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REVIEW

The unfolded protein response signaling pathways in molluscs

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Abstract

Unfolded protein response (UPR) as collective signal transduction pathways is essential for surviving the endoplasmic reticulum (ER) stress in vertebrates and invertebrates. Upon accumulation of unfolded proteins in the ER lumen, UPR increases the degradative and protein-folding capacities of cells and decreases global protein synthesis to maintain the cell homeostasis. Because of their importance in cellular stress and protein folding process, UPR signaling pathways receive increasing attentions, and their components and multiple regulation functions have been well characterized in mammals, fly, and worm etc. Molluscs are widely distributed in various environments with high species diversity, which exhibit remarkable capacity for adaptation and survival upon diverse stressors. Because of the homeostatic role in response to ER stress, the knowledge about UPR would be helpful for understanding the wide distributions, living habits and adaptability to the environment of molluscs. This review summarizes the UPR signaling pathways in molluscs with the emphasis on recent research progresses about the characteristics of molluscan UPR signaling pathway members and their expression profiles in response to various environmental stressors.

Key Words: molluscs, unfolded protein response (UPR), signaling pathway, molecular components, expression profile, stress response

Introduction

Unfolded protein response (UPR) is a collection of phylogenetically conserved signaling pathways to monitor the conditions in endoplasmic reticulum (ER), including insufficiency of ER's protein-folding ability and the excessive misfolding, and then initiates the consequent signal transduction pathways (Walter and Ron, 2011). When protein folding in the ER is inhibited, UPR increases the biosynthetic capacity and decreases the biosynthetic burden of the ER to maintain the homeostasis of cell (Schröder and Kaufman, 2005). In mammals, UPR is composed of three signaling pathways represented by three ER transmembrane protein sensors, inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (Cao and Kaufman, 2012). The signal transduction mechanisms of the three signaling pathways are different: ATF6 by regulating proteolysis, PERK by translational control, and IRE1 by nonconventional mRNA splicing (Fig. 1) (Walter and Ron, 2011). UPR plays an important role in immunity and inflammation, and is involved in various prevalent diseases such as metabolic syndrome, neurodegenerative disorders, inflammatory bowel disease, and cancer (Grootjans *et al.*, 2016).

The phylum Mollusca is second only to the arthropods with numbers of more than 100,000 species (Telford and Budd, 2011). They distribute extensively in marine intertidal zone, freshwater and terrestrial ecosystems, and even in extreme environments such as deep-sea hydrothermal vents, 40 °C freshwater, and permanent ice areas (Haszprunar and Wanninger, 2012). Molluscs have evolved major stress-response pathways such as oxidation or anti-oxidation, apoptotic pathways and UPR signaling pathways to respond stresses and survive in extreme conditions (Zhang *et al.*, 2012; Zhang *et al.*, 2016a). The activation of UPR pathways is considered to be crucial to respond

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cellular stresses in ER and maintains the cellular homeostasis under various stressors (Walter and Ron, 2011), such as temperature elevation (Wang *et al.*, 2012; Zhang *et al.*, 2012; Li *et al.*, 2017), air exposure (Kawabe and Yokoyama, 2010), as well as metal bioaccumulation (Poynton *et al.*, 2014).

The aim of this review is to outline the molecular components of UPR signaling pathways so far identified in molluscs, their expression profiles under various stressors, and the related downstream response pathways, as well as their possible significance in the stress response of molluscs.

The molecular components of UPR signaling pathway in molluscs

Recently, UPR and its response to various stressors in molluscs have been paid increasing attentions. The core members in IRE1, PERK and ATF6 signaling pathways have been described in several molluscan species (Table 1; Fig. 2) (Mori, 2009; Vinther *et al.*, 2012; Hollien, 2013; Janssens *et al.*, 2014; Tanner *et al.*, 2017). ER chaperone BiP (Binding immunoglobulin protein) and other important ER molecules including calreticulin (CRT), calnexin (CNX), protein disulfide isomerase (PDI) and peptidyl-prolyl cis-trans isomerases (PPI) (Kennedy *et al.*, 1992; Kawabe and Yokoyama, 2010; Leung *et al.*, 2011; Mu *et al.*, 2015; Clark *et al.*, 2016) involved in molluscan UPR also receive widespread attention. The molecular characteristics of IRE1, PERK and ATF6 signaling pathway members as well as other important ER members involved in molluscan UPR are discussed in this section.

BiP (GRP78)

Binding immunoglobulin protein (BiP), also referred as 78 kDa glucose regulated protein (GRP78) (Hendershot, 2004), is an ER molecular chaperone of the heat shock protein HSP70 family (Ting and Lee, 1988; Hendershot *et al.*, 1994). It is well known for its activity to bind hydrophobic patches on nascent polypeptides within the ER and its role in UPR signaling pathways (Quinones *et al.*, 2008). Structurally, BiP is highly conserved across species (Quinones *et al.*, 2008) with two conserved functional domains, a nucleotide-binding domain (NBD) at the N-terminus and a substrate-binding domain (SBD) at the C-terminus. The NBD binds and hydrolyzes ATP to ADP, and the SBD binds hydrophobic polypeptides in an extended conformation as substrates (Yang *et al.*, 2015). The BiP genes have been so far described in some molluscan species including intertidal gastropod *Littorina littorea* (Storey *et al.*, 2013), sea hare *Aplysia californica* (Kuhl *et al.*, 1992), sea snail *Nacella concinna*, Antarctic clam *Laternula elliptica* (Clark *et al.*, 2008; Clark *et al.*, 2016), Pacific oyster

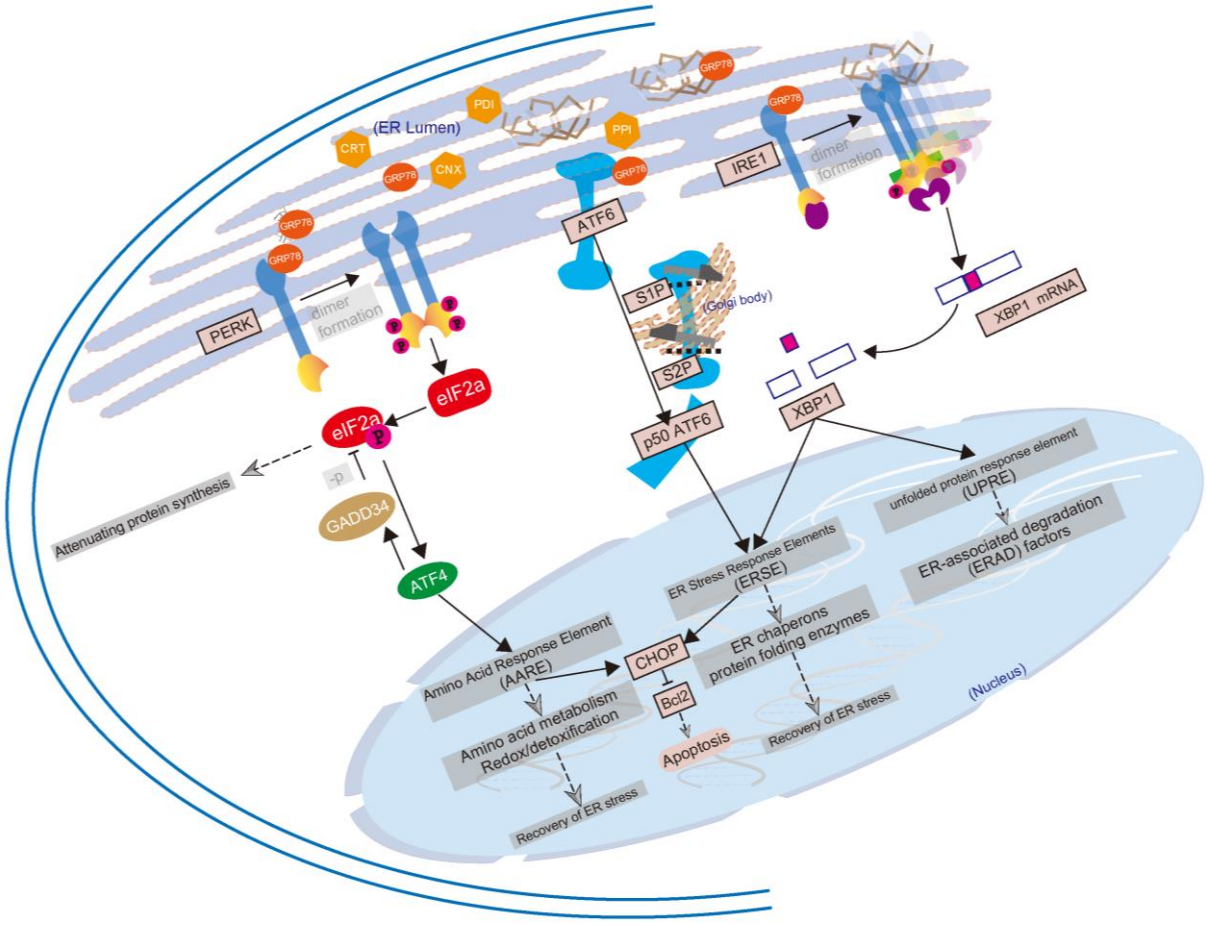

Fig. 1 Schematic diagram of UPR

Table 1 The UPR members and expression profiles in mollusca species

	Species	Stresses	Response	Tissues	References
BiP (GRP78)					
GRP78	*L. littorea*	20 h anoxia exposure	↑	foot muscles	Storey *et al.*, 2013
GRP78	*A. californica*	3, 7, 18 h glucose starvation	↑	central nervous system	Kuhl *et al.*, 1992
GRP78	*N. concinna*	2 h heat shock for 10°C, 15°C or 20°C	—	feet	Clark *et al.*, 2008
GRP78	*L. elliptica*	2 h heat shock 15°C	↑	gills, mantles and siphons	Clark *et al.*, 2008
GRP78	*L. elliptica*	12 h acute heat treatment at 3°C	↑	siphons (younger animals compared with older animals)	Clark *et al.*, 2016
GRP78	*C. gigas*	24 h and 48 h heat treatment for 35°C	↑	gills and adductor muscles	Yokoyama *et al.*, 2006
GRP78	*C. gigas*	12 h pb treatment 500 μg/L	↑	gills	G. Zhang *et al.*, 2012
GRP78	*C. gigas*	6, 12, 24 h acute heat stress 25°C	↑	haemocytes	Yang *et al.*, 2017
GRP78	*C. hongkongensis*	1h heat treatment 37°C	↑	gills	Li *et al.*, 2017
GRP78	*G. demissa*	1 h acute heat stress 40°C	One isoform↓ Another isoform↑	gills	Fields *et al.*, 2012a
GRP78	*G. demissa*	1 h heat stress for 45°C	↑	gills	Fields *et al.*, 2016
GRP78	*M. trossulus*	4 weeks chronic temperature stress at 19°C	↑	gills	Fields *et al.*, 2012b
GRP78	*V. lienosa*	6 days heat treatment for 29±2°C	↑	adductor muscles, mantles, and gills	Wang *et al.*, 2012
GRP78	*P. viridis*	24 h benzo(a)pyrene stimulation 400 μg/L	↑	embryos	Jiang *et al.*, 2016
IRE1–XBP1 signaling pathway					
IRE1	*C. gigas*	12 h pb treatment 500 μg/L	↑	gills	G. Zhang *et al.*, 2012
IRE1	*C. gigas*	6, 12, 24 h acute heat stress 25°C	↑	haemocytes	Yang *et al.*, 2017
IRE1	*V. lienosa*	6 days heat treatment for 29±2°C	↑	adductor muscles, mantles, and gills	Wang *et al.*, 2012
XBP1	*V. lienosa*	6 days heat treatment for 29±2°C	↑	adductor muscles, mantles, and gills	Wang *et al.*, 2012
XBP1	*O. edulis*	heavy bonamiosis	↑	haemolymph cells	Martín-Gómez *et al.*, 2014
XBP1	*O. edulis*	heavy disseminated neoplasia (DN)	↑	haemolymph cells	Martín-Gómez *et al.*, 2014
XBP1	*O. edulis*	light DN	↑	mantles and digestive organs	Martín-Gómez *et al.*, 2014
XBP1	*O. edulis*	light DN	↓	gills	Martín-Gómez *et al.*, 2014
XBP1	*M. edulis*	2 weeks Pb treatment 0.54 μM	↑	gills	Poynton *et al.*, 2014
XBP1	*M. edulis*	2 weeks (Pb and Cd) mix-treatment 0.54 μM	↑	gills	Poynton *et al.*, 2014
PERK–eIF2a–ATF4–CHOP signaling pathway					
PERK	*C. gigas*	9 days air exposure	↑	gills	G. Zhang *et al.*, 2012
PERK	*C. gigas*	6, 12, 24 h acute heat stress 25°C	↑	haemocytes	Yang *et al.*, 2017
PERK	*C. hongkongensis*	1h heat treatment 37°C	↑	gills	Li *et al.*, 2017
PERK	*V. lienosa*	6 days heat treatment for 29±2°C	↑	adductor muscles, mantles, and gills	Wang *et al.*, 2012
eIF2α	*V. lienosa*	6 days heat treatment for 29±2°C	↑	adductor muscles, mantles, and gills	Wang *et al.*, 2012
eIF2α	*C. gigas*	12 h heat treatment 35°C	↑	gills	G. Zhang *et al.*, 2012
Phospho-eIF2α	*L. littorea*	20 h anoxia exposure	↑	foot muscles	Storey *et al.*, 2013
Phospho-eIF2α	*Mercenaria*	18 h anoxia treatment	↑	adductor muscles	Ivanina *et al.*, 2016
ATF4	*L. littorea*	20 h anoxia exposure	↑	foot muscles	Storey *et al.*,

					2013
ATF4	*V. lienosa*	6 days heat treatment for 29±2°C	↑	muscles, mantles and gills	Wang *et al.*, 2012
BCL2	*C. gigas*	5 days air exposure	↑	muscles	G. Zhang *et al.*, 2012
GADD34	*L. littorea*	20 h anoxia exposure	↓	foot muscles	Storey *et al.*, 2013
GADD153	*L. littorea*	20 h anoxia exposure	↑	foot muscles	Storey *et al.*, 2013
ATF6 signaling pathway					
p90 ATF6	*L. littorea*	20 h anoxia exposure	—	foot muscles	Storey *et al.*, 2013
p50 ATF6	*L. littorea*	20 h anoxia exposure	↓	foot muscles	Storey *et al.*, 2013
Others members					
CRT	*A. californica*	3, 7, 18 h glucose starvation	↑	central nervous system	Kennedy *et al.*, 1992
CRT	*C. gigas*	7 days air exposure 20°C	fluctuated	adductor muscles, mantles, gills	Kawabe *et al*, 2010
CRT	*C. gigas*	15 days air exposure 4°C	fluctuated	adductor muscles, mantles, gills	Kawabe *et al*, 2010
CRT	*C. gigas*	12 h pb treatment 500 µg/L	↑	gills	G. Zhang *et al.*, 2012
CRT	*C. hongkongensis*	1h heat treatment 37°C	↑	gills	Li *et al.*, 2017
CRT	*P. viridis*	14 days Cd treatment 0.5 ppm	fluctuated	hepatopancreas	Leung *et al.*, 2011
CRT	*P. viridis*	24 h benzo(a)pyrene stimulation 400 µg/L	↑	embryos	Jiang *et al.*, 2016
CNX	*C. gigas*	7 days air exposure 20°C	fluctuated	adductor muscles, mantles, gills	Kawabe *et al*, 2010
CNX	*C. gigas*	15 days air exposure 4°C	fluctuated	adductor muscles, mantles, gills	Kawabe *et al*, 2010
CNX	*C. gigas*	12 h pb treatment 500 µg/L	↑	gills	G. Zhang *et al.*, 2012
CNX	*C. hongkongensis*	1 h heat treatment 37°C	↑	gills	Li *et al.*, 2017
PDI	*P. diffusa*	3 weeks chronic heat treatment 35°C	↑	hepatopancreas	Mu *et al.*, 2015
PDI	*P. canaliculata*	3 weeks chronic heat treatment 35°C	↑	hepatopancreas	Mu *et al.*, 2015
PDI	*C. hongkongensis*	1 h heat treatment 37°C	↑	gills	Li *et al.*, 2017
PDI	*C.gigas*	6, 12, 24 h acute heat stress 25°C	↑	haemocytes	Yang *et al.*, 2017
PDI	*P. viridis*	14 days Cd treatment 0.5 ppm	↑	hepatopancreas	Leung *et al.*, 2011
PDI	*P. viridis*	24 h benzo(a)pyrene stimulation 400 µg/L	↑	embryos	Jiang *et al.*, 2016
PPI	*G. demissa*	1 h acute heat stress 40°C	↑	gills	Fields *et al.*, 2012a
PPI	*G. demissa*	1 h heat stress for 45°C	↑	gills	Fields *et al.*, 2016
PPI	*L. elliptica*	12 h acute heat treatment at 3°C	↑	siphons (younger animals compared with older animals	Clark *et al.*, 2016

↑: up-regulation ↓: down-regulation —: no significant change

Crassostrea gigas (Yokoyama *et al.*, 2006; Zhang *et al.*, 2012; Yang *et al.*, 2017), oyster *C. hongkongensis* (Li *et al.*, 2017), Atlantic ribbed mussel *Geukensia demissa* (Fields *et al.*, 2012a; Fields *et al.*, 2016), blue mussel *Mytilus trossulus* (Fields *et al.*, 2012b), freshwater mussel *Villosa lienosa* (Wang *et al.*, 2012), and green mussel *Perna viridis* (Jiang *et al.*, 2016). However, most of the recent researches on molluscan BiP are mainly focused on the gene cloning and sequence analysis, and mainly from bivalve and gastropod. The protein sequences of molluscan BiP are found to be relatively conserved among molluscs, and even comparing with other invertebrates and vertebrates (Mamady and Storey, 2006; Song *et al.*, 2015). For instance, the oyster BiP (GenBank: BAD15288.1) shares 89%, 87% and 83% similarities of amino acid sequences with that from bivalve *Mizuhopecten yessoensis* (Accession: XP_021349015.1), gastropod *A. californica* (Accession: NP_001191581.1), and mammal *Homo sapiens* (Accession: NP_005338.1), respectively (Yokoyama *et al.*, 2006). Oyster BiP contains an ATPase domain and a peptide binding domain, which denotes the

protein translocation, ER calcium stores, and unfolded protein response activation as mammalian BiP (Hendershot, 2004). Under homeostatic conditions, the ER-luminal domains of ER stress sensors (PERK and IRE1, ATF6) in UPR signaling pathways are maintained in an inactive state through association with BiP, and the sensors can be activated by releasing from BiP when the misfolded proteins are accumulated in ER lumen (Cao and Kaufman, 2012; Grootjans *et al.*, 2016). Although many homologues of BiP have been identified in molluscs, the knowledge about the detailed structure and function of molluscan BiPs are still limited, and further studies are needed to evaluate their regulation mechanism in UPR to maintain the homeostasis under ER stress.

IRE1–XBP1 signaling pathway members

IRE1–XBP1 (X-box binding protein 1) signaling pathway is the most conserved UPR pathway in yeasts, nematodes, insects, and mammals (Mori, 2009; Cao and Kaufman, 2012). The molecular components of IRE1 signaling mainly include IRE1 and its substrate XBP1 (Liu and Kaufman, 2003). IRE1 is a stress receptor of ER localized type I transmembrane protein with a serine/threonine kinase domain on its cytosolic portion and an endoribonuclease (RNase) domain (Grootjans *et al.*, 2016). Similar to other two ER stress sensors (PERK and ATF6), IRE1 can be activated directly by BiP dissociation. Moreover, yeast IRE1 can also directly bind to unfolded proteins to activate the UPR (Gardner and Walter, 2011). So far, two IRE1s have been identified from Pacific oyster *C. gigas* (Zhang *et al.*, 2012; Yang *et al.*, 2017) and freshwater mussel *V. lienosa* (Wang *et al.*, 2012). Molluscan IRE1 owns low conserved protein sequence compared with the ones identified from invertebrate and vertebrate. For example, the deduced amino acid sequence of oyster IRE1 (GenBank: EKC40550.1) shares 52% and 48% identity with that from echinoderm *Acanthaster planci* (XP_022103307.1) and mammal *Bos mutus* (ELR62219.1), respectively. Conserved domain (Marchler-Bauer *et al.*, 2014) alignments showed that the oyster IRE1 contained a luminal domain, a catalytic domain of the serine/threonine kinase and an RNase domain, which are conserved from human to yeast IRE1s (Tirasophon *et al.*, 1998). UPR is initiated by ER-localized transmembrane receptors with lumenal domains that sense misfolded proteins, and then the cytosolic effector domains pass the signal to the downstream components (D and SR, 2008). The presence of conserved lumenal and cytosolic effector domains in the molluscan IRE1 suggested that it might play important roles in the initiation of UPR in molluscs (Zhang *et al.*, 2012; Marchler-Bauer *et al.*, 2014; Yang *et al.*, 2017). Dual copies of IRE1 (IRE1α and IRE1β) have been discovered in vertebrates such as mammals (Mori, 2009) and *Xenopus* embryos (Yuan *et al.*, 2008), whereas only one IRE1 has been identified in *C. elegans* and *D. melanogaster* (Mori, 2009). Similar to other invertebrates, the unique isoform of IRE1 was recently found in molluscs (Wang *et al.*, 2012; Zhang *et al.*, 2012; Yang *et al.*, 2017).

XBP1 mRNA is the substrate of IRE1α and IRE1β in mammals, which encodes a basic leucine zipper containing transcription factor. Three XBP1 genes were recently identified in molluscs, including flat oyster *Ostrea edulis* (Martín-Gómez *et al.*, 2014), blue mussel *M. edulis* (Poynton *et al.*, 2014), and freshwater mussel *V. lienosa* (Wang *et al.*, 2012). Although all the XBP1s from mollusc contain a basic leucine zipper (bZIP) domain, their amino acid sequences display low conservation compared with that in other organisms. XBP1 from oyster *C. gigas* (GenBank: EKC34044.1) shares 59%, 51%, 41% and 35% identity with that in mollusca bivalvia *Mytilus edulis* (Accession: ABA43316.1) and *Mizuhopecten yessoensis* (Accession: OWF42676.1), vertebrate fish *Danio rerio* (Accession: AAI52196.1), and mouse *Cricetulus griseus* (Accession: EGW12286.1), respectively. The splicing of XBP1 mRNA is initiated by the RNase activity of IRE1 to generate mature XBP1 mRNA (Liu and Kaufman, 2003). XBP1 induces the expression of a wide range of genes that orchestrate ER protein folding, secretion, quality control and endoplasmic-reticulum-associated protein degradation (ERAD), and activates phospholipid biosynthesis and ER expansion upon ER stress (Cao and Kaufman, 2012). The identification of IRE1 and XBP1 in oyster and mussel suggests that IRE1–XBP1 signaling pathway also exists in the molluscs. Nevertheless, the detailed mechanisms of XBP1 mRNA maturation and the induction of XBP1 to the downstream genes in molluscs still need further investigations.

PERK–eIF2α–ATF4–CHOP signaling pathway members

The PERK signaling pathway usually attenuates the initiation of translation by activating PERK to phosphorylate α subunit of eukaryotic translation initiation factor 2 (eIF2α) to reduce the ER protein-folding burdens in mammals (Walter and Ron, 2011). PERK, eIF2α, the activating transcription factor 4 (ATF4) and CCAAT/enhancer binding protein homologous protein (CHOP) are identified as the main members of PERK signaling pathway in mammals (Cao and Kaufman, 2012). PERK is a type I transmembrane protein with a cytosolic serine/threonine kinase domain, which shares similar luminal domain and activation machinery with IRE1α (Grootjans *et al.*, 2016). In response to ER stress, the activation of PERK is triggered by a large number of signals to phosphorylate the α subunit of eIF2α, blocks the recycling of GDP to GTP, and finally leads to translation attenuation (Liu and Kaufman, 2003; Donnelly *et al.*, 2013). The recently reports about molluscan PERK–eIF2α–ATF4–CHOP signaling pathway are mainly from bivalve and gastropod. Three molluscan PERKs have been identified in oyster *C. gigas* (Zhang *et al.*, 2012; Yang *et al.*, 2017), *C. hongkongensis* (Li *et al.*, 2017), and freshwater mussel *V. lienosa* (Wang *et al.*, 2012). The oyster PERKs are low-conservative in comparison with their homologues from other invertebrates and vertebrates. The deduced amino acid sequence of oyster PERK (GenBank: EKC35408.1) shares 43%, 38% and 36% identity with that from *Mizuhopecten yessoensis* (Accession:

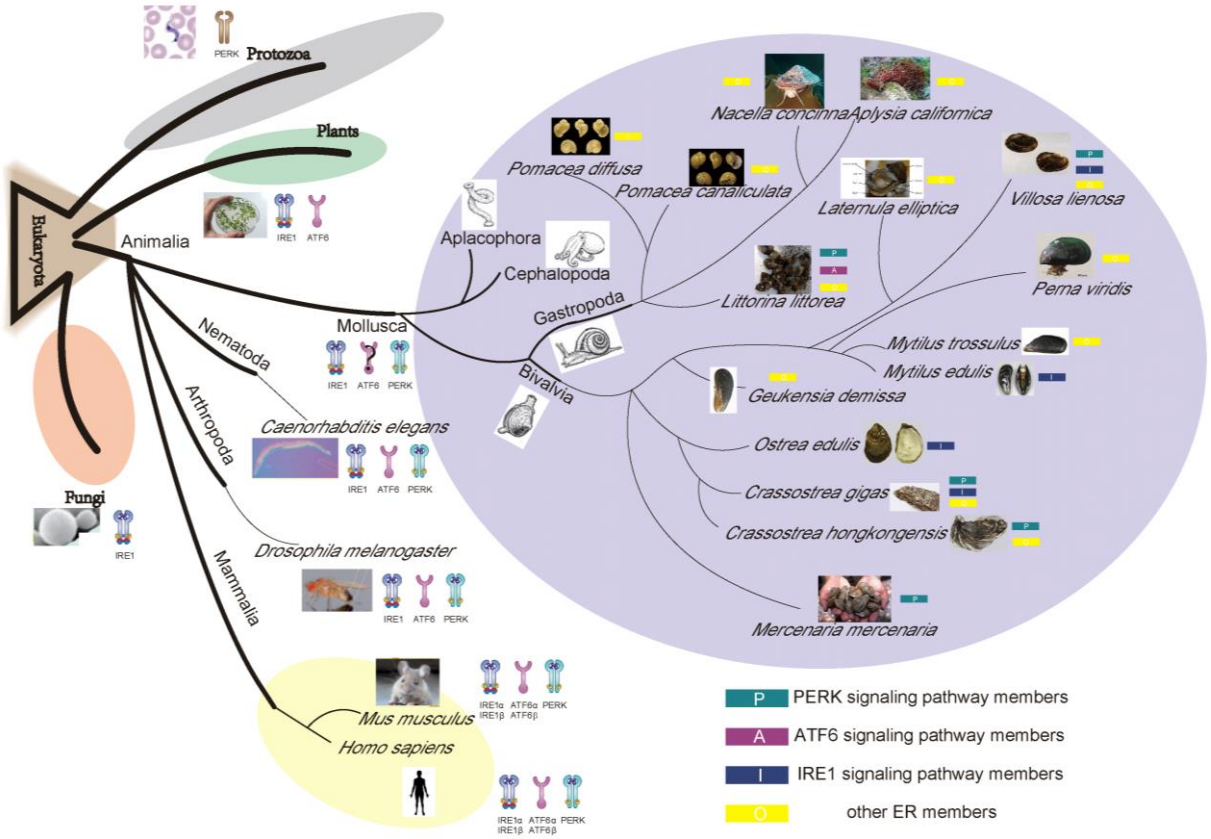

Fig. 2 Family tree of molluscan UPR

OWF40340.1), arthropoda *Limulus polyphemus,* and *Homo sapiens* (AAI26355.1). The oyster PERK protein possesses luminal dimerization domain, and catalytic domains of serine/threonine-specific and tyrosine-specific protein kinases, indicating the conserved sensing and activation machinery of PERK in molluscs. eIF2α genes have also been discovered in molluscs, including intertidal gastropod *L. littorea* (Storey *et al.*, 2013), oyster *C. gigas* (Zhang *et al.*, 2012), clam *Mercenaria mercenaria* (Ivanina *et al.*, 2016), and *V. lienosa* (Wang *et al.*, 2012). The amino acid sequences of eIF2αs are relatively conservative in molluscs, other invertebrates and vertebrates. The deduced amino acid sequence of oyster eIF2α (GenBank: EKC31327.1) contains a conserved eukaryotic translation initiation factor 2 alpha subunit，which shares 86% and 88% identity with that in mollusca bivalve *M. yessoensis* (Accession: XP_022341697.1) and gastropod *A. californica* (Accession: XP_005105117.1), and 79% with human (Accession: NP_004085.1). Phosphorylation/dephosphorylation of eIF2α at Ser-51 is the major regulatory activity site in protein synthesis of eukaryotic cells and this site in molluscan eIF2α also deserves further study (Nonato *et al.*, 2002).

In mammalian PERK signaling pathway, ATF4 induces the transcriptions of BiP and GRP94, and stimulates autophagy gene expression. CHOP is the downstream targets of eIF2α–ATF4, and it is an important transcription factor of ER stress-induced apoptosis to increase the protein synthesis by inducing transcription of growth arrest and DNA damage 34 (GADD34) (Cao and Kaufman, 2012; Grootjans et al., 2016). The induced GADD34 recruits protein phosphatase 1 (PP1) to dephosphorylate eIF2α-P and reverses the translational attenuation (Liu and Kaufman, 2003). ATF4 has been identified in mussel *V. lienosa* under elevated temperature exposure (Wang *et al.*, 2012). ATF4, CHOP and GADD34 were further identified in intertidal gastropod *L. littorea,* suggesting that PERK sensor could mediate UPR response under anoxia to suppress protein synthesis and elevate ER chaperones, which would contribute to cell survival during prolonged anaerobiosis (Storey *et al.*, 2013). The increasing reports about the components in PERK–eIF2α–ATF4–CHOP signaling pathway in different molluscs suggests that this signaling pathway may be evolutionarily conservative in molluscs, and play indispensable contribution in the adaptive evolution of diverse molluscan species (Pomar *et al.*, 2003; Cao and Kaufman, 2012; Kitamura, 2013; Krivoruchko and Storey, 2013).

The ATF6 signaling pathway members

In mammals, the activation of ATF6 signaling pathway is to cleave inactive 90 kDa ATF6 to active

50 kDa ATF6. As a type II ER transmembrane protein, ATF6 harbors a CREEB/ATF basic leucine zipper (bZIP) transcription factor in its cytosolic domain (Cao and Kaufman, 2012). There are two closely related factors, ATF6α and ATF6β, in mammals. It is reported that ATF6α but not ATF6β plays important role in transcriptional control (Adachi *et al.*, 2008). Upon accumulation of unfolded proteins in ER, 90 kDa ATF6 is released from BiP and transferred to the Golgi apparatus to be cleaved into 50 kDa ATF6 by site-1 protease (S1P) and site-2 protease (S2P). 50 kDa ATF6 is then transported to nucleus to activate gene expression (Grootjans *et al.*, 2016).

The oyster ATF6 (Accession: XM_022470698.1) and other molluscan ATF6s exhibit low conservation compared with that in other invertebrates and vertebrates. The deduced amino acid of oyster ATF6 exhibits 42% identity with that in bivalve *Mizuhopecten yessoensis* (Accession: XP_021375264.1), 34% identity with that in arthropod *Limulus polypohemus* (Accession: XP_013792454.1) and fish *Ctenopharynqodon Idella* (Accession: AKZ87017.1). The basic region leucine zipper (bZIP) domain of oyster ATF6 protein mediates its sequence-specific DNA-binding, which is required for dimerization of leucine zippers (Hurst, 1994). The information about molluscan ATF6 is extremely limited so far, and the inactive 90 kDa and active 50 kDa ATF6 (produced via protease cleavage) in intertidal gastropod *L. littorea* are the only identified molluscan ATF6 members. The relative expression levels of the inactive 90 kD ATF6 and active 50 kD form under anoxia exposure were detected and they were found not involved in resisting anoxia exposure (Storey *et al.*, 2013). The presence of these two molecules suggests that molluscan ATF6 may possess the similar mechanisms for protease cleavage and activation of gene expression as its homologues in vertebrates (Ye *et al.*, 2000; Sommer and Jarosch, 2000; Shen *et al.*, 2005b). There is one ATF6 in *C. elegans* and *D. melanogaster*, while two ATF6 (ATF6α and ATF6β) in mammals, mice and fish (Mori, 2009; Ishikawa *et al.*, 2013), suggesting that the ER stress sensors/transducers have experienced structural and functional differentiation during evolution. The identification of ATF6 in molluscs also provides vital clues to understand the evolution of these molecules as well as the UPR pathways.

CRT/CNX/PPI/PDI

Some other important UPR related molecules, such as calreticulin (CRT), calnexin (CNX), protein disulfide isomerase (PDI) and peptidyl-prolyl cis-trans isomerases (PPI) have also been identified from molluscs. CRT and CNX are lectin-like ER molecular chaperones induced by various ER stresses, and they are involved in ER quality control. CRT is a soluble protein, while CNX is a type I transmembrane protein in the ER (Kawabe and Yokoyama, 2010). CRT and CNX have been identified from oysters *C. gigas* (Kawabe and Yokoyama, 2010; Zhang *et al.*, 2012) and *C. hongkongensis* (Li *et al.*, 2017). There is a signal sequence at the N-terminus of oyster CRT and CNX, while a KDEL peptide motif and a transmembrane domain locate at the C-terminus of CRT and CNX, respectively, which shares high similarity with vertebrate CNXs and CRTs. CRT genes were also found in sea hare *A. californica* (Kennedy *et al.*, 1992) and green mussel *Perna viridis* (Jiang *et al.*, 2016; Leung *et al.*, 2011). The protein disulfide isomerase (PDI) and peptidyl-prolyl cis-trans isomerases (PPI) are able to catalyze the reactions of protein folding (Schröder and Kaufman, 2005). The PDI have been found in freshwater apple snail *Pomacea canaliculata* and freshwater snail *P. diffusa* (Mu *et al.*, 2015), oysters *C. gigas* (Yang *et al.*, 2017), *C. hongkongensis* (Li *et al.*, 2017), and *P. viridis* (Leung *et al.*, 2011; Jiang *et al.*, 2016). Two PPIs have been discovered in Atlantic ribbed mussel *Geukensia demissa* (Fields *et al.*, 2012a; Fields *et al.*, 2016) and Antarctic clam *L. elliptica* (Clark *et al.*, 2016). These findings suggest a rigorous regulation mechanism on the ER quality control may exist in molluscs.

The protein sequences in molluscan CRT/CNX/PPI/PDI have been analyzed in comparison with their homologues in other animals. As expected, the molecular conservation between molluscs and other invertebrates is higher than that between molluscs and vertebrates. For instance, the CRT in oyster *C. gigas* (Accession: BAF63639.1) shares slightly higher identity with that in other invertebrate than vertebrate. The same situations have also been found in oyster CNX (GenBank: BAF63638.1), PPI (Accession: ABY27347.1) and PDI (Accession: EKC29663.1), which display 70%-80% identity with that in invertebrates and 50%-60% with that in vertebrates, respectively. These data indicate that CRT/CNX/PPI/PDI in molluscs, especially in oysters, exhibit relatively evolutional conservation, which provides intriguing pointcut for further protein structure and biological function investigations in molluscan UPR regulation.

Although several UPR members have been described in molluscs, it is only an overview and the knowledge of the structural diversity of molluscan UPR system is still quite meagre and not comparable with that in other invertebrate and vertebrate (Chen *et al.*, 2014). Gratifyingly, there are numerous predicted isoforms available in the data base of NCBI, owing to the increasingly release of the molluscan genome (Zhang *et al.*, 2012; Albertin *et al.*, 2015; Wang *et al.*, 2017), which will shed lights for the further structure and function studies of molluscan UPR pathways.

The response of molluscan UPR signaling pathway under various stressors

As a master ER surveillance system, the UPR regulates essentially all aspects of ER function, and continuously coordinates the activity and participation of the processing and degradation pathways for unfolded proteins (Hampton, 2000). Therefore, the UPR is a paradigm to establish and maintain the cell homeostasis (Walter and Ron, 2011). The majority of researches on molluscan UPR are focused on the expression profiles of key molecules in response against environmental stresses and immune challenges. Different UPR signaling pathways display different expression

patterns under various stressors, and they play different roles in the response to diverse stressors. Here we present the expression patterns of molluscan UPR signaling pathways under different stressors.

Expression profiles of IRE1–XBP1 signaling pathway after heat, metal stresses, and immune stimulations

IRE1-XBP1 signaling pathway is the most well studied pathway, and it plays an important role in a wide spectrum of biological processes, including differentiation, metabolism, inflammation, tumorigenesis, and neurodegeneration (Patil and Walter, 2001; Cao and Kaufman, 2012; Chen and Brandizzi, 2013). Previous reports in vertebrates indicate that IRE1-XBP1 signaling pathway responds against different stresses. For instance, cadmium and Ultraviolet A (UVA) could activate IRE1-XBP1 pathway by splicing XBP1 mRNA (Yokouchi *et al.*, 2007; Komori *et al.*, 2012; Kitamura, 2013). In mammalian cells, cadmium induced the activation of the IRE1-XBP1 pathway, which played proapoptotic roles in cadmium-induced apoptosis (Yokouchi *et al.*, 2007).

As the oldest and most-conserved branch of the UPR in eukaryotes (D and SR, 2008), IRE1-XBP1 signaling pathway has been identified in invertebrates, which responds against tunicamycin and/or dithiothreitol (DTT) treatments (Travers *et al.*, 2000; Shen *et al.*, 2001; Plongthongkum *et al.*, 2007). In DDT treated *Drosophila melanogaster* cells, the IRE1 mediating XBP1 mRNA splicing mechanism was extremely conserved and exerted a critical role for modulating XBP1 protein synthesis (Plongthongkum *et al.*, 2007). In virus challenged shrimp *Litopenaeus vannamei,* the expression profiles of IRE1 and XBP1 were detected, which confirmed that the IRE1-XBP1 pathway was important for *L. vannamei* environmental stress resistance (Chen *et al.*, 2012).

The activation of IRE1-XBP1 signaling pathway in response against thermal, metal stresses and immune challenges such as parasites and neoplasia has also been evidenced in molluscs. Under heat treatment, a significant increase of BiP mRNA was observed in both gill and adductor muscle of *C. gigas*, and the mRNA transcripts of BiP, PDI and IRE1 were also up-regulated in *C. gigas* hemocytes (Yokoyama *et al.*, 2006; Yang *et al.*, 2017). Similarly, BiP, IRE1 and XBP1 were all up-regulated in muscle, mantle and gill of *V. lienosa* exposed to heat treatment (Wang *et al.*, 2012). In the metal bioaccumulation toxicology experiment, BiP, CNX, CRT and IRE1 were up-regulated in *C. gigas* gills exposed to plumbum (Pb) treatment (Zhang *et al.*, 2012). XBP from *M. edulis* also showed significant up-regulation in gills under Pb or mixture of Pb and cadmium (Cd) treatments (Poynton *et al.*, 2014), suggesting that IRE1, XBP1 and BiP were involved in the response of molluscs against the environmental stressors. In flat oyster *O. edulis*, bonamiosis is a lethal haemocyte infection caused by *Bonamia* genus protozoan parasites, and disseminated neoplasia (DN) is a malignant proliferation of circulating cells resembling leukaemia. The expressions of XBP1 in *O. edulis* with bonamiosis or DN were significantly increased in hemocytes, mantle, and digestive gland. Remarkably, the expression of XBP1 was significant down-regulation in the gill under slight DN infection (Martín-Gómez *et al.*, 2014). The previous reports about responses of molluscan IRE1-XBP1 signaling are mainly focused on the heat stimulation at temperature range of 20-30 °C both in short term as several hours (Yang *et al.*, 2017) and in long term for a few days (Wang *et al.*, 2012). Single metal stimulation with short time (Zhang *et al.*, 2012) and mixture metal for long term (Poynton *et al.*, 2014) are also concerned in oyster and mussels. The severe and slight immune treatments with parasites and neoplasia (Martín-Gómez *et al.*, 2014) are another interesting research related to the response of IRE1-XBP1 signaling (Table 1).

In general, the IRE1-XBP1 signaling pathway is believed to respond rapidly to different stressors and functions as early stress indicator, and the regulation mode of IRE1-XBP1 signaling pathway is suspected to be relatively conservative in molluscs. The fluctuations of IRE1 and XBP mRNA expression under various stressors indicates that IRE1 activity-dependent XBP1 mRNA splicing under ER stress also exist in molluscs (Calfon *et al.*, 2002; Back *et al.*, 2005), but further assays are need to investigate the biology functions of activated and un-activated XBP1 and the detailed activation and regulation mechanisms of IRE1-XBP1 signaling pathway in molluscs.

The activation of PERK–eIF2α–ATF4–CHOP signaling pathway under anoxia and heat stress

PERK-mediated eIF2α phosphorylation contributes to transcriptional activation in UPR and decreases global protein synthesis to reduce the ER load (Ron and Walter, 2007). Previous studies in mammals shows that PERK–eIF2α signaling pathway is dominant over the IRE1-XBP1 and ATF6 signaling pathway (Oyadomari and Mori, 2004) and it is activated by many kinds of viruses (He, 2006) and malnutrition like glucose (Glc) deprivation (Fernandez *et al.*, 2002; Kitamura, 2013). For instance, PERK was activated by an increase of PERK auto-phosphorylation when mammalian cells were infected with herpes simplex virus and it played a key role in limiting viral replication (He, 2006). PERK was also responsible for the phosphorylation of eIF2α induced by glucose deprivation and the translational regulation via eIF2α phosphorylation was important in response to cellular stress (Fernandez *et al.*, 2002). Additionally, ER stress responses leading to eIF2α phosphorylation and XBP1 splicing were detected in heat stressed rat cortex as well (Liu *et al.*, 2012).

As for invertebrates, PERK–eIF2α signaling pathway has also been reported. In shrimp, PERK–eIF2α signaling pathway was activated by white spot syndrome virus (WSSV) (Xu *et al.*, 2014; Zhang *et al.*, 2016b), tunicamycin and cycloheximide (Aparna *et al.*, 2003) by inducing eIF2α phosphorylation. The promoter of pro-apoptotic protein endoplasmic reticulum oxidoreductin was activated by the key transcription factor ATF4 of PERK–eIF2α pathway during WSSV stimulation. WSSV infection enhanced the phosphorylation of

eIF2α and the PERK–eIF2α pathway activation was important for innate immune during WSSV infection in shrimp. It also indicated that WSSV could induce apoptosis via the PERK–eIF2α pathway (Xu *et al.*, 2014; Zhang *et al.*, 2016b). The tunicamycin and low concentrations of cycloheximide promoted eIF2α phosphorylation in *Spodoptera frugiperda* ovarian cells but without apoptosis. These findings therefore suggested that eIF2α phosphorylation was not always necessary to induce apoptosis, but it was a characteristic hallmark of stressed cells and also of cells undergoing apoptosis (Aparna *et al.*, 2003).

In molluscs, the main components of PERK–eIF2α–ATF4–CHOP signaling pathway have been found to response against various stresses, such as anoxia and thermal stress. PERK from *C. gigas* was up-regulated in gills with air exposure treatment (Zhang *et al.*, 2012). PERK in oyster hemocytes as well as eIF2α in gills were also up-regulated after heat treatment (Zhang *et al.*, 2012; Yang *et al.*, 2017). The mRNA expressions of BiP, CNX, CRT, PDIs and PERK were up-regulated when *C. hongkongensis* was subjected to 37 °C heat treatment (Li *et al.*, 2017), suggesting there was a complex cellular response to thermal stress in this species. A transcriptome analysis of freshwater mussel *V. lienosa* indicated that PERK, eIF2α and ATF4 were up-regulated in muscle, mantle and gill during thermal stress, and the KEGG analysis revealed a nearly complete PERK–eIF2α pathway in canonical UPR system (Wang *et al.*, 2012). It indicates that molluscs may evolve PERK–eIF2α signaling pathway to cope with thermal stress in high temperature living regions. The phosphorylation of eIF2α has also been observed in molluscs responded upon the anoxia exposure. The expressions of BiP, phosphorylated eIF-2α and ATF4, and GADD153 in foot muscles of *L. littorea* were significantly up-regulated after anoxia exposure, while down-regulations of GADD34 were observed at 20 h after anoxia exposure (Storey *et al.*, 2013). In *M. mercenaria*, the protein expression level of eIF2α was not affected by hypoxia stress, but the phosphorylated eIF2α increased at 18 h (Ivanina *et al.*, 2016). The significance of eIF2α phosphorylation in the induction of UPR is highlighted in mammals by a number of studies. Phosphorylated eIF2α is essential for optimal expression of many UPR genes and also critical for the maintenance of cellular homeostasis (Roy and Lee, 1999; Harding *et al.*, 2000). The increased phosphorylated eIF2α in *M. mercenaria* after hypoxia exposure showed a greater suppression of energy-consuming processes such as protein turnover, indicating that inactivation of eIF2α might be essential for molluscan hypoxia tolerance (Ivanina *et al.*, 2016). In mammals, de-phosphorylation of eIF2α mediated by GADD34, as a negative feedback loop, can promote recovery from translational inhibition in the UPR (Novoa *et al.*, 2001). The up-regulation of phosphorylated eIF-2α and down-regulations of GADD34 give a hint that the negative feedback mechanism may dedicate to the regulation of PERK–eIF2α signaling pathway in molluscs, as part of the adaptive response of cells to ER stress. The knowledge about the activation and responses of molluscan PERK–eIF2α–ATF4–CHOP signaling pathway is mainly from the investigations on air exposure (Zhang *et al.*, 2012), heat treatments (Wang *et al.*, 2012; Li *et al.*, 2017), and anoxia exposure (Storey *et al.*, 2013; Ivanina and Sokolova, 2016), from hours to days and 20 ℃ to 30 ℃ (Table 1). The accumulated evidences indicate that the PERK–eIF2α signaling pathway plays crucial roles for molluscs to defend the environment stresses. And it is necessary to study the involvement and regulation mechanism of molluscan PERK–eIF2α signaling pathway in malnutrition and pathogen infection to compare with that in vertebrate.

Expression profiles of ATF6 signaling pathway in molluscs

As a transcription factor, ATF6 is initially synthesized as an ER-resident transmembrane protein bearing a large ER-luminal domain. In response to ER stress, ATF6 is translocated from ER to Golgi apparatus and cleaved to the active form by site 1 and 2 proteases (S1P and S2P) in Golgi apparatus (Haze *et al.*, 1999; Okada *et al.*, 2002; Shen *et al.*, 2005a; Adachi *et al.*, 2008; Walter and Ron, 2011). It has been reported that mammalian ATF6 enters nucleus and activates the transcription of its target genes such as ER chaperone genes to increases ER protein folding capacity under stress (Yoshida *et al.*, 2000, 2001; Namba *et al.*, 2007). For example, *Shigella dysenteriae* toxins treatment induced the activation of ATF6 in human myelogenous leukaemia cell line, and the nuclear translocation of ATF-6 increased highly in riboflavin-deficient cells (Manthey *et al.*, 2005; Lee *et al.*, 2008; Kitamura, 2013). The stimulation of the myelogenous leukaemia cell line with purified shiga toxins could induce the ER stress response and increased the activation of the ER stress sensors IRE1, PERK, and ATF6. The degradation of the ATF6 90 kDa proteins and decrease of immune-reactive ATF6 proteins were observed in monocytic cells treated with shiga toxins (Lee *et al.*, 2008). Nuclear translocation of ATF6 was enhanced and associated with activation of the unfolded protein response in riboflavin-deficient cells (Manthey *et al.*, 2005).

In shrimps, ATF6 was found to be vital for WSSV replication, and UPR in shrimp could facilitate WSSV infection (Yuan *et al.*, 2017; Xu *et al.*, 2018). The expression of ATF6 increased in kuruma shrimp *Marsupenaeus japonicus* after WSSV challenge. When ATF6 was knocked down by RNA interference, the cumulative mortality of shrimp decreased with presence of WSSV infection (Yuan *et al.*, 2017; Xu *et al.*, 2018).

The previous researches on molluscan UPR are mainly focus on gene cloning and mRNA expression (Kuhl *et al.*, 1992; Yokoyama *et al.*, 2006). In foot muscles *L. littorea*, the relative level of the inactive 90 kDa ATF6 did not change significantly, while a significant decrease of active 50 kDa ATF6 level was observed under anoxia exposure (Storey *et al.*, 2013). On the contrary, the level of active p50 ATF6 in mammals increased significantly under stress (Haze *et al.*, 1999). These results indicated that the ATF6 signaling pathway might not be part of an anoxia-responsive activation of the UPR in *L. littorea*

(Storey *et al.*, 2013). In *C. elegans*, IRE1–XBP1 was demonstrated to play predominant role in UPR signaling pathways, while ATF6 was less important (Ma and Hendershot, 2001). It is suspected that IRE1α–XBP1 signaling pathway is activated to response against thermal, metal and immune stressors, while PERK–eIF2α–ATF4–CHOP signaling pathway is inferred to involve in the response against anoxia and thermal stress in molluscs. Although the active/inactive forms of ATF6 have been found in molluscs, there is no solid evidence for its involvement in molluscan UPR (Table 1). In model organisms, the UPR is differently regulated in different cell types (Walter and Ron, 2011), the characters in various cell types and its underlying mechanism of ATF6 signaling pathway in molluscs deserve to further study.

The pathways co-expressed with molluscan UPR and the possible downstream immune response

The pathways co-expressed with molluscan UPR

In mammals, several elements in pleiotropic pathways such as p38 mitogen-activated protein kinase, nuclear factor NF-kappa-B (NF-κB) and c-Jun N-terminal kinase (JNK) are found to be involved in IRE1α–XBP1 and PERK–eIF2α signaling pathways (Cao and Kaufman, 2012; Grootjans *et al.*, 2016). Multiple immune pathways, such as apoptosis and inflammatory response, have been retrieved in the heat-treated gastropod and bivalve (Zhang *et al.*, 2012; Clark *et al.*, 2016; Fields *et al.*, 2016; Li *et al.*, 2017; Yang *et al.*, 2017), and the heavy metal-treated bivalve (Poynton *et al.*, 2014). Although there are some reports about the downstream immune pathways of UPR, the enrichment terms are still partial due to the incomplete reference database and lack of comprehensive investigation. Further investigations are urgently needed to reveal the correlations between UPR and immune pathways in molluscs.

The possible downstream immune response of UPR

The chronic or unresolved mammalian ER stress, such as prolonged activation of IRE1 and CHOP, can trigger cell apoptosis (Tabas and Ron, 2011). The molecular mechanisms of apoptosis in molluscs have also been studied under different stressors. For instance, Arg–Gly–Asp (RGD) peptides could inhibit integrin–ligand interactions and active caspase-3 to induce cell death (Buckley *et al.*, 1999), and the apoptosis was triggered in hemocytes of the pacific oyster when it was treated with RGD peptides (Terahara *et al.*, 2005; Kiss, 2010). The large-scale apoptotic and anti-apoptotic genes annotated in molluscan genome suggested that apoptosis and anti-apoptotic mechanisms might play important roles in molluscan adaptive survival (Zhang *et al.*, 2012; Zhang *et al.*, 2016a). Recently, UPR has been reported to involved in autophagy (Yeganeh *et al.*, 2015), central nervous system (CNS) development (Godin *et al.*, 2016), ribosomal proteins ubiquitylation (Higgins *et al.*, 2015), cell transformation, tumor development and aggressiveness (Dejeans *et al.*, 2015), and inflammation (Janssens *et al.*, 2014; Grootjans *et al.*, 2016). In-depth study of the UPR pathway and its mediated pathways especially apoptosis will provide important reference for better understanding of the diversity of molluscs and their evolutionary adaptation mechanisms, as well as the complicated physiological and biochemical principle.

Conclusion

A preliminary map of molluscan UPR signaling pathways has been outlined in the last decades with conserved constitution similar to that in other eukaryotes. The molluscan UPR signaling pathways exhibit different response to stressors at mRNA and protein levels, among which IRE1α–XBP1 and PERK–eIF2α–ATF4–CHOP signaling pathways are speculated to devote enormously in adaptive response of molluscan cells to ER stresses. Meanwhile, numerous response and pathways, including apoptosis, anti-apoptosis, metabolic pathways, cytoskeletal, protein de-phosphorylation and ubiquitination, tissue regeneration, DNA repair, antigen presentation, complement cascades, defense and immune responses, have been discovered paralleling with UPR signaling pathways in molluscs. At the same time, the function and detailed regulation mechanism of molluscan UPR signaling and the crosstalk between UPR and other signaling pathways still need further investigations. In future, synergistic approaches including engineering of genetic, cell, protein, enzyme, and biochemical will make molluscan UPR a promising field for evolutionary stress response.

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