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p38 MAPK is involved in the immune response to pathogenic *Vibrio* in the clam *Meretrix petechialis*

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ABSTRACT

p38 mitogen-activated protein kinases (MAPKs) are involved in the response to various extracellular stimuli via regulating gene expression. In the present study, a *p38 MAPK* gene (*MpP38*) was identified from the clam *Meretrix petechialis*. The full-length cDNA of *MpP38* measures 1,720 bp, consisting of a 134-bp 5′-UTR, a 1,095-bp ORF and a 491-bp 3′-UTR. Both the mRNA and protein expression levels of *MpP38* increased after *Vibrio* challenge, implying that *MpP38* is involved in clam immunity. Based on our previous study, a transcription factor activated by p38 MAPK, i.e. microphthalmia-associated transcription factor (MITF), participated in clam immunity by regulating the expression of phenoloxidase (PO). Coupled with other related reports, the mechanism underlying the involvement of *MpP38* in clam immunity is most likely that pathogen stimuli induce the phosphorylation of p38 MAPK and thus activate MITF to regulate the expression of the immune-related gene *PO*. The results obtained in this study support this mechanism. First, we found that the MpP38 phosphorylation level increased in response to *Vibrio* challenge. Second, as revealed by a yeast two-hybrid assay, there was a direct interaction between MpP38 and MITF. Meanwhile, inhibiting the phosphorylation of MpP38 decreased the phosphorylation level of MpMITF, implying that MpP38 phosphorylation is required for MpMITF activation. Additionally, our results showed that there was a regulatory relationship between MpP38 phosphorylation level and *PO* expression level. With increased MpP38 phosphorylation level, the *PO* expression level was also increased after *Vibrio* challenge; when MpP38 phosphorylation was inhibited, the *PO* expression level was significantly decreased. This study describes the immune function of p38 MAPK in the clam for the first time and analyses its potential underlying mechanism, which will help to elucidate the immune mechanism in the clam *M. petechialis*.

1. Introduction

The clam, *Meretrix petechialis*, an important commercial species of marine bivalve molluscs, is widely distributed on the southeastern coast of Asia [1]. With the development of artificial breeding, *M. petechialis* has become one of the most widely cultured molluscs in China [2]. In recent years, the mass mortality of clams caused by pathogens has been a threat to the mollusc farming industry. Among these pathogens, *Vibrio* has been reported to cause outbreaks of disease in clams [3]. The clam *M. petechialis*, as an invertebrate species, responds to *Vibrio* challenge primarily relying on the innate immune system to execute cellular and humoral immune reactions [4,5]. It is necessary to investigate the mechanism of the pathogen resistance of *M. petechialis*.

The mitogen-activated protein kinases (MAPKs) are members of discrete signalling cascades that play important roles in response to extracellular stimuli [6]. Four subgroups of the MAPK family have been described, including extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinase (JNK/SAPK), ERK5/big MAP kinase 1 (BMK1) and p38 group of protein kinases [7]. Each MAPK family is involved in biochemically distinct signalling cascades. p38 MAPKs have been found to play an essential role in regulating many cellular processes, including inflammation, apoptosis, cell

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differentiation, cell growth and death [8]. p38 MAPKs can be activated by many stimuli, including hormones, growth factors, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), and environmental stresses [6,9].

p38 MAPK (p38) contains a conserved serine/threonine protein kinase catalytic domain (S_TKc) that includes an activation loop with a dual phosphorylation site of the Thr-Gly-Tyr (TGY) motif and several specific-binding sites for ATP and substrates [10]. The conserved dual phosphorylation sites Thr180 and Tyr182 in the TGY motif can be phosphorylated and activated by upstream MAP kinase kinases (MKKs) [11]. Pathogen stimuli can induce the phosphorylation of p38 [6]. Some transcription factors, such as transcription factor 1 (ATF1), serum response factor (SRF) and microphthalmia-associated transcription factor (MITF), can be activated by phosphorylated p38 and thereby regulate the expression of downstream genes [6,12]. As a transcription factor activated by p38, MITF is involved in regulating cell proliferation, survival and immune defence [13]. MITF participates in immune defence by regulating a number of target genes involved in innate immune signalling pathways, e.g., phenoloxidase (*PO*) [14]. According to the previous reports on the p38 MAPK signalling pathway [6,14,15], we include that p38 is involved in the response to pathogen stimuli; and one potential mechanism is that pathogen stimuli induce the phosphorylation of p38 and then activate MITF by phosphorylation, then the activated MITF binds to the promoter of an immune-related gene *PO* to regulate its expression (Fig. 1).

In this study, a *p38* gene (*MpP38*) was identified from the clam *M. petechialis*. The expression profile of *MpP38* during infection implies the involvement of *MpP38* in the immune response to the *Vibrio* challenge. Our previous study has shown that MITF is involved in the immune response to *Vibrio* infection in *M. petechialis* [16] by regulating the downstream immune-related gene PO [14]. Thus, a regulatory relationship among *MpP38*, *MpMITF* and *PO* probably exists in *M. petechialis*, which is probably the mechanism underlying the *MpP38* involvement in clam immunity. To verify this mechanism in the clam, we first detected the influence of *Vibrio* infection on MpP38 phosphorylation level, then the interaction between MpP38 and MpMITF, and finally the regulatory relationship between the MpP38 phosphorylation level, MITF phosphorylation level and PO expression level. This research will provide information about the role of p38 in clam innate immunity.

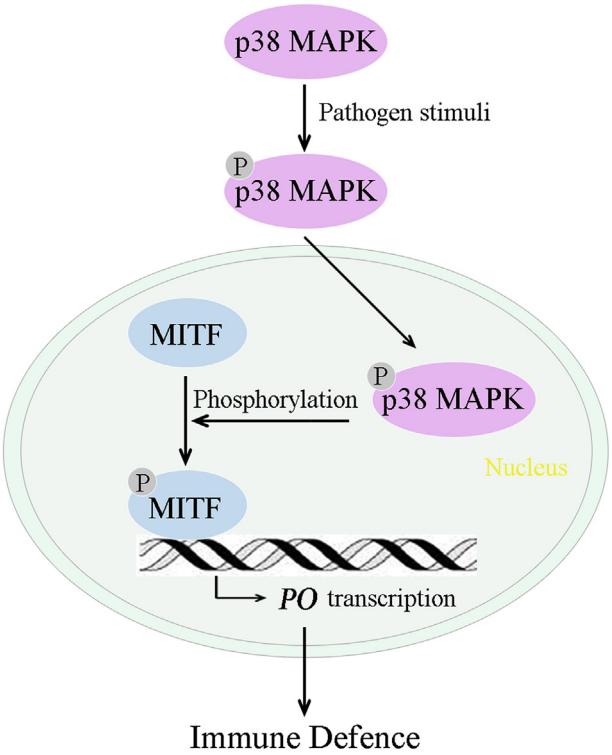

Fig. 1. Schematic illustration of one potential mechanism underlying the involvement of p38 in the immunity. p38 resides in the cytoplasm of resting cells. Pathogen stimuli induce the phosphorylation of p38, and then phosphorylated p38 translocates into the nucleus and activates downstream MITF by phosphorylation. Finally, activated MITF binds to the promoter of *PO* to regulate its expression. The letter (P) represents the phosphorylation of proteins.

2. Materials and methods

2.1. Experimental clams, Vibrio challenge and tissue collection

The adult clams applied to the *Vibrio* challenge were bought from an aquatic market in Qingdao, China. These clams were supplied with filtered aerated seawater and microalgae and were acclimated to laboratory conditions for one week. For the tissue distribution analysis, five healthy clams were collected, and the hepatopancreas, mantle, foot, gill and adductor muscle tissues were separately dissected. For the *Vibrio* challenge assay, clams were challenged by immersion in seawater with a concentration of 1×10^7 CFU mL^{-1} of *V. parahaemolyticus* [17]. Five clams were separately sampled at days 0, 1, 2, 3, 5 and 12 post-challenge. The hepatopancreas of each clam were dissected and applied to the RNA extraction for mRNA expression analysis. Meanwhile, the hepatopancreas of five clams was separately collected at days 0, 2 and 5 post-challenge and applied to the protein extraction for Western blot analysis.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from each sampled tissue using a total RNA Kit (Omega, USA) according to the manufacturer's instructions. The extracted total RNA was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, USA). The first-strand cDNA was synthesized from total RNA with a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. The cDNA used for quantitative real-time PCR (qRT-PCR) was synthesized from total RNA using a PrimeScript™ RT reagent Kit with a gDNA Eraser (TaKaRa, Japan).

2.3. Molecular cloning and bioinformatic analysis

The SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to clone the full-length cDNA of *MpP38* based on the fragment from our NGS transcriptomic data of *M. petechialis*. Briefly, specific primers MpP38-5′GSP, MpP38-5′NGSP, MpP38-3′GSP and MpP38-3′NGSP were used to amplify the fragments by nested PCR (Table 1). The PCR reaction conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 40 s, annealing at a suitable temperature depending on primers for 30 s and 72 °C for 2 min followed by another 10 min of final extension at 72 °C. The whole assembled sequence was confirmed by PCR using cMpP38-F and cMpP38-R primers (Table 1) and sequencing.

The ORF was predicted with ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The nucleotide sequence was translated into the amino acid sequence with DNAMAN 8.0 software. The molecular weight (MW) of the predicted protein was calculated using the online software ExPASy Server (http://www.expasy.org/vg/index/Protein). Multiple sequence alignments and a neighbor-joining (NJ) phylogenetic analysis were conducted using the software ClustalX and MEGA 6.0, respectively [18]. The domain architecture of MpP38 was predicted by online software SMART (http://smart.embl-heidelberg.de/).

2.4. Quantitative real-time PCR (qRT-PCR)

The mRNA expression levels of genes were detected by qRT-PCR

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Efficiency	Tm (°C)	Product size
Actin-F	TTGTCTGGTGGTTCAACTATG	96%	57.3	180 bp
Actin-R	GACTGATTTCTTACGGATG		53.0	
EF1α-F	CTGGAAGAGATGCCAAAGGT	100%	59.3	128 bp
EF1α-R	ATGTCACGCACAGCAAAACG		61.5	
P38-RT-F	TTGATGTCTGGTCAGTTGGGTGTA	100%	63.3	237 bp
P38-RT-R	CTATGGCATGTGGATTTGCTCC		61.1	
PO-RT-F	TGGGACTCTACCCTAGATGACC	91%	61.7	165 bp
PO-RT-R	CGTGCCACCTGTACCAGAATAT		61.7	
cMpP38-F	GCCATAATCCGGAAGTTTCTCG		60.7	1649 bp
cMpP38-R	CAATAACAGCATGCTAGCACTC		58.9	
MpP38-ORF-F	ATGTCGAATAAGCAGCAACTGAAA		60.8	1095 bp
MpP38-ORF-R	TCATTCCATTGCATCCATCCCG		62.7	
MpMITF-ORF-F	GCAGATTCAGGCATTGACATAGA		60.2	1365 bp
MpMITF-ORF-R	TAAGCCATAATCCATGCTATCATCGT		61.7	
MpP38-5′GSP	GGCTTGCTCTGCCGTGATTCGT		68.3	——
MpP38-5′NGSP	TTGGCATGAATTGCTGACTGAAAGG		67.4	——
MpP38-3′GSP	CGCAAGACAGTGACGGGATGGA		67.6	——
MpP38-3′NGSP	GTGTTGCTGCCTTTAATTCATA		55.5	

using appropriate primers. A QuantiNova SYBR Green PCR Kit (Qiagen, Germany) was used in the qRT-PCR with an ABI 7500 Fast Real-Time Thermal Cycler machine (Applied Biosystems, USA). *β-Actin* (GenBank accession No. JN084197) was used as the internal reference to normalize the expression levels between samples [26], and the results were then verified by another internal reference *EF1α* [19,20]. The primers used in this experiment are listed in Table 1. The efficiency of each primer pair was analysed with serial 10-fold dilutions of cDNA (1, 10^{-1}, 10^{-10}, 10^{-100} and 10^{-1000}). Three repeats of each sample were run in a 10-μl reaction volume containing 20 ng of template, 0.3 μM of each primer and 5 μl of QuantiNova SYBR Green PCR Master Mix. The PCR parameters were 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The $2^{-\Delta\Delta CT}$ method was used to analyse the relative gene expression levels according to the procedure reported in Livak et al. and Bustin et al. [21,22]. For the expression analysis among different tissues, the expression level in the gill tissue was set as the calibrator; for the expression analysis during the *Vibrio* challenge, the expression level at D0 was defined as the calibrator; for the expression analysis between the p38 inhibition group and control group, the expression level in the control clams was defined as the calibrator.

2.5. Western blot analysis

Western blotting was conducted to detect the protein expression according to our previous procedure [19] using a rabbit anti-p38 antibody (catalogue No. A10832. ABClonal, USA), a rabbit anti-phospho-p38 antibody (catalogue No. AP0297. ABClonal, USA) or a rabbit anti-phospho-MITF antibody (catalogue No. AF3027. Affinity biosciences, USA). Briefly, the total protein was extracted from the hepatopancreas or haemocytes using a Total Protein Extraction Kit (BestBio, China). The protein concentration was quantified using a BCA Protein Assay Kit (BestBio, China) following the manufacturer's instructions. Equal amounts of total protein samples were separated by SDS-PAGE (12%) and transferred onto a PVDF membrane. The membrane with transferred proteins was immersed in 5% nonfat milk in TBST for 2 h at room temperature. Then, the membrane was incubated with the diluted primary antibody (1:1,000) at 4 °C overnight. After washing three times for 10 min with TBST, the membrane was incubated with the diluted secondary HRP-antibody (1: 5,000). The target protein signals were detected with the Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen, China).

2.6. Yeast two-hybrid assay

The full-length cDNA sequences of *MpP38* and *MpMITF* were amplified by PCR using specific primers MpP38-ORF-F/R and MpMITF-ORF-F/R, respectively (Table 1). The cDNA encoding MpP38 was cloned into a vector pGBKT7 to construct a recombinant plasmid pGBKT7-p38 that allows the expression of the fused protein MpP38 and DNA binding domain (BD), while the cDNA encoding MpMITF was cloned into a vector pGADT7 to construct a recombinant plasmid pGADT7-MITF that allows the expression of the fused protein MITF and activation domain (AD). The two recombinant plasmids were co-transfected into the Y2HGold yeast strain using the YeastMaker™ yeast transformation system 2 (Clontech, USA). Then, the transfected yeast cells were grown on SD/-Ade/-His/-Leu/-Trp/AbA/X-α-Gal medium (SD medium without Ade, His, Leu and Trp but supplemented with AbA and X-α-Gal). If the two expressed proteins MpP38 and MpMITF interact in the yeast cells, BD and AD are brought in close distance and thus become able to activate the transcription of the reporter gene LacZ, leading to the growth of blue bacterial colonies on the medium. Yeast cells co-transfected with pGBKT7-53 and pGADT7-T were used as positive controls. Yeast cells co-transfected with pGBKT7-p38 and empty pGADT7 were the negative control.

2.7. p38 inhibition experiment

To analyse the relationship among p38, MITF and PO, a p38 inhibition experiment was performed using the p38-specific inhibitor SB203580 (Beyotime Biotechnology, China). This inhibitor can inhibit the phosphorylation of p38 and thus block the activation of downstream genes. Briefly, the haemocytes extracted from healthy clams were treated with SB203580 (the p38 inhibitor group) or DMSO (the control group) for 12 h at 18 °C. The haemocytes were washed three times with PBS and then stored at −70 °C for RNA extraction and protein extraction. The phosphorylation levels of MpMITF in the p38 inhibitor group and the control group were detected by Western blotting using an anti-phospho-MITF antibody (see section 2.5). In addition, the relative expression level of *MpPO* was compared between the p38 inhibitor group and the control group by qRT-PCR.

2.8. Statistical analysis

All data are presented as the means ± S.D. Statistically significant differences were analysed by one-way ANOVA using SPSS 19.0 software. The significance level was set as $P < 0.05$.

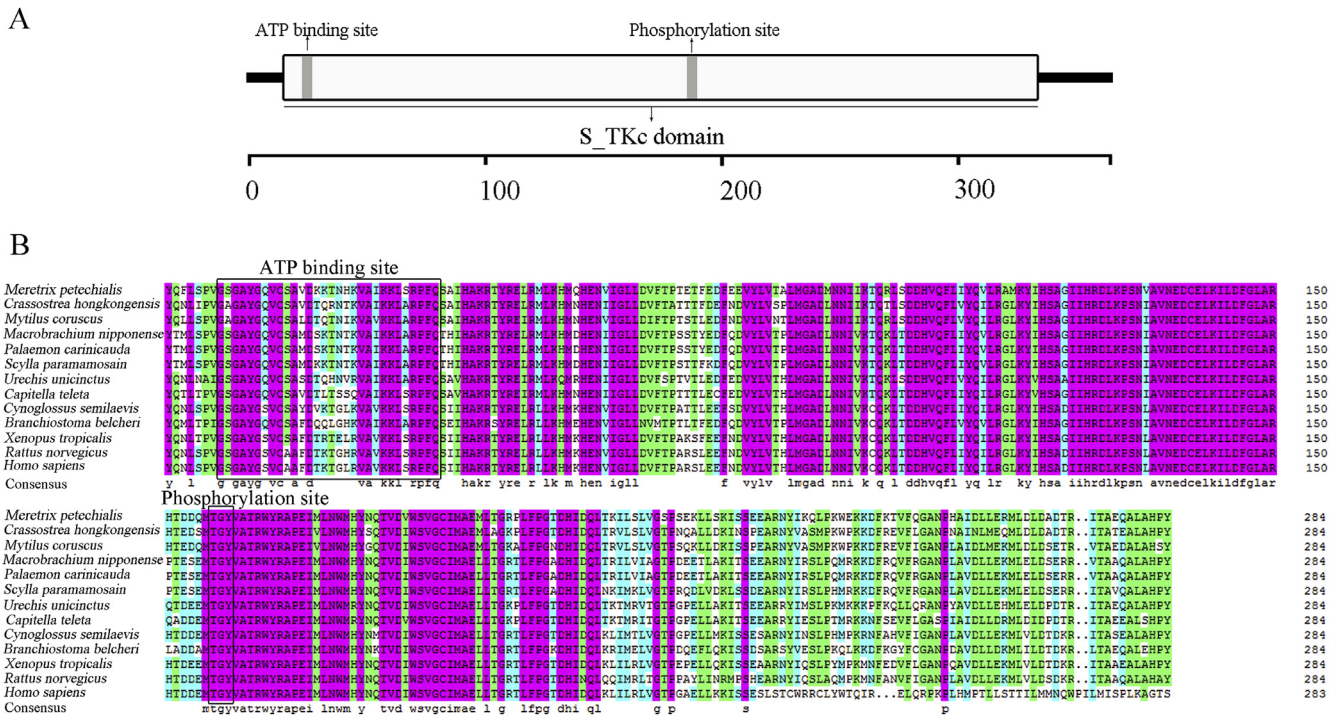

Fig. 2. (A) Domain architecture of the MpP38 protein, predicted by SMART (http://smart.embl-heidelberg.de/). (B) Multiple alignments based on amino acid sequences of MpP38 and p38s of other species. GenBank accession numbers and species for the used p38 sequences: MK558275 (*Meretrix petechialis*), APC65297.1 (*Crassostrea hongkongensis*), AGW27417.1 (*Mytilus coruscus*), ASM46958.1 (*Macrobrachium nipponense*), ASU54245.1 (*Palaemon carinicauda*), AHH29322.1 (*Scylla paramamosain*), ALF99798.1 (*Urechis unicinctus*), ELU14889.1 (*Capitella teleta*), XP_008318491.1 (*Cynoglossus semilaevis*), XP_019645887.1 (*Branchiostoma belcheri*), NP_001005824.1 (*Xenopus tropicalis*), NP_112282.2 (*Rattus norvegicus*), NP_620583.1 (*Homo sapiens*).

3. Results

3.1. Sequence analysis of MpP38

The full-length cDNA sequence of *MpP38* was obtained from *M. petechialis*, which measures 1,720 bp (GenBank: MK558275), consisting of a 134-bp 5′-UTR, a 1,095-bp ORF and a 491-bp 3′-UTR. The nucleotide sequence of *MpP38* was predicted to encode a polypeptide of 365 amino acid residues with a molecular weight of 41 kDa. The nucleotide and putative amino acid sequences are shown in Supplemental Fig. 1 (Fig. S1). A conserved S_TKc domain with a conserved TGY phosphorylation site and an ATP-binding site were detected in the MpP38 protein sequence (Fig. 2A). In addition, the results of multiple sequence alignments showed that MpP38 was similar in the protein sequence of S_TKc domain to the p38 of other species, including oyster *Crassostrea hongkongensis*, scallop *Mytilus coruscus*, river prawn *Macrobrachium nipponense*, white prawn *Palaemon carinicauda*, mud crab *Scylla paramamosain*, spoon worm *Urechis unicinctus*, segmented worm *Capitella teleta*, tongue sole *Cynoglossus semilaevis*, amphioxus *Branchiostoma belcheri*, clawed frog *Xenopus tropicalis*, rat *Rattus norvegicus* and human *Homo sapiens* (Fig. 2B). Based on the above p38 proteins and MpP38, a phylogenetic tree was conducted (Fig. 3), showing that MpP38 has the closest evolutionary relationship with those from bivalves, e.g., oyster and scallop.

3.2. Changes in both mRNA and protein expression of MpP38 during Vibrio challenge

The mRNA expression of *MpP38* in the hepatopancreas, mantle, foot, gill and adductor muscle of clams was analysed by qRT-PCR. The results showed that *MpP38* mRNA was most highly expressed in the hepatopancreas followed by the mantle and adductor. The lowest expression of *MpP38* was detected in the gill (Fig. 4, Fig. S2). The hepatopancreas is the main immune organ in clams [23]; therefore, the hepatopancreas was selected for further study.

To investigate the response of *MpP38* to *Vibrio* challenge, we analysed changes in both mRNA and protein expression of MpP38 during a *Vibrio* challenge. As shown in Fig. 5A and Fig. S2, the mRNA expression level of *MpP38* increased at 2 days post-infection (dpi), peaked at 3 dpi, and then decreased to the base level at 12 dpi. A significant difference was detected at 3 dpi compared to that at 0 dpi ($P < 0.05$). By Western blot analysis, we found that the protein expression of MpP38 increased at 2 dpi and 5 dpi compared to 0 dpi (Fig. 5B). These results imply that MpP38 is associated with the immune response against *Vibrio* infection.

3.3. Interaction between MpP38 and MpMITF

To investigate whether MpP38 directly interacts with MpMITF, a yeast two-hybrid assay was performed. As shown in Fig. 6, the yeast strain co-transfected with pGBKT7-p38 and pGADT7-MITF activated the reporter gene and thus exhibited blue colonies on the selective SD/-Ade/-His/-Leu/-Trp/AbA/X-α-Gal medium; the yeast strain co-transfected with pGBKT7-p38 and the empty pGADT7 vector did not grow on the selective medium. The yeast two-hybrid results indicate that MpP38 is able to directly bind to MpMITF.

3.4. Influence of Vibrio challenge on the MpP38 phosphorylation level

We found a TGY dual phosphorylation site in the protein sequence of MpP38 (Fig. 2), suggesting that MpP38 can be phosphorylated. Pathogen stimuli can induce the phosphorylation of p38 [24,25]. To verify whether *Vibrio* challenge can cause MpP38 phosphorylation, Western blotting was performed. Almost no phosphorylated MpP38 was detected before *Vibrio* infection (0 dpi), while the phosphorylation level of MpP38 was increased after *Vibrio* infection, with the highest level being at 2 dpi (Fig. 7). These results indicate that *Vibrio* challenge is able to induce the phosphorylation of MpP38 in clams.

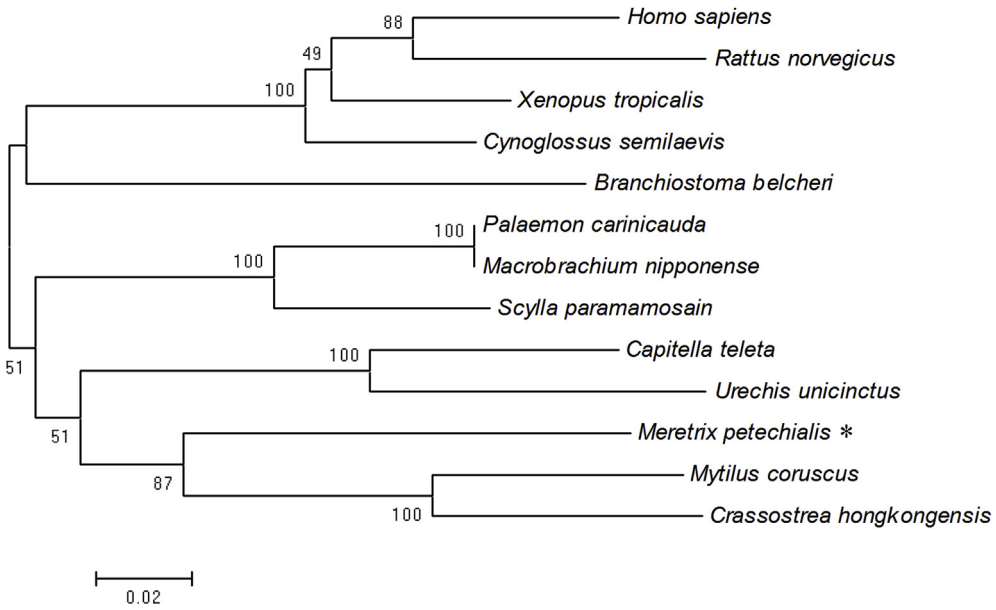

Fig. 3. Neighbor-joining phylogenetic tree based on p38 protein sequences. The p38 sequences used are the same as those analysed in multiple alignments. The numbers refer to the bootstrap values.

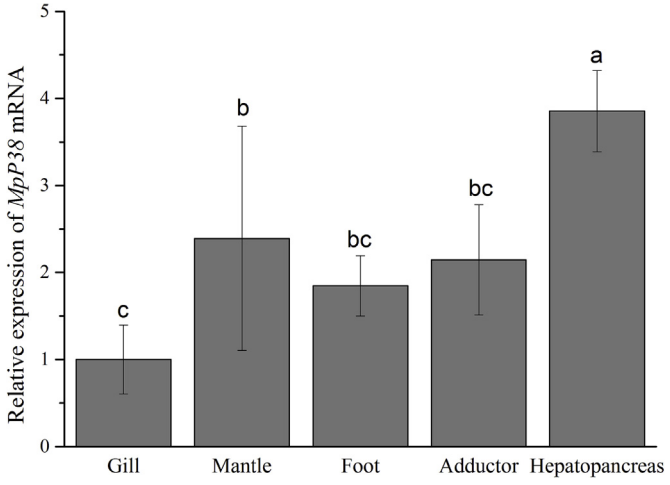

Fig. 4. Relative mRNA expression of MpP38 in different tissues of *M. petechialis*. Error bars represent the SD. Different letters indicate significant differences in expression level ($P < 0.05$).

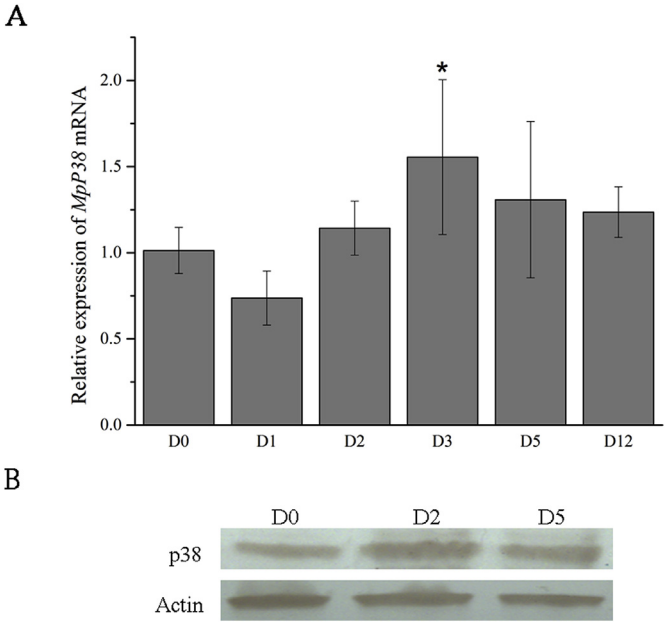

Fig. 5. MpP38 expression in the hepatopancreas of *M. petechialis* during the *Vibrio* challenge. (A) Relative mRNA expression levels measured by qRT-PCR. Error bars represent SD. The asterisk (*) represents a significant difference found when compared to 0 dpi ($P < 0.05$). D0/1/2/3/5/12 means 0/1/2/3/5/12 days post *Vibrio* infection. (B) Protein expression, measured by Western blotting. β-Actin was used to confirm that equal amounts of protein were run on gel. D0/2/5 means 0/2/5 days post *Vibrio* infection.

3.5. Regulatory relationships between the MpP38 phosphorylation level, MpMITF phosphorylation level and MpPO expression level

To clarify the influence of MpP38 phosphorylation on the activation of MpMITF, we inhibited MpP38 phosphorylation and then analysed changes in the MpMITF phosphorylation level by Western blot. The results showed that the phosphorylated MpMITF was decreased in the p38 inhibitor group compared to the control group (Fig. 8A), implying that phosphorylated MpP38 can activate MpMITF by kinase activity.

Our previous research has demonstrated that *PO* is an immune-related target gene of MITF [14]. In this study, to investigate whether the expression of *PO* was related to the phosphorylation level of MpP38, some experiments were performed. First, *MpPO* expression levels were detected during the *Vibrio* challenge. Similar to the changes in MpP38 phosphorylation level (Fig. 5B), the expression level of *MpPO* also significantly increased at 2 dpi compared to 0 dpi ($P < 0.05$) (Fig. 8B, Fig. S2). In addition, changes in the expression of *MpPO* were detected after MpP38 phosphorylation was inhibited. As shown in Fig. 8C and Fig. S2, the expression of *MpPO* was significantly decreased in the p38 inhibitor group compared to the control group ($P < 0.05$). Our results imply that phosphorylated MpP38 increases the MpMITF phosphorylation level and ultimately upregulates the expression level of *MpPO*.

4. Discussion

p38 mediates a wide variety of cellular behaviours in response to extracellular stimuli and plays an important role in regulating gene expression [6]. The functions of p38 have been reported in vertebrate species, especially in mammals [9]; however, few studies on p38 have

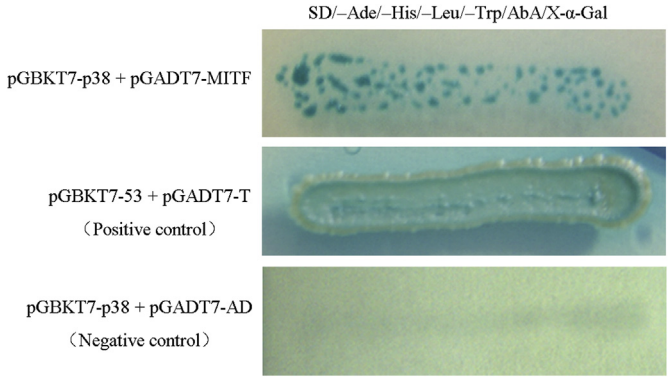

Fig. 6. Interaction between MpP38 and MpMITF, measured by the yeast two-hybrid assay. “SD/-Ade/-His/-Leu/-Trp/AbA/X-α-Gal” is the selective medium used in the yeast two-hybrid assay. “pGBKT7-p38 + pGADT7-MITF” represents the pGBKT7-p38 plasmids, and pGADT7-MITF plasmids were co-transfected into the yeast strain. The pair of pGBKT7-53 and pGADT7-T is a positive control, indicating the interaction between the p53 and T7 proteins. The pair of pGBKT7-p38 and pGADT7-AD is a negative control, showing the absence of an interaction lacking MITF protein.

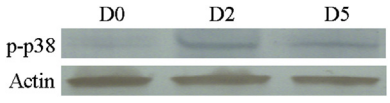

Fig. 7. Phosphorylated MpP38 (p-p38) expression in the hepatopancreas of *M. petechialis* during the *Vibrio* challenge, measured by Western blot. β-actin was used as the internal reference to normalize the total protein quantity between samples. D0/2/5 means 0/2/5 days post *Vibrio* infection.

been published to date in molluscs. In this study, we identified a *p38* (*MpP38*) gene from the clam *M. petechialis* and revealed its immune function, which may help to elucidate the immune mechanism in the clam *M. petechialis*.

The full-length cDNA and predicted protein sequence of MpP38 were obtained (Supplemental Fig. 1). p38 reported in other species contains an S_TKc domain. For instance, p38 in the Chinese mitten crab *Eriocheir sinensis* has an S_TKc domain containing active sites, such as an activation loop and an ATP binding site [26]. Similarly, a conserved S_TKc domain with an activation loop and several specific binding sites is also detected in the p38 of rock bream *Oplegnathus fasciatus* [27]. A multiple sequence alignment showed a high similarity in the sequences of the S_TKc domain between MpP38 and p38s of some species, including invertebrates and vertebrates (Fig. 2). To the best of our knowledge, all known MAPKs can be categorised based on the sequence of the Thr-Xaa-Tyr (TXY) motif [28]. The ERK group possesses a Thr-Glu-Tyr (TEY) motif. The JNK group has a Thr-Pro-Tyr (TPY) motif. p38s have a Thr-Gly-Tyr (TGY) motif [29]. Each MAPK group has a distinct substrate specificity and is regulated by a separate signal transduction pathway [30]. In the S_TKc domain of MpP38, a TGY motif containing a dual phosphorylation site was found (Fig. 2). In summary, MpP38 is highly conserved and may have an equivalent function with other reported p38 MAPKs.

p38s are ubiquitously expressed in different tissues, such as the kidney, lung, testis and muscle, in mammals [31]. We investigated the tissue expression pattern of *MpP38* and found that *MpP38* was widely expressed in all five detected tissues, including hepatopancreas, mantle, foot, gill and adductor muscle, and the highest expression level was detected in hepatopancreas (Fig. 4). As hepatopancreas is an important organ involved in the immune response in invertebrates [23], hepatopancreas was selected for further study. In mammals, p38 plays a critical role in the cellular immune response to different environmental stressors and pathogen infections [32]. Moreover, the p38 pathway participates in *Drosophila* host defence in response to microbial

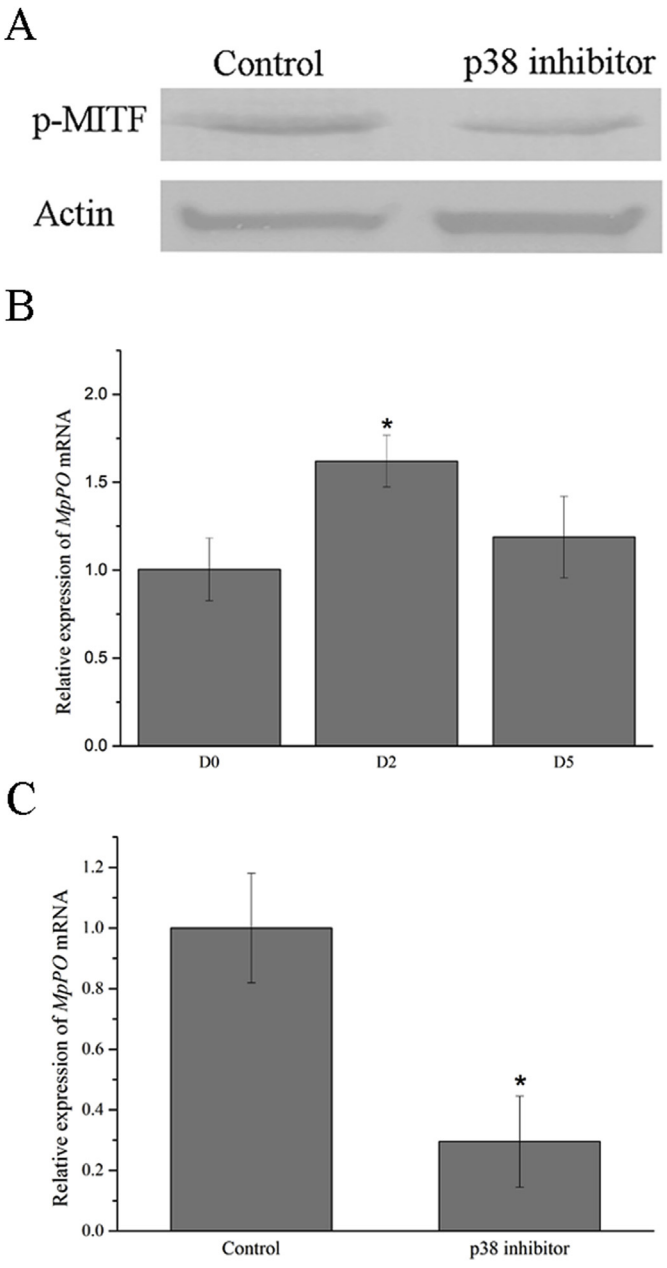

Fig. 8. (A) Phosphorylated MpMITF (p-MITF) expression in the haemocytes of *M. petechialis* in the control group and p38 inhibitor group, as measured by Western blot. β-actin was used as the internal reference to normalize the total protein quantity between samples. (B) Relative mRNA expression of *MpPO* in the hepatopancreas of *M. petechialis* during the *Vibrio* challenge, measured by qRT-PCR. The asterisk (*) represents a significant difference found when compared to 0 dpi ($P < 0.05$). D0/2/5 means 0/2/5 days post *Vibrio* infection. (C) Relative mRNA expression of *MpPO* in the haemocytes of *M. petechialis* in the control group and p38 inhibitor group, measured by qRT-PCR. Error bars represent SD. The asterisk (*) represents a significant difference found between the control group and p38 inhibitor group ($P < 0.05$).

infection [33]. p38 mediating resistance to bacterial infection has also been well-established in *C. elegans* [34]. In this study, we found that *MpP3*8 mRNA expression was significantly increased during the *Vibrio* challenge and that MpP38 protein expression also showed the same trend ($P < 0.05$) (Fig. 5). Similarly, the expression of p38 is increased in response to different immune stimuli in vertebrates, such as rock bream *Oplegnathus fasciatus* and grass carp *Ctenopharyngodon idella* [27,35], and invertebrates, such as shrimps [36,37]. These data suggest that MpP38 participates in the immune response of the clam *M.*

petechialis.

As a further question, we need to know how MpP38 functions in the clam immune response. Previous studies have demonstrated the interaction between p38 and MITF, which is essential for the activation of the latter and the resulting regulation of the downstream genes [12,38]. In this study, we performed a yeast two-hybrid assay to detect the interaction between MpP38 and MpMITF, and the results indicate that MpP38 can directly bind to MpMITF (Fig. 6). *PO*, an important immune-related gene of molluscs [39], has been demonstrated to be a downstream gene of MITF in mouse and molluscs [14,40]. In *M. petechialis*, *MpMITF* participates in the clam immune defence [16] by directly regulating the expression of downstream immune-related genes, e.g., *PO* [14]. Thus, it is suggested that in *M. petechialis,* MpP38 is involved in clam immune defence by activating MpMITF and thereby influencing the expression of the downstream immune-related gene *MpPO*.

Previous studies have demonstrated that only phosphorylated p38 can activate MITF and thus influence the expression of downstream genes [12,41]. We analysed the phosphorylation level of MpP38 during *Vibrio* infection by Western blot analysis, and the results showed that MpP38 phosphorylation levels obviously increased after *Vibrio* infection (Fig. 7). This result was consistent with that observed in oyster *Crassostrea gigas*. The phosphorylation level of p38 in oyster haemocytes significantly increased after LPS stimulation [24]. A similar result was also observed in *Drosophila*, showing that the phosphorylation level of p38 increased after exposure to environmental toxicants [25]. Our results suggest that *Vibrio* infection induces the phosphorylation of MpP38.

Phosphorylated p38 can activate MITF by kinase activity, thereby upregulating the transcriptional expression of the downstream gene [42]. To investigate whether MpMITF is activated by phosphorylated MpP38, the relationship between the MpP38 phosphorylation level and the MpMITF phosphorylation level was detected. We found that the MpMITF phosphorylation level was decreased after blocking the phosphorylation site in MpP38 by a specific inhibitor (Fig. 8A). Bhattacharyya et al. also demonstrated that phosphorylated p38 increased the MITF phosphorylation level [43]. To further clarify the influence of phosphorylated p38 on MpMITF and its downstream gene, we analysed the regulatory relationship between the *MpPO* expression level and the MpP38 phosphorylation level. Our results showed that during *Vibrio* infection, the mRNA expression of *MpPO* increased with the increase of phosphorylated MpP38 (Fig. 8B); when the phosphorylation of MpP38 was inhibited, the mRNA expression level of *MpPO* was significantly decreased (Fig. 8C). Similarly, the inhibited p38 MAPK reduced the mRNA expression of PO in B16 cells [44]. These results imply that *MpPO* expression is influenced by the phosphorylation level of MpP38 via activating MpMITF.

In conclusion, a p38 gene (*MpP38*) was identified from the clam *M. petechialis*. *MpP38* is involved in the clam immune response against *Vibrio,* and the underlying mechanism is most likely that *Vibrio* infection induces the phosphorylation of *MpP38* and ultimately increases the expression of the immune-related gene *MpPO* via activating MpMITF. This study implies the immune function of p38 in the clam for the first time and may help to elucidate the immune mechanism of the clam *M. petechialis*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.048.

References

[1] B. Liu, B. Dong, B. Tang, T. Zhang, J. Xiang, Effect of stocking density on growth, settlement and survival of clam larvae, *Meretrix meretrix*, Aquaculture 258 (1–4) (2006) 344–349.
[2] B. Tang, B. Liu, G. Wang, T. Zhang, J. Xiang, Effects of various algal diets and starvation on larval growth and survival of *Meretrix meretrix*, Aquaculture 254 (1–4) (2006) 526–533.
[3] X. Yue, B. Liu, J. Xiang, J. Jia, Identification and characterization of the pathogenic effect of a *Vibrio parahaemolyticus*-related bacterium isolated from clam *Meretrix meretrix* with mass mortality, J. Invertebr. Pathol. 103 (2) (2010) 109–115.
[4] R. Medzhitov, C.A. Janeway, Innate immunity: the virtues of a nonclonal system of recognition, Cell 91 (3) (1997) 295–298.
[5] M.r. Arala-Chaves, T. Sequeira, Is there any kind of adaptive immunity in invertebrates? Aquaculture 191 (1) (2000) 247–258.
[6] K. Ono, J. Han, The p38 signal transduction pathway activation and function, Cell. Signal. 12 (1) (2000) 1–13.
[7] G.L. Johnson, R. Lapadat, Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases, Science 298 (5600) (2002) 1911.
[8] L. New, J. Han, The p38 MAP Kinase pathway and its biological function, Trends Cardiovasc. Med. 8 (5) (1998) 220–228.
[9] J.M. Kyriakis, J. Avruch, Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update, Physiol. Rev. 92 (2) (2012) 689–737.
[10] M. Ressurreição, D. Rollinson, A.M. Emery, A.J. Walker, A role for p38 MAPK in the regulation of ciliary motion in a eukaryote, BMC Cell Biol. 12 (1) (2011) 6.
[11] T. Zarubin, J. Han, Activation and signaling of the p38 MAP kinase pathway, Cell Res. 15 (2005) 11.
[12] K.C. Mansky, U. Sankar, J. Han, M.C. Ostrowski, Microphthalmia transcription factor is a target of the p38 MAPK pathway in response to receptor activator of NF-kappa B ligand signaling, J. Biol. Chem. 277 (13) (2002) 11077–11083.
[13] C.L. Hershey, D.E. Fisher, Genomic analysis of the microphthalmia locus and identification of the MITF-J/Mitf-J isoform, Gene 347 (1) (2005) 73–82.
[14] S. Zhang, X. Yue, J. Yu, H. Wang, B. Liu, MITF regulates downstream genes in response to *Vibrio parahaemolyticus* infection in the Clam *Meretrix Petechialis*, Front. Immunol. 10 (2019) 1547.
[15] K. Flores, S.S. Yadav, A.A. Katz, R. Seger, The nuclear translocation of mitogen-activated protein kinases: molecular mechanisms and use as novel therapeutic target, Neuroendocrinology 108 (2) (2019) 121–131.
[16] S. Zhang, X. Yue, F. Jiang, H. Wang, B. Liu, Identification of an MITF gene and its polymorphisms associated with the *Vibrio* resistance trait in the clam *Meretrix petechialis*, Fish Shellfish Immunol. 68 (2017) 466–473.
[17] B. Liang, F. Jiang, S. Zhang, X. Yue, H. Wang, B. Liu, Genetic variation in *vibrio* resistance in the clam *Meretrix petechialis* under the challenge of *Vibrio parahaemolyticus*, Aquaculture 468 (2017) 458–463.
[18] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (24) (1997) 4876–4882.
[19] X. Yue, B. Liu, Q. Xue, An i-type lysozyme from the Asiatic hard clam *Meretrix meretrix* potentially functioning in host immunity, Fish Shellfish Immunol. 30 (2) (2011) 550–558.
[20] F. Jiang, X. Yue, H. Wang, B. Liu, Transcriptome profiles of the clam *Meretrix petechialis* hepatopancreas in response to *Vibrio* infection, Fish Shellfish Immunol. 62 (2017) 175–183.
[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (4) (2001) 402–408.
[22] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (2009) 611–622.
[23] T. Rőszer, The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism, Cell Tissue Res. 358 (3) (2014) 685–695.
[24] J. Sun, L. Wang, Z. Wu, S. Han, L. Wang, M. Li, Z. Liu, L. Song, P38 is involved in immune response by regulating inflammatory cytokine expressions in the Pacific oyster *Crassostrea gigas*, Dev. Comp. Immunol. 91 (2019) 108–114.
[25] J.E.S. Batista, L.R. Sousa, I.K. Martins, N.R. Rodrigues, T. Posser, J.L. Franco, Data on the phosphorylation of p38 MAPK and JNK induced by chlorpyrifos in *Drosophila melanogaster*, Data Brief 9 (2016) 32–34.
[26] J.C. Zhao, Y.L. Wang, Q. Li, M. Zhu, W.J. Sun, T.M. Wu, Q. Wang, L. He, Molecular cloning and characterization of p38gene in the Chinese Mitten Crab, *Eriocheir sinensis*, Aquacult. Res. 47 (4) (2016) 1353–1363.
[27] N. Umasuthan, S.D. Bathige, J.K. Noh, J. Lee, Gene structure, molecular characterization and transcriptional expression of two p38 isoforms (MAPK11 and MAPK14) from rock bream (*Oplegnathus fasciatus*), Fish Shellfish Immunol. 47 (1) (2015) 331–343.
[28] S.K. Hanks, T. Hunter, Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification, FASEB J. 9 (8) (1995) 576–596.
[29] X.S. Wang, K. Diener, C.L. Manthey, S.-w. Wang, B. Rosenzweig, J. Bray, J. Delaney,

C.N. Cole, P.-Y. Chan-Hui, N. Mantlo, H.S. Lichenstein, M. Zukowski, Z. Yao, Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase, J. Biol. Chem. 272 (38) (1997) 23668–23674.
[30] R.J. Davis, MAPKs: new JNK expands the group, Trends Biochem. Sci. 19 (11) (1994) 470–473.
[31] Y. Jiang, C. Chen, Z. Li, W. Guo, J.A. Gegner, S. Lin, J. Han, Characterization of the structure and function of a new mitogen-activated protein kinase (p38 MAPK), J. Biol. Chem. 271 (30) (1996) 17920–17926.
[32] R.J. Hoefen, B.C. Berk, The role of MAP kinases in endothelial activation, Vasc. Pharmacol. 38 (5) (2002) 271–273.
[33] J. Chen, C. Xie, L. Tian, L. Hong, X. Wu, J. Han, Participation of the p38 pathway in Drosophila host defense against pathogenic bacteria and fungi, Proc. Natl. Acad. Sci. U.S.A. 107 (48) (2010) 20774–20779.
[34] D.H. Kim, R. Feinbaum, G.Î. Alloing, F.E. Emerson, D.A. Garsin, H. Inoue, M. Tanaka-Hino, N. Hisamoto, K. Matsumoto, M.-W. Tan, F.M. Ausubel, A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity, Science 297 (5581) (2002) 623.
[35] F. Qu, J. Tang, J. Liao, B. Chen, P. Song, W. Luo, D. Xiong, T. Liu, Q. Gao, S. Lu, Z. Liu, Mitogen-activated protein kinase kinase 6 is involved in the immune response to bacterial di-/tripeptide challenge in grass carp *Ctenopharyngodon idella*, Fish Shellfish Immunol. 84 (2019) 795–801.
[36] H. Yan, S. Zhang, C.Z. Li, Y.H. Chen, Y.G. Chen, S.P. Weng, J.G. He, Molecular characterization and function of a p38 MAPK gene from *Litopenaeus vannamei*, Fish Shellfish Immunol. 34 (6) (2013) 1421–1431.
[37] Y. He, W. Yao, P. Liu, J. Li, Q. Wang, Expression profiles of the p38 MAPK signaling pathway from Chinese shrimp *Fenneropenaeus chinensis* in response to viral and bacterial infections, Gene 642 (2018) 381–388.
[38] S.M. Sharma, A. Bronisz, R. Hu, K. Patel, K.C. Mansky, S. Sif, M.C. Ostrowski, MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation, J. Biol. Chem. 282 (21) (2007) 15921–15929.
[39] A. Luna-Acosta, M. Breitwieser, T. Renault, H. Thomas-Guyon, Recent findings on phenoloxidases in bivalves, Mar. Pollut. Bull. 122 (1–2) (2017) 5–16.
[40] F. Murisier, S. Guichard, F. Beermann, The tyrosinase enhancer is activated by Sox10 and Mitf in mouse melanocytes, Pigment Cell Res. 20 (3) (2007) 173–184.
[41] Y. Mizutani, N. Hayashi, M. Kawashima, G. Imokawa, A single UVB exposure increases the expression of functional KIT in human melanocytes by up-regulating MITF expression through the phosphorylation of p38/CREB, Arch. Dermatol. Res. 302 (4) (2010) 283–294.
[42] H.J. Kim, J.S. Kim, J.T. Woo, I.S. Lee, B.Y. Cha, Hyperpigmentation mechanism of methyl 3,5-di-caffeoylquinate through activation of p38 and MITF induction of tyrosinase, Acta Biochim. Biophys. Sin. (Shanghai) 47 (7) (2015) 548–556.
[43] S. Bhattacharyya, L. Feferman, J.K. Tobacman, Inhibition of phosphatase activity follows decline in sulfatase activity and leads to transcriptional effects through sustained phosphorylation of transcription factor MITF, PLoS One 11 (4) (2016) e0153463.
[44] N. Hirata, S. Naruto, K. Ohguchi, Y. Akao, Y. Nozawa, M. Iinuma, H. Matsuda, Mechanism of the melanogenesis stimulation activity of (ΓêÊ)-cubebin in murine B16 melanoma cells, Bioorg. Med. Chem. 15 (14) (2007) 4897–4902.