ISJ 22: 78-90, 2025 ISSN 1824-307X

RESEARCH REPORT

# Involvement of GSK-3β in regulating endoplasmic reticulum stress-mediated apoptosis in *Patinopecten yessoensis* under high temperature stress

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 *Accepted July 22, 2025*

## Abstract

Glycogen synthase kinase-3β (GSK3β), a conserved regulator involved in glycogen metabolism and signal transduction, was identified in Yesso scallop (*Patinopecten yessoensis*) as *Py*GSK-3β, containing a conserved S_TKc domain and phosphorylation sites at Ser9 and Tyr216. The gene expression, protein phosphorylation and apoptosis under high temperature (HT) stress were assessed by using quantitative real-time PCR, Western blotting, and immunohistochemistry. *Py*GSK-3β mRNA was ubiquitously expressed in all the examined tissues and haemocytes. HT stress significantly upregulated the mRNA expression of *Py*GSK-3β, *Py*GRP78, *Py*IRE1, *Py*JNK, and *Py*Caspase-3, as well as the phosphorylation level of *Py*GSK-3β (Tyr216) and *Py*JNK (Thr183) in gills, and decreased the mRNA expression of *Py*BCL-2. Furthermore, the GSK-3 inhibitor SB216763 significantly suppressed the mRNA expression of *Py*GSK-3β, *Py*GRP78, *Py*JNK and *Py*Caspase-3, decreased the phosphorylation level of *Py*GSK-3β (Tyr216) and *Py*JNK (Thr183), and reduced HT stress-induced cell apoptosis after HT treatment. However, SB216763 had no effect on the mRNA expression of *Py*BCL-2. These findings highlight the pivotal role of *Py*GSK-3β in ER stress-mediated apoptosis and offer insights into scallop high temperature stress adaptation, suggesting its potential as a molecular target for breeding or further functional studies in aquaculture.

**Key Words**: *Patinopecten yessoensis*; GSK-3β; high temperature stress; endoplasmic reticulum stress; apoptosis

## Introduction

Glycogen synthase kinase (GSK) 3 is a serine/threonine kinase that plays a central role in regulating a wide range of cellular functions, such as cell signaling, division, differentiation, proliferation, growth, and apoptosis (Ali *et al*., 2001; Choi *et al*., 2007). In *Rattus norvegicus*， GSK-3 exists as two closely related isoforms, GSK-3α and GSK-3β. Although they share a high degree of structural homology in their kinase domains, they differ in other regions and are encoded by distinct genes. GSK-3β, compared to GSK-3α, exhibits a broader and more potent functional profile, impacting a wider array of cellular processes and signaling pathways (Sayas *et al*., 2012).

GSK-3β, initially identified as a rate-limiting enzyme in glycogen metabolism, has since become recognized for its unique structural features and its critical role in regulating diverse cellular processes. Structurally, GSK-3β consists of a negatively regulated N-terminal domain, a kinase domain comprising the ATP-binding and catalytic sites, and a C-terminal domain with additional negative regulatory functions (Jacobs *et al*., 2012). Unlike other kinases, GSK3β employs a unique activation mechanism where its substrates generally require pre-phosphorylation by another kinase. The phosphorylation at Ser21 or Ser9 for the α and β isoforms inhibits GSK3's kinase activity, which in turn

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**Table 1** Sequences of the primers used in the present study

| Primer | Sequence (5'-3') |
|---|---|
| **Clone primers** | |
| *Py*GSK-3β-F | ATGAGTGGTCGTCCAAGGACAAC |
| *Py*GSK-3β-R | CGCGTTGCCGGCAGCTGCAG |
| **RT-PCR primers** | |
| *Py*GSK-3β-RT-F | CCTGCCACTCGTCTACCAAG |
| *Py*GSK-3β-RT-R | TCCTCCGGTTGTGGCATTTG |
| *Py*IRE1-RT-F | AATAGATTTCAACCCTAACGA |
| *Py*IRE1-RT-R | CTAGAATATGGAGTGTGCCCC |
| *Py*GRP78-RT-F | TGAGGTCGTAGCAACTAATGG |
| *Py*GRP78-RT-R | CCTGGTGAGTGTTTCAGAGAA |
| *Py*JNK-RT-F | GGCCATCTCCAGACTCCTAT |
| *Py*JNK-RT-R | TGCAGCTCCAACAGCATGG |
| *Py*Caspase3-RT-F | GCATGGAGGAACACAGCGAG |
| *Py*Caspase3-RT-R | TGCGACTTCGCGATTGACAC |
| *Py*BCL-2-RT-F | TTGGGATGGTTTCGTGGAATTCTA |
| *Py*BCL-2-RT-R | CACATTTCCCCCCCATTTTTTA |
| β-actin -RT-F | CATCACCATCGGAAACGAGCGTTT |
| β-actin -RT-R | CAATGATCTTGATTTTCATTGTGC |

activates glycogen synthase, enhancing glycogen synthesis (Cross *et al*., 1995; Lee and Kim, 2007). In contrast, phosphorylation at Tyr216 enhances the catalytic activity of GSK-3β, further strengthening its role in cell signaling (ter Haar *et al*., 2001). Once activated, GSK-3β influences cell fate by regulating downstream proteins to respond to various stimuli (Watcharasit *et al*., 2002; Dokken *et al*., 2008; Koizumi *et al*., 2023). In particular, GSK-3β plays a critical role in the unfolded protein response (UPR), which is mediated by Inositol-Requiring Enzyme 1 (IRE1) and two other ER membrane sensors, and is essential for managing cellular stress (Kohno, 2010; Liu *et al*., 2017; Zheng *et al*., 2019). However, when the prolonged stress caused serious damage, IRE1 usually regulates GSK-3β to interact with multiple key molecules related to apoptosis and promote apoptosis (Kim *et al*., 2015b). For example, GSK-3β-mediated c-Jun N-terminal kinase (JNK) signaling played an important role in regulating glucose regulatory protein 78 (GRP78) and contributes to ER stress-induced apoptosis (Oh and Nam, 2019). Overall, GSK-3β plays a crucial role in regulating apoptosis induced by ER stress, though the specific mechanisms under different stress conditions still require further investigation.

GSK-3β has been identified in numerous aquatic species, and its involvement in key biological processes, including growth, development, and immune response, has been preliminarily elucidated (Sun *et al*., 2021; Yu *et al*., 2023). Aquatic GSK-3βs are generally conserved across species, possessing a typical S_TKc domain. Nevertheless, interspecies variations exist in the number of exons within GSK-3β encoding genes (Bian *et al*., 2020; Zhang *et al*., 2020). Key regulatory residues, such as Ser9 and Tyr216 are highly conserved, and in *Ctenopharyngodon idella*, phosphorylation at these sites has been shown to regulate GSK-3β activity (Xu *et al*., 2019). Despite structural conservation, GSK-3β exhibits diverse tissue distribution and expression patterns, likely reflecting functional adaptation to species-specific physiological demands. In *Danio rerio*, GSK-3β is predominantly localized in the nervous system, spinal cord, and somites, with its expression gradually expanding to other tissues during development (Tsai *et al*., 2000). This suggests a role for GSK-3β in nervous system development and embryonic morphogenesis. GSK-3β of *Crassostrea ariakensis* is highly expressed in the gills and adductor muscles, and its expression shows a negative correlation with glycogen content, indicating its importance in regulating energy metabolism in aquatic species (Wang *et al*., 2023). Furthermore, in *Magallana gigas*, GSK-3β expression levels exhibit significant sex- and season-dependent variation, implying a potential link to reproductive cycles or environmental adaptation (Hwang *et al*., 2023). Aquatic GSK-3βs have also been implicated in the regulation of inner ectoderm development in *Strongylocentrotus purpuratus* (Emily-Fenouil *et al*., 1998), growth and molting in *Litopenaeus vannamei* (Pang *et al*., 2021), and cellular phagocytosis in *Eriocheir sinensis* (Li *et al*., 2017). In the context of

**Table 2** Sequences used for the GSK-3β alignment and phylogenetic analysis

| Protein name | Organism | Accession number |
|---|---|---|
| GSK-3β | *Crassostrea gigas* | XP_011452760.1 |
| GSK-3 | *Danio rerio* | NP_571456.1 |
| GSK-3β | *Drosophila pseudoobscura* | XP_033235571.1 |
| GSK-3β | *Eriocheir sinensis* | ANZ22981.1 |
| GSK-3β | *Penaeus vannamei* | XP_027228608.1 |
| GSK-3β | *Siniperca chuatsi* | XP_044072399.1 |
| GSK-3β | *Homosapiens* | NP_002084.2 |
| GSK-3β | *Sus scrofa* | NP_001121915.1 |
| GSK-3β | *Mus musculus* | NP_062801.1 |
| GSK-3β | *Crassostrea virginica* | XP_022324238.1 |
| GSK-3β | *Patinopecten yessoensis* | XP_021353495.1 |
| GSK-3β | *Xenopus laevis* | AAA84444.1 |

antibacterial immune regulation, GSK-3β activates signaling pathways such as NF-κB and MAPK, and enhances bacterial clearance through modulation of the Nrf2-mediated antioxidant system and autophagy-related molecules like LC3 and Beclin-1, thereby consolidating its central role in innate immunity (Zou *et al*., 2025). Moreover, studies in *Crassostrea gigas* have shown that antioxidant responses to high temperature stress involve the AKT/GSK-3β and Nrf2 pathways, providing valuable insights into redox regulation mechanisms (Liu *et al*., 2025). These results suggest that GSK-3β may modulate redox signaling during environmental stress. However, its specific role in regulating apoptosis and thermal tolerance in aquatic invertebrates - especially mollusks - remains poorly understood.

The Yesso scallop *Patinopecten yessoensis*, a typical coldwater shellfish thriving at temperatures ranging from 5 °C to 20 °C, is an important aquaculture species with significant potential in the northern Yellow Sea of China (Jiang *et al*., 2016). However, mass mortality events among farmed scallops frequently occur during summer, primarily due to environmental stress, pathogen infection, and physiological imbalances (Jie *et al*., 2005). Although GSK-3β is known to participate in ER stress and apoptosis, its specific role in HT stress responses in aquatic invertebrates, particularly mollusks remains poorly understood. The connection between GSK-3β, ER stress, and apoptosis has yet to be fully elucidated. Given the increasing threat of heat-induced mortality in bivalves, understanding how GSK-3β contributes to thermal tolerance is critical. In this study, GSK-3β was identified from *P. yessoensis* (*Py*GSK-3β). The main objectives of this study are: (1) to analyze its sequence and structural characteristics, (2) to investigate its expression and activation patterns under HT stress, and (3) to explore its regulatory role in ER stress-induced apoptosis under HT stress.

## Material and methods

*Animals, treatment and sample collection*

Two-year-old Yesso scallops with an average shell length of 6-8 cm were collected from a commercial farm in Dalian, China. Adult scallops were chosen for this study as they possess fully developed immune and metabolic systems. The scallops were cultured in aerated seawater at 18 °C and fed with diatom once a day for seven days before the following experiments. All experiments were performed in accordance with the approval and guidelines of the Ethics Review Committee of Dalian Ocean University.

Tissues including gills, gonad, adductor muscle, hepatopancreas and mantle were collected from six scallops as parallel samples, and haemolymph from these scallops were extracted and centrifuged at 800×*g*, 4 °C for 10 min to harvest the haemocytes. After addition of 1 mL Trizol reagent (Invitrogen), all the samples were stored at -80 °C for RNA extraction.

Forty-eight scallops were used for the HT treatment experiment. They were initially cultured in aerated seawater at 18 °C (Blank group) for seven days, then 42 scallops were transferred to seawater at 25 °C (HT treatment group). Gills were collected from six randomly selected scallops at 1, 3, 6 and 12 h after HT treatment as well as in the Blank group. A submersible heater (GR, Sunsun, China) was used to raise the temperature, and a digital controller with a temperature probe (WK-SM, KEDIBO, China) was employed to monitor and maintain it. The temperature was kept at 25 ± 0.5 °C throughout the experiment. The collected samples were used for testing the expression of *Py*GSK3β, *Py*GRP78, *Py*Caspase3, *Py*JNK and *Py*Bcl-2 mRNA, the expression of *Py*GSK3β protein, and the phosphorylation level of *Py*GSK3β and *Py*JNK.

Twenty-seven scallops were used for the GSK-3 inhibitor SB216763 (SC0384, Beyotime) combined

with HT treatment experiment. These scallops were randomly divided into three groups, including the Blank group, DMSO+HT group, and SB216763+HT group. Scallops in the DMSO+HT and SB216763+HT groups individually received an injection of 100 μL DMSO (1%, dissolved in PBS) and 100 μL SB216763 (5 mM, diluted in sterile seawater), respectively. At 24 h after injection, the scallops were treated at 25 °C for 6 h, and gills were collected afterward. Some gills were fixed in paraformaldehyde for immunohistochemistry experiments, some were soaked in Trizol and stored at -80 °C for RNA extraction and qPCR analysis, while the others were subjected to native protein extraction, with the prepared protein samples stored at -80 °C for Western blot analysis.

*RNA isolation, cDNA synthesis and gene cloning of PyGSK-3β*

Total RNA isolation, cDNA synthesis and gene cloning were performed according to previous reports (Yang *et al*., 2017). Briefly, total RNA was extracted from haemocytes and tissues using TRIzol reagent (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) with oligo(dT)-adaptor primers, following the manufacturer's instructions. The primers of *Py*GSK-3β-F and *Py*GSK-3β-R (Table 1) were designed based on the full CDS sequence of *Py*GSK-3β (GenBank accession No. XM_021497820) to amplify its open reading frame (ORF). The PCR product was gel-purified, cloned into pMD19-T simple vector (Takara), and confirmed by DNA sequencing.

*Sequence analysis*

The sequence homology analysis of *Py*GSK-3β was conducted using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ExPASy Server (https://www.expasy.org/) was used to predict the isoelectric point and molecular weight of *Py*GSK-3β. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional domains of *Py*GSK-3β, and multiple sequence alignments were conducted using the ClustalW program (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Sequence information of the GSK-3 is listed in Table 2.

*Quantitative real-time PCR (qRT-PCR)*

The SYBR Green qRT-PCR was carried out on an ABI 7500 Real-time Thermal Cycler platform according to the manufacturer's protocol (TaKaRa). All primers used in this assay were listed in Table 1. The β-actin gene (XM_021511578.1) was employed as an internal control, and the relative expression levels of *Py*GSK-3β, *Py*GRP78 (MF318508.1), *Py*IRE1 (MF318507.1), *Py*JNK (XM_021491077.1), *Py*BCL-2 (XM_021496753), and *Py*Caspase-3 (XM_021490863.1) were analyzed using the comparative Ct method ($2^{-\triangle\triangle Ct}$ method).

*Western blot analysis*

The specificity of the commercial antibodies, including Anti-GSK-3β Polyclonal Antibody (rabbit, Solarbio, K106607P), Phospho-GSK-3β (Tyr216) Polyclonal Antibody (rabbit, Beyotime, AF5833), and Phospho-JNK1 (Thr183) Polyclonal Antibody (rabbit, Beyotime, AF5860), against *Py*GSK-3β and *Py*JNK in gills was examined using Western blot analysis as previously described (Jia *et al*., 2015). The proteins were extracted from the gills of scallops in Blank, HT treatment, DMSO+HT treatment, and SB216763+HT treatment groups. Protein concentrations were determined using a BCA protein assay kit (Beyotime, China). Equal amounts of protein were then separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes using a mini transfer tank. After blocking with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 3 h, the membranes were incubated with 1:1000 diluted antibody against *Py*GSK-3β in TBST containing 5% nonfat milk at room temperature for 3 h. For phospho-specific antibodies (p-*Py*GSK-3β and p-*Py*JNK), blocking and incubation were performed in TBST containing 5% BSA (Bovine Serum Albumin) instead, following the previously described protocol (Liu *et al*., 2023). Following incubation with the primary antibodies, the membranes were incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibodies, including goat anti-mouse and goat anti-rabbit IgG (both at 1:2000 dilution; Sangon Biotech). The immune-blotted protein bands were then visualized using the Western Lighting ECL chemiluminescence substrate system (Thermo Scientific). The results were visualized using Amersham Imager 600 (GE Healthcare). α-Tubulin (Rabbit, Beyotime) was employed as an internal control.

*TUNEL assay*

Gills from the Blank, DMSO+HT, and SB216763+HT treatment groups were sampled and fixed in 10 mL of 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned according to the method described previously (Yue *et al*., 2024). The paraffin sections were dewaxed to water and treated with proteinase K working solution (proteinase K: PBS = 1:9). To permeabilize cell membranes, 0.1% Triton was used to cover the tissue, followed by incubation at room temperature for 10 min. A mixture of TDT enzyme, dUTP, and buffer from the TUNEL assay kit (Servicebio, G1501) was prepared at a ratio of 1:5:50 and applied to cover the tissues. After incubating at 37°C for 2 h, the sections were washed three times with PBS (pH 7.4). The slides were stained with DAPI solution for 10 min at room temperature in the dark, followed by three PBS washes. After air-drying, the samples were mounted with anti-fluorescence quenching mounting medium and imaged using a fluorescence microscope (Zeiss AxioImager A2).

*Statistical analysis*

All data were analyzed using ANOVA followed by multiple comparisons. Prior to ANOVA, data were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test to ensure the appropriateness of parametric analysis. The results were given as mean ± SD ($N \geqslant 4$), and statistically significant difference was designated at $p < 0.05$.

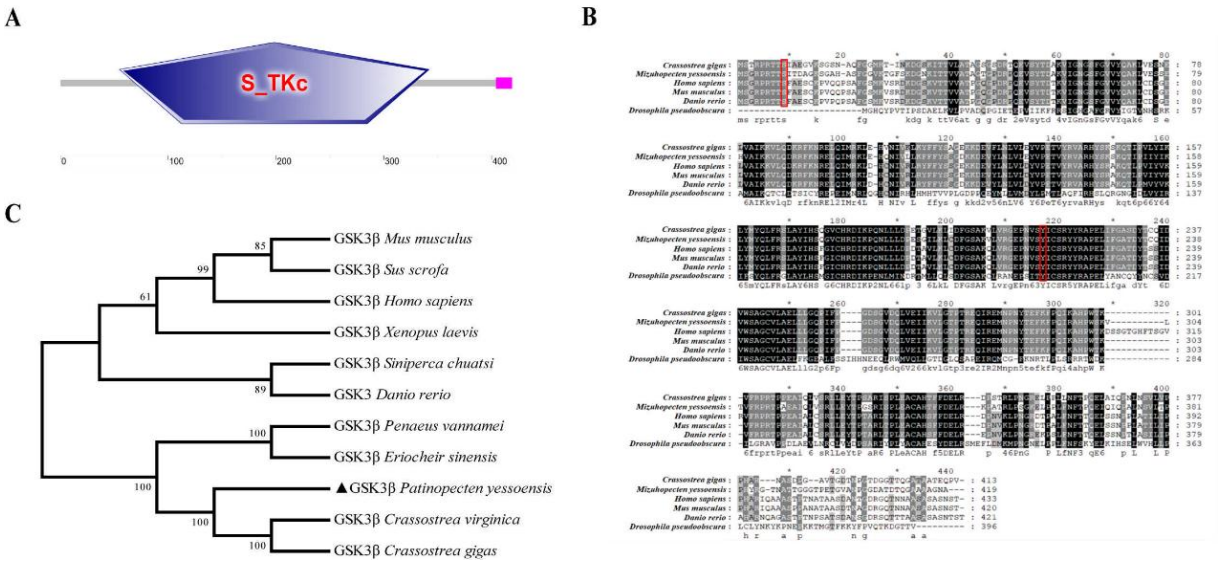


**Fig. 1** Protein domain analysis, multiple sequence alignments and phylogenetic analysis of the GSK-3β proteins from *P. yessoensis* and other organisms. (A) The domain structure of *Py*GSK-3β analyzed using SMART analysis. (B) The identical amino acid residues of GSK-3βs are shaded in black, and similar amino acids are shaded in dark gray. The highly conserved phosphorylation sites (Ser9 and Tyr216 in *P. yessoensis*) are marked with red boxes. (C) The phylogenetic tree of GSK-3βs was constituted using the neighbor-joining method in MEGA X. The number at each branch indicates the percentage bootstrap values. The amino acid sequences were obtained from NCBI. Sequence information of the GSK-3βs is listed in Table 2

## Results

*Molecular characteristics of PyGSK-3β*

The open reading frame (ORF) of *Py*GSK-3β was of 1257 bp, encoding a polypeptide of 419 amino acid residues with an isoelectric point (pI) of 9.02 and molecular weight (MW) of 46.00 kDa. There was a conserved S_TKc in the amino terminal of *Py*GSK-3β, which includes the active sites for phosphorylation at Tyr216 (Fig. 1A).

Multiple sequence alignment of GSK-3β sequences from various species revealed that *Py*GSK-3β shared the highest homology with GSK-3βfrom *Crassostrea gigas* (81.71%). It also showed significant similarity with GSK-3β from vertebrates such as *Mus musculus* (73.52%), *Homo sapiens* (72.06%) and *Danio rerio* (73.82%), whereas it exhibited relatively low similarity with GSK-3β from *Drosophila pseudoobscura* (24.47%). Additionally, two key phosphorylation sites of GSK-3β, Ser9 and Tyr216 were highly conserved across a range of species, suggesting that they are evolutionarily and functionally significant (Fig. 1B).

Eleven GSK-3β sequences from various species in vertebrates and invertebrates were selected for phylogenetic analysis. The phylogenetic tree revealed two distinct branches, the vertebrate GSK-3βs and the invertebrate GSK-3βs. *Py*GSK-3β initially clusters with GSK-3βs from mollusks, specifically *Crassostrea gigas* and *Crassostrea virginica*, indicating a closer evolutionary relationship. Then, it groups with GSK-3βs from other invertebrates, further supporting its classification within the invertebrate branch (Fig. 1C).

*Tissue distribution and temporal expression of PyGSK-3β after HT treatment*

The mRNA transcripts of *Py*GSK-3β were detected in haemocytes and all tested tissues, including gonad, mantle, hepatopancreas, gills, and adductor muscle. The mRNA expression level of *Py*GSK-3β was highest in the mantle and lowest in haemocytes, with the expression in the mantle being 231.67-fold higher than that in haemocytes ($p < 0.05$) (Fig. 2A).

The expression profiles of *Py*GSK-3β in gills after HT treatment at 25 °C were investigated using qRT-PCR. After HT treatment, the mRNA transcripts of *Py*GSK-3β at 1 h and 3 h showed a downward and slight upward trend, which was 0.55- and 1.17-fold of that in the Blank group, respectively, however, there was no significant difference ($p > 0.05$). The *Py*GSK-3β transcripts significantly up-regulated at 6 h after HT treatment (3.43-fold of that in Blank group, $p < 0.05$), and decreased to normal level at 12 h (2.22-fold, $p > 0.05$) (Fig. 2B).

*Temporal mRNA expression of UPR-related genes in gills after HT treatment*

The expression levels of UPR-related molecules, including *Py*IRE1 and *Py*GRP78, in gills were investigated using qRT-PCR at 1, 3, 6 and 12 h after HT treatment. The results showed that the mRNA transcripts of *Py*IRE1 were significantly up-regulated at 3 h and 6 h after HT treatment, which were 1.70-fold and 2.55-fold of that in the Blank group ($p < 0.05$), and then decreased to normal level at 12 h (Fig. 3A). On the other hand, the mRNA transcripts of *Py*GRP78 were significantly up-regulated at 3 h, and

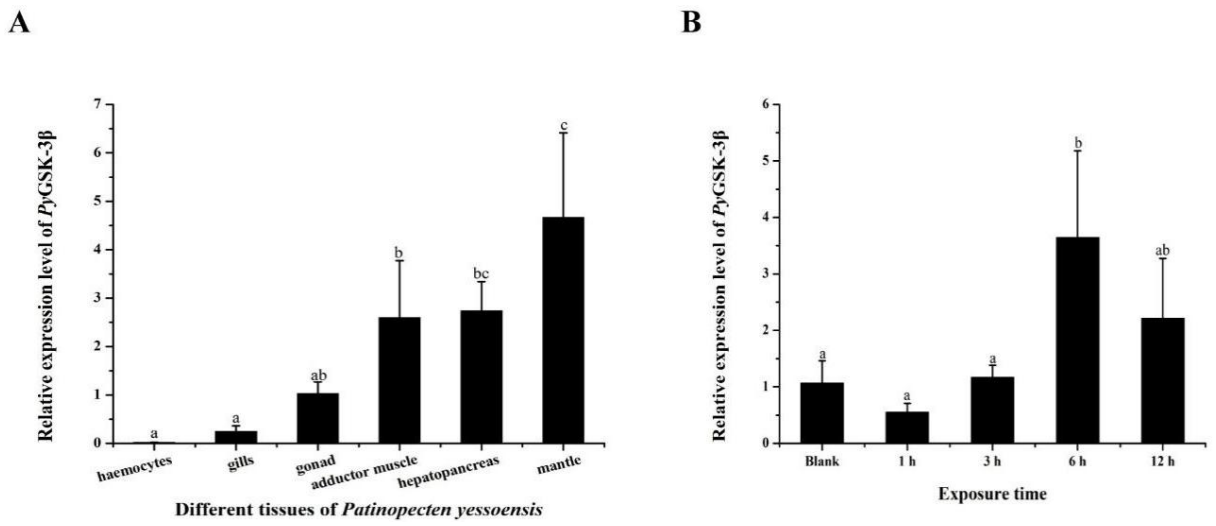


**Fig. 2** The distribution of *Py*GSK-3β mRNA in tissues and the temporal expression of *Py*GSK-3β mRNA in gills after HT treatment. (A) The relative mRNA expression levels of *Py*GSK-3β among different tissues are normalized to that of *Py*EF-α, with significant differences indicated by different letters (a, b, and c). (B) The relative mRNA expression level of *Py*GSK-3β among different tissues is normalized to that of *Py*EF-α, and the significant difference is indicated by different letters (a and b)

decreased significantly at 6 h after HT treatment, which was 1.89- and 0.09-fold of that in Blank group, respectively ($p < 0.05$) (Fig. 3B).

*Temporal expression of apoptosis-related genes and proteins in gills after HT treatment*

The expression levels of apoptosis-related molecules, including *Py*JNK, *Py*Caspase-3, and *Py*BCL-2, in gills of scallops at 1, 3, 6, and 12 h after HT treatment were investigated using qRT-PCR. The results showed that the mRNA transcripts of *Py*JNK in gills rapidly increased at 6 h (3.01-fold of that in Blank group, $p < 0.05$), and then decreased to normal levels at 12 h (Fig. 4A). Similarly, the mRNA transcripts of *Py*Caspase-3 rapidly increased at 3 h (15.08-fold of that in Blank group, $p < 0.05$), and then decreased to normal levels at 12 h (Fig. 4B). The mRNA transcripts of *Py*BCL-2 significantly increased at 3 h (2.37-fold of that in Blank group, $p < 0.05$), and then significantly decreased at 6 h (0.35-fold of that in Blank group, $p < 0.05$), and finally recovered to normal level at 12 h (Fig. 4C).

The protein levels of *Py*GSK-3β and the phosphorylation levels of *Py*GSK-3β and *Py*JNK were examined using Western blot analysis. The protein and the phosphorylation levels of *Py*GSK-3β (Tyr 216) were both significantly up-regulated at 1, 3 and 6 h after HT treatment, and then returned to normal level at 12 h. The phosphorylation level of *Py*JNK (Thr183) significantly increased at 1 h after HT treatment, returned to normal level at 3 h, and then significantly increased again at 6 h and 12 h (Fig. 4D).

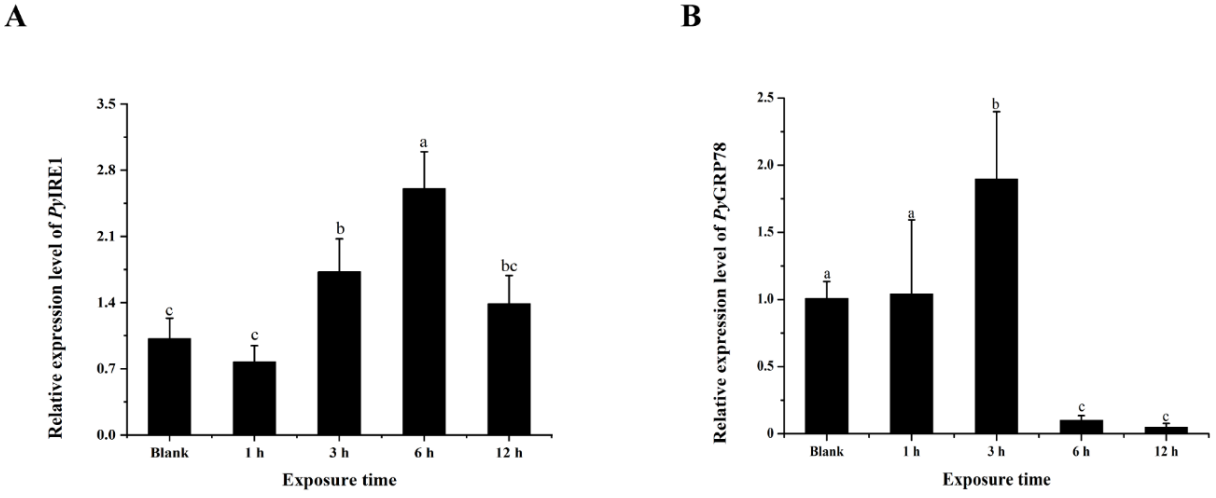


**Fig. 3** The temporal expression of *Py*IRE1 (A) and *Py*GRP78 (B) mRNA in gills after HT treatment. Relative mRNA expression levels of *Py*IRE1 and *Py*GRP78 in gills were normalized to that of *Py*EF-α, and the significant difference is indicated by different letters (a, b, and c). Data are shown as mean ± S.D. (N ≥ 4)

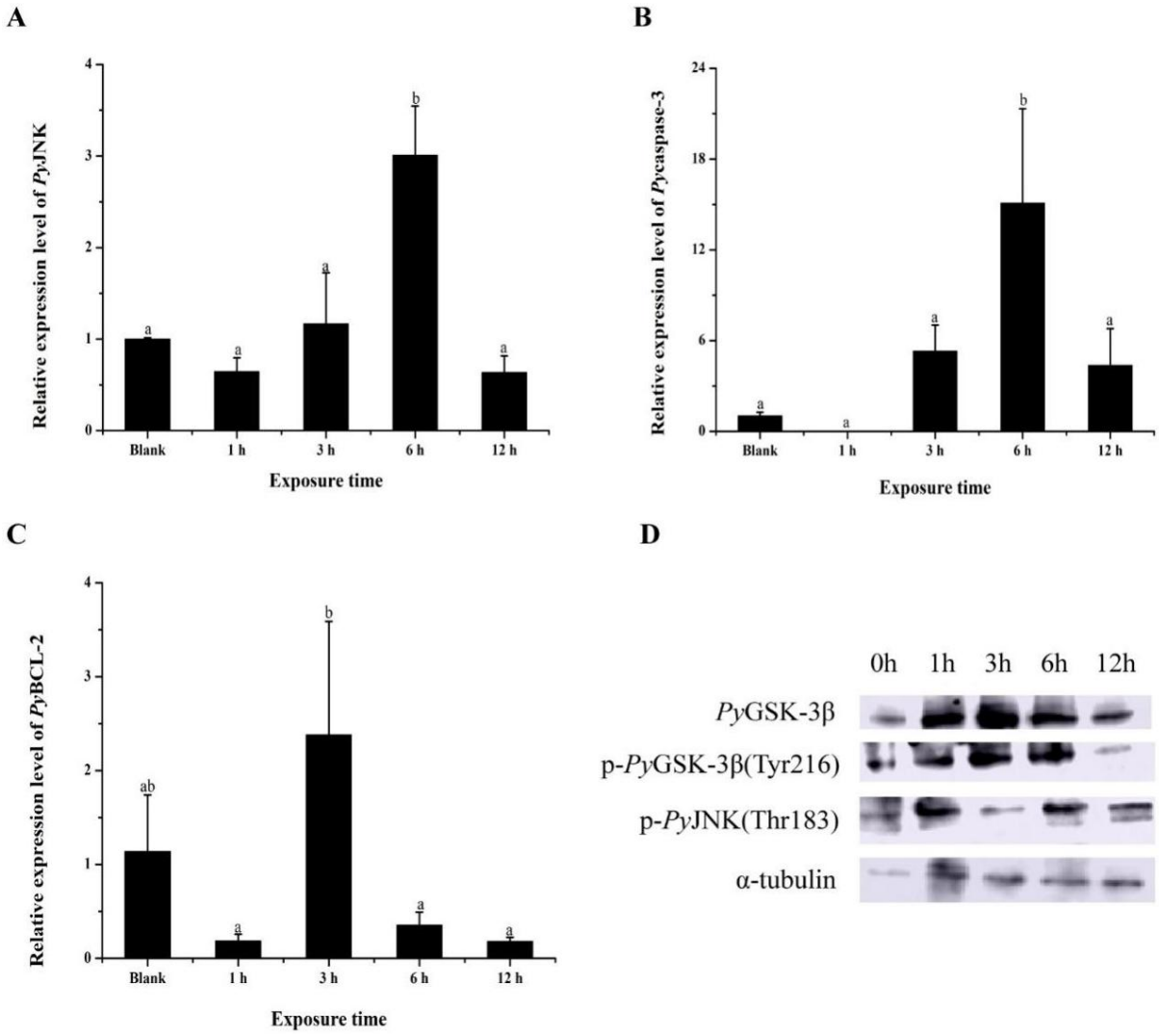


**Fig. 4** The temporal expression of *Py*JNK (A), *Py*Caspase-3 (B), and *Py*BCL-2 (C) mRNA, and the protein levels of *Py*GSK-3β and *Py*JNK (D) in gills after HT treatment. Relative mRNA expression levels of *Py*JNK, *Py*Caspase-3, and *Py*BCL-2 in gills were normalized to that of *Py*EF-α. Significant differences are indicated by different letters (a, b, and c). Data are presented as mean ± S.D. (N ≥ 4). Western blot analysis was performed for *Py*GSK-3β, p-*Py*GSK-3β (Tyr216), and p-*Py*JNK (Thr183)

*The UPR in gills of PyGSK-3β-inhibition scallop after HT treatment*

The relative mRNA expressions of *Py*GSK-3β and *Py*GRP78 in gills were evaluated following SB216763 combined with HT treatment using qRT-PCR. The results indicated that SB216763 treatment significantly suppressed the mRNA expression of both *Py*GSK-3β and *Py*GRP78 after HT treatment. Specifically, in the DMSO+HT group, the *Py*GSK-3β mRNA levels demonstrated a dramatic increase. However, in the SB216763+HT group, the *Py*GSK-3β mRNA level was significantly reduced (0.10-fold of that in the DMSO+HT group, $p < 0.001$) (Fig. 5A). Furthermore, the *Py*GRP78 mRNA level in the SB216763+HT group was markedly lower (0.05-fold of that in the DMSO+HT group, $p < 0.05$) (Fig. 5B).

*Apoptosis-related indicators in gills of PyGSK-3β-inhibition scallop after high temperature treatment*

The relative mRNA expressions of *Py*JNK, *Py*Caspase-3, and *Py*BCL-2 in gills after SB216763 combined with HT treatment were investigated. The results showed that the relative expression levels of *Py*JNK and *Py*Caspase-3 mRNA decreased significantly after SB216763 combined with HT treatment. In the SB216763+HT group, the relative expression level of *Py*JNK mRNA was 0.01-fold of that in the DMSO+HT group ($p < 0.05$) (Fig. 6A), and the relative expression level of *Py*Caspase-3 mRNA was 0.01-fold of that in the DMSO+HT group (Fig. 6B). In contrast, the relative expression level of *Py*BCL-2 mRNA showed no significant difference between the SB216763+HT group and the DMSO+HT group (Fig. 6C).

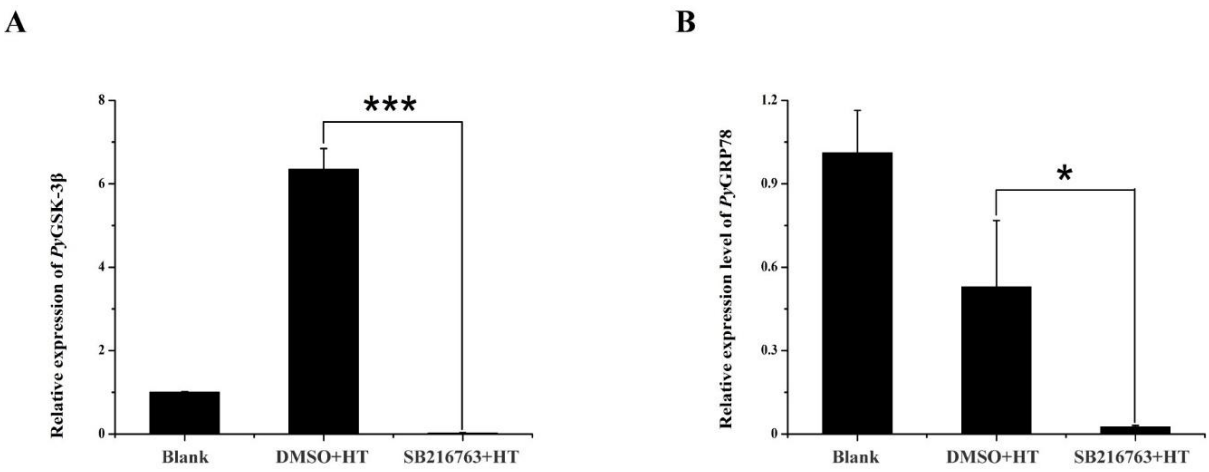


**Fig. 5** The mRNA expressions of *Py*GSK-3β (A) and *Py*GRP78 (B) in gills of *Py*GSK3 inhibitor-treated scallops after HT treatment. Relative mRNA expression levels of *Py*GSK-3β and *Py*GRP78 were normalized to that of *Py*EF-α. Significant differences are indicated by different letters (a, b, and c). Data are presented as mean ± S.D. (N ≥ 4)

The phosphorylation levels of *Py*GSK-3β and *Py*JNK were examined using Western blot analysis after SB216763 combined with HT treatment. The Western blot results showed that the phosphorylation level of *Py*GSK-3β (Tyr216) in the SB216763+HT group was significantly lower compared to the DMSO+HT group, and also lower compared to the Blank group. Similarly, the phosphorylation level of *Py*JNK (Thr183) in the SB216763+HT group was significantly lower compared to the DMSO+HT group, and also lower compared to the Blank group (Fig. 6D).

The changes of apoptosis in gills were detected and compared between the SB216763+HT group and the DMSO+HT group using immunofluorescence histochemistry. The results showed no apoptotic signals detected in the Blank group. In the DMSO+HT group, significant positive signals were detected, indicating that HT treatment induced cell apoptosis. However, in the SB216763+HT group, the positive signals were significantly reduced, suggesting that the *Py*GSK-3β inhibitor SB216763 reduced HT stress-induced cell apoptosis (Fig. 7).

## Discussion

GSK-3β plays an important role in multiple signal transduction pathways and physiological processes (Grimes and Jope, 2001; Plyte et al., 1992). By far, the role of GSK-3β in ER stress-induced apoptosis has been well demonstrated in vertebrates (Huang et al., 2009). However, there was few related studies on invertebrate GSK-3β, especially under HT stress. In this study, the homologous gene of GSK-3β was identified from Yesso scallops (*Py*GSK3β), and its amino acid sequence is highly conserved with other species, particularly the two key phosphorylation sites (Ser 9 and Tyr 216), which were evolutionarily conserved and regulated GSK-3β activity (Park et al., 2013). This high conservation suggests that *Py*GSK3β likely plays a conserved and crucial functional role across these different species. Furthermore, *Py*GSK3β shares a closer evolutionary relationship with mollusks before branching out to other invertebrates and vertebrates, highlighting the evolutionary conservation and divergence of the GSK-3β gene across different species. This supports the view that although certain conserved domains contribute to high sequence similarity with vertebrate GSK-3βs, the overall evolutionary context places *Py*GSK-3β closer to other marine invertebrates, consistent with its taxonomic position.

Gene expression in different tissues and under various stressors is typically closely linked to its function. It was reported that the expression of GSK-3β was closely associated with glycogen metabolism in oysters (Bacca et al., 2005). In this study, *Py*GSK-3β is expressed across various tissues, with particularly high levels in the mantle and hepatopancreas, both of which play crucial roles in immune defense and glycogen metabolism under normal physiological conditions (Liao et al., 2023). This is consistent with previous studies that have highlighted the hepatopancreas as key tissue for energy storage and metabolism in bivalves (Lama et al., 2013; Young-Jae et al., 2015). In bivalves, gills are highly exposed and sensitive tissue that directly interact with the surrounding environment, allowing for rapid responses to changes in seawater temperature (Jiang et al., 2018), which makes them an ideal target tissue for studying acute stress responses. The expression level of *Py*GSK-3β mRNA in gills significantly increased at 6 h after HT treatment. The low expression level of *Py*GSK-3β mRNA in gills under normal physiological conditions and its significant upregulation after HT treatment indicated that *Py*GSK-3β not only participated in the regulation of glycogen metabolism, but also played an important role in other signaling pathways during stress conditions. Considering the activation of GSK-3β during ER stress (Kim et al., 2015b), it is hypothesized that HT may induce stress responses by modulating *Py*GSK-3β expression.

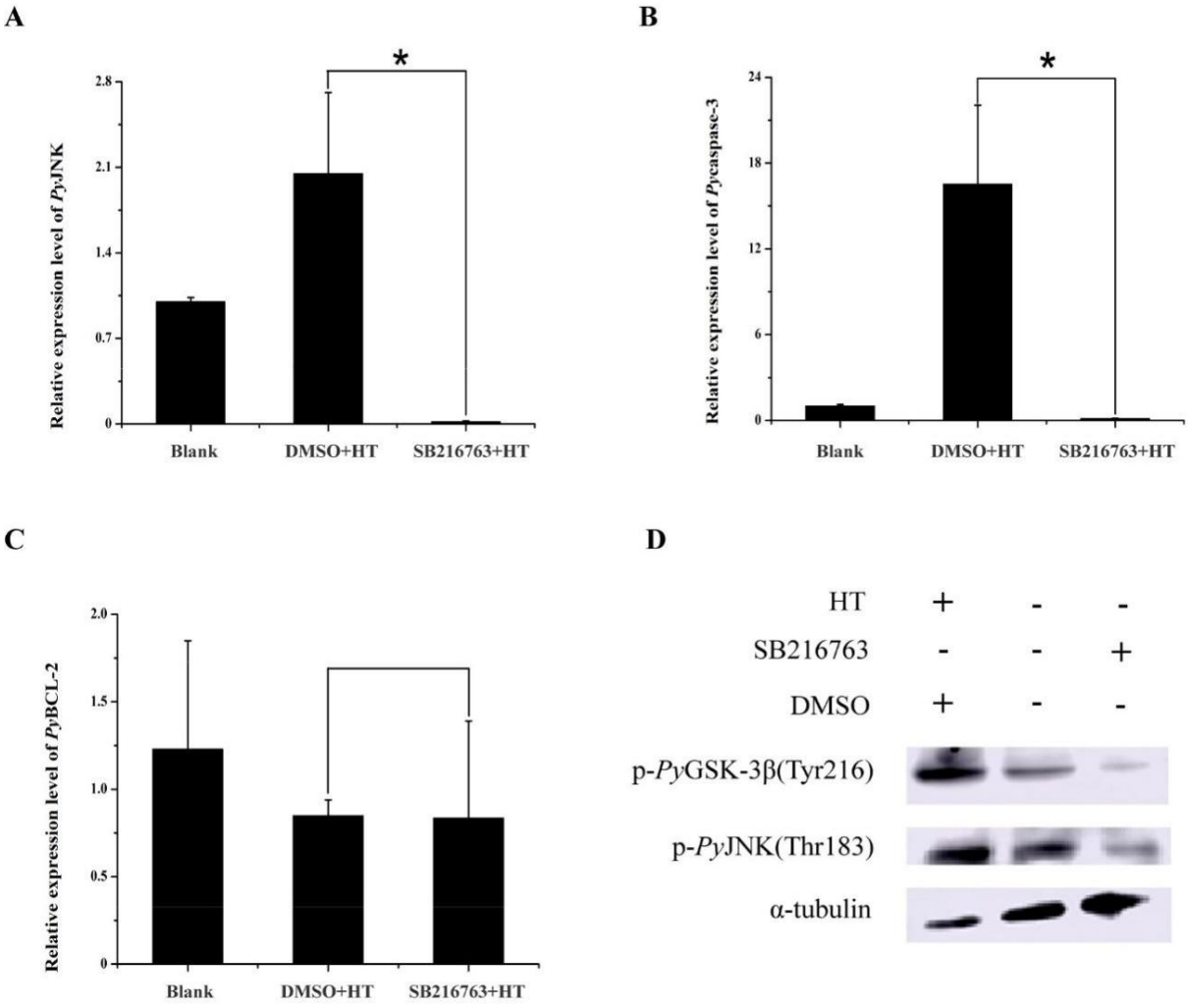


**Fig. 6** The mRNA expressions of *Py*JNK (A), *Py*Caspase-3 (B) and *Py*BCL-2 (C), as well as the protein levels of p-*Py*GSK-3β and p-*Py*JNK (D), in gills of *Py*GSK3 inhibitor-treated scallops after HT treatment. Relative mRNA expression levels of *Py*JNK, *Py*Caspase-3, and *Py*BCL-2 in gills were normalized to that of *Py*EF-α. Significant differences are indicated by different letters (a, b, and c). Data are presented as mean ± S.D. (N ≥ 4). Western blot analysis was performed for p-*Py*GSK-3β (Tyr216) and p-*Py*JNK (Thr183)

UPR regulates protein folding and degradation mechanisms to maintain cellular homeostasis and enable cellular adaptation to environmental stress or damage (Hetz, 2012). When misfolded or unfolded proteins accumulate in the ER due to such stressors, BIP/GRP78 is activated to assist in their modification and facilitate their degradation via the ER-associated degradation (ERAD) pathway, thereby alleviating cellular stress (Carrara et al., 2015; Chen et al., 2020). In this study, a significant increase was observed in the expression levels of *Py*IRE1 and *Py*GRP78 in gills following HT treatment, indicating the activation of UPR. Specifically, *Py*GRP78 mRNA levels rose markedly at 3 h after HT treatment and declined at 6 h, suggesting its crucial role in the early stress response and alignment with previous studies on GRP78's role in early UPR activation (Preissler and Ron, 2019). Under *Py*GSK3β inhibition, *Py*GRP78 mRNA levels significantly decreased, indicating that *Py*GSK3β may contribute to the regulation of the UPR by modulating *Py*GRP78. Upon dissociation from BIP/GRP78, IRE1 oligomerizes and activates itself through autophosphorylation (Lee et al., 2003). *Py*IRE1 mRNA levels rapidly increased at 3 h and peaked at 6 h after HT treatment, suggesting that the activation of IRE1 is closely related to the progression of the heat stress response. Furthermore, previous studies in *P. yessoensis* have demonstrated that *Py*IRE1 plays a critical role in regulating apoptosis under heat stress by modulating the expression of pro-apoptotic molecules such as *Py*Bax and caspase-3. Inhibition of *Py*IRE1 significantly reduced the levels of these markers, confirming its importance as an ER stress sensor involved in apoptotic regulation (Wu et al., 2018). This aligns with the proposed role of GSK-3β in regulating UPR, possibly through modulation of the IRE1 pathway under stress conditions (Kim et al.,

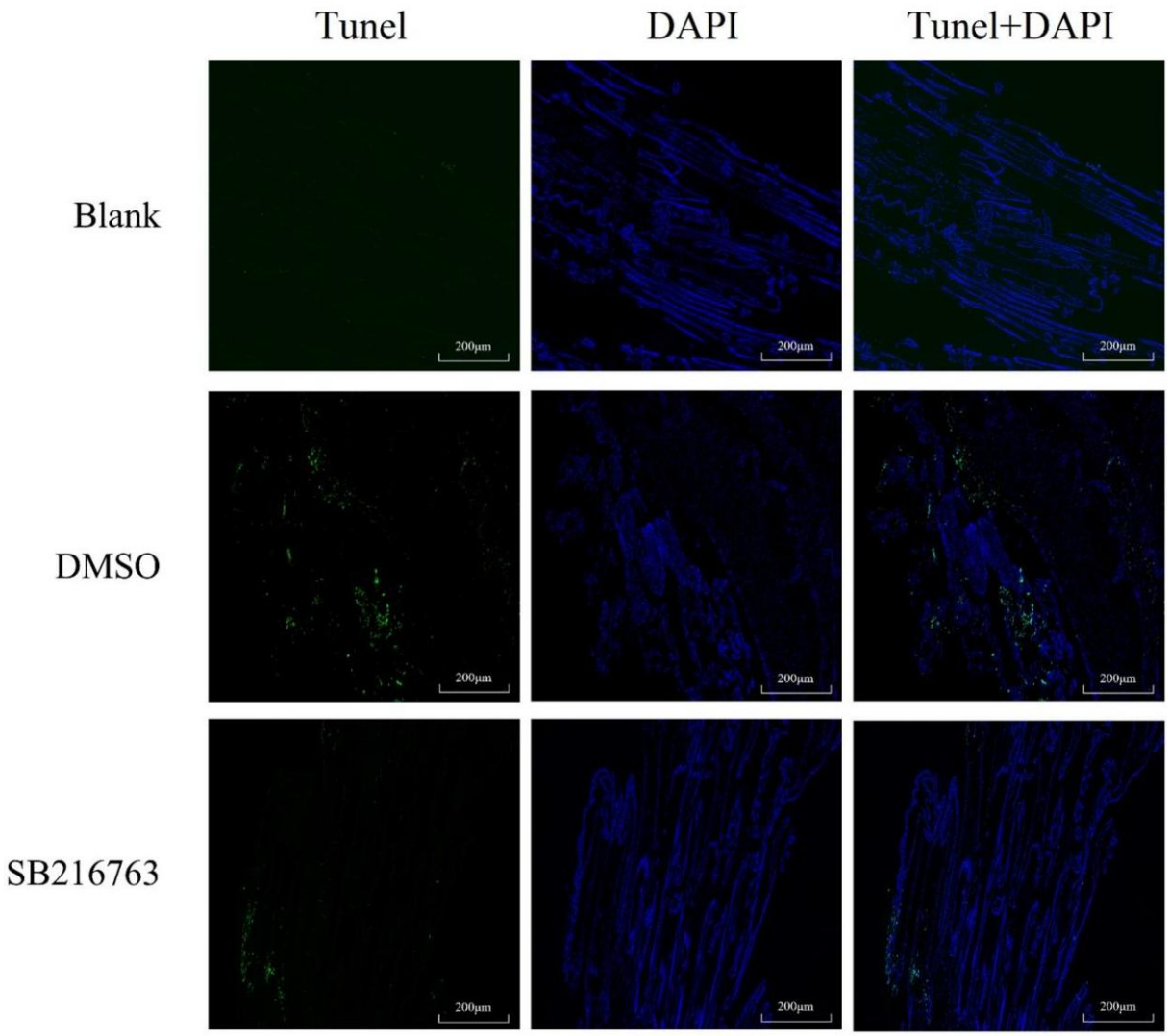


**Fig. 7** Changes in apoptosis in gills of *Py*GSK-3β inhibitor-treated scallops before and after HT treatment. TUNEL results are shown with green fluorescence, indicating apoptotic cells, while DAPI results are shown with blue fluorescence, indicating normal cells (Scale bar = 200 μm)

2015a). Taken together, these results suggest that *Py*GSK-3β is critically involved in UPR regulation, particularly in modulating GRP78 and IRE1 signaling pathways under heat stress conditions.

Apoptosis removes damaged cells to prevent their accumulation and protect tissues from further harm (Elmore, 2007). Under prolonged or excessive stress, the UPR fails to restore ER homeostasis, eventually leading to apoptosis (Tabas and Ron, 2011). IRE1 activates JNK through a series of signaling pathways, and the activated JNK subsequently phosphorylates its substrate BCL-2, thereby abrogating its anti-apoptotic function (Siwecka et al., 2021). In this study, *Py*JNK mRNA levels were significantly elevated at 6 h after HT treatment, whereas *Py*BCL-2 mRNA levels increased at 3 h but decreased at 6 h, suggesting that Yesso scallops initially activated protective mechanisms to maintain cell survival but shifted towards apoptosis as stress persisted. Additionally, as a key executioner, *Py*Caspase-3 mRNA levels increased at 3 h and peaked at 6 h, further supporting this observation. Previous studies have shown that GSK-3β is involved in ER stress-induced apoptosis and is associated with the JNK pathway (Srinivasan et al., 2005). The significant increase in *Py*GSK-3β and *Py*JNK phosphorylation levels under HT conditions further suggests that *Py*GSK-3β may participate in UPR-mediated apoptosis by regulating *Py*JNK. Following *Py*GSK-3β gene inhibition, *Py*JNK phosphorylation levels were significantly reduced, whereas *Py*BCL-2 mRNA levels remained unchanged, and *Py*Caspase-3 mRNA levels were significantly decreased, indicating that GSK-3β plays a critical role in the JNK-mediated apoptotic pathway. In addition to BCL-2, GSK-3β has also been reported to regulate other BCL-2 family proteins such as Bax and Mcl-1 in vertebrates, which are critical in the

execution of apoptosis (Qiu et al., 2023). Previous studies have demonstrated that inhibiting GSK-3β reduces JNK phosphorylation and alleviates ER stress-induced apoptosis in the dorsal hippocampus of rats (Oh and Nam, 2019). Furthermore, immunohistochemical analysis showed that, compared to the control group, the apoptotic fluorescence signal was significantly reduced in the *Py*GSK-3β inhibition group, further confirming that GSK-3β promotes ER stress-mediated apoptosis. This finding is consistent with studies in mice, where GSK-3β inhibition mitigated ER stress-induced apoptosis following tunicamycin treatment (Shirakawa et al., 2013). GSK-3β has also been shown to be involved in other stress pathways, such as oxidative stress via the β-catenin/C/EBPα axis and cell survival through mTOR signaling, thereby highlighting its broader regulatory role beyond ER stress (Liu et al., 2018; Urbanska et al., 2018). In summary, *Py*GSK-3β activity is upregulated by ER stress. *Py*GSK-3β affects the phosphorylation of the key component *Py*JNK in the ER stress-mediated apoptosis pathway, thereby influencing the expression of apoptotic markers and regulating cell apoptosis.

In conclusion, *Py*GSK-3β with conserved S_TKc domain and active sites Ser9 and Tyr216 was identified in Yesso scallops. It could be activated by *Py*GRP78-*Py*IRE1 pathway under HT stress, and sequentially activate *Py*JNK, ultimately promoting apoptosis by upregulating the expression of *Py*Caspase-3 and *Py*BCL-2. On the contrary, inhibiting *Py*GSK-3β partly alleviated apoptosis. These findings uncover a previously uncharacterized regulatory mechanism and provide novel insights into the molecular basis of high temperature adaptation in bivalves, with potential applications for enhancing stress resilience in aquaculture.

However, this study still has limitations in fully elucidating the interactions and dynamic regulation among these key signaling molecules. Future work should explore the roles of *Py*GSK-3β in other tissues and under different stressors, as well as investigate its upstream and downstream signaling pathways, including post-translational modifications, to further clarify its role in thermal adaptation and support bivalve aquaculture resilience.

**Acknowledgements**

This research was supported by funds for CARS-49 in MARA, Natural Science Foundation of Liaoning, China (2024-MSLH-056), the innovation team of Aquaculture Environment Safety from Liaoning Province (LT202009), and the Dalian High Level Talent Innovation Support Program (2017RQ014 and 2022RG14).

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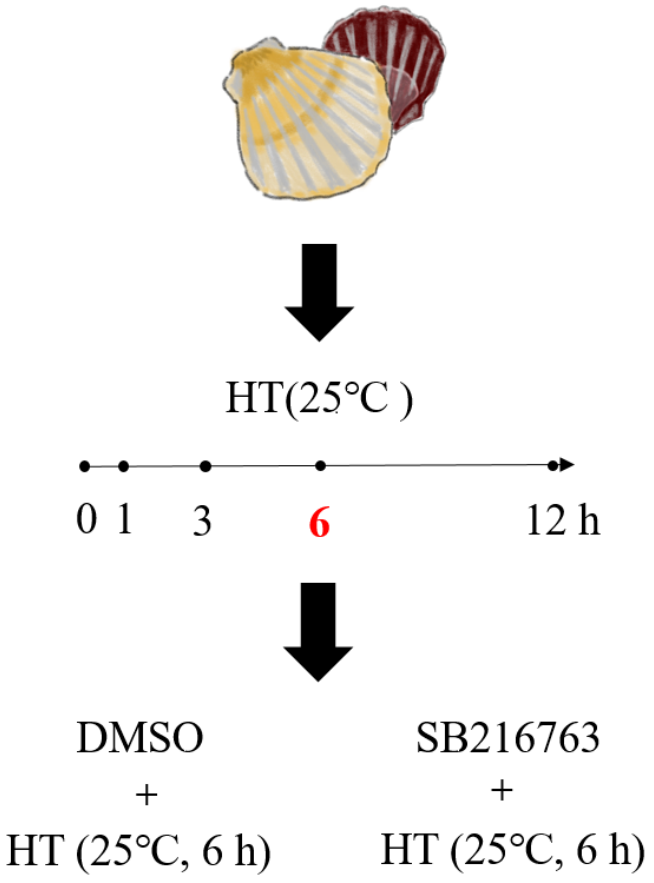


**Fig. S1**