The purine-rich element-binding protein *ChPur-α* negatively regulates *Hsc70* transcription in *Crassostrea hongkongensis*

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Abstract *ChPur-α*, a purine-rich element-binding protein, was discovered showing affinity to the *ChHsc70* promoter in *Crassostrea hongkongensis* by DNA affinity purification and mass spectrometry analysis. Direct interaction between purified *ChPur-α* and the *ChHsc70* promoter region was demonstrated by electrophoretic mobility shift assay in vitro. *ChPur-α* reduction led to clear enhancements of *ChHsc70* transcription in the hemocytes of *C. hongkongensis*. Consistently, *ChPur-α* over-expression in heterologous HEK293T cells correlated with repressive phenotype in *ChHsc70* promoter expression. *ChHsc70* transcription was responsive to heat shock or CdCl₂ stress by RT-PCR, signifying an inducible feature of *ChHsc70* transcription by physical/chemical stress despite its constitutive nature. *ChPur-α* transcription was also induced by the two stressors. This indicates a plausible association between *ChHsc70* and *ChPur-α* in the stress-induced genetic regulatory pathway. This study discovered a negatively regulatory role of *ChPur-α* in controlling *ChHsc70* transcription in *C. hongkongensis*, and contributed to better understanding the regulatory mechanisms in control of *Hsc70* transcription.

Keywords Pur-α · *Crassostrea hongkongensis* · *Hsc70* · Transcriptional regulator · Negative regulation

Introduction

Molecular chaperones of HSP70 family are conserved stress proteins and ubiquitously present in the entire spectrum of organisms from bacteria to humans (Saibil 2013; Whitley et al. 1999). They are composed of both stress-inducible (Hsp70) and constitutive (Hsc70) forms and essential for survival and repair of stress-induced damage as well as during normal cellular homeostasis (Whitley et al. 1999). Hsp70 synthesis is rapidly and strongly induced from basal level by a variety of stressors so as to restore the proper folding of impaired proteins and thus prevent the formation of cytotoxic aggregates, whereas Hsc70 is synthesized at relative higher level under normal conditions and most dedicated to house-keeping functions such as assisting the correct folding of newly synthesized polypeptides, refolding or elimination of aberrant proteins, and transport of proteins into cellular organelles (Deshaies et al. 1988). Hsc70 expression has been previously acknowledged to change little upon burden of stressors (Giebel et al. 1988); however, there are a few of reports arguing their stress-inducible feature despite its constitutive nature. For instance, *Hsc70* transcription in Chinese hamster cells was demonstrated to increase at least tenfold after a mild heat shock (Chen et al. 1996). Lencioni et al. recently reported a time-dependent upregulation of midge *Hsc70* upon pesticide exposure regardless of pesticide concentration (Lencioni et al. 2016). These pieces of evidence indicate that the response manner of the *Hsc70* to external stresses is host-dependent and plausibly involves distinct regulatory mechanisms.

The mechanisms triggering the *Hsp70* induction has been extensively investigated at the transcriptional level, involving

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the combined regulation from both general regulators, such as GAGA factor, TBP (TATA-binding protein), STAT (signal transducer and activator of transcription), and gene-specific regulators like HSF-1 (heat shock factor 1) (Mason and Lis 1997; Pirkkala et al. 2001). In contrast, presumably due to the low sensitivity to stress, the reports regarding the regulation of Hsc70 expression are relatively less despite its physiological importance, although putative transcriptional elements have been demonstrated to be conservatively present in *Hsc70* promoter region, which include TATA box, CAT box, and heat shock elements (HSEs) (Chen et al. 1996). The latter is supposed to be recognized by HSF-1 trimer upon stressed conditions, so as to fulfill the transcription activation (Stephanou et al. 1999). Only few transcription factors have been reported to participate in the Hsc70 expression regulation so far, including Sp1-like protein and KLF4 (Liu et al. 2008; Wilke et al. 2000). Sp1 was shown to specifically bind to the *Hsc70* promoter at the Sp1 site so as to mediate ethanol induction of Hsc70 in neural cells (Wilke et al. 2000). KLF4 was discovered to perform a positive regulatory role on Hsc70 expression in murine myoblast cells (Liu et al. 2008), while whether the regulation is through direct protein-DNA interaction was not determined yet. The above information indicates that despite physiological and biological significance of Hsc70, the regulatory mechanisms governing its expression, particularly its host-dependent stress response, remain largely unknown.

In this study, transcriptional factors that show affinity to the *ChHsc70* promoter region were isolated from gills of *Crassostrea hongkongensis*, the economically valuable oysters inhabiting mainly in the coast and estuary of south China. Purine-rich element-binding protein *ChPur-α* was identified, and its specific interaction with *ChHsc70* promoter region was demonstrated in vitro. A negative regulatory role of *ChPur-α* on *ChHsc70* messenger RNA (mRNA) expression was discovered in oyster native host as well as in heterologous HEK293T cells. Both *ChHsc70* and *ChPur-α* transcriptions were induced by external physical or chemical stresses, and distinct induction patterns were shown.

Materials and methods

Oyster maintenance and treatment

One-year-old oysters (*C. hongkongensis*) with a mean shell length of 9 ± 1 cm were collected from a farm located in the Yangxi suburb, in Guangdong, China. The oysters were allowed to acclimate for 2 weeks in aerated artificial seawater as described previously (Miao et al. 2016). For heavy metal stress treatment, the oysters were selected at random and evenly transferred to 25-l plastic tanks filled with artificial sea water complemented either with CdCl_2 at 50 $\mu\text{g/l}$ or with no supplements. The seawater was renewed every other day with

the original CdCl_2 concentration. For thermal stress treatment, the acclimated oysters were relocated to a 37 °C water bath and kept for 1 h, followed by recovery under the original conditions for 3 h.

DNA affinity purification

DNA affinity purification was performed as described previously (Miao et al. 2016). Briefly, biotin-labeled *ChHsc70* promoter that was amplified from *C. hongkongensis* genomic DNA using primers C70 promoter up and C70 promoter down (Table 1) was designed according to the *ChHsc70* sequences deposited in GenBank with accession number KY906022. The resulting DNA products were immobilized to streptavidin-coated magnetic beads (Cat. No. 88816; Thermo) in DNA-binding buffer (10 mM Tris-HCl, 1 mM EDTA, and 2 M NaCl, pH 7.5) following the manufacturer's instructions. After the washing steps, the nuclear protein extracts that were previously prepared from the gills of the stressed and the control oysters were introduced individually. Then, the reaction mixtures were incubated at room temperature for 15 min. After a second washing step, high-salt buffer (25 mM Tris-HCl and 200 mM NaCl, pH 7.5) was applied and the proteins showing affinity to the promoter were eluted. The latter was then subject to SDS-PAGE analysis. The identity of the band of interest was clarified by mass spectrometry as described in the supplementary materials.

Full-length *ChPur-α* cDNA sequencing

Total RNA was isolated from oyster gills by the TRIzol (Cat. No. 15596-026; Invitrogen) following the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA using oligo(dT) primer at 55 °C for 30 min with 200 U M-MLV Reverse Transcriptase (Promega). The resultant cDNA pool was then used as template to amplify the *ChPur-α* coding sequence with the *ChPur-α*-up and *ChPur-α*-down primers (Table 1). The resulting products were ligated into the pMD18-T (TaKaRa) vector, and the inserted *ChPur-α* coding sequence was confirmed by nucleotide sequencing (Liuhe BGI). The 5' and 3' cDNA end sequence of *ChPur-α* was determined using RACE technology as described previously (Zhang and Zhang 2012).

Electrophoretic mobility shift assay

The *ChPur-α* coding sequence was amplified from the cDNA pool of *C. hongkongensis* using *ChPur-α*-F-NdeI and *ChPur-α*-R-HindIII primers by PCR. The PCR product was cloned into the pET22b(+) vector, and the resulting recombinant plasmid was transformed into *E. coli* BL21. The 6-histidine-tagged *ChPur-α* (His₆-*ChPur-α*) was overexpressed and purified as described previously (Xu et al. 2010). The 5'

FITC-labeled *ChHsc70* promoter region (−457 to +41 relative to the predicted transcriptional start as +1) was amplified from *C. hongkongensis* genomic DNA by PCR using primers C70 promoter up and C70 promoter down. The labeled DNA probe was incubated with purified His₆-ChPur- α at various concentrations for 15 min at room temperature in binding buffer (10 mM Tris-HCl, 50 mM KCl, and 1 mM DTT, pH 7.5). Extra reactions were set in parallel using excess amounts of unlabeled *ChHsp70* promoter and irrelevant DNA probes that act as specific and non-specific competitors, respectively. After incubation, the complexes were resolved on a 4% native polyacrylamide gel that had been pre-run at 100 V for 30 min and then subsequently run for 90 min at 100 V. The DNA bands were visualized by fluorescence imaging using an UVI Alliance 4.7 imager (UK).

ChPur- α knockdown

Approximately 300 ml of hemolymph was extracted from the posterior adductor muscle of the oysters using syringes, followed by centrifugation at 3000 rpm for 2 min. The collected hemocytes were washed with PBS three times and then resuspended in 50 ml complete DMEM (Dulbecco's modified Eagle's medium comprising 10% heat-inactivated fetal bovine serum [FBS] and supplemented with 0.4% NaCl, 100 units/ml penicillin, and 100 μ g/ml streptomycin). The resulting hemocyte solution was plated on 24-well plates at a cell density of 1×10^5 cells/well, followed by incubation at 28 °C in a humidified incubator supplied with 5% CO₂ for 24 h, so as to achieve 30–50% cell confluence. Subsequently, small interfering RNA (siRNA) and control RNA prepared by Sangon were individually transfected into the hemocytes using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The cells were allowed to recover at 28 °C for 24 h before collecting the first sample. The collected hemocytes were subsequently applied to RT-PCR analysis.

Dual-luciferase reporter assay

The *ChPur- α* coding sequence was amplified from the *C. hongkongensis* cDNA pool by PCR using the ChPur- α -F-KpnI and ChPur- α -R-XhoI primers (Table 1). The resulting PCR products were digested with the corresponding restriction enzymes and cloned into the pcDNA3.1 vector to yield pcDNA3.1-*ChPur- α* . The *ChHsc70* promoter was amplified from *C. hongkongensis* genomic DNA using primers C70-F-KpnI and C70-R-XhoI (Table 1). The resulting PCR products were digested with *Kpn* I and *Xho* I restriction enzymes and cloned into the promoter probe vector pGL3-basic at the same sites to yield pGL3-*ChHsc70p*. HEK293T cells were cultivated according to the procedures described above for hemocyte cultivation. A 90–95% cell confluence was reached after plating onto 24-well plates. The dual-luciferase reporter assay was

conducted as described previously (Miao et al. 2016). Briefly, the pGL3-*ChHsc70* (200 ng/well), pRL-TK (20 ng/well, Promega), pcDNA3.1-*ChPur- α* (200 ng/well), or empty pcDNA3.1 (200 ng/well) plasmids were co-transfected into HEK293T cells using Lipofectamine™ 2000 (Invitrogen) in the DMEM medium lacking FBS and antibiotics in accordance with the manufacturer's instructions. An incubation was maintained at 37 °C for 4.5 h, and the medium was changed back to complete DMEM, followed by an additional incubation for 24 h prior to the collection of the first sample. A Dual-Luciferase Reporter Assay System (Promega) was employed to determine the luciferase activities following the manufacturer's instructions. Renilla luciferase activities were applied for correcting the transfection efficiency.

Quantitative real-time PCR

Total RNA was isolated from hemocytes transfected with either siRNA or control RNA as well as from gills of oysters that were either non-treated or treated by heat or CdCl₂ at various time points using the TRIzol reagent (Cat. No. 15596-026; Invitrogen). First-strand cDNA was synthesized from 2 μ g of total RNA of each sample using MLV Reverse Transcriptase (Promega) and oligo(dT) primer. Quantitative real-time PCR was performed as described previously (Zhang and Zhang 2012).

Statistical analysis

The data were processed by one-way ANOVA followed by the Tukey HSD test. Differences were considered as statistically significant at $P < 0.05$. IBM SPSS Statistics 19.0 was used for the statistical analysis.

Nucleotide sequence accession number

The *ChPur- α* cDNA sequence was deposited in the GenBank with accession number KY982276.

Results

Isolation and identification of proteins showing affinity to the *ChHsc70* promoter in *C. hongkongensis*

The transcription factors that show affinity to the *ChHsc70* promoter region were isolated by DNA affinity purification as described in the “Materials and Methods” section. The results are shown in Supplementary Fig. 1. A number of protein bands were visually detected under stressed or non-stressed conditions, while distinctive band patterns were shown. The band that is noticeably visible right below the 35 kDa marker was specifically appeared in the control and

Cd-stressed lanes, but not in the heat-stressed lane. The nature of the band was subsequently revealed by mass spectrometry as Pur- α (Supplementary Fig. 2). The latter is a member of the Pur family of nucleic acid-binding proteins, which have been well known for the regulatory roles in a variety of DNA-dependent processes including transcription (Gallia et al. 2000). The stress-dependent affinity of Pur- α to the *ChHsc70* promoter was of interest, and was therefore preferred for further study.

Characterization of the *ChPur- α* cDNA

The *ChPur- α* cDNA is 1503 bp in length, containing an open reading frame of 747 bp, as well as 87 and 669 bp of 5' and 3' untranslated regions, respectively (Fig. 1a). The peptide sequence consists of 248 amino acids, with a molecular weight of 28 kDa and an isoelectric point of 6.79. The three-dimensional structure was predicted by online SWISS-

MODEL software (<http://swissmodel.expasy.org/>) using the *ChPur- α* amino acid sequence. The deduced protein model revealed the presence of three repeats of the Pur domains (Fig. 1a), which are dedicated to bind DNA, and the structure of the previous two Pur domains is shown in Fig. 1b.

ChPur- α directly interacts with the *ChHsc70* promoter region in vitro

In order to test the ability of *ChPur- α* to directly interact with the *ChHsc70* promoter region, electrophoretic mobility shift assay (EMSA) was performed using a FITC-labeled *ChHsc70* promoter region and prokaryotically expressed/purified His₆-*ChPur- α* . As shown in Fig. 2, the addition of *ChPur- α* clearly retarded the mobility of *ChHsc70* promoter in a concentration-dependent manner (lanes 2 to 5). When excess amounts of unlabeled *ChHsc70* promoter were introduced, the shifted band was eliminated (Fig. 2, lane 6), indicating a successful specific

Table 1 Oligonucleotides used in this study

Primers name	Sequence 5' → 3'	Start site to end site	Usage
Oligo(dT)18	TTTTTTTTTTTTTTTTT		Reverse transcription
C70 promoter up	ATCATTCACAGATCTAGAGGGGGG	-457 to -480	Used to amplify ChHsc70 promoter fragment for DNA affinity purification and EMSA
C70 promoter down	TTTCTCCCTTTCCCTTACT	+19 to +41	
ChPur- α -up	ATGTCGGATAGAGAGAG	1~17	Used to amplify ChPur- α fragment for RACE
ChPur- α -down	CTACTGACTTTCTTCACC	730~747	
UP	CGGCAGTGGTATCAACGCAGAGTAC		Used with 3GSP2 and 5GSP2, UP (universal primer)
UP(dT)	CGGCAGTGGTATCAACGCAGAGTAC(T)18		Synthesis of first-strand cDNA for 3' RACE
3GSP 1	CAGATGACCATGAGGGACAGAATG	485~508	Used with 3-Down (dT) for first PCR of 3' RACE
3GSP 2	CGTGGTGTGTACCTCAGAATTTCTGAAGT	577~605	Used with UP for nested PCR of 3' RACE
UP(dG)	CGGCAGTGGTATCAACGCAGAGTAC(G)10		Used for first PCR of 5' RACE
5GSP 1	GTCCCTCATGGTCATCTGTCC	482~502	Used with UP for nested PCR of 5' RACE
5GSP 2	CTGAGCAGGAATGGCAATCTGAGAGC	407~432	
ChPur- α -F-NdeI	ATAAAACATATGTCGGATAGAGAGAGTGGGGAC	4~24	Used to amplify ChPur- α fragment for clone and expression
ChPur- α -R-HindIII	ATAAAAAAGCTTCTGACTTTCTTCACCCCT	726~744	
C70-F-KpnI	ATAAAAGGTACCATCATTCACAGATCTAGAGG	-457 to -476	Used to amplify ChHsc70 promoter fragment for Dual-luciferase reporter assay
C70-R-XhoI	ATAAACTCGAGTTTCTTCCCCTTTCCCCT	+24 to +41	
ChPur- α -F-KpnI	ATAAAAGGTACCATGTCGGATAGAGA GAGTGG	1~20	Used to amplify ChPur- α fragment for Dual-luciferase reporter assay
ChPur- α -R-XhoI	ATAAACTCGAGCTACTGACTTTCTTCACCCC	728~747	
Actin-Up	CTGTGCTACGTTGCCCTGGACTT	+701 to +723	Used to amplify β -actin fragment for real-time PCR
Actin-Down	TGGGCACCTGAATCGCTCGTT	+829 to +849	
RT-C-Up	GGACAAGGCCAGATCCACGACA	993~1015	Used to amplify ChHsc70 fragment for real-time PCR
RT-C-Down	CAGAATGGCGGCCTGGACAGCT	1134~1155	
RT-P-Up	TGGATCAAACAGACGTGGTGTG	564~585	Used to amplify ChPur α fragment for real-time PCR
RT-P-Down	AGCCCTGCACTGGGTTTAC	701~720

Fig. 1 **a** The cDNA sequence and deduced amino acid sequence of *ChPur-α* in *C. hongkongensis*. The nucleotides and amino acids are numbered along both margins. Three repeats of Pur domains are marked by *single lines*, *dashed lines*, and *double underlines*,

respectively. The putative DNA-binding sites are marked by *triangles*. The translation stop codon is indicated with an *asterisk*. **b** Three-dimensional structure of *ChPur-α*

competition. However, the introduction of an irrelevant DNA fragment (+25 to +424 relative to the translational start on the *ChPur-α* coding sequence) led to largely compromised competition (Fig. 2, lane 7). These results indicated that *ChPur-α* interacts specifically with the *ChHsc70* promoter *in vitro*.

ChPur-α knockdown led to enhanced *ChHsc70* transcription in *C. hongkongensis* hemocytes

In order to discover the regulatory role of *ChPur-α* on *ChHsc70* transcription, *ChHsc70* mRNA expression was

determined in both control- and siRNA-treated hemocytes of *C. hongkongensis* by RT-PCR. The results are shown in Fig. 3. Upon siRNA treatment, the mRNA expression of *ChPur-α* was depleted drastically at all tested time points, validating the effective gene knockdown of *ChPur-α*. In conditions of *ChPur-α* depletion, *ChHsc70* transcription was apparently increased in all tested time points within 24 h. These results demonstrated that *ChPur-α* negatively regulated *ChHsc70* mRNA expression in native *C. hongkongensis* host. Another noticeable point was the induced profile of the basal as well as the siRNA-treated *ChHsc70* transcription until 12 h. While taking into account the

Fig. 2 Electrophoretic mobility shift assay of the *ChHsc70* promoter with prokaryotically purified His₆-*ChPur-α*. Fifty picomoles of FITC-labeled *ChHsc70* promoter was added to all the lanes. The band positions of the shifted DNA-protein complexes and free DNA are designated by *arrows*

in vitro cultivation of the oyster hemocytes, this induction might be very likely due to the experienced stress caused by the disturbed biological context.

***ChPur- α* overexpression repressed *ChHsc70* transcription in heterologous host HEK293T cells**

The regulatory effects of overexpressed *ChPur- α* on *ChHsc70* transcription were tested in HEK293 cells using luciferase reporter assay as described in the “Materials and methods” section. The results are shown in Fig. 4. The overexpressed *ChPur- α* clearly repressed the *ChHsc70* mRNA expression particularly at 0, 6, 12,

and 24 h. These results demonstrated that constitutively expressed *ChPur- α* played a negative regulatory role on the *ChHsc70* transcription in the heterologous HEK293T host, which is consistent with that in the oyster native hemocytes. Noticeably, the *ChHsc70* promoter activity in the control cells clearly decreased at 3 h. However, this repressed state was soon overturned since a clear increase in the *ChHsc70* promoter expression was observed at 6 h. Interestingly, such oscillating expression manner (decrease followed by overturn) was presented again in the following time points. These results indicate that the mechanisms underlying the basal promoter expression are intrinsically complex in the heterologous HEK293 host.

Fig. 3 *ChHsc70* and *ChPur- α* steady-state RNA levels by RT-PCR after the introduction of either control RNA (control) or siRNA (RNAi). All values are expressed as the means $\pm$ SD ($n = 3$). Asterisks above the bars indicate that values are significantly different from the individual controls ($*P < 0.05$ and $**P < 0.01$). Bars on the same group with different letters are statistically significant from one another

Fig. 4 Luciferase activity driven by the *ChHsc70* promoter in HEK293T cells upon the introduction of pcDNA3.1-*ChPur- α* and pcDNA3.1. All values are presented as the means $\pm$ SD ($n = 3$). Asterisks above the bars indicate that the values are significantly different from the individual controls ($*P < 0.05$ and $**P < 0.01$). Bars on the same group with different letters are statistically significant from one another

ChHsc70 and *ChPur- α* transcription profiles upon heat shock and CdCl₂ stresses

In order to discover the relationship between *ChHsc70* and *ChPur- α* in response to the external physical or chemical stress, the *ChHsc70* and *ChPur- α* transcriptions were determined by RT-PCR in the gills of the oysters treated with heat or CdCl₂ at 50 μ g/l, the concentration of which has been previously demonstrated to be suitable for induction (data not shown). The results are shown in Fig. 5. The mRNA expression of *ChHsc70* was clearly induced upon heat and Cd stresses, with the induced peaks at 6 h and 2 days, respectively. The *ChPur- α* transcription was also induced upon either of the two stresses. However, a different induction pattern was shown since the induction peaks were accumulated at 24 and 4 h, respectively, both of which were slightly delayed in comparison with that of the *ChHsc70*. These results demonstrated that the mRNA expressions of both *ChHsc70* and *ChPur- α* were inducible by external stresses, indicating a correlated association between the two genes in the stress-induced regulatory pathway.

Discussion

Pur- α is a highly conserved nucleic acid-binding protein that is ubiquitously expressed from bacteria through humans (Gallia et al. 2000). Human Pur- α was characterized to bind preferentially to a purine-rich single-strand element that is present upstream of the c-MYC gene (Bergemann and Johnson 1992;

Bergemann et al. 1992). A consensus Pur- α binding sequence of GGNNAGGGAGARRRR has been derived, and its presence was confirmed at origins of replication and in gene flanking regions in various eukaryotes (Bergemann and Johnson 1992). Pur- α is known with multiple cellular functions including the initiation of DNA replication and control of mRNA translation and transcription, and thus plays important roles in regulating cell cycle and proliferation (Gallia et al. 2000). Pur- α has been demonstrated to positively or negatively regulate the transcription of diverse target genes such as α -MHC (α -myosin heavy chain) (Gupta et al. 2003), PL (placental lactogen) (Limesand et al. 2004), A β PP (Darbinian et al. 2008), and BC1 RNA (Kobayashi et al. 2000). In this study, *ChHsc70* was discovered as a novel target of *ChPur- α* , and the negative regulatory role of *ChPur- α* on *ChHsc70* transcription was characterized in *C. hongkongensis*.

Analysis of the *ChPur- α* amino acid sequence showed the presence of three repeats of Pur domains, each of which contains an aromatic-basic section and a leucine-acidic section (Fig. 1). Such a unique and structurally conserved domain is known to be involved in DNA binding, especially to the single-stranded element with the sequence GGN (N is for any nucleotide) (Gallia et al. 2000). It has been previously demonstrated that Pur α binds to a purine-rich negative regulatory (PNR) element in competition with an Ets-related protein to repress the transcription and translation of α -MHC gene (Gupta et al. 2003). When analyzing the *ChHsc70* promoter, two sites that show 87.5% similarity (only one nucleotide difference) with the palindrome (TTCC...GGAA) in PNR were indeed discovered at the region

Fig. 5 *ChHsc70* and *ChPur-α* expression levels by RT-PCR after **a** heat or **b** CdCl₂ stress. All values are expressed as the means $\pm$ SD ($n = 3$). Asterisks above the bars indicate that the values are significantly different

between two HSEs (Supplementary Fig. 3), which were therefore deemed highly possible to be the target nucleotide sites of *ChPur-α* on the *ChHsc70* promoter.

RNAi analysis clearly demonstrated a negative regulatory function of *ChPur-α* on *ChHsc70* transcription in the oyster native host (Fig. 3). Considering together with the previous EMSA data (Fig. 2), these results suggest that *ChPur-α* negatively regulates the *ChHsc70* mRNA expression through direct binding to its promoter region. Since *ChPur-α* depletion had a positive impact on *ChHsc70* transcription, it was logical to suppose that conversely, *ChPur-α* overexpression should associate with a repressive phenotype. Indeed, in comparison with the control, the *ChHsc70* mRNA expression was apparently decreased upon *ChPur-α* overexpression at most tested time points; however, such phenotype was not steadily shown since the repression was largely compromised at 3 and 18 h (Fig. 4). It has been previously noted that in addition to DNA and/or RNA binding, an important aspect of Pur-α function is to recruit and interact with cooperative transcription factors, affect their DNA-binding capabilities, and finally manipulate the transcription of target

genes. For example, the association of Pur-α with E2F-1 showed to inhibit the DNA-binding capability of E2F-1 and resulted in a drastic decrease in the ability of E2F-1 in activating its target promoter DHFR (Darbinian et al. 1999). It is therefore reasonable to expect the emergence of any intrinsic repressors whose DNA-binding ability was intermittently disturbed by overexpressed *ChPur-α*, so as to lead an irregular repressive phenotype.

In spite of the constitutive nature of Hsc70, a lot of evidence has shown that the *Hsc70* expression is inducible by heat shock and/or heavy metal stress in various hosts such as sea urchin and midge larvae (Bernabo et al. 2011; Lencioni et al. 2016; Yoshimi et al. 2009). In this study, the expression of *ChHsc70* of *C. hongkongensis* was demonstrated to be significantly induced by both heat and Cd stresses by RT-PCR analysis, presenting the induction profiles of induction accumulation, peaking, decline, and recovery (Fig. 5). Such profiles resemble that of the stress-inducible *ChHsp70* which was determined previously (Miao et al. 2016; Zhang and Zhang 2012) and likely reflect the transition of the

physiological states of the oysters, which was driven by the demands of the molecular chaperones at different stages upon external stresses. *ChPur- α* transcription was also responsive to the two stresses, while the accumulated *ChPur- α* expression peak induced by either heat or Cd appeared at the point that was slightly delayed when compared with the corresponding *ChHsc70* peak. Taking into account the previously discovered negative regulatory function, this tardy accumulated *ChPur- α* might be very likely to contribute to the recovery of the induced *ChHsc70* expression back to its original level. Besides heat and heavy metal (Cd) stresses, a similar induction pattern was observed to both *ChHsc70* and *ChPur- α* transcriptions when exposed to NP (nonylphenol) and TBT (tributyltin) (Supplementary Fig. 4), which are notorious endocrine-disrupting chemicals that are frequently found but not readily decomposable in aquatic environments (Lee et al. 2010; Soares et al. 2008). These results indicate that *ChHsc70* and *ChPur- α* transcriptions might respond universally to common aquatic pollutants.

In summary, we demonstrate a clear negative regulatory role of *ChPur- α* on *ChHsc70* mRNA expression through direct protein-DNA interaction in *C. hongkongensis*. The unsteady *ChPur- α* regulatory effects on *ChHsc70* transcription in a heterologous HEK293T host cells suggest a complex mechanism mediated by *ChPur- α* and likely involve the collaboration of other factors. This study discovered a novel regulatory target for Pur- α and presents the first evidence of a transcriptional factor in regulating the *Hsc70* expression in *Crassostrea*.

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Author contributions We would like to declare that the present work is a product of the intellectual environment of the entire team, and all members have contributed in various degrees to the analytical methods used, to the data collection/interpretation, to the research concept, to the experiment design, and to the drafting/critical revision of the article. The detailed roles or tasks of each author are listed:

1. Delin Xu

Constructed the idea and hypothesis for research, planned the methodology to reach the conclusions, logically interpreted the results, completed the literature review, constructed/drafted the entire manuscript, critically revised the article before submission, and approved the final version of the article to be submitted

2. Di Wang

Managed the experimental materials and reagents, executed the experiments, managed and reported the data, presented and interpreted the results, reviewed the article mainly for spelling and grammar before submission, and approved the final version of the article to be submitted

3. Miao Cui

Managed the experimental materials and reagents, executed the experiments, managed and interpreted the data, completed the literature review, organized the course of the project, reviewed the article for both grammar and intellectual content before submission, and approved the final version of the article to be submitted

4. Qizhong Zhang

Provided the personnel, environmental, and financial support and tools and instruments that are vital for the project; constructed the idea or hypothesis for the research and manuscript; supervised the course of the project and article; reviewed the article for both grammar and intellectual contents before submission; and approved the final version of the article to be submitted

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