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Molecular characterization of transcription factor CREB3L2 and CREB3L3 and their role in melanogenesis in Pacific oysters (*Crassostrea gigas*)

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ABSTRACT

Colorful shells in mollusks are commonly attributable to the presence of biological pigments. In Pacific oysters, the inheritance patterns of several shell colors have been investigated, but little is known about the molecular mechanisms of melanogenesis and pigmentation. cAMP-response element binding proteins (CREB) are important transcription factors in the cAMP-mediated melanogenesis pathway. In this study, we characterized two *CREB* genes (*CREB3L2* and *CREB3L3*) from Pacific oysters. Both of them contained a conserved DNA-binding and dimerization domain (a basic-leucine zipper domain). *CREB3L2* and *CREB3L3* were expressed highly in the mantle tissues and exhibited higher expression levels in the black-shell oyster than in the white. Masson-Fontana melanin staining and immunofluorescence analysis showed that the location of CREB3L2 protein was generally consistent with the distribution of melanin in oyster edge mantle. Dual-luciferase reporter assays revealed that CREB3L2 and CREB3L3 could activate the *microphthalmia-associated transcription factor* (*MITF*) promoter and this process was regulated by the level of cAMP. Additionally, we found that cAMP regulated melanogenic gene expression through the CREB-MITF-TYR axis. These results implied that *CREB3L2* and *CREB3L3* play important roles in melanin synthesis and pigmentation in Pacific oysters.

1. Introduction

Body color polymorphism exists widely in almost all animal groups in nature, and plays an important role in camouflage, thermoregulation, light protection, social interaction, and environmental adaptation (Andrade et al., 2019; Gong et al., 2012; Hu et al., 2020; Slominski et al., 2004). The difference in pigment content and distribution is an important reason for the color polymorphisms in animals (Williams, 2017). As one of the most widely distributed pigments in nature, melanin is a crucial material basis for the formation of body color, and its synthesis is under complex regulatory control by multiple agents interacting *via* numerous pathways (Slominski et al., 2004). The regulatory pathways of melanin synthesis in mammals and teleosts are conserved and are well understood. There are four key regulatory pathways, SCF/KIT, Wnt/β-catenin, MAPK, and cAMP, that regulate melanocyte differentiation, development, and maturation in fish (Cal et al., 2017; Luo et al., 2021). In mammals, melanin synthesis is regulated by multiple pathways, including cAMP, Wnt, melanogenesis, and tyrosine metabolism signaling pathways (Wan et al., 2011).

cAMP-response element binding proteins (CREB) play an important role in the cAMP-mediated melanogenesis pathway (Arora et al., 2021). In that process, cAMP serves as a key messenger in regulating the expression of *microphtalmia-associated transcription factor* (*MITF*) and melanin synthesis by activating CREB (Stolnitz, 2002). *MITF* is a key gene in the melanogenesis signaling pathways, responsible for aggregating upstream pathway information (Qiu et al., 2022). The increase in *MITF* expression up-regulates tyrosinase expression and melanin synthesis (Bertolotto et al., 2001). It was reported that a high level of intracellular cAMP could upregulate melanin synthesis in melanocytes (Bertolotto et al., 2001). The inhibition of CREB downregulates the expression of MITF and TYR-related proteins (Chiang et al., 2014). These studies therefore proposed a cAMP regulated melanogenesis pathway. However, while the function of CREB has been well understood in vertebrate pigmentation (Bertolotto et al., 2001; Park et al., 2006), research on the role of CREB in melanogenesis in invertebrates, including mollusks, is sparse.

Pacific oysters (*Crassostrea gigas*) are the most widely cultured and high-yield economic shellfish in the world (Jiang et al., 2024a), and during the last few decades, many researchers have made significant efforts toward the genetic improvement of commercially important

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traits in Pacific oysters (Langdon et al., 2003; Li et al., 2011; Jiang et al., 2013; De Melo et al., 2018). Some strains with fast growth and stable shell colors (white, black, gold, and orange) have been developed through selective breeding (Ge et al., 2016; Xing et al., 2018; Xu et al., 2019). These strains provide valuable materials for the research on the molecular mechanism of oyster pigmentation. Several functional genes involved in melanin synthesis have been identified in Pacific oysters, including *MITF*, *Tyrosinase* (*TYR*), *Tyrosinase related-protein* (*TYRP*), *B* (0,+)*-type amino acid transporter 1* (*B-aat1*), *Cystathionine beta-synthase* (*Cbs*), and *paired-box 7* (*Pax 7*) (Zhu et al., 2022; Li et al., 2023a, 2023b). However, these studies only focus on single gene function, and the molecular mechanism of pigmentation in oysters is still poorly understood. In our previous study, a higher level of cAMP was found in the black shell oyster mantle than in the white (Jiang et al., 2024b). Supporting this, the cAMP pathway was significantly enriched in the black *vs.* white shell oyster transcriptome (Jiang et al., 2024b). These results suggest that the cAMP-CREB axis may be related to melanogenesis in oysters.

In this study, we identified two *CREB* genes (*CREB3L2* and *CREB3L3*) from Pacific oysters and characterized their expression patterns in different tissues and different shell color strains. We analyzed the role of CREB3L2 and CREB3L3 in activating the *MITF* promoter, and the regulation of cAMP in this process. Additionally, the regulation pattern of cAMP on the CREB-MITF-TYR melanogenesis pathway was investigated. This study provided novel insights into the molecular basis for melanin synthesis and pigmentation in Pacific oysters.

2. Materials and methods

2.1. Experimental animals

Black, white, and normal shell color strains were collected from a farm in Rongcheng, Shandong Province, China (Fig. 1). These are varieties with stable shell color after multiple generations of selection. As shown in Fig. 1, the oyster mantle exhibited consistency with the shell color. All oysters were acclimated in seawater (32 ppt) at 18 ± 1 °C and pH 8.0 ± 0.1 for one week.

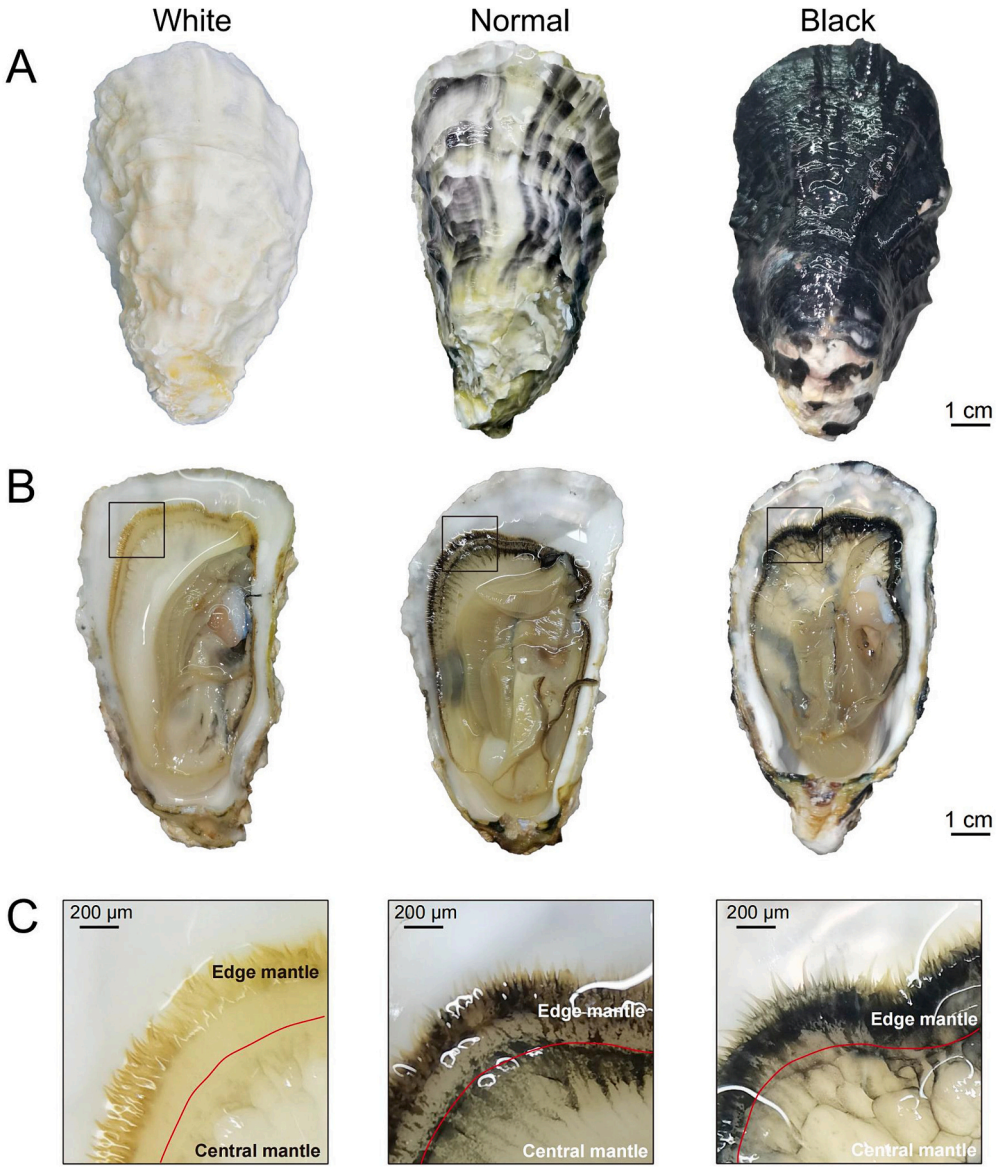

Fig. 1. Photographic illustration of white, normal, and black shell strains of Pacific oysters. (A) Three oyster strains with different shell colors. (B) The anatomy of three oyster strains. (C) The mantle structure and regions of Pacific oysters. Oyster mantle could be divided into two regions (edge mantle and central mantle) according to their position on the shell.

2.2. Gene identification and sequence analysis

CREB3L2 and *CREB3L3* gene sequences were obtained from the Pacific oyster genome (NCBI accession: GCF_902806645.1). Gene structure and position information were obtained from NCBI and presented using GSDS2.0 (https://gsds.gao-lab.org/Gsds_help.php) and MG2C_v2.1 (http://mg2c.iask.in/mg2c_v2.1/), respectively. The full length of *CREB3L2* and *CREB3L3* was amplified and sequenced. Multiple sequence alignment was performed using Clustal X 1.83 software and displayed with ESPript 3.0. The conserved domains and tertiary structures of CREB3L2 and CREB3L3 were predicted using SMART and SWISS-MODEL. Phylogenetic trees based on the protein sequences of CREB3L2 and CREB3L2 from *C. gigas* and other species were constructed using the neighbor-joining (NJ) method. The accession number of those proteins is presented in Table S1.

2.3. Gene expression analysis

RNA-seq data generated from Pacific oyster different tissues and black and white shell oyster strains (Zhu et al., unpublished data) were used to analyze the expression patterns of *CREB3L2* and *CREB3L3*. Briefly, ten tissues from normal shell color strain were collected, including adductor muscle (Amu), digestive gland (Dgl), female gonad (Fgo), male gonad (Mgo), gill (Gil), hemolymph (Hem), labial palp (Lpa), hepatopancreas (Pan), left mantle (Man1), and right mantle (Man2). The left shell of oysters commonly attaches with the surface of rocks or other individuals (Zhang et al., 2022). According to their position in the shell, the oyster mantle was divided into two parts, the edge mantle and central mantle (Fig. 1C). To compare the difference between the white shell oyster and the black, their edge mantle and central mantle were collected. All the collected tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using an RNA-easy Isolation Reagent (Vazyme, China) according to the manufacturer's protocol. The RNA-seq analysis was performed as described previously (Li et al., 2022a).

The edge mantle from three shell color strains, white (W), normal (N), and black (B) shell strains, was used to analyze the gene expression by Quantitative Real-time PCR (qPCR). cDNA was synthesized using an Evo M-MLV RT Kit (Accurate Biology, China). qPCR primers used in this study are shown in Table S2. qPCR was performed on a LightCycler 480 instrument (Roche, USA) using SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China). *Elongation factor Iα* (*EF-Iα*) was used as an internal control due to its stable expression in Pacific oysters (Feng et al., 2019). The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.4. Masson-Fontana staining

A Masson-Fontana staining kit (Cat. No. DJ0021, Leagene, China) was used to perform the Masson-Fontana Staining assay. The collected edge mantle tissues were fixed in a 10% Neutral Formalin Fix Solution (Sangon Biotech, China). The mantle tissue was dehydrated through an ethanol gradient (75%, 85, 95%, and 100%), embedded in paraffin, and then sliced into 7 mm sections. After the dewaxing process, the sections were stained with Fontana ammoniac silver solution and incubated at 56 °C for 40 min. The sections were washed with water five times and then placed in Hypo solution for five min. Finally, the sections were counterstained with the neutral red solution (0.5%) for three min and were examined with a light microscope.

2.5. Immunofluorescence

The edge mantle was fixed in paraformaldehyde (4%) for 24 h, dehydrated *via* an ethanol gradient, and embedded in paraffin. The sections (7 mm) were incubated with Improved Citrate Antigen Retrieval Solution (Beyotime, China) at 100 °C for 15 min, and blocked with 5% Bovine Serum Albumin (BSA) (Servicebio, China) for one hour. After washing, the sections were incubated with CREB3L2 rabbit pAb (ABclonal, China) for 12 h. The negative control was incubated with unimmunized rabbit serum. The positive control was incubated with TYR pAb (Li et al., 2023a). The sections were then incubated with Cy3-conjugated goat anti-rabbit IgG (Sangon Biotech, China) and 4′,6-diamidino-2-phenylindole (DAPI) (Sangon Biotech, China), respectively. Finally, fluorescence was detected under confocal microscopy (TCS SP98, Leica, Germany).

2.6. Subcellular location

To identify the distribution of CREB3L2 and CREB3L2 in cells, we performed a subcellular localization assay. CREB3L2 and CREB3L3 sequence from oyster was subcloned into pEGFP-N1 plasmid. Primers used in this experiment are presented in Table S2. The constructed plasmid was identified by sequencing. Then the plasmids were extracted using an endotoxin-free plasmid extraction kit (Omega, USA). As oyster cells cannot be subcultured *in vitro*, we used HEK-293 cells in this experiment. HEK-293 cells were cultured with Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA) containing 10% fetal bovine serum (Hyclone, USA) and 1% Penicillin-Streptomycin solution at 37 °C with 5% CO_2. Before the experiment, HEK293T cells were plated in a confocal dish (Leica, USA). When the HEK293T cells had grown to 70% confluence, pEGFP-CREB3L2 and pEGFP-CREB3L3 were transfected into cells using Lipofectamine 3000 (Invitrogen, USA) for 48 h. Then cells were stained with DAPI for 15 min and the image capture was performed using confocal microscopy.

2.7. Dual-luciferase reporter assays

A dual-luciferase reporter assay was performed to identify the activity of CREB3L2 and CREB3L3 in activating *MITF* promoters. Eight CREB binding sites were predicted upstream of *MITF* (−1930/+ 23) by JASPER software. Based on the position of those predicted binding sites (−167/+23, −389/+23, −1004/+23, −1229/+23, −1435/+23, −1829/+23, −1930/+23), seven truncated promoter regions of *MITF* were subcloned into a pGL3-Basic vector. *CREB3L2* and *CREB3L3* sequences were subcloned into pcDNA3.1(+) vector. Primers used in this experiment are presented in Table S2. HEK-293 cells were pre-cultured in 24-well plates. When the cell confluence reached 80%, plasmid combinations containing the constructed pcDNA3.1(+) vectors, pRL-TK vector, and the constructed pGL3 vectors were co-transfected into HEK-293 cells for 48 h. Luciferase activity was detected using a dual-luciferase reporter assay system (Promega, Madison, USA) and a multifunctional microplate reader Synergy™ H1 (BioTek, USA).

Forskolin (MCE, USA) is an inducer of cAMP. Rp-cAMPS (MCE, USA) is a competitive antagonist of cAMP. To detect the influence of cAMP level on CREB3L2 and CREB3L3 activation of the *MITF* promoter, cAMP (MCE, USA), forskolin, and Rp-cAMPS were used to treat HEK-293 cells after transfection. Briefly, after transfected with plasmid combinations for 24 h, two concentrations of cAMP (50 and 500 μM), forskolin (50 and 200 μM), and Rp-cAMPS (5 and 20 μM) were independently added into the culture medium and incubated for 24 h. Then the luciferase activity was detected and analyzed as above.

2.8. Mantle cell culture and treatment

To determine the crosstalk among cAMP, CREB3L2, CREB3L3, and melanogenic genes, oyster mantle cell culture and treatment assays were conducted. The oysters used in this study were two years old with black shells. All oysters were cleaned and sustained with filtered seawater for seven days. Before dissecting, oysters were maintained in filtered seawater containing an antibiotic combination of Penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (50 μg/mL) under a UV lamp for two hours. The oyster mantles were collected, then washed with PBS

six times, and washed with primary medium (M199 and L15 medium in a 1: 1 volume ratio) (Gibco, USA). Then the mantles were minced using sterile scissors and cultured with the primary medium supplemented with 5% fetal bovine serum in 12-well plates at 27 °C. After culturing for 48 h, gradient concentrations of cAMP (5, 10, 50, 100, and 500 μm), forskolin (2, 10, 50, 100, and 500 μm), and Rp-cAMPS (0.5, 1, 5, 10, and 50 μm) were respectively added and incubated cells for 16 h. The gradient concentrations of cAMP, forskolin, and Rp-cAMPS were set according to the manufacturer's instructions, respectively. Finally, the cultured cells were collected and washed with PBS, then frozen in liquid nitrogen, and stored at −80 °C. qPCR was performed as described above.

2.9. Statistical analysis

SPSS 20.0 software (IBM, USA) was used for statistical analysis. Data normality (Kolmogorov-Smirnov's test) and homogeneity of variance (Levene's test) were both examined and confirmed. One-way analysis of variance (ANOVA) and Tukey multiple comparison tests were used to determine differences. $P < 0.05$ was considered to be statistically significant. All experiments were carried out in at least triplicate. All the data were expressed as the mean ± SD.

3. Results and discussion

3.1. Sequence and phylogenetic analysis

Several signaling pathways are known to regulate melanogenesis (Lee et al., 2018). CREB is a crucial transcription factor in the cAMP-mediated melanogenesis signal pathway (Arora et al., 2021). In this study, we identified two *CREB* genes (*CREB3L2* and *CREB3L3*) from Pacific oysters. *CREB3L2* and *CREB3L3*, known as CREB-related proteins, are two members of the CREB family (De Cesare et al., 1999). *CREB3L2* is located on chromosome 5 and contains six introns and seven exons (Fig. 2A-B). *CREB3L3* is located on chromosome 3 and contains five introns and six exons (Fig. 2A-B). The full open reading frame (ORF) of CREB3L2 was 1260 bp encoding a 420 amino acid (aa) protein. The full ORF of CREB3L3 was 1674 bp encoding a 558 aa protein. The CREB3L2 and CREB3L3 amino acid sequences were aligned with their

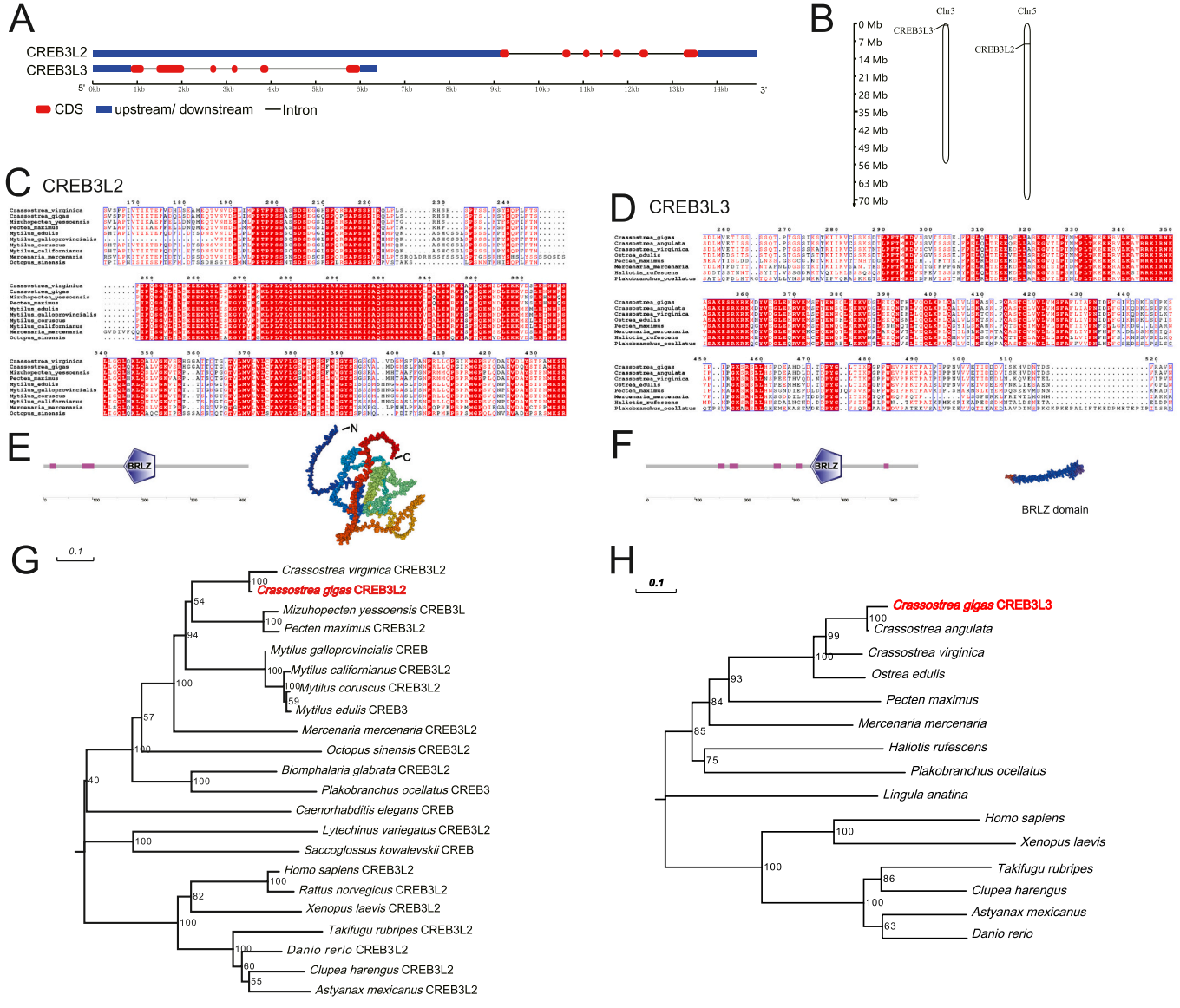

Fig. 2. Gene structure, multiple alignment, and phylogenetic analysis. (A) Gene structure of *CREB3L2* and *CREB3L3*. (B) The position of *CREB3L2* and *CREB3L3* on chromosomes. (C-D) Alignment of the amino acid sequences of CREB3L2 and CREB3L3 with CREB from other mollusks. (E-F) The conserved domains and tertiary structure of CREB3L2 and CREB3L3. Oyster CREB3L2 and CREB3L3 contained a BRLZ domain. (G-H) Phylogenetic tree of CREB3L2 and CREB3L3. The accession numbers of these proteins are shown in Table S1. The neighbor-joining phylogenetic trees were constructed using MEGA 10.0 with a 1000 boot-strap test.

counterparts in mollusks (Fig. 2C-D). CREB3L2 was highly homologous with CREB3L2 from other mollusks. CREB3L2 and CREB3L3 contained a basic-leucine zipper (bZIP) domain (Fig. 2E-F) that is a DNA-binding and dimerization domain (Qiu et al., 2022).

The neighbor-joining phylogenetic trees were constructed with the CREB3L2 and CREB3L3 protein sequences from Pacific oysters and from other species by MEGA 10.0 with a 1000 boot-strap test (Fig. 2G-H). CREB3L2 proteins were independently divided into two branches, vertebrates and mollusks. It firstly clustered with *C. virginica*, *Mizuhopecten yessoensis*, and *Pecten maximus*, and then with other mollusks, like *Mytilus galloprovincialis*, *M. californianus*, *Mercenaria mercenaria*, *Octopus sinensis, Biomphalaria glabrata, etc* (Fig. 2G). Similar to CREB3L2, CREB3L3 proteins also were divided into two branches (Fig. 2H). It first clustered with other oysters, including *C. angulata, C. virginica,* and *Ostrea edulis,* then clustered with other mollusks, like *P. maximus, M. mercenaria, Haliotis rufescens,* and *Plakobranchus ocellatus*. The phylogenetic analyses showed that the phylogenetic relationships of CREB3L2 and CREB3L3 were generally consistent with the traditional taxonomy.

3.2. Expression and distribution characteristics

Using transcriptomic data, we analyzed the expression of *CREB3L2* and *CREB3L3* in different tissues and two shell color strains. *CREB3L2* expression was highly expressed in the left mantle (3.8-fold), right mantle (3.0-fold), and digestive gland (3.1-fold) relative to hemolymph. *CREB3L3* was highly expressed in hemolymph (12.0-fold), left mantle (7.6-fold), labial palp (7.0-fold), and gill (5.8-fold) relative to hepatopancreas. Generally, *CREB3L2* and *CREB3L3* were widely expressed in different tissues indicating their potential role in many physiological processes (De Cesare et al., 1999). The mantle of mollusks is considered responsible for shell formation and shell pigmentation (Saenko and Schilthuizen, 2021). Here, we found that *CREB3L2* and *CREB3L3* expression was relatively higher in the mantle tissues (Fig. 3A). Compared with white shell oysters, both *CREB3L2* and *CREB3L3* were highly expressed in the central mantle (BC) and edge mantle (BE) of black shell oyster (Fig. 3B).

Quantitative PCR was performed to detect mRNA expression of *CREB3L2*, *CREB3L3*, *MITF*, and *TYR* in the edge mantle of different shell color strains. In this study, the internal control gene (*EF-Iα*) showed stable expression characteristics. The amplification efficiencies of specific primers ranged from 0.92 to 1.05 (Table S2). The qPCR results presented that *CREB3L2* and *CREB3L3* were more highly expressed in the edge mantle of black and normal shell oysters than in the white ($P < 0.05$). *MITF* and *TYR* are well-known pigmentation-related genes in bivalves (Zhu et al., 2022). It has been reported that MITF is involved in shell pigmentation by activating tyrosinase-mediated melanin synthesis in Pacific oysters (Zhu and Li, 2024). Here, we found that the expression patterns of *CREB3L2* and *CREB3L3* in white, normal, and black shell oysters were consistent with *MITF* and *TYR* expression.

The edge mantle has a three-fold structure, including the inner-middle-, and outer folds, which is responsible for secretion and shell formation (Clark et al., 2020). In this study, we performed Masson-Fontana melanin staining and immunofluorescence assays to determine the localization of melanin and CREB3L2 protein in the edge mantle. Our results showed that a large amount of melanin was distributed in the inner fold (if), middle fold (mf), and epithelium (ep) (Fig. 4A). Many melanin granules (black tangle) were found in the outer fold (of). The immunofluorescence assay showed that there was no positive signal in the negative control (Fig. S1A). The positive control showed TYR protein was mainly distributed in the inner- and outer fold (Fig. S1B). CREB3L2 protein was mainly located in the inner- (if), middle- (mf), and outer fold (of) of the mantle (Fig. 4B). Some positive signals of CREB3L2 protein also were presented in the epithelium (ep). Similarly, some melanin synthesis-related genes, like TYRP-1, TYRP-3,

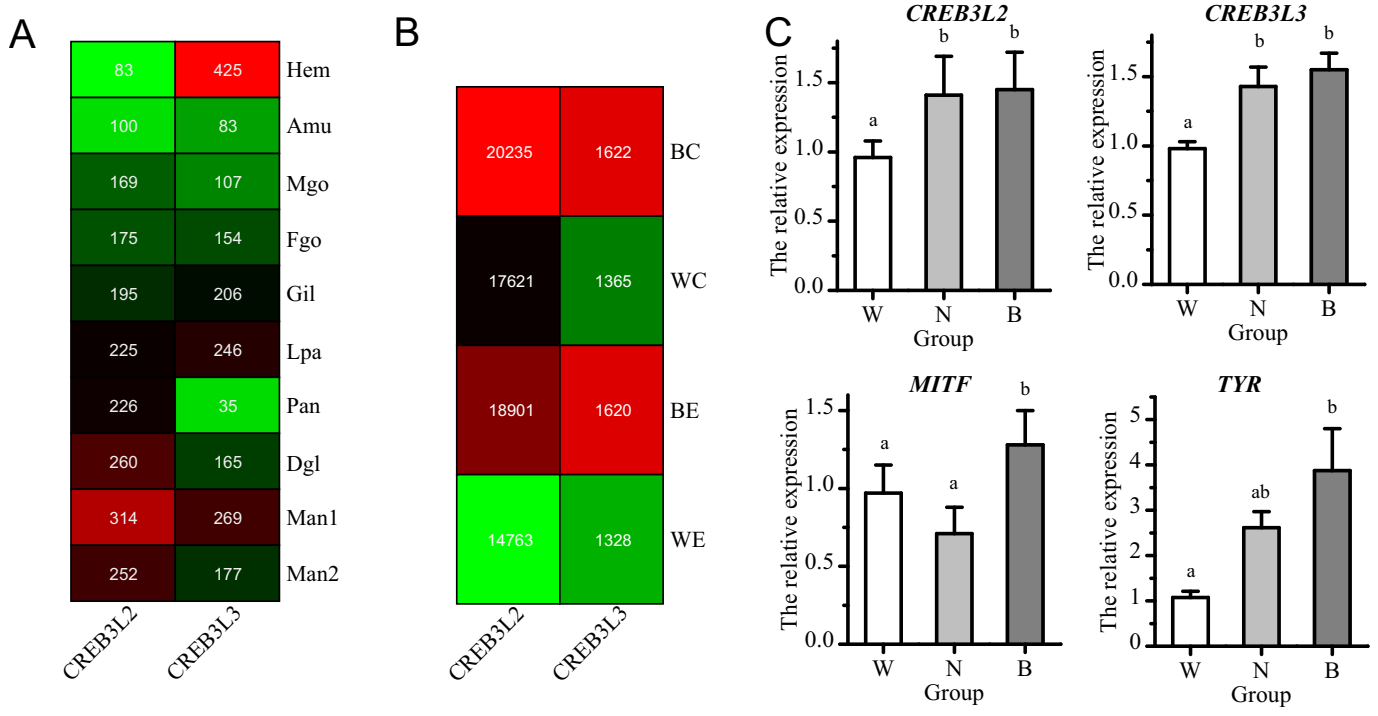

Fig. 3. Gene expression and distribution analysis using transcriptomic data and qPCR. (A) The heatmap shows *CREB3L2* and *CREB3L3* Transcripts Per Kilobase per Million mapped reads (TPM) values in different tissues, Hem (hemolymph), Amu (adductor muscle), Mgo (male gonad), Fgo (female gonad), Gil (Gill), Lpa (labial palp), Pan (hepatopancreas), Dgl (digestive gland), Man1 (left mantle), Man2 (right mantle). (B) Heatmaps showing *CREB3L2* and *CREB3L3* TPM values in black and white shell oysters, BC (central mantle of black shell oyster), WC (central mantle of white shell oyster), BE (edge mantle of black shell oyster), WE (edge mantle of white shell oyster). (C) Gene expression of *CREB3L2*, *CREB3L3*, *MITF*, and *TYR* in the edge mantel of white (W), normal (N), and black (B) shell strains. Data are presented as mean ± SD ($n = 3$). Differences in gene expression levels among the three strains were assessed by one-way ANOVA. Bars with different letters indicate significant differences ($P < 0.05$) between groups.

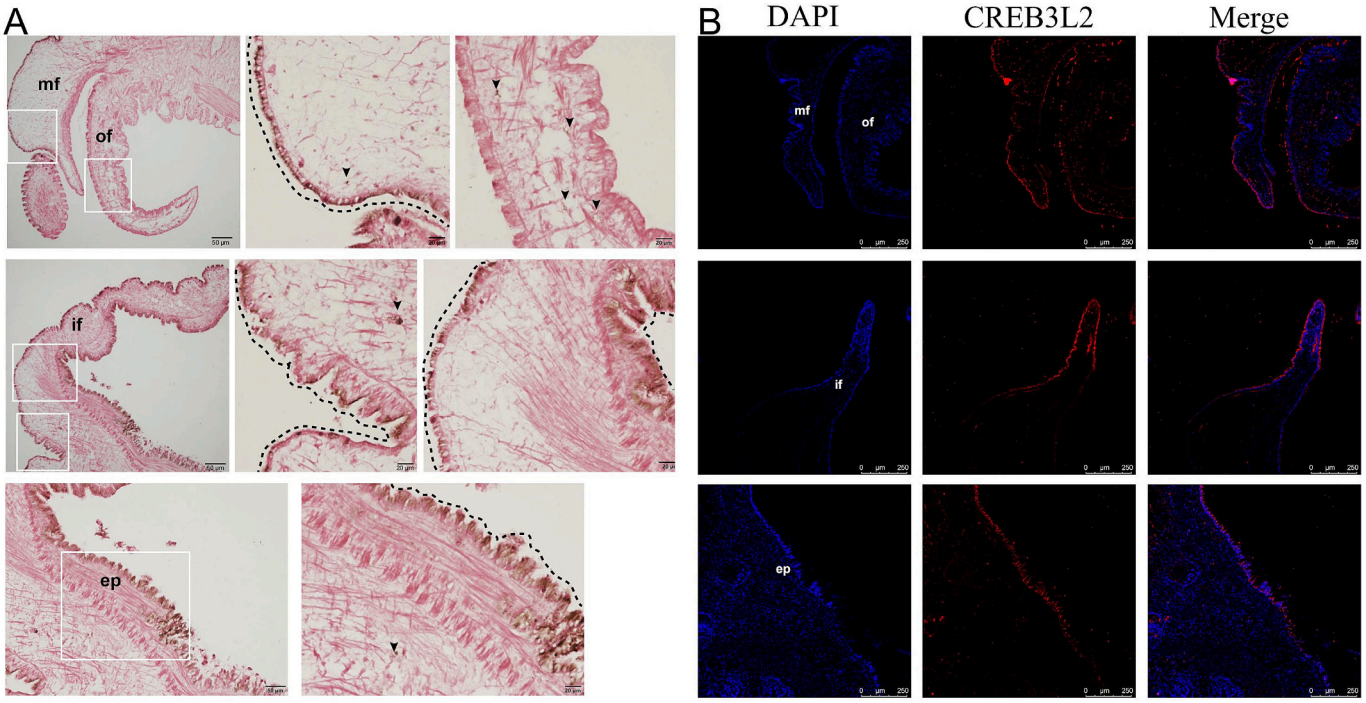

Fig. 4. Melanin distribution and CREB3L2 protein localization analysis. (A) Masson Fontana staining of melanin in mantle tissue. Brown or black particles indicate melanin. (B) Immunofluorescence examination of CREB3L2 proteins in the mantle, mf (middle fold), of (outer fold), if (inner fold), ep (epithelium). Cy3-conjugated goat anti-rabbit IgG and DAPI were used to stain the target protein and nucleus, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Pax7, have also been found distributed in oyster edge mantle (Li et al., 2022b; Zhu et al., 2022). The location of CREB3L2 protein was generally consistent with the distribution of melanin in the edge mantle of oysters, suggesting that CREB3L2 may influence melanin synthesis and pigmentation in oysters.

3.3. Transcription factor CREB3L2 and CREB3L3 activate the MITF promoter

The HEK cell line has been widely used as an expression tool in some non-model species that have no cell lines (Jiang et al., 2019). To analyze the location of CREB proteins, pEGFP-N1-CREB3L2 and pEGFP-N1-CREB3L2 were transfected into HEK-293 cells, respectively. The subcellular location results showed that both CREB3L2 and CREB3L3 proteins were located in the nucleus of HEK293 cells (Fig. 5A), suggesting that they are transcription factors and function in the nucleus.

The dual-luciferase reporter assay was conducted to identify the capacity of CREB3L2 and CREB3L3 in activating *MITF* promoters. As shown in Fig. 5B, CREB3L2 significantly activated *MITF* promoter fragments (−1229/+23 and − 1004/+3) relative to the control. CREB3L3 only significantly activated *MITF* promoter fragment −1829/+23. Previous research has found that *MITF* promoters contain a partial recognition motif for bZIP factors (Chauhan et al., 2022; Hartman and Czyz, 2015). In this study, both CREB3L2 and CREB3L3 contain a bZIP domain. It has previously been reported that CREB can recognize the *MITF* promoter and then regulate *MITF* expression and pigmentation in melanoma (Chauhan et al., 2022; Goding and Arnheiter, 2019). Taken together, our results demonstrated that CREB3L2 and CREB3L3 can activate *MITF* promoters and might be involved in regulating *MITF* expression.

3.4. cAMP regulates CREB3L2 and CREB3L3 to activate MITF

Forskolin is an inducer of intracellular cAMP production (Rodriguez et al., 2013). Rp-cAMPS is an analog of cAMP and useful as a tool for inhibiting cAMP-kinase in cells (Gjertsen et al., 1995). Here, we analyzed the influence of cAMP, forskolin, and Rp-cAMPS on *CREB* activation of *MITF*. Our results revealed that cAMP (50 and 500 μM) and forskolin (50 μM) significantly enhanced CREB3L2 activation of the *MITF* promoter (Fig. 6A-B), while Rp-cAMPS showed no significant influence (Fig. 6C). forskolin (50 μM) significantly promoted CREB3L3 activation of the *MITF* promoter ($P < 0.05$) and Rp-cAMPS inhibited the activation of the *MITF* promoter ($P > 0.05$). Similar results have also been reported in previous studies. For example, interferon-c (IFN-c) inhibits CREB binding to the *MITF* promoter (Son et al., 2014), and forskolin increases *MITF* expression and promotes melanogenesis (Arora et al., 2021; Jian et al., 2011). In this study, our results revealed cAMP and forskolin promoted CREB3L2 and CREB3L3 activation of the *MITF* promoters. cAMP plays an important role in cAMP-mediated melanogenesis (Zhou et al., 2018). In our previous study, the cAMP signaling pathway was significantly enriched in the black *vs.* white shell oyster transcriptome (Jiang et al., 2024b). In addition, the black shell oyster showed a higher level of cAMP in the edge mantle than that in the white (Jiang et al., 2024b). Taken together, these data suggest that melanin synthesis in oysters may be regulated by cAMP mediated melanogenesis.

3.5. cAMP regulates MITF and TYR expression by activating CREB3L2 and CREB3L3

Primary cell and tissue cultures provide useful model systems used in a wide range of biological applications (Potts et al., 2020). In mollusks, mantle tissue is the key organ responsible for shell calcification pigmentation (Sun et al., 2016). Here, we cultured oyster mantle cells and performed incubation assays using three exogenous substances (cAMP, forskolin, and Rp-cAMPS) to determine the effect of cAMP on the CREB-MITF-TYR axis in Pacific oysters. After incubation with different concentrations of those substances, we analyzed the mRNA expression of *CREB3L2*, *CREB3L3*, *MITF*, and *TYR* genes. cAMP treatment significantly regulated the expression of these genes relative to the control (Fig. 7A). The expression of *CREB3L2* was significantly promoted by 5–50 μM

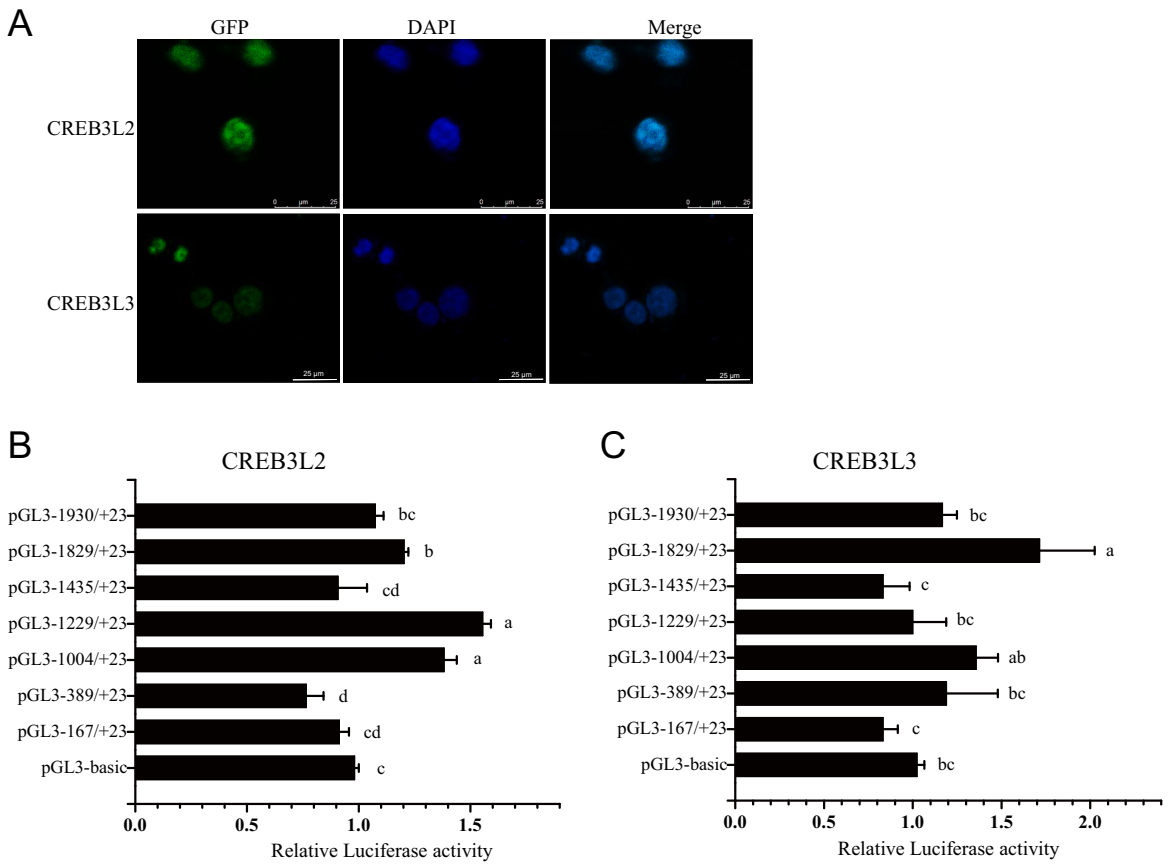

Fig. 5. Subcellular location and transcription factors activity analysis. (A) Subcellular localization of CREB3L2 and CREB3L3 in HEK293T cells. The blue fluorescent signals show the nuclei and the green fluorescent signal represents the location of the fusion protein. (B) CREB3L2 and (C) CREB3L3 transcription factors activated *MITF* promoter fragment. The luciferase activity of each construct was compared with RL-TK transcription activity. Data are shown as the mean ± SD (n = 3). Significant differences were determined using one-way ANOVA. Bars with different letters indicate significant differences ($P < 0.05$) between groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

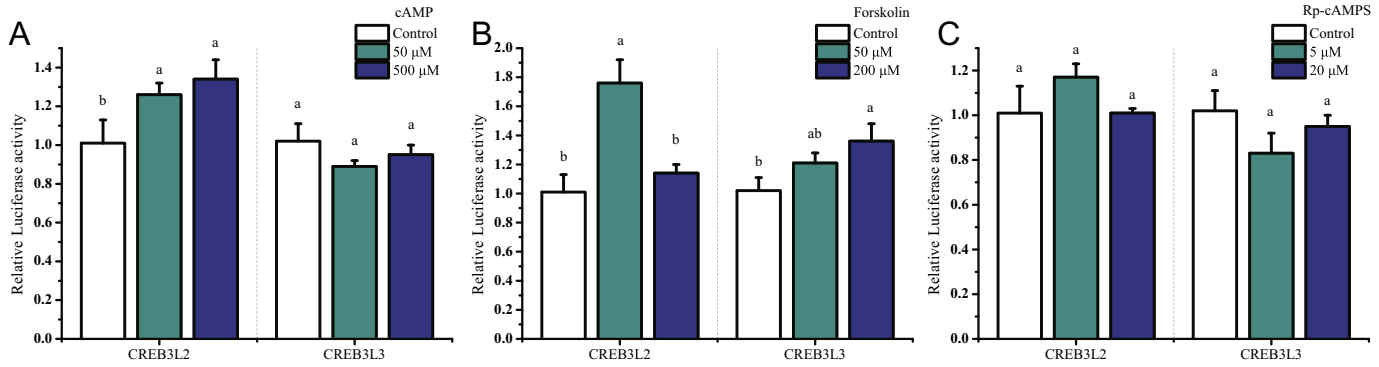

Fig. 6. The activation of *MITF* promoter under cAMP regulation. HEK293T cells were transfected with reporter vectors and then treated with different concentrations of cAMP (A), forskolin (B), and Rp-cAMPS (C). Data are shown as the mean ± SD (n = 3). Significant differences were determined *via* one-way ANOVA. Bars with different letters indicate significant differences ($P < 0.05$) between groups.

cAMP ($P < 0.05$). *CREB3L3* and *TYR* expression were up-regulated under the treatment of 10–50 μM cAMP ($P < 0.05$). 5–50 μM cAMP significantly increased *MITF* expression ($P < 0.05$). Forskolin induced a significant increase in *CREB3L3* expression at concentrations of 10–500 μM, *MITF* expression at 10–100 μM, and *TYR* expression at concentrations <50 μM (Fig. 7B). Rc-cAMPS had no significant effect on *MITF* or *TYR* gene expression (Fig. 7C).

cAMP stimulates the translocation of protein kinase A (PKA) into the nucleus, activating CREB (Chen et al., 2019; Lee et al., 2022). CREB then positively modulates the expression of *MITF* (Hartman and Czyz, 2015). *MITF* is a key gene in the regulation of melanin synthesis, which aggregates information from all upstream pathways and controls the expression of tyrosinase family genes and melanin synthesis (Estrada et al., 2022). The relationship between CREB and MITF has been well understood in some vertebrates, however, the interaction between CREB and melanogenic genes is barely reported in Mollusca. In this study, our results showed that the expression of *CREB*, *MITF*, and *TYR* was under the regulation of cAMP, cAMP inhibitor, and cAMP activator. A moderate concentration of cAMP activated the CREB-MITF-TYR axis. These results indicated that cAMP regulates oyster melanogenesis by

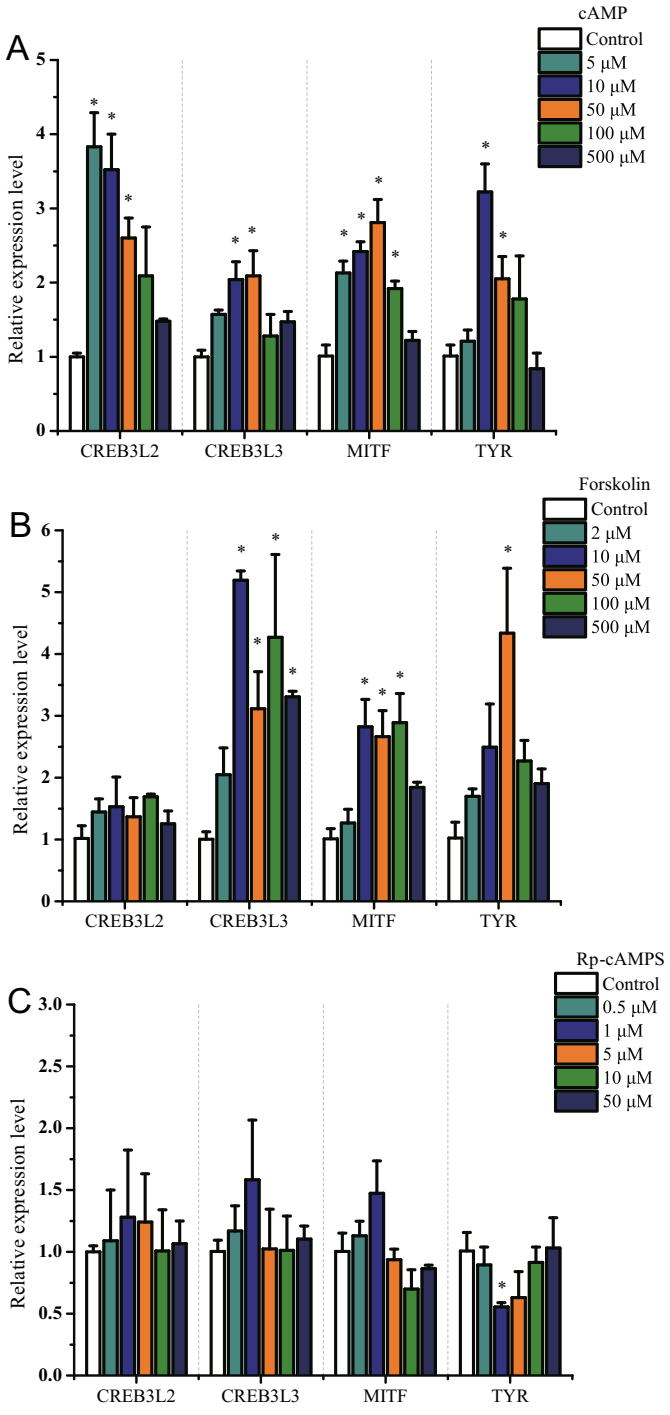

Fig. 7. cAMP regulates melanogenic genes expression through CREB-MITF-TYR axis. The expression analysis of *CREB3L2*, *CREB3L3*, *MITF*, and *TYR* in mantle cells after treatment with cAMP (A), forskolin (B), and Rp-cAMPS (C). Data are presented as the mean ± SD ($n = 3$). One-way ANOVA and Tukey multiple comparison tests were used to determine differences. *: $P < 0.05$.

activating CREB3L2 and CREB3L3 (Fig. 8).

4. Conclusions

In this study, we identified two transcription factors (*CREB3L2* and *CREB3L3*), analyzed their expression and distribution characteristics, and determined their role in regulating oyster melanogenesis. Our results showed that *CREB3L2* and *CREB3L3* were highly expressed in the edge mantle. Their expression level in the black shell oysters was higher than that in the white. Transcription factors CREB3L2 and CREB3L3 activated the *MITF* promoter under cAMP regulation. In addition, cAMP regulated melanogenic gene expression through CREB3L2 and CREB3L3 activation of the *MITF* promoter. These observations indicated a cAMP regulated CREB-MITF-TYR axis plays an important role in melanin synthesis and pigmentation in Pacific oysters.

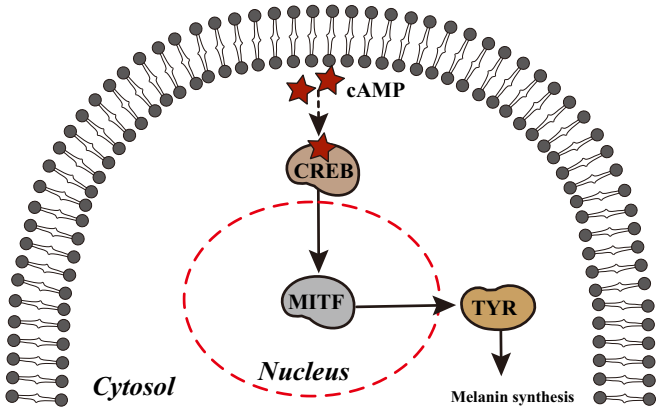

Fig. 8. The proposed model for CREB-MITF-TYR melanogenesis pathway in Pacific oysters.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2024.110970.

CRediT authorship contribution statement

Kunyin Jiang: Writing – original draft, Methodology, Investigation, Formal analysis. **Hong Yu:** Resources. **Lingfeng Kong:** Resources. **Shikai Liu:** Resources. **Qi Li:** Writing – review & editing, Supervision, Formal analysis.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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