovisacs. As shown in Figure 3(B), clear signals from Arp-CBP-A, Arp-CBP-B and Arp-CBP-C mRNA were observed in the embryos of oviparous ovisacs. By contrast, the signal was absent when hybridized with a sense control probe. These ISH results revealed that Arp-CBP mRNAs are expressed in the embryos of oviparous Artemia.

Arp-CBP distribution throughout the cyst shell

To accurately determine the distributions of Arp-CBP-A, Arp-CBP-B and Arp-CBP-C peptides in the cyst shell, immunohistochemical localization in combination with TEM were performed. In general, when the embryo enters diapause, it is surrounded by a complex cyst shell [Figure 4A(a)] consisting of a non-cellular CL and a cellular ECL [Figure 4A(b)]. TEM revealed that the ECL consists of a complex OCM, an FL and a distinctive ICM, thus forming a tripartite structure [Figure 4A(c)].

After incubation with an antibody against Arp-CBP-A, gold nanoparticles were observed with back-scattered electron imaging in the ECL of the cyst shell. The black spot signals of gold nanoparticles were densely distributed throughout the FL and ICM. Furthermore, after incubation with antibodies against Arp-CBP-B and Arp-CBP-C, a large number of gold nanoparticles were observed in the ICM and OCM of the cyst shell, and weak signals were observed in the FL (Figure 4B). Arp-CBP-A, Arp-CBP-B or Arp-CBP-C was not detected in the CL. These results showed that Arp-CBPs are specifically distributed in the cyst shell and that each has a distinct distribution pattern in the ECL.

In vivo knockdown of Arp-CBP by RNAi

To clarify the structural roles of these proteins in cyst shell formation, knockdown of Arp-CBP-A, Arp-CBP-B or Arp-CBP-C, or all three, was performed. The 180-bp Arp-CBP-A, 182-bp Arp-CBP-B, 192-bp Arp-CBP-C and 359-bp GFP dsRNA fragments were designed on the basis of their cDNA sequences and injected into the body cavity of oviparous Artemia just before
ovarian development. Arp-CBP expression levels, measured at 6 days after oocytes development began, decreased with increasing doses of the respective dsRNAs (Figure 5A). Following injection with 500 ng of dsRNA for Arp-CBP-A, Arp-CBP-B or Arp-CBP-C, or all three Arp-CBP s together, the mRNA and protein expression levels were reduced to less than 10% of those of the GFP dsRNA-injected group (Figure 5A).

Although none of the Arp-CBP RNAi-treated Artemia exhibited a significantly abnormal phenotype, the ECL of the cysts shell exhibited marked variations. TEM revealed obvious changes in the OCM structure of ECL of single Arp-CBP RNAi-treated cyst shells in comparison with GFP RNAi-treated cyst shells. The dark region of the OCM became markedly brighter, the lamellar region became visibly thinner and the distinct membrane layers were noticeably irregular (Figure 5B). However, the cysts of Arp-CBP RNAi-treated Artemia did not show any obvious abnormal changes with regard to resistance to environmental stress or hatching rate.
Chitin-binding proteins participate in formation of *Artemia* cyst shells

**Figure 4** Structure of the cyst shell and immunohistochemical localization of Arp-CBPs

(A) Structure of the cyst shell. a, Inverted microscope at magnification ×400. Scale bar = 30 μm. b, TEM at magnification ×8000. Scale bar = 2 μm; c, ECL of cyst shell obtained by TEM at magnification ×12,500. Scale bar = 0.5 μm. (B) Immunohistochemical localization of Arp-CBP-A, Arp-CBP-B or Arp-CBP-C obtained by TEM image analysis using gold nanoparticles in slices of cyst shell. Arrowheads indicate the gold nanoparticle signals at magnification ×39,000. Scale bars = 0.2 μm.

Moreover, in the triple *Arp-CBP* RNAi-treated cyst shells, the most remarkable changes were observed in the ECL. The ultrastructure of the OCM adopted a fuzzy appearance and the distinct layers of the OCM also became markedly irregular. Observations using TEM revealed that the ICM of the ECL had completely disappeared and that the width of the FL was stretched to more than twice the width of the FL in *GFP* RNAi-treated cyst shells (Figure 5B). In addition, the ECL tore easily during the preparation of the sections for TEM.

Overall, the structure of ECL was dramatically changed after the silencing of *Arp-CBPs*, indicating that Arp-CBPs participate in the formation of the ECL in *Artemia* cysts.

**In vitro protein-binding activity**

To confirm the carbohydrate-binding activity of Arp-CBPs, the *E. coli* pGEX vector was used to produce proteins fused to the C-terminus of GST. The purified proteins were incubated with beads, washed and eluted, as described in the Materials and methods section. The binding affinity of purified recombinant proteins for chitin, cellulose, xylan and chitosan were examined by SDS/PAGE.

As shown in Figure 6(A), a significant amount of purified Arp-CBP-A bound to chitin and cellulose, whereas GST protein alone failed to bind. Purified Arp-CBP-B bound to chitin, cellulose, xylan and chitosan, and was observed in the eluted lane in all
Figure 5  Phenotype of the ECL following RNAi-mediated suppression of Arp-CBP expression

(A) The expression levels of Arp-CBP mRNAs were determined by quantitative real-time RT–PCR following RNAi treatment. The expression levels of Arp-CBP mRNA in the GFP dsRNA-injected group were used as control. Arp-CBP protein expression was analysed by Western blotting and α-tubulin was used as a loading control. (B) The phenotypes of all RNAi-treated cysts were determined by observation by TEM. Black arrows indicate the width of the FL. The boxes highlight the phenotypes of the OCM. Scale bars = 0.5 μm. AL, alveolar layer.
Our deduction that Arp-CBPs are involved in the formation of the cyst shell was on the basis of the unique expression characteristics of Arp-CBPs. First, the expression of Arp-CBP mRNAs is both abundant and up-regulated in oviparous oocytes that subsequently develop into diapause embryos. Secondly, the accumulation and dispersal of Arp-CBP mRNA occurs in synchrony with the synthesis of shell materials. The amount of mRNA increased over time during oviparous development and was accumulated in significant amounts at the ovisac stage. This is synchronous with the chorion formation of the cyst shell by brown granules secreted from the shell gland. Thirdly, Arp-CBP proteins are specifically expressed and abundantly accumulated in embryos at the ovisac stage, which is the final period of embryo assembly in the uterus before cyst release. In brief, these expression characteristics indicate that Arp-CBPs are closely associated with the formation of encysted gastrulae.

Our conclusion that Arp-CBPs participate in formation of the cyst shell was supported by the results of protein distribution and carbohydrate-binding affinity analyses. Arp-CBP proteins were specifically distributed in the ECL of the cyst shell. Arp-CBP-A was densely distributed throughout the FL and ICM, whereas Arp-CBP-B and Arp-CBP-C were chiefly present in the ICM and OCM. These different protein distributions reflect the potentially distinct contribution of each Arp-CBP to shell formation.

In the Artemia cyst shell, the FL of the ECL is loose and contains primarily carbohydrates and proteins [3,4]. Chitin and chitosan have been reported to be present in the cyst shell [5,9]. In the present study, all Arp-CBPs possessed apparent carbohydrate-binding affinity. These results indicate that the function of Arp-CBP is probably to hold polysaccharide fibrils and other proteins together, which is necessary to form an organized fibrous lattice.

The assertion that Arp-CBPs function during the formation of cyst shells was mainly deduced from the results of RNAi, in which the formulation of the cellular ECL of the cyst shell changed dramatically after the silencing of Arp-CBP expression. In single Arp-CBP RNAi-treated cyst shells, TEM revealed that the dark region of the OCM became irregular and thinner. Ultrastructurally, the OCM is the most complex of the membranes. Owing to its ionic selectivity, the OCM may be important for maintaining salt balance [4]. However, structural changes in the dark-staining layer of the OCM suggest that the function of the OCM as a permeability barrier may be defective following Arp-CBP knockdown. In addition, the ionic selectivity of the cyst shell and the osmotic properties of the diapause cysts may also be changed. Those possibilities remain to be examined in future work.

In triple Arp-CBP RNAi-treated cyst shells, significant changes occurred in the ECL. The structure of the FL appeared looser when observed using TEM, and the ICM had completely disappeared. Therefore we presume that the silencing of all three Arp-CBP genes distinctly reduced carbohydrate binding and microfibril binding, and thus influenced the material composition of the fibrous matrix and completely destroyed the structure of the ECL. Disruption of the fibrous matrix may also have profound effects on the shell tensile strength, permeability and the capacity to appropriately process environmental signals. In addition, the ICM is the innermost layer and typically separates during prenauplius emergence [3,4]. Disappearance of the ICM after Arp-CBP RNA silencing reduces the integrity of ECL and may influence hatching.

Within the cyst shell of Artemia, the cellular ECL is formed by blastoderm cells and protects the cyst by acting as a biochemical barrier between the inner embryo and outer environment [3,4]. Disappearance of the ICM could influence the transfer of water and other volatile factors in to or out of the cysts. Therefore Arp-CBPs are possibly involved not only in connecting proteins.

**DISCUSSION**

Artemia undergoes oviparous development to release encysted embryos able to withstand harsh environments and ensure species survival. The reproductive paths and gene expression profiles of Artemia oocytes have already been determined in the oviducts. Identification of the differentially expressed genes in oocytes at cyst formation. Disappearance of the ICM could influence the transfer of water and other volatile factors in to or out of the cysts. Therefore Arp-CBPs are possibly involved not only in connecting proteins.
and scaffolding material, but also in construction of the ECL and processing environmental signals.

In conclusion, we successfully elucidated the expression pattern and localization of three Arp-CBPs from oviparous oocytes of *A. parthenogenetica*. These diapause-destined chitin-binding proteins play an important role in the binding of microfibrils and fibrous lattice formation. The results of the present study improve our understanding of ECL formation in *Artemia* cyst shells.

**AUTHOR CONTRIBUTION**

Wei-Jun Yang and Wen-Ming Ma conceived and designed the experiments; Wen-Ming Ma and Hua-Wei Li performed the experiments; Zhong-Min Dai and Fan Yang analysed the experiment data; Wei-Jun Yang, Wei-Ming Ma, Hua-Wei Li and Jin-Shu Yang wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Chitin-binding proteins of Artemia diapause cysts participate in formation of the embryonic cuticle layer of cyst shells

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Table S1 Nucleotide sequences and positions of primers used in PCRs

F and R indicate the forward and reverse directions respectively. The underlined regions represent the adscititious recognition sequences of restriction endonucleases.

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1 These authors contributed equally to the study, and are joint first authors for this paper.
2 To whom correspondence should be addressed (email w_jyang@cls.zju.edu.cn).
Figure S1  Nucleotide and deduced amino acid sequences of the cDNAs encoding Arp-CBPs  

(A–C) The complete cDNAs of Arp-CBPs and their deduced amino acid sequences. The nucleotide and amino acid residue numbers are indicated on the left. The asterisk denotes termination of amino acids and the putative CBDs are underlined. The putative SP is shown in bold and italic font and the predicted N-glycosylated site of Arp-CBP-A is boxed.