Characterization of *Cherax quadricarinatus* prohibitin and its potential role in spermatogenesis

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**A B S T R A C T**

Prohibitin (PHB) proteins have diverse functions, such as cellular signaling, transcriptional control and mitochondrial biogenesis. In this study, we characterized PHB gene and its protein expression in *Cherax quadricarinatus*. PHB cDNA comprises 1472 nucleotides with an open reading frame of 828 bp, which encodes 275 amino acid residues. The highest transcript levels were found during the spermatogonial developmental phase, with the lowest levels detected during the resting phase in the reproductive cycle. Western blot analysis revealed that PHB is an approximately 30 kDa protein, and occurs in a number of unexpected isoforms, ranging from 30 kDa to greater than 180 kDa in the testes of different developmental phases, which may be the ubiquitinated substrates. The strongest immunolabeling signal was found in spermatogonia, with lower levels of staining in secondary spermatocytes, and weak or absent expression in mature sperm. Immunogold electron microscopy results confirmed the localization of PHB in the inner mitochondrial membranes. The results showed that PHB is a substrate protein for spermatogenesis, with a potential reproductive function involving sperm ubiquitination in invertebrates.

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1. Introduction

Prohibitin (PHB) is an evolutionally conserved protein belonging to a Band-7 or PHB domain family (Merkwirth and Langer, 2009; Mishra et al., 2006) and mainly localized in mitochondria (Artal-Sanz and Tavernarakis, 2009; Ilonen et al., 1995; Terashima et al., 1994; Thompson et al., 1999). Two highly homologous forms of this protein exist termed PHB1 and PHB2 (Mishra et al., 2006). Besides the initially proposed role in cell cycle progression (Cummins, 1998; Mishra et al., 2006; Nijtmans et al., 2000; Nuell et al., 1991; Piper et al., 2002; Sharma and Qadri, 2004; Theiss et al., 2007), PHBs have also been implicated in transcriptional regulation (Sun et al., 2004), sister chromatid cohesion regulation (Schmittgen and Livak, 2008), cellular signaling (Mishra et al., 2006; Rajalingam et al., 2005), apoptosis (Fusaro et al., 2003) and mitochondrial biogenesis (Artal-Sanz and Tavernarakis, 2009; Berger and Yaffe, 1998; Nijtmans et al., 2000). PHB1 mRNA and protein were reported to be expressed differentially during germ cell development. Subsequently, it was suggested that PHB1, a conserved 30 kDa component of the inner mitochondrial membrane, is also expressed as an unusual, high-molecular-mass isoform in mammalian spermatozoa (Sutovsky, 2003). However, little is known regarding the functional significance of PHB1 in the male germ cell in invertebrate animals.

The mitochondrion is a complex sub-cellular organ present in the cytoplasm of all animal and plant cells (Sanz et al., 2003). Several studies have indicated an important role for mitochondria in spermatogenesis and fertility (Sharma and Qadri, 2004; Shivaji et al., 2009). Mitochondria are a double-membrane structure consisting of outer and inner membranes (IM) separated by an inter-membrane space. The IM forms numerous folds inside the mitochondrial matrix known as “cristae” and is unusually high in protein content. PHB1 and PHB2 associate to form a macromolecular structure of approximately 1 MDa at the mitochondrial IM (Nijtmans et al., 2002). The PHB complex has been implicated in regulation of membrane protein degradation by the mitochondrial m-AAA protease (Langer, 2000). Ubiquitination is a versatile and universal mechanism for protein recycling, through which misfolded or aged proteins are tagged for degradation by the covalent attachment of one or more molecules of ubiquitin (Sutovsky, 2003). Mitochondrial DNA mutations have been reported in mitochondrial...
respiration defects giving rise to meiotic arrest and abnormalities in sperm morphology (Ankel-Simons and Cummins, 1996; Cummins, 1998; Merkworth et al., 2008; Spiropoulos et al., 2002).

Cherax quadricarinatus is a native species in the north of Queensland (Australia) and the southeast of Papua New Guinea (Jones, 1997). It is an important cultured species in aquaculture that has been reared for human consumption since 1985. Knowledge of the reproductive biology of male C. quadricarinatus is mainly limited to the anatomy of the reproductive system and the effects of nutrition or neuroendocrine regulation on fecundity (López Greco and Lo Nostro, 2008). Hence, a better understanding of the molecular mechanisms of spermatogenesis is important for understanding the molecular mechanisms of crayfish and invertebrates.

2. Materials and methods

2.1. Animals

Fifteen male C. quadricarinatus (70–110 g) were purchased from Shanghai Jinshan aquaculture farm during May (spring), July (summer) September (autumn) and December (winter) in 2010, and then placed in an ice bath for 3 to 5 min until lightly anesthetized prior to sacrifice. Various tissues were dissected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted using Trizol reagent (RNA Extraction Kit, CA, USA) according to the protocol provided by the manufacturer. The concentration and quality of total RNA were estimated by spectrophotometry (absorbance at 260 nm) and agarose gel electrophoresis, respectively. Total RNA (2 μg) isolated from testes was reverse transcribed using the SMART™ cDNA kit (Clonetech, CA, USA) for cDNA cloning and using the Prime Script™ RT-PCR Kit (TaKaRa, Japan) for semi-quantitative RT-PCR (RT-qPCR) analysis or the PrimeScript Real-time PCR Kit (TaKaRa, Japan) for real-time quantitative RT-PCR (RT-qPCR) analysis.

2.3. PHB gene isolation

Table 1: Sequences of primers.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primers for target PHB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'-ACATGNYCTCGGTTGTATCGMGA-3'</td>
<td>DP-R</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-GGGATGAGYNINTACAGACA-3'</td>
<td>DP-F</td>
</tr>
<tr>
<td>Gene-specific primers for PHB cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RACE gene-specific primer pairs for PHB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHB 5' primer</td>
<td>5'-TGGCGGAAAGTCGACAGCTATTA-3'</td>
<td>RT-R</td>
</tr>
<tr>
<td>PHB 3' primer</td>
<td>5'-GGATGCTCTCCATAACTGGCAGT-3'</td>
<td>RT-F</td>
</tr>
<tr>
<td>qRT-PCR primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHB 5' primer</td>
<td>5'-TCAAGACATCATACGCTATAT-3'</td>
<td>Real-R</td>
</tr>
<tr>
<td>PHB 3' primer</td>
<td>5'-TATCTATTACTACCTGATACCCG-3'</td>
<td>Real-F</td>
</tr>
<tr>
<td>18S RNA primers for RT-PCR and qRT-PCR</td>
<td></td>
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<tr>
<td>18S RNA reverse</td>
<td>5'-GGAGGTAGCTAGCAGAAAAACCC-3'</td>
<td>18S-R</td>
</tr>
<tr>
<td>18S RNA forward</td>
<td>5'-GGAGCGCTGACAGCCACATCGG-3'</td>
<td>18S-F</td>
</tr>
</tbody>
</table>

which were designed based on the obtained cDNA partial sequence of Cq-PHB. The full-length Cq-PHB cDNA was obtained using the RACE cDNA amplification kit according to the protocol provided by the manufacturer (Bioscience Clontech, CA, USA).

2.4. Alignment of multiple sequences and phylogenetic analysis

Multiple alignments of the full-length PHB sequence were performed with PHB sequences from other species. Amino acid sequences of various crustacean species were retrieved from the NCBI GenBank and analyzed using the ClustalW 2.0 Multiple Alignment program. Phylogenetic analyses were conducted using maximum likelihood (ML) approach (Holmes, 2003; Huelsenbeck and Rannala, 1997). It is known to be robust to a number of systematic biases of phylogenetic reconstruction. A ML phylogenetic tree was constructed using MEGA software version 5.0 (Holmes, 2003; Tamura et al., 2011). Jones–Taylor–Thornton (JTT) model was used as substitution model for phylogenetic tree construction (Jones et al., 1992). Reliability of nodes was estimated by ML bootstrap percentages (BPML) (Sullivan, 2005) obtained after 1000 pseudo replications, using the previously estimated ML parameters.

2.5. PHB mRNA expression patterns

Quantitative analysis of tissue- and testes developmental cycle-dependent mRNA expression was conducted via Real-time RT-qPCR. First-strand cDNA was prepared as described in Section 2.2. RT-qPCR primers (Q-R and Q-F, Table 1) were designed based on the cloned Cq-PHB cDNA to produce a 310 bp amplicon. PCR reactions were performed according to the PrimeScript Real-time PCR Kit protocol. The primers 18S-F and 18S-R were designed based on the sequence of C. quadricarinatus 18S rRNA (AF235968) to amplify a 232 bp fragment. Samples were run in triplicate and normalized to the selected control gene, 18S rRNA. All PCR reactions were performed in triplicate using extracted RNA (pooled) of the same concentration. Cq-PHB expression levels were calculated by the 2−ΔΔCt comparative CT method (Cobal et al., 2002). Mean and standard deviation values were calculated from triplicate experiments, and presented as fold differences in expression relative to 18S rRNA. Data were analyzed using the CFX Manager™ software version 1.0.
2.6. Preparation of anti-PHB antibody

The purified recombinant Cq-PHB protein was prepared using a previously described method (Zhou et al., 2010) and was then used to raise antibodies in a New Zealand white rabbit. Approximately 1 mg of purified PHB was emulsified in Freund’s complete adjuvant and injected subcutaneously at multiple sites. Animals were subsequently boosted with 0.5 mg antigen mixed with Freund’s incomplete adjuvant delivered on in three separate injections at intervals of 2 weeks (total, 1.5 mg antigen). One week after the final booster, blood was collected and serum was prepared. Serum from the same rabbit collected prior to immunization was used as a control. An increase in the titers of peptide-specific antibodies was verified by ELISA. The anti-sera were aliquoted and stored at −80 °C.

2.7. Western blot analysis

Testes tissues were homogenized in SDS sample buffer supplemented with 1 mM PMSF. Proteins were resolved on a 12.5% polyacrylamide gel under reducing conditions and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was treated in blocking solution (Roche, China) and incubated with primary antibody at 1:1000 for 1 h at room temperature. After washing with Tris-buffered saline (20 mM Tris-HCl, 0.9% NaCl, pH 7.4) containing 0.1% Tween-20, the membrane was incubated with a second antibody (goat anti-rabbit serum) labeled with alkaline phosphatase (Bio-Rad) at 1:10,000 for 1 h. Detection was performed using NBT/BCIP (Roche) as a substrate.

2.8. Immunofluorescence (IF)

Freshly dissected testes were fixed in 4% paraformaldehyde and embedded in OCT compound (Sakura Finetek, Torrance, CA). The cryosections (8 μm in thickness) were mounted on glass slides, washed in phosphate-buffered saline (PBS), and immersed in 3% BSA for 1 h to block non-specific binding. These slides were then incubated with primary antibodies against PHB at dilutions of 1:100 for 18 h at 4 °C, washed twice in PBS/Tween-20 solution, incubated with a fluorescein isothiocyanate (FITC)- conjugated secondary antibody, and viewed using a fluorescence microscope.

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *C. quadricarinatus* full-length cDNA. The transmembrane domain is shown in gray, the PHB domain is underlined and the coiled-coil domain is shown in bold. The CCAAT box is shown in the rectangle.
antibody for 1 h at room temperature, and photographed with a laser scanning confocal microscope (Leica, Germany).

2.9. Immunogold electron microscopy (IEM)

Fresh testes were fixed in a mixture of 0.25 % glutaraldehyde and 2.5 % paraformaldehyde in 10 mM PBS (pH 7.4) for 2 h at room temperature. The samples were embedded in Epon resin and ultrathin sections (60 nm) were collected on 200 mesh nickel grids. Slices were treated (2 × 15 min) with 3 % sodium metaperiodate, quickly rinsed in distilled water and treated (3 × 5 min) in citrate buffer (15 mg citric acid/10 ml) pH 6 at room temperature. A final incubation (10 min, 95 °C) in citrate buffer was carried out. After washing in distilled water (3 × 5 min), sections were incubated for 15 min in blocking solution Tris buffered saline (TBS) pH 7.4, 1 % BSA and finally incubated overnight with the primary antibody diluted 1:250 in blocking solution. Grids were then washed in...

![Fig. 2. Comparison of deduced amino acid sequence of PHB proteins. Three different domains are boxed in the corresponding colored rectangles.](image)

![Fig. 3. Phylogenetic analysis of PHB1 and PHB2 subfamily proteins. A maximum likelihood phylogenetic tree was constructed using MEGA software version 4.0, and the reliability of the branching was tested using bootstrap re-sampling (with 1000 pseudo replicates). PHB1 and PHB2 are clustered in two branches. The putative protein GenBank accession number is shown in parentheses.](image)
TBS (5 x 2 min), in TBS 0.5% BSA (5 x 2 min) and finally in TBS 1% BSA (15 min). Immunocomplexes were visualized by incubation (1 h) with goat anti-rabbit immunoglobulins conjugated to colloidal gold (20 nm diameter) diluted 1:200 in TBS. After a repeat series of washings, sections were stained with uranyl acetate for 15 min and analyzed using a Leo 912 Zeiss electron microscope. Controls were incubated with primary antibody preabsorbed with 10^-6 M of the corresponding peptide (1 V peptide/10 V TBS). Negatives were scanned by Umax Powerlook 3000 flat-bed scanner (Umax Technologies, CA, USA) and edited with Adobe Photoshop CS 3.0.

2.10. Statistical analysis

A multiple comparisons (Duncan’s) test was used to compare significant differences in Cq-PHB gene expression between control and tested samples using SPSS15.0 software. P < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of Cq-PHB

Using degenerate PCR amplification, a cDNA fragment of 302 bp was obtained (Fig. 1; GenBank accession number: JN168787) that showed 95% similarity to PHB of Litopenaeus vannamei and Scylla paramamosain. Full-length cDNA of Cq-PHB was 1472 bp in length and contained a predicted open reading frame (ORF) of 828 bp, beginning with a methionine codon at position 161 and ending with a TGA termination codon at position 987. The 3′-untranslated region was 485 bp in length and contained a polyadenylation signal (AATAAA) and a poly (A) tail of 23 bp. The complete sequence was then deposited in GenBank (accession number: HM345982). The encoded 275 amino acid polypeptide had a calculated molecular mass of 30.3 kDa and a predicted isoelectric point of 6.37.

Using the InterPro search database, the primary structure of PHB protein was shown to lack motifs typical of signal sequences, nuclear localization signals, ATP-binding sites or transcription factors. Comparisons with PHB genes from other species revealed eight highly conserved regions, four of which corresponded to binding sites of known transcriptional control proteins (CCAAT box, ‘SV40’ sites and two Spl sites; Fig. 2).

Blast analysis revealed that Cq-PHB shared high similarity with other PHBs from other species, including L vannamei (88%), S. paramamosain (86%), Drosophila melanogaster (74%), Danio rerio (73%), Gallus gallus (72%), Salmo salar (71%), Bos taurus (71%), Mus musculus (71%), Homo sapiens (71%) and Sus scrofa (71%). Furthermore, Cq-PHB shared >60% identity with PHBs from certain other species. The phylogeny of these PHBs is shown in Fig. 3. The phylogenetic tree contained two distinct clusters, where C. quadricarinatus PHB belongs to PHB1 subfamily and clusters with other ectotherms, while mammals, birds and primates formed a separate cluster.

3.2. Expression patterns of Cq-PHB transcripts

The mRNA transcript of Cq-PHB was expressed universally in all the tissues investigated, including the brain, gills, heart, hemocytes, hepatopancreas, testes, stomach and muscles (Fig. 4). Expression was greater in the brain and testes, with lower levels in the other tissues.

Temporal expression of Cq-PHB in the testes during the crayfish reproductive cycle was measured using RT-qPCR with 18S rRNA acting as the internal control. A single peak was observed for both Cq-PHB and 18S rRNA genes at the corresponding melting temperature in the dissociation curves, indicating that the PCR specifically amplified a single gene. The temporal expression of the Cq-PHB mRNA transcripts is presented in Fig. 5. During the developmental cycle in the testes, expression of Cq-PHB mRNA was upregulated during the developmental phase, and high expression was maintained during the spermatogonial multiplication period. Subsequently, expression was downregulated during the mature sperm stage, and lower expression levels were found during the resting phase (P < 0.05).

3.3. Western blot analysis of testes proteins

Anti-PHB and anti-serum antibodies also recognized components in a crude extract of adult C. quadricarinatus testes. Probing of protein extracts transferred to a nitrocellulose membrane with anti-PHB revealed a series of ladder bands characteristic of Cq-PHB. Anti-PHB probing produced bands of approximately 30 kDa to 180 kDa. Cq-PHB protein expression was higher in the developmental and multiplication phases compared with the absence and mature phases. No protein components of the C. quadricarinatus extracts were detected using the control serum from the pre-immunized rabbit (Fig. 6).

3.4. Localization of Cq-PHB

Localization of the Cq-PHB protein was studied by IF and IEM. Whole sections of testes stained with hematoxylin and eosin (H&E) and with anti-PHB immunolabeling (counterstained with H&E) are shown in Figs. 7A and B. Positive immunoreactive signals (green fluorescence) for the PHB protein were detected in primary spermatocytes (I), secondary spermatocytes (II) and spermatids (III) of normal mature testes. Within the testes, the strongest signals for Cq-PHB were found in primary spermatocytes, with lower positive signals in secondary spermatocytes, and weak or absent signals in spermatids or mature sperm (Fig. 7, D, III). Moreover, the Cq-PHB protein was concentrated mainly in the cytoplasm of developmental sperm cells. No positive signals were detected in the negative control, which was incubated with pre-immune rabbit serum (Fig. 7B).

Prohibitin was found mainly on the mitochondrial inner membrane of the spermatozoa, with some labeling black particles in the matrix and...
free mitochondria (Fig. 8, arrows showed) using colloidal gold immunochemistry. PHB was also observed on the spermatozoa nucleus. No positive signals were detected in negative preparations generated by omission of the primary antibody (Fig. 8).

4. Discussion

Genetic studies using different organism models have provided strong evidence for an important biological role of PHB in mitochondrial
function, cell proliferation, and development (Nijtmans et al., 2002). PHB has been identified as a candidate ubiquitination substrate within the sperm mitochondrial membrane (Thompson et al., 1999). PHB transcripts were previously detected in the rat spermatogenic cell, although the protein was no longer apparent by the time that spermiation was completed in the testis (Choongkittaworn et al., 1993). In this study, the Cq-PHB gene primary structure, together with the PHB protein ultrastructural localization, were characterized in testes of C. quadricarinatus.

Using the InterPro database and NCBI blast analysis showed that Cq-PHB is a highly conserved protein belonging to band-7 super family (Figs. 1, 2 and 3). It shares an evolutionarily conserved stomatin/prohibitin/flotillin/Hflk/C (SPFH) domain with significant homology to several eukaryotic and prokaryotic proteins (Tavernarakis et al., 1999). Members of this protein family are membrane-associated and implicated in cellular processes (Roskams et al., 1993; Tamhane, 1977). Furthermore, this protein comprises an N-terminal hydrophobic transmembrane domain, followed by the well-conserved PHB domain, and a C-terminal coiled-coil region (Fig. 2). The archetypal PHB domain-containing protein is a mitochondrial inner membrane protein that has been proposed to act as a chaperone for the stabilization of mitochondrial proteins (Ikonen et al., 1995; Merkwirth and Langer, 2009; Nijtmans et al., 2002; Schwartz and Vissing, 2002).

In C. quadricarinatus, Cq-PHB transcripts were detected in all the tissues that were examined (Fig. 4). The wide distribution of PHBs among different tissue types may result from its fundamental role in many biological functions, such as cell proliferation, mitochondrial functions and its role as a binding site for ubiquitin (Sutovsky, 2003). Further, RT-qPCR data showed that the Cq-PHB mRNA transcripts were present in the testes throughout the whole reproductive cycle of C. quadricarinatus. In the developmental reproductive cycle, higher Cq-PHB transcript synthesis was seen during spermatogonial developmental and the multiplication phases, which suggests that Cq-PHB is critical for spermatogonial multiplication and spermatogenesis (Fig. 5). The high levels of Cq-PHB mRNA observed in the spermatogonial multiplication period may imply that Cq-PHB is also an essential protein in crayfish spermatogenesis, which would support its enhancement of cell proliferation and regulation of sperm metabolism in vertebrates (Merkwirth and Langer, 2009; Merkwirth et al., 2008). Taken together, marked transformations and cellular differentiation take place during all these development periods, indicating that spermatogenesis is associated with dynamic expression levels of Cq-PHB.

The male crayfish reproductive system consists of paired testes, vasa deferentia, and genital appendices (appendices masculinae) at the base of the fifth pereiopods (An et al., 2011; López Greco and Lo Nostro, 2008). Each testis consists of numerous testicular lobes and a non-branched collecting duct. Germ cells undergoing a single stage of spermatogenesis are found in each seminal acinus and spermatogenesis is synchronous within each acinus but not between acini (An et al., 2011; López Greco and Lo Nostro, 2008). Following meiosis, spermatids undergo a complicated metamorphosis until aflagellate spermatozoa are formed. Spermatogenesis is a well-defined synchronous and spatial arrangement process that provides a good model system for investigating the regulation and sequential activation of genes during germ cell differentiation (Rajalingam et al., 2005). In C. quadricarinatus there are numerous diffuse mitochondria in spermatogonia, round or oval-shaped ridges of developing mitochondria in spermatocytes and only a few visible mitochondrial pellets in spermatids (López Greco and Lo Nostro, 2008). Spermatogonia and spermatocytes are mainly distributed in the developmental and proliferation phases, respectively. Western blot analysis showed that the PHB mRNA transcript expression was higher in these two phases compared with

Fig. 8. Colloidal gold immunocytochemical detection of prohibitin in sperm mitochondria. Colloidal gold particles are mainly present on the mitochondrial membranes of the sperm cytoplasm. The black particles with arrows show the immunolabeled mitochondria and the gray mitochondria show the negative controls. E: endoplasmic reticulum, M: mitochondria. (A): Secondary spermatocyte; (B): early spermatids; (C): enlargement of the immunolabeled mitochondria; D: the negative control with omission of the primary antibody for the secondary spermatocyte. Bar length for (A), (B) and (D) is 1.0 μm, for (C) is 0.5 μm.

of different molecular weight forms of macromolecular complexes through the spermatogenesis process. It also indicated that the different levels of ubiquitination occur in the process of spermatogenesis. PHB protein acts as an ubiquitin binding site (Figs. 7 and 8). Ubiquitinated proteins are degraded in the proteasome, thus suggesting a relationship between PHB and protein degradation. This then leads to mitochondrial degradation, suggesting that PHB is related to the maternal inheritance of mtDNA. This result is consistent with a previous study on bull and rhesus spermatooza (Sutovsky, 2003; Tatsuta et al., 2005). Thus, these data indicate that PHB has a similar biological function in crustaceans and other invertebrate animals.

This study demonstrated that PHB is a mitochondrial protein localized in the inner membrane and that it exhibits dynamic changes in spermatogenesis. It is also known that mitochondria exhibit similar dynamic changes during spermatogenesis. Thus, it can be speculated that PHB may serve as a molecular biomarker of mitochondria. However, the crustacean sperm is immotile and has less mitochondria than vertebrates, whether a similar mitochondrial inheritance mechanism in the crustacean and invertebrates exists or not needs further research to confirm.

5. Conclusion

In the present study, we characterized the molecular structure, expression pattern and some functions of the novel mitochondrial-related gene PHB in the crayfish C. quadricarinatus. This provides an initial step towards understanding ubiquitination during spermatogenesis and the mitochondrial inheritance mechanism in aquatic invertebrate animals. The results showed that Cq-PHB regulates spermatogenesis, and may explain the molecular mechanisms underlying ubiquitination of the crayfish sperm. Given the apparent new function for PHB identified in this investigation, namely, the maintenance of mtDNA, degradation of PHB by the ubiquitin–proteasome pathway could facilitate the degradation of paternal mtDNA and enhance the inheritance of maternal mtDNA. Further research is required to elucidate the exact mechanism by which Cq-PHB maintains mtDNA and the biological significance of this regulation.

Acknowledgments

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