Transcriptional regulation of the matrix protein Shematrin-2 during shell formation in pearl oyster

Received for publication, August 15, 2018, and in revised form, September 21, 2018 Published, Papers in Press, October 3, 2018, DOI 10.1074/jbc.RA118.005281

Yan Chen‡, Jing Gao‡, Jun Xie, Jian Liang, Guilan Zheng, Liping Xie, and Rongqing Zhang‡

From the Protein Science Laboratory of the Ministry of Education, School of Life Sciences, Tsinghua University, Beijing 100084 and the Department of Biotechnology and Biomedicine, Yangtze Delta Region Institute of Tsinghua University, Jiaxing 314006, Zhejiang Province, China

Edited by Qi-Qun Tang

The molluscanshell is a fascinating biomineral consisting of a highly organized calcium carbonate composite. Biomineralization is elaborately controlled and involves several macromolecules, especially matrix proteins, but little is known about the regulatory mechanisms. The matrix protein Shematrin-2, expression of which peaks in the mantle tissues and in the shell components of the pearl oyster Pinctada fucata, has been suggested to be a key participant in biomineralization. Here, we expressed and purified Shematrin-2 and explored its function and transcriptional regulation. An in vitro functional assay revealed that Shematrin-2 binds the calcite, aragonite, and chitin components of the shell, decreases the rate of calcium carbonate deposition, and changes the morphology of the deposited crystal in the calcite crystallization system. Furthermore, we cloned the Shematrin-2 gene promoter, and analysis of its sequence revealed putative binding sites for the transcription factors CCAAT enhancer–binding proteins (Pf-C/EBPs) and nuclear factor-Y (NF-Y). Using transient co-transfection and reporter gene assays, we found that cloned and recombinantly expressed Pf-C/EBP-A and Pf-C/EBP-B greatly and dose-dependently up-regulate the promoter activity of the Shematrin-2 gene. Importantly, Pf-C/EBP-A and Pf-C/EBP-B knockdowns decreased Shematrin-2 gene expression and induced changes in the inner-surface structures in prismatic layers that were similar to those of antibody-based Shematrin-2 inhibition. Altogether, our data reveal that the transcription factors Pf-C/EBP-A and Pf-C/EBP-B up-regulate the expression of the matrix protein Shematrin-2 during shell formation in P. fucata, improving our understanding of the transcriptional regulation of molluscan shell development at the molecular level.

A vast array of organisms can convert inorganic ions into solid minerals by a process termed biomineralization. Due to their superior performance as materials and their medical benefits, products of biomineralization, such as teeth, bones, oto-liths, spicules, shells, and pearls, have intrigued many investigators from life science, biophysics, and materials science (1). As the understanding of the mechanisms of shell and pearl formation may improve pearl quality or lead to the generation of new medical materials (2, 3), these mechanisms have been extensively studied by researchers in various fields.

The pearl oyster Pinctada fucata, the shell of which is composed of an inner nacre and an outer prismatic layer, is a major seawater pearl shellfish found in the southeast of China. Both nacre and prism are mainly composed of calcium carbonate; less than 5% of their components are organic macromolecules, including matrix proteins, polysaccharides, and lipids (4). Matrix proteins, such as nacrein (5), Pif (6), the KRMP family (7), and the Shematrin family (8), have been proven to be the major components responsible for nucleation, orientation, morphology, and organization during the shell formation process in P. fucata (9). The Shematrin family, one of the two main families that participate in the shell formation process, is highly expressed in the mantle of P. fucata and other pearl oyster species (Pinctada maxima and Pinctada margaritifera) (10, 11), which indicates their significant and conserved roles in shell formation. The Shematrin-2 protein, a member of the Shematrin family, is abundantly expressed at the edge of the mantle and is supposed to function as a framework protein in the formation of the prismatic layer of the shell (8). However, information on the function and transcriptional regulation of Shematrin-2 is limited. The characteristic structural organization of the shell is of interest; although the mechanisms of calcification that matrix proteins participate in have been sought by many investigators, little is known about how the upstream transcription factors regulate the downstream matrix protein genes (12–15).

The C/EBP family consists of six isoforms (α, β, γ, δ, ε, and ζ), and the isoforms are structurally similar, with a highly conserved basic leucine zipper domain at the C terminus, which facilitates dimerization and DNA binding (16, 17). However,
Transcriptional regulation of matrix protein Shematrin-2

C/EBP isoforms are functionally distinct, as their transcriptional activation domains are less well conserved. This difference gives rise to a wide range of responses in which C/EBP isoforms have been implicated, including cell proliferation and differentiation, the immune response, the cell cycle, and cancer development, hematopoiesis, and osteoclast formation (16, 18–23). In mammals, the C/EBP isoforms have been reported to participate in various biological processes, especially in the formation of teeth and bones. Previous studies have shown that C/EBPα can active the transcription of the amelogenin gene, which is essential for enamel biomineralization (24, 25). In addition, C/EBPβ and C/EBPδ participate in bone formation by activating the transcription of osteocalcin (26). Although homologous genes of C/EBP have been identified in some mollusk species (27, 28), their function in biomineralization has not been further explored.

In this study, we expressed and purified the Shematrin-2 protein for the first time and explored its function during shell formation in P. fucata. In addition to the cloning and identification of C/EBP transcription factors, we further explored the upstream transcription mechanisms of Shematrin-2 in P. fucata. Our results provided the foundation for understanding the transcriptional regulation of matrix proteins and shed light on the mechanisms of shell formation at the molecular level.

Results

The distribution, expression, and purification of Shematrin-2

The quantitative PCR results showed that the relative expression level of Shematrin-2 in mantle tissues was maintained at an extremely high level compared with the expression of three other matrix proteins, namely, ACCBP, MIS60, and N19 (Fig. 1A), which was in accordance with the results from transcriptome sequencing (8). The high expression level in mantle tissues indicated the significant role that Shematrin-2 plays during the process of shell formation. However, its function has not been identified yet due to difficulties in protein expression. In our study, a soluble MBP label was added to the N terminus of Shematrin-2 to enhance the amount of soluble protein expressed. The SDS-PAGE results showed that the purified Shematrin-2–MBP recombinant protein and the MBP label protein appeared as clear and distinct bands on the gel (Fig. 1B).

Next, to explore the exact distribution of Shematrin-2 in the shell layers, polyclonal antibodies raised against recombinant Shematrin-2 were used for the immunodetection of native Shematrin-2 in EDTA-soluble matrix and EDTA-insoluble matrix (EISM) extracts of separated nacre and prisms and in the extrapallial fluid. The Western blotting results showed that Shematrin-2 was present in the EISM of the nacreous and prismatic layers of the shell and in the extrapallial fluid (Fig. 1C).

According to previous reports on matrix proteins (29, 30), EISM proteins are conventionally believed to be responsible for the construction of the structural organic framework of the shell and for the physical properties of the shell during calcification. Moreover, the extrapallial fluid is considered the place where shell biomineralization occurs, and some of the matrix proteins play direct regulatory roles in the extrapallial cavity (30–32). Therefore, it is logical to assume that Shematrin-2 may be not only a structural matrix protein but also a regulatory factor affecting crystalline deposition in shell biomineralization.

The effects of Shematrin-2 on shell formation in vitro

To confirm the hypothesis that Shematrin-2 plays an important role in calcium carbonate crystallization, we conducted a series of experiments. First, we investigated the binding properties of Shematrin-2 with the main components of the shell by the crystal-binding assay. As shown in Fig. S1, compared with the soluble MBP-labeled protein, Shematrin-2–MBP recombinant protein was found to be bound to calcite, aragonite, and
The control groups with or without 40 μg/ml precipitation crystals were all typical calcite rhombohedra in the negative control. In the calcite crystallization system, the different concentrations of the Shematrin-2–MBP recombinant protein significantly slowed the process of precipitation 2 min after the reaction, and this effect occurred in a concentration-dependent manner (Fig. 2A).

To further characterize the role that Shematrin-2 plays in the growth of calcium carbonate crystals, we performed calcite and aragonite crystallization experiments in the presence of 10 and 40 μg/ml of Shematrin-2–MBP recombinant protein in vitro; 40 μg/ml of MBP protein was used as a negative control. In the calcite crystallization system, the precipitated crystals were all typical calcite rhombohedra in the control groups with or without 40 μg/ml of MBP (Fig. 2, B and C), and the Raman spectra showed that the crystals were calcite (Fig. 2, F and G). With 10 μg/ml of Shematrin-2–MBP recombinant protein, the morphologies of the deposited crystals were modified: the edges of the crystals were no longer regular, and irregular depositions, similar to the multinucleation site superpositions, were observed (Fig. 2D). As the concentration of the recombinant protein increased, there was increased formation of irregular crystals and a decrease in the number of crystals that formed (Fig. 2E). However, analysis of the Raman spectra showed that in the presence of 10 and 40 μg/ml of Shematrin-2–MBP recombinant protein, the crystals were still calcite (Fig. 2, G and H). These results suggested that the Shematrin-2 protein could regulate the morphology of calcite crystallization rather than crystal formation. In contrast to calcite, the different concentrations of the Shematrin-2–MBP recombinant protein had little effect on aragonite formation (Fig. S2).

The effects of Shematrin-2 on shell formation in vivo

Due to the abundance of Shematrin-2 in the extrapallial fluid, where shell biomineralization occurs, an in vivo antibody inhibition assay was performed to explore the specific physiological role of Shematrin-2 in pearl oysters. The affinity-purified polyclonal antibody against Shematrin-2 (anti-Shematrin-2) was injected into the extrapallial space of P. fucata to disrupt the physiological functions of Shematrin-2. In contrast to the negative group that was treated with preimmune rabbit serum (Fig. 2J), the surface of the prismatic layer in the low-dosage antibody-injected group demonstrated incomplete calcite crystallization with obvious holes (Fig. 2K). An abnormal phenomenon turned out to be more significant in the high-dosage group (Fig. 2L), where the regular polygon and the organic framework disappeared. Interestingly, no significant differences in the nacreous layer were observed after antibody injection, which was in agreement with the results of the aragonite crystallization assay in vitro. These results suggest that the prismatic layer was perturbed to some extent when the function of Shematrin-2 was inhibited, which agrees well with the observed functional experiments in vitro.

Cloning and transcriptional activities of the Shematrin-2, ACCBP, MSI60, and N19 gene promoters

To further explore the regulation mechanism, the promoters of the matrix protein genes Shematrin-2, ACCBP, MSI60, and N19 were cloned and identified (Fig. 3A). The sequences of the promoters of Shematrin-2, ACCBP, MSI60, and N19 were submitted to the GenBankTM database under the accession numbers KM519602, KM519603, KM519604, and KM519605, respectively. The cloned promoter region of Shematrin-2 was a 1616-bp fragment, and intron 1 was 384 bp. The lengths of the ACCBP, MSI60, and N19 promoters that were cloned were 2283, 2233, and 2020 bp, respectively. The intron lengths of MSI60 and N19 were 924 and 1685 bp, respectively.

To detect the transcriptional activities of the above four promoters, the obtained gene promoters were subcloned into the pGL4.10 luciferase vector individually, and then, the combined plasmids were transiently transfected into HEK293T cells. The dual luciferase reporter system results showed that the relative luciferase activity of the Shematrin-2 gene promoter was obviously higher than that of the other three gene promoters and ~100 times higher than that of the pGL4.10 control group (Fig. 3B). The results demonstrate that the promoter of Shematrin-2 has a relatively high transcriptional activity in HEK293T cells, which was consistent with the high expression of Shematrin-2 in mantle tissues (Fig. 1B).

Cloning and phylogenetic analysis of C/EBPs and NF-Y

Through sequence analysis of the Shematrin-2 promoter, a reverse CCAAT box was found in the promoter region; the CCAAT box can be recognized by several transcription factors, such as CCAAT transcription factor/nuclear factor 1 (33–35), CCAAT displacement protein (36, 37), C/EBP (38, 39), and NF-Y (nuclear factor-Y) (40). In mammals, the transcription factors NF-Y and C/EBP activate mineralization-related genes such as Amelogenin and Osteocalcin (24–26). Therefore, the cDNA sequences of Pf-C/EBP-A, Pf-C/EBP-B, Pf-C/EBP-γ, Pf-NF-YA, Pf-NF-YB, and Pf-NF-YC were cloned and encoded 318-, 269-, 128-, 358-, 200-, and 384-bp amino acids, respectively. Detailed information about each sequence is described in the supporting materials (Fig. S3–S8).

To confirm our classification and to further understand the evolutionary position of Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ, a phylogenetic tree was constructed. The results showed that Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ were clustered with their homologs in Crassostrea gigas, which has a close relationship with pearl oyster (Fig. 3C). Similarly, the results of the phylogenetic tree for Pf-NF-YA, Pf-NF-YB, and Pf-NF-YC showed that the three proteins clustered in different branches, and they each clustered with homologs from other species (Fig. 3D). Furthermore, Pf-NF-YA and Pf-NF-YC also clustered with their homologs in C. gigas.

The expression patterns of Pf-C/EBPs and Pf-NF-Ys among different tissues and during embryonic and larval development

The mRNA expression levels of three Pf-C/EBPs members and three Pf-NF-Ys members among seven tissues in pearl oyster were measured by real-time PCR. The results showed that
Transcriptional regulation of matrix protein Shematrin-2

A

![Graph showing absorbance at 570nm over time](image)

- **Control**
- MBP 8μg/ml
- SH-2 2μg/ml
- SH-2 4μg/ml
- SH-2 8μg/ml

Time (min)

0 1 2 3 4 5 6 7

Absorbance at 570nm

0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20

B, C, D, E, F, G, H, I, J, K, L

Micrographs and Raman shift graphs

- B, C, D, E, F, G, H, I, J, K, L

10μm, 100μm, 50μm
Transcriptional regulation of matrix protein Shematrin-2

the tissue distribution patterns of the six genes were totally different (Fig. S9), which suggests that they might be involved in different physiological activities of *P. fucata*. In addition, the relative expression levels of the six genes in mantle tissues were compared, as mantle tissues play a key role in shell formation. The results showed that Pf-C/EBP-A and Pf-C/EBP-B had higher expression levels in mantle tissues (Fig. 3E) than the other four genes, which implied that Pf-C/EBP-A and Pf-C/EBP-B were more likely to participate in the process of shell formation.

The development of embryos and larvae is accompanied by the formation of shells in *P. fucata*; prodissocoench is formed during the early D-shape stage, and the morphology of the juvenile stage is almost the same as that of the adult pearl oyster. Therefore, the genes that showed higher expression in the D-shape stage and juvenile stage were more likely to play prominent roles in the process of shell formation. In this study, the mRNA expression levels of the six transcription factor genes were also determined in the oosperm, trochophere stage, D-shape stage, umbonal stage, and juvenile stage (Fig. S10). The data in Fig. 3F showed the relative expression levels of the six genes in the D-shape stage and juvenile stage. Intriguingly, in the D-shape stage and juvenile stage, the expression levels of Pf-C/EBP-A and Pf-C/EBP-B were significantly higher than those of the other four genes. Altogether, these findings support the hypothesis that transcription factors Pf-C/EBP-A and Pf-C/EBP-B play a role in shell formation.

### Analysis of the regulatory effects of Pf-C/EBP-A and Pf-C/EBP-B on Shematrin-2 in vitro

To further explore whether Pf-C/EBPs and/or Pf-NF-Y participate in the transcriptional regulation of Shematrin-2, co-transfection studies followed by dual-luciferase assays and Western blotting experiments were performed. As shown in Fig. 4, Pf-C/EBP-A and Pf-C/EBP-B significantly enhanced the promoter activities of Shematrin-2, and the activating effects were apparently dose-dependent, whereas Pf-C/EBP-γ had no effects on the promoter activity of the Shematrin-2 gene. Similarly, the three members of Pf-NF-Y, alone or in combination, had no obvious effect on the promoter activity of the Shematrin-2 gene. These results suggest that the transcriptional factors Pf-C/EBP-A and Pf-C/EBP-B considerably enhanced the promoter activities of the Shematrin-2 gene.

### Regulation effect of Pf-C/EBP-A and Pf-C/EBP-B on Shematrin-2 during shell formation in vivo

Shells were slightly notched to investigate the functions and the correlation of Pf-C/EBP-A, Pf-C/EBP-B, and Shematrin-2 during shell formation. As shown in Fig. 5, in the process of shell regeneration, the expression patterns of Pf-C/EBP-A, Pf-C/EBP-B, and Shematrin-2 were extremely consistent; the expression level increased after shell notching, reached its highest point at 24 h after shell injury, and then decreased slowly. In addition, the statistical analysis results showed that the expression of the matrix protein Shematrin-2 was significantly correlated with Pf-C/EBP-A and Pf-C/EBP-B (Table 1). The expression patterns of KRMP and nacrein were in agreement with previous results (Fig. S11) (41), which excluded artificial errors. These results demonstrate that Pf-C/EBP-A and Pf-C/EBP-B may play essential roles in shell regeneration by regulating Shematrin-2.

To further elucidate the regulatory function of Pf-C/EBP-A and Pf-C/EBP-B on Shematrin-2 during shell formation in vivo, we performed a knockdown experiment of Pf-C/EBP-A and Pf-C/EBP-B individually by RNAi. Compared with the group injected with Milli-Q water, the expression levels of Pf-C/EBP-A and Pf-C/EBP-B were decreased by nearly 50% after dsRNA injection (Fig. 6, A and B). No significant variation in the Pf-C/EBP-A and Pf-C/EBP-B expression levels was observed in the negative control group injected with GFP dsRNA. In addition, the expression levels of Shematrin-2 were suppressed by nearly 70 and 80% after the injection of Pf-C/EBP-A and Pf-C/EBP-B dsRNA, respectively. In contrast, the expression levels of Shematrin-2 only slightly decreased in the group injected with GFP dsRNA (Fig. 6, A and B). Altogether, these data support the hypothesis that Pf-C/EBP-A and Pf-C/EBP-B regulate the expression of the matrix protein gene Shematrin-2 in pearl oysters.

The inner surface structures of both the prismatic and nacreous layers of the groups treated with GFP, Pf-C/EBP-A, and Pf-C/EBP-B dsRNA were also observed by SEM. In the GFP dsRNA-injected group, the surfaces of the prismatic and nacreous layers were normal, which was the same result seen in the untreated pearl oysters (Fig. 6, C and D). The normal, sail-like surface of the nacreous layer was not obvious in either the Pf-C/EBP-A dsRNA- or the Pf-C/EBP-B dsRNA-injected groups, and the organic framework was also disrupted (Fig. 6D). The Pf-C/EBP-A dsRNA-injected group showed abnormal formation of the organic framework in the prismatic layer, where the lacuna became larger and some tiny holes appeared. In the Pf-C/EBP-B dsRNA-injected group, the changes in the prismatic layer were more obvious; the organic framework gradually disappeared, and an irregular polygon emerged (Fig. 6C). The effect of knocking down the transcription factors Pf-C/EBP-A and Pf-C/EBP-B on calcite growth during shell mineralization was similar to that of blocking Shematrin-2 in the extrapallial fluid. All the results indicated that Pf-C/EBP-A and Pf-C/EBP-B could control the formation of the organic framework of the prismatic and nacreous layers by regulating Shematrin-2 during shell formation in vivo.

Figure 2. The roles that Shematrin-2 plays in calcium carbonate crystallization in vitro and in vivo. A. inhibitory activity of Shematrin-2 on calcium carbonate precipitation. Changes in the turbidity of the assayed solutions are shown. SH-2, shematrin-2–MBP recombinant protein; O, negative control; ▲, 8 μg/ml of MBP; ◄, 2 μg/ml of Shematrin-2–MBP recombinant protein; △, 4 μg/ml of Shematrin-2–MBP recombinant protein; ■, 8 μg/ml of Shematrin-2–MBP recombinant protein. *p value <0.05. B–E, SEM images of crystals formed in calcite crystallization experiments in the presence of Shematrin-2. Crystals formed in the blank control (B), in the presence of 40 μg/ml of MBP (C), 10 μg/ml of Shematrin-2–MBP (D), and 40 μg/ml of Shematrin-2–MBP (E). Images displayed in the upper right corner of B–E are high-magnification images of the associated particles indicated by white arrows. F–I, Raman spectra of the crystals formed in these experiments. J, SEM image of the inner surface of the preimmune rabbit serum–treated group in the oyster *P. fucata*. K, SEM image of the inner surface of the shell in the high-dosage anti-Shematrin-2–treated group. Scale bars: 100 μm in B–D; 10 μm in the high-magnification images; 50 μm in J and L.
Transcriptional regulation of matrix protein Shematrin-2

A

Shematrin-2

promoter

TSS

ATG

B

N19

-2020

promoter

TSS

ATG

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

Transcriptional regulation of matrix protein Shematrin-2

A

Shematrin-2

promoter

TSS

ATG

B

N19

-2020

promoter

TSS

ATG

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z
Transcriptional regulation of matrix protein Shematrin-2

Figure 3. Basic information of both matrix protein and transcription factor Pf-C/EBPs and Pf-NF-Ys. A, structure diagrams of the promoters and partial genome sequences of the Shematrin-2, ACCBP, MSI60, and N19 genes (GenBank accession numbers KMS19602, KMS19603, KMS19604, and KMS19605, respectively). In 1 is the intron of each gene. TSS represents the transcription start site. B, the relative luciferase activities of the four matrix protein genes (Shematrin-2, ACCBP, MSI60, and N19) in HEK293T cells determined using the dual luciferase assay system 36 h after transfection. Values are the mean ± S.D. of three independent experiments. C, phylogenetic analysis of the P. fucata transcription factors Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ with C/EBP family members from other species. The numbers at the branch nodes indicate the percentage recovery of the branch in 10,000 bootstrap replicates. Pf-C/EBP-A, Pf-C/EBP-B, Pf-C/EBP-γ, Pf-NF-YA, Pf-NF-YB, and Pf-NF-YC are indicated by filled triangles. The species abbreviations that the sequences use are as follows: H. sapiens, Homo sapiens; M. musculus, Mus musculus; G. gallus, Gallus; X. laevis, Xenopus laevis; D. rerio, Danio rerio; X. tropicalis, Xenopus tropicalis; C. gigas, Crassostrea gigas; H. vulgaris, Hydra vulgaris; A. queenslandica, Amphimedon queenslandica. The GenBank accession numbers are provided in the supporting information (Table S2). D, relative expression levels of Pf-NY-YA, Pf-NY-YB, Pf-NY-NC, and Pf-C/EBP-γ mRNA in mantle tissues measured using real-time PCR with β-actin as an internal standard and calculated using the 2^-ΔΔCt method. E, the relative expression levels of Pf-C/EBP-A, Pf-NY-YA, Pf-C/EBP-B, Pf-NY-YB, and Pf-NY-NC mRNA during the D-shape and juvenile stages, as measured by real-time PCR using 18S rRNA as an internal standard and calculated using the 2^-ΔΔCt method. Values are the mean ± S.D. of three samples.

Discussion

According to previous sequence information (GenBank accession no. AB244420.1), the full-length of Shematrin-2 sequence has been acquired, which contains glycin- and tyrosine-rich domains, 41.2% of the amino acids is glycine and 8.1% of which is tyrosine. The 6 repetitive sequence elements are GGGYG, they are arranged in tandem with highly conserved repeats, making Shematrin-2 one of the most repetitive molluscan shell proteins. Although the mechanisms of calcification that matrix proteins participate in have been sought by many investigators, information on the function of Shematrin-2 is limited, as the difficulty in expressing this protein in vitro. Here, we identify the dual roles of Shematrin-2 wherein it acts as a structural matrix protein and as a regulatory factor that affects crystalline deposition. In addition, we demonstrate that the role Shematrin-2 plays during shell formation is regulated by transcription factors Pf-C/EBP-A and Pf-C/EBP-B.

Figure 4. Dose-dependent activation of the Shematrin-2 gene promoter by Pf-C/EBP-A and Pf-C/EBP-B in HEK293T cells. A, dual luciferase assay of cells co-transfected with the promoter of the matrix protein gene Shematrin-2 and pcDNA3.1-myc, Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, or Pf-C/EBP-γ-pcDNA3.1-myc expression vector alone or Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, and Pf-C/EBP-B-pcDNA3.1-myc together. Up to 500 ng of recombinant/empty pcDNA3.1(+) myc expression vector, 100 ng of recombinant/empty pGL4.10 luciferase vector, and 2 ng of pRL-TK were used in each well of a 48-well plate. The cells were lysed and analyzed 36 h after transfection. B and C, detection of the dose effect of Pf-C/EBP-A and Pf-C/EBP-B on the activation of the Shematrin-2 gene promoter. Values are the mean ± S.D. of three independent experiments. D and E, expression of Pf-C/EBP-A and Pf-C/EBP-B at the protein level was verified by Western immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Transcriptional regulation of matrix protein Shematrin-2

Figure 4. Dose-dependent activation of the Shematrin-2 gene promoter by Pf-C/EBP-A and Pf-C/EBP-B in HEK293T cells. A, dual luciferase assay of cells co-transfected with the promoter of the matrix protein gene Shematrin-2 and pcDNA3.1-myc, Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, or Pf-C/EBP-γ-pcDNA3.1-myc expression vector alone or Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, and Pf-C/EBP-B-pcDNA3.1-myc together. Up to 500 ng of recombinant/empty pcDNA3.1(+) myc expression vector, 100 ng of recombinant/empty pGL4.10 luciferase vector, and 2 ng of pRL-TK were used in each well of a 48-well plate. The cells were lysed and analyzed 36 h after transfection. B and C, detection of the dose effect of Pf-C/EBP-A and Pf-C/EBP-B on the activation of the Shematrin-2 gene promoter. Values are the mean ± S.D. of three independent experiments. D and E, expression of Pf-C/EBP-A and Pf-C/EBP-B at the protein level was verified by Western immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

According to previous sequence information (GenBank accession no. AB244420.1), the full-length of Shematrin-2 sequence has been acquired, which contains glycin- and tyrosine-rich domains, 41.2% of the amino acids is glycine and 8.1% of which is tyrosine. The 6 repetitive sequence elements are GGGYG, they are arranged in tandem with highly conserved repeats, making Shematrin-2 one of the most repetitive molluscan shell proteins. Although the mechanisms of calcification that matrix proteins participate in have been sought by many investigators, information on the function of Shematrin-2 is limited, as the difficulty in expressing this protein in vitro. Here, we identify the dual roles of Shematrin-2 wherein it acts as a structural matrix protein and as a regulatory factor that affects crystalline deposition. In addition, we demonstrate that the role Shematrin-2 plays during shell formation is regulated by transcription factors Pf-C/EBP-A and Pf-C/EBP-B.

Figure 4. Dose-dependent activation of the Shematrin-2 gene promoter by Pf-C/EBP-A and Pf-C/EBP-B in HEK293T cells. A, dual luciferase assay of cells co-transfected with the promoter of the matrix protein gene Shematrin-2 and pcDNA3.1-myc, Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, or Pf-C/EBP-γ-pcDNA3.1-myc expression vector alone or Pf-NF-YA-pcDNA3.1-myc, Pf-NF-YB-pcDNA3.1-myc, and Pf-C/EBP-B-pcDNA3.1-myc together. Up to 500 ng of recombinant/empty pcDNA3.1(+) myc expression vector, 100 ng of recombinant/empty pGL4.10 luciferase vector, and 2 ng of pRL-TK were used in each well of a 48-well plate. The cells were lysed and analyzed 36 h after transfection. B and C, detection of the dose effect of Pf-C/EBP-A and Pf-C/EBP-B on the activation of the Shematrin-2 gene promoter. Values are the mean ± S.D. of three independent experiments. D and E, expression of Pf-C/EBP-A and Pf-C/EBP-B at the protein level was verified by Western immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Transcriptional regulation of matrix protein Shematrin-2

Currently, dozens of matrix proteins have been identified, and their roles in mineralization have been characterized. It is well known that multifunctionality is a common characteristic of many shell matrix proteins, for example, MS160 (42), nacrein (5), and Prisilkin-39 (30); we infer that Shematrin-2 shares this characteristic. The extracted matrix proteins can be classified as soluble proteins and insoluble proteins after the decalcification treatment by EDTA. Even though the insoluble matrix proteins were generally thought to be structural proteins that are responsible for the formation of the organic framework, some of these proteins have been reported to participate in the regulation of shell biomineralization (30, 43). Here, we observed that the matrix protein Shematrin-2 is present in the EISM of shell nacreous and prismatic layers and in the extrapallial fluid where shell biomineralization occurs (Fig. 1C), suggesting that Shematrin-2 might act as both a structural protein and regulatory factor. To confirm this hypothesis, the biomineralization function of this protein was tested both in vivo and in vitro. In the calcium carbonate crystallization assay in vitro (Fig. 2B–I), the recombination protein Shematrin-2–MBP influenced the morphology of calcite crystallization, instead of aragonite crystallization, in a concentration-dependent manner, and it significantly inhibited the rate of calcium carbonate precipitation (Fig. 2A). Consistent with the above results, the in vivo observation showed that Shematrin-2 is essential for the integrity of the prismatic layer. Hence, we infer that Shematrin-2 is one of the matrix proteins that acts as both a component and a regulator of calcite growth. In addition, crystal binding experiments (Fig. S1) further revealed that the Shematrin-2 recombinant protein could bind calcite, aragonite, and chitin. Thus, the specific role of Shematrin-2 in aragonite formation needs to be further investigated as the regulation of crystal growth is subtle and complex.

What is the regulatory role of Shematrin-2 during shell formation? Our study first demonstrated that Pf-C/EBP-A and Pf-C/EBP-B, the transcription factor C/EBP homologs, are involved in transcriptional regulation of the matrix protein gene Shematrin-2 in the pearl oyster. In the present study, three members of the C/EBP family, namely, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBPγ, and three subunits of NF-Ys, namely, Pf-NF-Yα, Pf-NF-YB, and Pf-NF-YC, were cloned and characterized. These transcription factors also play important roles in the formation of biomineralization products such as bones or teeth in mammals (24–26). Our results demonstrate that in contrast to the other four transcription factors, Pf-C/EBP-A and Pf-C/EBP-B could bind the promoter of the Shematrin-2 gene to enhance the transcriptional activity, and the activating effects were dose-dependent at both mRNA and protein levels (Fig. 4). These data were in agreement with the significantly higher expression levels of Pf-C/EBP-A and Pf-C/EBP-B genes in the mantle tissue and during the D-shape and juvenile stages of P. fucata development than those of the other four transcription factors (Fig. 3, E and F). Furthermore, RNAi experiments showed that Pf-C/EBP-A or Pf-C/EBP-B knockdown lead to a decrease in Shematrin-2 gene expression (Fig. 6, A and B). These results presented above are direct evidence that Pf-C/EBP-A and Pf-C/EBP-B could regulate the transcriptional expression level of the matrix protein Shematrin-2. In addition, the correlation assays show that Pf-C/EBP-A and Pf-C/EBP-B have close relationships with Shematrin-2 during shell regeneration, which further validates our model of the regulatory mechanism. Intriguingly, the expression pattern of Shematrin-2 in the shell regeneration process of P. fucata was similar to that in the scallop Chlamys farreri (44), which suggests that the role that Shematrin-2 plays in biomineralization among different species might be similar. However, the SEM images of Pf-C/EBP-A and Pf-C/EBP-B knockdown demonstrate that these two transcription factors are related to the formation of the prismatic and nacreous layers. As the C/EBP family members are involved in multiple signaling pathways and can regulate various factors in mammals, for example, NF-κB (45), EAK (46), NF-Y (24), HOX and MEIS (23), we speculate that Pf-C/EBP-A and Pf-C/EBP-B, together with other transcription factors, might synergistically regulate some other matrix proteins, such as Aspein, Prismalin-14, and Pearlin. The Aspein and Prismalin-14 matrix proteins participate in the formation of the prismatic layer, whereas Pearlin functions in the formation of the nacreous layer (47–49). Thus, our experiments demonstrate the pivotal roles of Pf-C/EBP-A and Pf-C/EBP-B in the transcriptional regulation of Shematrin-2 and some other factors, which are critical upstream factors in biomineralization.

Conclusions

In summary, in this study, we report the first systematic exploration of the function and regulatory mechanisms of Shematrin-2. We found that Shematrin-2 has dual roles in shell biomineralization and provide evidence of the important roles that Pf-C/EBP-A and Pf-C/EBP-B play in regulating the tran-
scription of Shematrin-2 in vitro and in vivo. In addition, further investigations are needed to understand the regulatory mechanism of Pf-C/EBP-A and Pf-C/EBP-B and to elucidate the regulatory network among different transcription factors and matrix proteins in *P. fucata*.

**Experimental procedures**

**Ethics statement**

This study was approved by the Animal Ethics Committee of Tsinghua University, China.

**Experimental animals**

The pearl oysters, *P. fucata*, were purchased from the Zhanjiang Pearl Farm (Guangdong Province, China) and maintained in an aerated artificial seawater tank for 5 days prior to experimentation.

**Preparation of polyclonal antibodies against Shematrin-2**

Total RNA samples were extracted from the mantle tissue of *P. fucata* using TRizol (Invitrogen, USA), and RNA quality was determined by agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Scientific, USA). Full-length cDNAs were synthesized using SMART MMLV reverse transcriptase (Clontech, Japan). The open reading frame (ORF) of Shematrin-2 (GenBank accession no. AB244420.1), without the signal peptide coding sequence, was amplified by the primers SH2-PET-F and SH2-PET-R (Table S1) using high-fidelity KOD polymerase (Toyobo, Japan) and then subcloned into the
Transcriptional regulation of matrix protein Shematrin-2

expression vector pET28a. Recombinant Shematrin-2 with a His6 tag at the C terminus was overexpressed in Escherichia coli BL21 (DE3) at 16 °C for 12 h after 0.4 M isopropyl 1-thio-β-d-galactopyranoside induction. The expressed recombinant Shematrin-2 protein was purified using a HisTrap HP column (GE Healthcare, USA) under denaturing conditions (4 M urea) according to the manufacturer's instructions. Polyclonal antibodies against Shematrin-2 were prepared by immunizing New Zealand rabbits following standard immunization procedures and purified using an Antibody Purification Kit (Protein A) (Abcam, UK) according to the manufacturer's instructions.

Detection of Shematrin-2 in shell extracts and extrapallial fluid

EDTA-soluble and EDTA-insoluble matrices from different shell layers were prepared as previously described (50). The extrapallial fluid was extracted as previously described (51) and then concentrated by ultrafiltration (Millipore, 3-kDa cut-off, Germany). The protein concentrations were determined by using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, Germany). The collected soluble recombinant Shematrin-2–MBP tag protein (~50 μg each) were added to 20 mg of chitin (Wako), calcite (Sigma), or aragonite (Alfa Aesar) that had been previously equilibrated with 0.5% ammonium bicarbonate and mixed in a shaker at 4 °C overnight. After removal of the solution by centrifugation, the mixture was washed with Milli-Q water and 200 mM NaCl (20 mM Tris, pH 7.4) three times each, and the supernatant was collected after the third wash. The final insoluble residue was boiled in 30 μl of 2% (w/v) SDS containing 20 (w/v) 2-mercaptoethanol for 10 min along with the supernatant collected above. Each wash or supernatant was subjected to SDS-PAGE on a 10% gel under reducing conditions. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Calcium carbonate precipitation assay

The experiment was examined according to the method of Suzuki et al. (6) with some modifications. Briefly, sample solution (10 μl) was mixed with 100 μl of NaHCO3 (100 mM, pH 8.5). Then, after the addition of 100 μl of CaCl2 (100 mM) to the mixed solution, the formation of CaCO3 precipitates was monitored by recording the changes in the turbidity every 30 s for 6 min based on the absorbance at 570 nm measured using a spectrophotometer (Bio-Rad 680). The protein solution buffer and the same concentration of MBP were chosen as negative controls.

In vitro calcium carbonate crystallization assay

Two types of crystallizing solutions were used to investigate the effect of Shematrin-2 on the morphology and polymorphism of calcium carbonate crystals. For calcite crystallization, saturated calcium bicarbonate solution was prepared as described by Xu et al. (54) with some modifications. CO2 gas was bubbled through a mixture of calcium carbonate (0.1 g/ml) and Milli-Q deionized water for 4 h, the excess solid CaCO3 was removed by filtration of a 0.22-μm filter, and the bubbling of the CO2 gas was maintained for 2 h. For the aragonite crystallization, 50 mM magnesium chloride was added to the above solution. Based on the designed experiments, different concentrations of protein were added to the two different crystal systems on a siliconized glass slide. After a 48-h crystallization in a sealed box, the solution was removed with Milli-Q water washing, and the crystals were air-dried for identification of the induced crystals using Raman spectroscopy analysis and for element analysis of crystals by using an FEI Sirion 2000 SEM equipped with an energy-dispersive X-ray spectroscopy system.

cDNA cloning of the NF-Y and C/EBP transcription factors

To obtain full-length cDNAs of Pf-NF-YA, Pf-NF-YB, Pf-NF-YC, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ, rapid amplification of cDNA ends (RACE) was performed using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Japan) according to the manufacturer’s instructions. Pairs of primers for RACE were designed based on expressed sequence tags from the mantle transcriptome of P. fucata.5 After RACE, the full-length cDNAs of Pf-NF-YA, Pf-NF-YB, Pf-NF-YC, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ were used as templates for the construction of full-length cDNAs.

5 Y. Chen, J. Gao, J. Xie, J. Liang, G. Zheng, L. Xie, and R. Zhang, unpublished data.
EVP-A, Pf-C/EBP-B, and Pf-C/EBP-γ were confirmed using high-fidelity KOD polymerase with the primer pairs YA-CF/YA-CR, YB-CF/YB-CR, YC-CF/YC-CR, CEBPA-CF/CEBPA-CR, CEBPB-CF/CEBPB-CR, or CEBPG-CF/CEBPG-CR, respectively. All primers used are listed in Table S1.

**Sequence analysis and phylogenetic tree construction of NF-Y and C/EBP transcription factors**

The conserved domains of Pf-NF-YA, Pf-NF-YB, Pf-NF-YC, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ were predicted from the deduced amino acid sequences using the online tool SMART (http://smart.embl-heidelberg.de/index2.cgi). The ClustalX program was used to align amino acid sequences. Based on multiple sequence alignments, phylogenetic trees were constructed using the neighbor-joining method in MEGA6 with 10,000 bootstrap replicates.

**Gene expressions of the NF-Y and C/EBP transcription factors during larval development**

The relative gene expression levels of Pf-NF-YA, Pf-NF-YB, Pf-NF-YC, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ were determined by real-time PCR on a StepOnePlus™ Real-time PCR system (ABI, USA). Larval culture and sample collection of five different developmental stages, the oosperm, trophophore stage, D-shape stage, umbonal stage, and juvenile stage, were modeled on a previous study (55). cDNA templates were prepared using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer’s instructions. The real-time PCR was performed using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Japan), and the expression levels were calculated using the 2^(-ΔΔCt) method. The 18S rRNA was used as an internal control (56). The primers used for real-time PCR are listed in Table S1.

**Tissue distributions detected by real-time PCR**

Tissue distributions of Pf-NF-YA, Pf-NF-YB, Pf-NF-YC, Pf-C/EBP-A, Pf-C/EBP-B, and Pf-C/EBP-γ mRNA among seven tissues, the adductor muscle, gonad, vescus, mantle pallial, mantle edge, gill, and foot, which were collected from three healthy pearl oysters, were also detected by real-time PCR. cDNA template preparation and real-time PCR were performed as described above. In addition, gene expression in the mantle tissue of the six transcription factors and four matrix proteins, Shematrin-2, MSI60, ACCBP, and N19, were also detected. GAPDH was used as an internal control. The primers used for real-time PCR are listed in Table S1.

**Cloning of the promoter regions of four matrix protein genes**

Genomic DNA was isolated from the adductor muscle of a pearl oyster using a TIANamp Marine Animals DNA Kit (Tiangen, China), and the quality of the DNA was determined by agarose gel electrophoresis and using a Nanodrop spectrophotometer. To obtain the promoter regions of the Shematrin-2 gene, ACCBP gene, MSI60 gene, and N19 gene, the genome-walking PCR was performed using the Genome Walking Kit as described by the manufacturer (TaKaRa, Japan). Three primers, namely, SH2-GW1R, SH2-GW2R, and SH2-GW3R, for Shematrin-2 gene promoter genome-walking PCR were designed based on the previously reported sequence scaffold 89285.1 (57). Three primers, namely, AC-GW1R, AC-GW2R, and AC-GW3R, for the ACCBP gene promoter genome-walking PCR were designed based on the previously reported sequence scaffold 13073.1 (57). The final sequences of the Shematrin-2 gene and the ACCBP gene promoter were amplified by two pairs of primers, namely, SH2-F and SH2-R, AC-F and AC-R, respectively, and were confirmed using KOD polymerase. Similarly, to obtain the promoter regions of the MSI60 gene and N19 gene, the partial gene promoter was first amplified by a pair of primers, namely, M60-1F and M60-1R, N19-1F and N19-1R, respectively. The primers of the MSI60 gene and N19 gene were designed based on the previously reported sequence scaffold 523.1 and sequence scaffold 16924.1, respectively (57). Then, a pair of primers, namely, M60-NF and M60-NR, was used to amplify the sequence of the first intron region of the MSI60 gene. For N19 gene, based on the sequence of the partial promoter and the first intron region, N19-GW1R, N19-GW2R, and N19-GW3R were designed for the first genome-walking PCR to amplify the longer promoter of the N19 gene. After the first genome-walking PCR, based on the obtained sequence, N19-GW4R, N19-GW5R, and N19-GW6R were designed for the second genome-walking PCR. Finally, two pairs of primers, namely, M60-F and M60-R, N19-F and N19-R, were used to verify the MSI60 gene and the N19 gene promoters by KOD polymerase, respectively. In addition, 5’-RACE was performed to verify the sequence of the first exon of the N19 gene using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Japan) according to the manufacturer’s instructions. All primers used are listed in Table S1. The transcription start sites of the promoters were predicted using the online BDGP software (http://www.fruitfly.org/seq_tools/promoter.html) (58).

**Plasmid construction for cell transfection**

The coding regions of Pf-C/EBP-A and Pf-C/EBP-B were subcloned into the expression vectors pcDNA3.1(+)-myc (Invitrogen) and Pcmv6-AC-GFP (Origene, Rockville, MD) for the expression of myc epitope fusion proteins and GFP fusion proteins, respectively. The full-length promoter regions of the Shematrin-2, ACCBP, MSI60, and N19 genes were subcloned into the pGL4.10 vector (Promega, Madison, WI) using four pairs of primers (SH2-PGL-F/SH2-PGL-R, AC-PGL-F/AC-PGL-R, MSI-PGL-F/MSI-PGL-R, and N19-PGL-F/N19-PGL-R, respectively). All constructs were verified by sequencing. The primers used are listed in Table S1.

**Cell culture and transfection**

The HEK293T cells (China Infrastructure of Cell Line Resources, Beijing, China) used in our experiments were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator. The transfection was conducted in accordance with the manufacturer’s instructions for VigoFect (Vigorous, 6 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site. 6
Transcriptional regulation of matrix protein Shematrin-2

Beijing, China). The detailed procedures were similar to those of our previous study (14).

Dual luciferase assay and Western blotting

Luciferase reporter analysis was performed 36 h after transfection. The transfected cells were collected in passive lysis buffer (Promega) after being rapidly washed with PBS at room temperature. The relative activity of the promoter was detected by using the dual luciferase assay system (Promega) and a Varioskan™ Flash multimode reader (Thermo Scientific). To determine the expression of Pf-C/EBP-A and Pf-C/EBP-B or their truncated forms at the protein level, the transfected cells were lysed by passive lysis buffer 36 h after transfection (Promega), and Western blotting was performed to analyze the cell lysate as described previously (52). Primary mouse anti-myc and secondary peroxidase-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Shell notching

Shell-notching assays were performed as described by Mount (59), with some modifications. A V-shaped notch was cut on the shell margin without disturbing the mantle tissue, so that the prismatic layer and the margin of the nacreous layer were damaged. Animals were divided into eight groups with five animals each and sacrificed at 0, 6, 12, 24, 36, 48, 72, or 96 h after notching. Mantle tissues of the same group with areas of ~0.5 cm² from the notch were pooled and stored in liquid nitrogen. Mantle tissues from five oysters without shell notching were collected in the same way and used as controls. The experiment was repeated three times.

Silencing of the Pf-C/EBP transcription factors

The RNAi assay in vivo was conducted as described by Suzuki et al. (6), with some modifications. The synthesis and purification of the Pf-C/EBP-A, Pf-C/EBP-B, and GFP dsRNAs were performed using a T7 RiboMAX™ Express RNAi System Kit (Promega) according to the manufacturer’s instructions. For GFP dsRNA synthesis, the vector pEGFP-C1 (Clontech) was used as the template. The primers used above are listed in Table S1. The dsRNA was diluted to 80 µg/200 µl using Milli-Q water (Merck Millipore, Billerica, MA). The dsRNA, together with PBS and GFP, was then injected into the adductor muscle of 2-year-old individuals with a shell length of 5–6 cm. Each treatment group contained nine individuals. Two days after treatment, total RNA samples were extracted from the mantle tissue. Preparation of the cDNA templates and determination of relative gene expression levels by real-time PCR were performed as previously described (14).

In vivo antibody inhibition assay

The purified antibody against Shematrin-2 (anti-Shematrin-2) was injected into the extrapallial space through the zone of the mantle tissue outside the pallial line at 0.5 µg (low dosage) and 1.5 µg (high dosage) per gram of wet weight per day. Each group contained 5 specimens, which were sacrificed 5 days after antibody injection. The preimmune rabbit serum of the prismatic layer and the margin of the nacreous layer were damaged. Animals were divided into eight groups with five animals each and sacrificed at 0, 6, 12, 24, 36, 48, 72, or 96 h after notching. Mantle tissues of the same group with areas of ~0.5 cm² from the notch were pooled and stored in liquid nitrogen. Mantle tissues from five oysters without shell notching were collected in the same way and used as controls. The experiment was repeated three times.

Statistical analysis

Statistical Package for the Social Sciences version 18.0 software (SPSS Inc., Chicago, IL) was used for the statistical analysis. Values are the mean ± S.D. of three independent experiments and were analyzed by Student’s t test to identify the differences between groups. A p value <0.05 was considered statistically significant. Spearman’s rank correlation was used to analyze the correlations between Pf-C/EBP-A, Pf-C/EBP-B, and Shematrin-2 during shell notching. p values <0.05 were considered statistically significant.

Author contributions—Y. C. and J. G. data curation; Y. C. and J. G. formal analysis; Y. C. writing—original draft; J. X. software; J. X. methodology; J. L. and G. Z. validation; J. L. project administration; G. Z., L. X., and R. Z. supervision; L. X. and R. Z. resources; R. Z. funding acquisition.

References

1. Arias, J. L., and Fernández, M. S. (2008) Polysaccharides and proteoglycans in calcium carbonate-based biomineralization. Chem. Rev. 108, 4475–4482 CrossRef Medline
2. Launspach, M., Gries, K. I., Heinemann, F., Hübner, A., Fritz, M., and Radmacher, M. (2014) Mapping nanomechanical properties of freshly grown, native, interlamellar organic sheets on flat pearl nacre. Acta Biomater. 10, 3986–3996 CrossRef Medline
3. Flausse, A., Henriponnet, C., Dossot, M., Dumas, D., Hupont, S., Pinzano, A., Mainard, D., Galois, L., Magdalou, J., Lopez, E., Gillet, P., and Rousseau, M. (2013) Osteogenic differentiation of human bone marrow mesenchymal stem cells in hydrogel containing nacre powder. J. Biomed. Mater. Res. A 101, 3211–3218 Medline
4. Lowenstam, H. A., and Weiner, S. eds (1989) On Biomineralization, Oxford University Press, New York
5. Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T., and Matsushiro, A. (1996) A carbonic anhydrase from the nacreous layer in oyster pearls. Proc. Natl. Acad. Sci. U.S.A. 93, 9657–9660 CrossRef Medline
6. Suzuki, M., Saruwatari, K., Kagure, T., Yamamoto, Y., Nishimura, T., Kato, N., and Nagasawa, H. (2009) An acidic matrix protein, Pif, is a key macromolecule for nacre formation. Science 325, 1388–1390 CrossRef Medline
7. Zhang, C., Xie, L., Huang, J., Liu, X., and Zhang, R. (2006) A novel matrix protein family participating in the prismatic layer framework formation of pearl oyster, Pinctada fucata. Biochim. Biophys. Res. Commun. 344, 733–740 CrossRef Medline
8. Yano, M., Nagai, K., Morimoto, K., and Miyamoto, H. (2006) Shematrin: a family of glycine-rich structural proteins in the shell of the pearl oyster Pinctada fucata. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 144, 254–262 CrossRef Medline
9. Marin, F., Luquet, G., Marie, B., and Medakovic, D. (2008) Molluscan shell proteins: primary structure, origin, and evolution. Curr. Top. Dev. Biol. 80, 209–276 Medline
10. Jackson, D. J., McDougall, C., Woodcock, B., Moase, P., Rose, R. A., Kube, M., Reinhardt, R., Rokhsar, D. S., Montagnani, C., Joubert, C., Piqueu, D., and Degnan, B. M. (2010) Parallel evolution of nacre building gene sets in molluscs. Mol. Biol. Evol. 27, 591–608 CrossRef Medline
11. Kinosita, S., Wang, N., Inoue, H., Maeyama, K., Okamoto, K., Nagai, K., Kondo, H., Hirono, I., Asakawa, S., and Watabe, S. (2011) Deep sequencing of ESTs from nacreous and prismatic layer producing tissues and a screen for novel shell formation-related genes in the pearl oyster. PLoS ONE 6, e21238 CrossRef Medline
layer formation from the oyster Pinctada fucata. J. Biol. Chem. 284, 10841–10854 CrossRef Medline

31. Xie, J., Liang, J., Sun, J., Gao, J., Zhang, S., Liu, Y., Xie, L., and Zhang, R. (2016) Influence of the extrapallial fluid of Pinctada fucata on the crystallization of calcium carbonate and shell mineralization. Crystal Growth Design 16, 672–680 CrossRef

32. Fitz, M., Belcher, A. M., Radmacher, M., Walters, D. A., Hansma, P. K., Stucky, G. D., Morse, D. E., and Mann, S. (1994) Flat pearls from biofabrication of organized composites on inorganic substrates. Nature 371, 49–51 CrossRef

33. Jones factors bind to the HSV thymidine kinase promoter in vitro. Cell 42, 559–572 CrossRef Medline

34. Zorbas, H., Reis, T., Krause, A., Hoffmann, K., and Winnacker, E. L. (1992) Nuclear factor I (NF-I) binds to an NF-I-type site but not to the CCAAT site in the human α-globin gene promoter. J. Biol. Chem. 267, 8478–8484 Medline

35. Osada, S., Daimon, S., Nishihara, T., and Imagawa, M. (1996) Identification of DNA binding-site preferences for nuclear factor 1-A. FEBS Lett. 390, 44–46 CrossRef Medline

36. Barberis, A., Superti-Furga, G., and Busslinger, M. (1987) Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. Cell 50, 347–359 CrossRef Medline

37. Superti-Furga, G., Barberis, A., Schaffner, G., and Busslinger, M. (1988) The −117 mutation in Greek HFPH affects the binding of three nuclear factors to the CCAAT region of the γ-globin gene. EMBO J. 7, 3099–3107 CrossRef Medline

38. Mischoulon, D., Rana, B., Bucher, N. L., and Farmer, S. R. (1992) Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBPα) gene expression during hepatocyte proliferation in the regenerating liver and in culture. Mol. Cell. Biol. 12, 2553–2560 CrossRef Medline

39. Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. (1996) DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J. Biol. Chem. 271, 3891–3896 CrossRef Medline

40. Dorn, A., Bollekins, J., Staub, A., Benoist, C., and Mathis, D. (1987) A multiplicity of CCAAT box-binding proteins. Cell 50, 863–872 CrossRef Medline

41. Pan, C., Fang, D., Xu, G., Liang, J., Zhang, G., Wang, H., Xie, L., and Zhang, R. (2014) A novel acidic matrix protein, PN44, stabilizes magnesium calcite to inhibit the crystallization of aragonite. J. Biol. Chem. 289, 2776–2787 CrossRef Medline

42. Sudo, S., Fujikawa, T., Nagakura, T., and Ohkubo, T. (1997) Structures of mollusc shell framework proteins. Nature 387, 563 CrossRef Medline

43. Liang, J., Xu, G., Xie, J., Lee, I., Xiang, L., Wang, H., Zhang, W., Xie, L., and Zhang, R. (2015) Dual roles of the lysine-rich matrix protein (KRMP)-3 in shell formation of pearl oyster, Pinctada fucata. PLoS ONE 10, e0131711 CrossRef Medline

44. Lin, Y., Jia, G., Xu, G., Su, J., Xie, L., Xu, H., and Zhang, R. (2014) Cloning and characterization of the shell matrix protein Shematrin in scallop Chlamys farreri. Acta Biochim. Biophys. Sin. 46, 709–719 CrossRef Medline

45. Pulido-Salgado, M., Vidal-Taboada, J. M., and Saura, J. (2015) C/EBPβ and C/EBPβ transcription factors: basic biology and roles in the CNS. Prog. Neurobiol. 132, 1–33 CrossRef Medline

46. Wen, C. L., Teng, C. L., Chiang, C. H., Chang, C. C., Hwang, W. L., Kuo, C. L., and Hsu, S. L. (2012) Methanol extract of Antrodia cinnamomea mycelia induces phenotypic and functional differentiation of HL60 into monocyte-like cells via an ERK/C/EBPβ signaling pathway. Phytomedicine 19, 424–435 CrossRef Medline

47. Takeuchi, T., Sarashina, I., Iijima, M., and Endo, K. (2008) In vitro regulation of CaCO3 crystal polymorphism by the highly acidic mollusk shell protein Aspein. FEBS Lett. 582, 591–596 CrossRef Medline

48. Suzuki, M., and Nagasawa, H. (2007) The structure-function relationship analysis of Prismalin-14 from the prismatic layer of the Japanese pearl oyster, Pinctada fucata. FEBS J. 274, 5158–5166 CrossRef Medline

49. Miyashita, T., Takagi, R., Okushima, S., Nakano, S., Miyamoto, H., Nishikawa, E., and Matsuhiro, A. (2000) Complementary DNA cloning and
Transcriptional regulation of matrix protein Shematrin-2

characterization of pearlin, a new class of matrix protein in the nacreous layer of oyster pearls. *Mar. Biotechnol. (NY)* **2**, 409–418

Fang, D., Pan, C., Lin, H., Lin, Y., Zhang, G., Wang, H., He, M., Xie, L., and Zhang, R. (2012) Novel basic protein, P1N23, functions as key macromolecule during nacre formation. *J. Biol. Chem.* **287**, 15776–15785

Ma, Z., Huang, J., Sun, J., Wang, G., Li, C., Xie, L., and Zhang, R. (2007) A novel extrapallial fluid protein controls the morphology of nacre lamellae in the pearl oyster, *Pinctada fucata*. *J. Biol. Chem.* **282**, 23253–23263

Hsu, Y. C., Liao, W. C., Kao, C. Y., and Chiu, I. M. (2010) Regulation of FGF1 gene promoter through transcription factor RFX1. *J. Biol. Chem.* **285**, 13885–13895

Inoue, H., Ohira, T., Ozaki, N., and Nagasawa, H. (2004) A novel calcium-binding peptide from the cuticle of the crayfish, *Procambarus clarkii*. *Biochem. Biophys. Res. Commun.* **318**, 649–654

Xu, G., Aksay, I. A., and Groves, J. T. (2001) Continuous crystalline carbonate apatite thin films: a biomimetic approach. *J. Am. Chem. Soc.* **123**, 2196–2203

Liu, J., Yang, D., Liu, S., Li, S., Xu, G., Zheng, G., Xie, L., and Zhang, R. (2015) Microarray: a global analysis of biomineralization-related gene expression profiles during larval development in the pearl oyster, *Pinctada fucata*. *BMC Genomics* **16**, 325

Miyazaki, Y., Nishida, T., Aoki, H., and Samata, T. (2010) Expression of genes responsible for biomineralization of *Pinctada fucata* during development. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **155**, 241–248

Takeuchi, T., Kawashima, T., Koyanagi, R., Gyoja, F., Tanaka, M., Ikuta, T., Shoguchi, E., Fujiwara, M., Shinzato, C., Hisata, K., Fujie, M., Usami, T., Nagai, K., Maeyama, K., Okamoto, K., et al. (2012) Draft genome of the pearl oyster *Pinctada fucata*: a platform for understanding bivalve biology. *DNA Res.* **19**, 117–130

Reese, M. G. (2001) Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput. Chem.* **26**, 51–56

Mount, A. S., Wheeler, A. P., Paradkar, R. P., and Snider, D. (2004) Hemocyte-mediated shell mineralization in the eastern oyster. *Science* **304**, 297–300