Investigation of the Possible Role of RAD9 in Post-Diapaused Embryonic Development of the Brine Shrimp Artemia sinica

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Abstract: Background: The cell cycle checkpoint protein RAD9 is a vital cell cycle regulator in eukaryotic cells. RAD9 is involved in diverse cellular functions by oligomer or monomer. However, the specific mechanism of its activity remains unknown in crustaceans, especially in embryonic diapause resumption of the brine shrimp Artemia sinica. Methods and Results: In the present article, a 1238 bp full-length cDNA of As–RAD9 gene, encoding 376 amino acids, was obtained from A. sinica. The expression pattern of As–RAD9 was analyzed by qPCR and Western blot. The mRNA expression level climbs to the top at the 10 h stage of embryo development, while the protein expression pattern is generally consistent with qPCR results. Moreover, the As–RAD9 related signaling proteins, As–RAD1, As–HUS1, As–RAD17, and As–CHK1, were also detected. Immunofluorescence assay showed that the location of As–RAD9 did not show tissue or organ specificity, and the intracellular expression was concentrated in the cytoplasm more than in the nucleus. We also explored the amount of As–RAD9 under the stresses of cold and high salinity, and the results indicate that As–RAD9 is a stress-related factor, though the mechanisms may be different in response to different stresses. Knocking down of the As–RAD9 gene led to embryonic development delay in A. sinica. Conclusions: All these results reveal that As–RAD9 is necessary for post-diapaused embryonic development in A. sinica.

Keywords: Artemia sinica; As–rad9; diapause embryo; cell cycle checkpoint protein; RAD9

1. Introduction

The brine shrimp Artemia sinica (arthropods, crustaceans, Branchiopoda, Anostaca, Artemia,) is extensively distributed in salt lakes in China. A. sinica is an excellent live food for shrimps, crabs, and fishes; hence, it is widely used in aquaculture and experimental research [1]. The diapause of A. sinica is one of the defense mechanisms against extreme environments, such as extreme temperature, water shortage, hypoxia, and high salinity. Some studies have shown that A. sinica will diapause in low temperature and high salt environment [2–4]. At the same time, the metabolic level of embryonic cells is significantly reduced, the cell cycle is arrested, and the life activity is almost completely stopped [5]. This state can be maintained for a long time, so that the Artemia cysts can withstand the damage of the harsh environment. After diapause termination, the embryo cells remain in a resting situation and resume developing only if the survival conditions are feasible [2–4]. However, the molecular
mechanism involved in cell cycle recovery during the restarting of diapause embryo is still unknown, which has become a research hotspot in this field.

Eukaryotic cell division can be delayed at cell cycle checkpoints because of cellular damage, exogenous stress signal, nutrients, or essential growth factors [6,7]. Proteins that function in the regulation of cell cycle checkpoints are critical for fate determination in different cells. RAD9 was first discovered in cell–cycle arrest induced by DNA damage in budding yeasts [8,9]. Deletion of RAD9 in mice showed checkpoint abnormalities and embryonic lethality [10]. Recent studies found that RAD9 is a versatile gene which is associated with apoptosis, DNA repair, genome integrity, cell cycle checkpoint control, radioresistance, transactivation of downstream genes, ribonucleotide metabolism, and telomere maintenance [8,11,12]. RAD9 interacts with a multitude of proteins directly or indirectly, which are involved in diverse physiologic functions. The indirect interaction is mostly mediated by the RAD9, RAD1, and HUS1 (9–1–1) complex. Extracellular stress-induced DNA damage can be repaired through the ataxia telangiectasia mutated (ATM) and ATR (ATM and RAD53-related) signaling pathways. At the same time, the 9–1–1 complex is phosphorylated by the loader complex RAD17–replication factor–C, and transferred to the impaired position in a replication protein A (RPA) dependent method, and then the heterotrimeric slides along DNA as a processivity factor for DNA polymerases [11]. The 9–1–1 complex can also bind to RHINO (RAD9, RAD1, Hus1 interacting nuclear orphan) [13,14] and TopBP1 [15] to further amplify the ATR signal, and participate in the activation of different downstream substrates, including CHK1, thereafter activating downstream effectors such as CDC25A and WEE1 [16,17]. In mammals, phosphorylation of RAD9 mediates the binding of 9–1–1 to TopBP1 and activates the ATR–CHK1 checkpoint pathway [18].

However, little research has been done on arthropods; in particular, the molecular mechanism and function of RAD9 are still unknown in *A. sinica*. In this study, the full-length cDNA of *As–RAD9* from *A. sinica* was cloned. Real-time qPCR and Western blotting were used to analyze the mRNA and protein expression levels during embryonic development. The expression location of *As–RAD9* was determined by immunofluorescence assay. The morphological changes and mRNA expression after interference were observed by siRNA interference assay. Our aim is to further explore the role of *As–RAD9* in the early embryonic development of *A. sinica* and response to exogenous stresses like cold and high salinity.

2. Results

2.1. Cloning and Bioinformatic Analysis of *As–RAD9*

The 1238 bp full-length cDNA of *As–RAD9* (GenBank accession number: MH797557) was obtained, which contains an 1131 bp open reading frame (ORF), and the length of 5′–UTR and 3′–UTR were 3 bp and 107 bp, respectively (Figure S1a). The encoding protein contains 376 amino acids, whose predicted molecular weight is 42.6 kDa, and the pl is 6.48. *As–RAD9* has 38 phosphorylation sites, indicating that it may frequently suffer post-translational modification (Table 1). The subcellular prediction showed that *As–RAD9* was most likely (60.9%) to be in the nucleus, 30.4% in the mitochondria, and 8.7% in the cytoplasm. *As–RAD9* is a non-secretory protein, since the SignalP 4.0 fails to find a signal peptide in the protein. Protoscale indicated that the putative protein was most likely to be hydrophilic (MIN: −3.100, MAX: 2.011). The TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) showed that *As–RAD9* has no transmembrane helices, indicating that *As–RAD9* is not a transmembrane protein.
Table 1. Predicted phosphorylation sites in As–RAD9.

| Name | Position | Context a | Score b | Name | Position | Context a | Score b |
|------|----------|-----------|---------|------|----------|-----------|---------|
| Ser  | 2        | —ASHLPY   | 0.002   | Thr  | 27       | GLLQTATKQ | 0.025   |
| 41   | PVVESKWR  | 0.350     |         | 29   | LQTATKQAE| 0.038     |
| 59   | WNFISGQY  | 0.004     |         | 45   | SKWRLEAF | 0.100     |
| 68   | LAGLSTLP  | 0.007     |         | 69   | AGLSTLPG | 0.028     |
| 78   | PIAASLMAF | 0.003     |         | 83   | LMAFASIT | 0.067     |
| 85   | AFTASITS  | 0.0079    |         | 87   | TASITPLT | 0.046     |
| 88   | ASITSPLT  | 0.662     |         | 91   | TPITLTTQ | 0.096     |
| 113  | LAPPSAASA | 0.032     |         | 92   | SPLITPLT | 0.192     |
| 116  | PSAASA*VFG | 0.114 |         | 95   | TQHLLFN  | 0.005     |
| 130  | AVGSIGLG  | 0.030     |         | 165  | EMPSTELD | 0.547     |
| 159  | FKVMSGEMP | 0.956     |         | 221  | HVSPHYVP | 0.212     |
| 164  | GEMPSTEDL | 0.996     |         | 234  | AARVTQILS| 0.174     |
| 177  | PAISPGAL  | 0.069     |         | 241  | LSLITQL  | 0.037     |
| 213  | IAFA*RNH  | 0.046     |         | 243  | SLITQILK | 0.015     |
| 219  | GNMVSPHY  | 0.294     |         | 259  | EDCSTPC– | 0.382     |
| 227  | YVPSDAAA  | 0.305     |         | 6    | SHLPYEIQ | 0.683     |
| 238  | TQILSSLIT | 0.003     |         | 63   | SGIQYLAG | 0.127     |
| 239  | QILSSLIT  | 0.157     |         | 144  | ILAGYTAG | 0.008     |
| 258  | NEDECSTPC-| 0.116     |         | 223  | SPLYVPS  | 0.714     |

a The sequences surrounding the phosphorylation sites. b The likelihood of the phosphorylation site being real.

The homology analysis of the As–RAD9 protein sequence revealed a highly conserved amino acid sequence between A. sinica and the other 12 species of GenBank (Figure S2); the result of multiple sequence alignment was further used for phylogenetic tree construction and evolutionary relationships analysis. The neighbor-joining (NJ) tree was built in Mega 4.1 software [19], with a bootstrapping value of 1000 (Figure S3).

2.2. Expression of As–RAD9 by qPCR

The mRNA expression patterns of As–RAD9 were tested by qPCR in different developmental stages of A. sinica (Figure 1). Results displayed that the amount of As–RAD9 mRNA began to increase rapidly from 0 h to 5 h at the developmental stage and reached a significantly high level at 10 h. Then, it declined from 15 h and maintained a relatively lower expression level. The expression levels of As–RAD9 in the different challenge of temperature (Figure 2) and salinity (Figure 3) in A. sinica were also detected. The expression levels of As–RAD9 mRNA peaked at 10 °C, while there was no significant difference between other challenges and control group. On the other hand, the expression tendency of As–RAD9 under stress of salinity is quite different. The expression level of As–RAD9 increased slightly at the tender stress of salinity (50%), and then slowly decreased with the increase of salinity stress. When the salinity reached 150%, the amount of As–RAD9 mRNA was the lowest, while the amount of As–RAD9 mRNA rebounded at 200%.

2.3. Expression Pattern of As–RAD9 Protein

The expression of RAD9 in different embryonic development stages of A. sinica was detected by western blotting and compared with other four related proteins (As–RAD17, As–RAD1, As–HUS1, As–CHK1) (Figure 5). The intensity was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression level of As–RAD9 increased from 0 h to 5 h at different developmental stages and reached the highest level. Since then, it gradually decreased in expression after 10 h. The other four cell cycle regulatory proteins associated with As–RAD9 showed a similar trend to As–RAD9.
Figure 1. Quantitative real-time qPCR analysis of the expression levels of As–RAD9 in different developmental stages of *A. sinica*. The 0h stage expression level of As–RAD9 was set as a control group, and the mRNA expression levels of As–RAD9 at different time points during development was measured. The x-axis represents the various phases (0 h to 7 days), and the y-axis represents the expression level relative to 0 h. Data are mean ± SD of three replicate experiments, and significant differences in different developmental stages (*P* < 0.05) were analyzed by one-way ANOVA and are indicated by lower case letters (a–d).

Figure 2. Quantitative real-time qPCR analysis of the expression level of As–RAD9 in different challenge temperatures. The expression level of As–RAD9 mRNA at 30 °C was set as a control group, and the expression level of As–RAD9 mRNA at five different temperatures was measured. Data are mean ± SD of three replicate experiments, and ** indicates a very significant difference compared to the control (*P* < 0.01).
Purification and Expression of As–RAD9 Protein

The As–RAD9 mRNA was cut by EcoRI and SalI, then ligated overnight. The recombinant plasmid pET28a–RAD9 was successfully constructed and confirmed by sequencing. The large-scale purification was conducted at the optimal condition of 1mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C (Figure 4). The As–RAD9 recombinant protein was first dissolved in 60 mM urea, then purified using ProteinIsol® Ni-NTA Resin column (TransGen, Beijing, China), and finally harvested after dialysis. The molecular weight of the fusion protein was approximately 47 kDa, agreeing with the ProtParam result.

Figure 3. Quantitative real-time qPCR analysis of the expression levels of As–RAD9 in different salinity challenge. The expression level of As–RAD9 mRNA was adjusted to the control group at 28‰ salinity, and the expression level of As–RAD9 mRNA was measured at four different salt concentrations. Data are mean ± SD of three replicate experiments, ** indicates a very significant difference compared to the control (P < 0.01).

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Figure 4. Expression and purification of the As–RAD fusion protein. (a) Different induction treatments of the As–RAD9 fusion protein. M: protein markers (14–116 kDa). Lanes 1–4: 1 mM IPTG at 37 °C and 30 °C; 0.25 mM IPTG at 37 °C and 30 °C, respectively, Lane 5–6 (control): non-induced pET–28a–RAD9 cells; Induced pET–28a cells. (b) Ultrasonication results of the recombinant pET–28a–RAD9 cells. Lane 1: Total proteins; Lane 2–3: Supernatant and sediment after ultrasonication and centrifugation of induced pET–28a–Rad9 cells; (c) Purification of the recombinant As–RAD9 protein. Lane 1: Total proteins extracted from induced pET–28a–RAD9 cells; Lane 2: flowed through proteins; Lanes 3–8: elution with 10 mM, 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM imidazole.
Figure 5. Western blot analyses of As–RAD9, As–RAD1, As–HUS1, As–RAD17, and As–CHK1 at different developmental stages (0 h–3 days) of A. sinica. (A) The intensities of the protein bands were normalized against those of GAPDH. (B) Values are expressed as arbitrary units of relative value. The x-axis indicates the different protein; the y-axis shows the relative expression level. Significant differences at different development stages ($P < 0.05$) were analyzed by one-way analysis of variance (ANOVA) and reported by lowercase letters (a–e).
Protein expression patterns of signaling proteins in different challenges of temperature and salinity were performed. In the stress test of different temperatures, the expression levels of As–RAD9 protein decreased as the temperatures declined from 25 °C to 15 °C. Then it showed an increasing trend when the temperatures continued descending, with the highest level appeared at 10 °C. Cell cycle regulatory proteins showed a similar pattern to As–RAD9. Moreover, with the decrease of temperature, the downstream signaling protein As–CHK1 showed a downward trend, and the expression level of the upstream protein As–RAD17 showed an upward trend and reached a peak at 15 °C (Figure 6). When it comes to the stress of salinity, the protein expression pattern was quite different. By this time, the expression levels of As–RAD9 protein went descending with the increase of salt concentration, and reached the minimum at the salt concentration of 200‰. Besides the 9–1–1 component As–RAD1, the expression pattern of cell cycle regulatory proteins (As–RAD17 and As–CHK1) was opposite to that of RAD9 (Figure 7).

**Figure 6.** Western blot analyses of As–RAD9, As–HUS1, As–RAD17, As–RAD1, and As–CHK1 proteins in response to temperature stresses. (A) The band intensity of the proteins is normalized to the band intensity of GAPDH. (B) The values are expressed as an arbitrary unit of relative value. Protein expression at 25 °C as control (blue), asterisk (**) indicates a statistical difference at \( P < 0.01 \), and (*) indicates \( 0.01 < P < 0.05 \).
Figure 7. Western blot analyses of As–RAD9, As–RAD17, As–RAD1, and As–CHK1 proteins of A. sinica under different salt concentration stresses. (A) The band intensity of the protein is normalized to GAPDH. (B) The values are expressed as an arbitrary unit of relative value. The expression of the protein at a salinity of 28‰ as control (yellow), and asterisk (**) indicates a statistically significant difference of $P < 0.01$, and (*) indicates a $0.01 < P < 0.05$.

2.5. Immunofluorescence Analysis of As–RAD9

To observe the localization of As–RAD9, we found embryos and adults using an immunofluorescence (IF) microscopy. The nucleus of A. sinica was labelled with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) in blue fluorescent, and the As–RAD9 was detected by fluoresceine isothiocyanate (FITC)-labeled secondary antibody in green. The combined images of the two fluorescences (Figure 8(A2,B2)) were displayed. The results showed that the reactivity of As–RAD9 was observed in the whole body of both embryo and adult stages, and the cytoplasmic–nuclear distribution is partly opposite to the prediction results.
Figure 8. Immunofluorescence analyses of As–RAD9 at the embryo and adult stages of A. sinica. The paraffin sections of different development stages were prepared for immunofluorescence microscopy. A (0 h), B (15 h), C (7 days), and D (10 h) represent single-labelled with polyclonal anti-RAD9; A1, B1, C1 and D1 represent single-labelled DAPI (nuclear blue fluorescent probe); A2, B2, C2, and D2 represent with the image overlay of control group samples dual-labelled with polyclonal anti-RAD9 and DAPI; A3, B3, and C3 represent the image overlay of the control group samples single-labelled with secondary antibody.

2.6. Small RNA Interference of As–RAD9

To further explore the role As–RAD9 in the post-diapause embryonic development of A. sinica, siRNA was used to knock down the expression of As–RAD9. Microscopic examination showed that after knocking down of As–RAD9, there was no significant difference between the experimental group and the control group from 0h to 5h. The main difference occurred after 10 h, especially when the A. sinica developed to nauplii, the individual growth and development rates were significantly slowed down, and both the abnormal rate and mortality increased (Figure 9A). The mortality rate of the control group was about 23.94%, while the mortality rate of the experimental group was up to 41.23%. The expression levels of As–RAD9 mRNA was confirmed by qPCR analysis, revealing that expression of RAD9 in the experimental group was less than that of the control group (0, 5, 10, 15, and 20 h; Figure 9B).
15–20 h corresponding to the nauplii stage, 40 h corresponding to the metanauplius larval stage, and was up-regulated from 0 to 10 h and peaked at 10 h. However, after 10 h, the expression level of (BH3) [23, 24]. According to the Signal P and Protscale analysis, TLK1B [25, 26] (Figure 10). The immunolocalization results also showed that the translocation of As–RAD9 gradually decreased and reached the lowest value at 40 h. The results indicate that As–RAD9 may rely on nuclear-cytoplasmic shuttling proteins like 14–3–3 and TLK1B [25, 26] (Figure 10). The immunolocalization results also showed that As–RAD9 may not be restricted by organ specificity.

3. Discussion

The embryonic diapause of A. sinica is a programmed developmental arrest [5]. During the diapause embryo restarting, the cell cycle recovery may need a lot of cell cycle checkpoint proteins. RAD9 plays a key role in cell cycle control, mediating cell cycle progression delay or exit in DNA damage or other physiologic stresses. [9, 20–22]. In the present study, the full-length cDNA sequence of RAD9 from A. sinica was cloned for the first time. As–RAD9 is a small gene with a full length of only 1238 bp cDNA, encoding a protein of 376 amino acids. As–RAD9 belongs to the RAD superfamily and is an important cell cycle checkpoint protein that contains Bcl–2 homeodomain 3 (BH3) [23, 24]. According to the Signal P and Protscale analysis, As–RAD9 lacks any signal peptide sequence and transmembrane region, indicating that As–RAD9 may not be a secretion protein. Followed immunolocalization analysis showed that As–RAD9 is widely distributed in all parts, but sometimes only in the cytoplasm or nucleus, which may be correlated with the different functions of RAD9. The translocation of As–RAD9 may rely on nuclear-cytoplasmic shuttling proteins like 14–3–3 and TLK1B [25, 26] (Figure 10). The immunolocalization results also showed that As–RAD9 was expressed in A. sinica at different developmental stages, suggesting that the regulation of As–RAD9 may not be restricted by organ specificity.

The development of A. sinica includes four main stages: 0–10 h corresponding to the embryo stage, 15–20 h corresponding to the nauplii stage, 40 h corresponding to the metanauplius larval stage, and 3 days to 7 days corresponding to the pseudo–adult stage [3]. Real-time qPCR and Western blotting of A. sinica embryos at different developmental stages showed that the level of As–RAD9 transcript was up-regulated from 0 to 10 h and peaked at 10 h. However, after 10 h, the expression level of As–RAD9 gradually decreased and reached the lowest value at 40 h. The results indicate that As–RAD9 plays an essential role in the early embryonic stage, which is the most active stage of cell division and differentiation. Our previous studies found that after diapause termination, the embryo cells remain in a resting situation [2–4], and the Artemia cysts cannot resume development without appropriate conditions. The resumption of resting embryo cells may be involved in a cell cycle control mechanism,
which is associated with the extracellular and endogenous cell signaling. RAD9 was found to play a vital role in the G1 cell cycle arrest [9] and the G2/M checkpoint exit [21] in response to environmental stresses, which corresponds to the cell cycle arrest occurred in the transitions of G1/S or G2/M during diapause [5,27]. The rising expression of RAD9 in early embryonic development of A. sinica may be associated with the exit of cell cycle checkpoint or maintaining genome stability under the expanding stress of rapid DNA replication during diapause embryo resumption.

Figure 10. The different functions that As–RAD9 may be involved in. (A) The checkpoint signaling pathway that may lead to cell and organic developmental arrest; (B) The CHK1/RAD9 complex in cycling cells; (C) Monomer As–RAD9 may interact with different proteins and be involved in various signaling pathways.

To further understand the functions of As–RAD9, the 9–1–1 component HUS1, RAD1, and the signaling proteins RAD17 and CHK1 during the early embryonic process of A. sinica were analyzed. Western blot results revealed that the expression levels of these proteins increased significantly at 0–5 h, which may be due to the rapid embryo reactivation, accelerated mitosis, expanded DNA replication, and RNA transcript, subsequently leading to an increase in the expression of cell cycle checkpoint control proteins. After 10 h, the mitosis process was stable, and the DNA damage or mispairing may be weakened. Therefore, the expression levels of these proteins were decreased. After 15 h, the Artemia larvae resumed growth and development, and the gene expression was further down-regulated. However, at 40 h, the expression levels of RAD17 and CHK1 increased. It is hypothesized that during the rapid development of the pseudo–adult, the synthesis of macromolecules like DNAs, RNAs, and proteins were greatly expanded, the regulation of cell cycle progression must be more complicated, and the role of 9–1–1 complex maybe not so crucial in the normal development as in the embryo restarting of A. sinica.
The evolution strategy for *A. sinica* living through the disadvantageous conditions benefits from the ability of females that produce diapause cysts before the onset of adverse environmental challenges, such as fluctuation of salinity and temperature due to oncoming winter [27–29]. To explore the role which RAD9 may be involved in during natural occurrence of diapause, in the present study, the high salinity and low-temperature conditions were repeated to study the expression of *As–RAD9* in these stress processes. As the temperature decreased to 10 °C, the expression level of *As–RAD9* was significantly increased, indicating that RAD9 might be involved in the cold response signaling pathway, and 10 °C might be a trigger condition for the corresponding mechanism. On the other hand, with the increase of salinity, the expression of *As–RAD9* decreased and ultimately rebounded at the salinity of 200%, indicating that the signaling pathways may be different, since the *Artemia* has to face diverse environmental stresses. When the salinity increased to 200%, the survival rate of *A. sinica* was meager, and the DNA replication stalling must have been so serious that the checkpoint signaling pathway would be recommissioned to maintain genome stability, so that the expression level of *AS–RAD9* rebounded. Moreover, the RAD9 is a versatile protein that interacts with a great diversity of proteins by monomer or polymer [11,13] (Figure 10). The different signaling pathways may be associated with complex protein–protein interaction or posttranslational modifications like ubiquitination, phosphorylation, and methylation in response to environmental changes [8,21,22,30–33], though the specific regulatory mechanism remains to be determined.

Knockdown of RAD9 in human and mouse cells with different RAD9 states results in extreme cellular sensitivities to various radiations and chemicals [10,34]. Deficient RAD9 and HUS1 in *Leishmania* led to significant opposite phenotypes, which suggests that RAD9 and HUS1 may display different functions in cell cycle control [35]. To verify the effect of *As–RAD9* deletion on *A. sinica*, we performed siRNA interference experiments. The results showed that knocking down of the *As–RAD9* gene would reduce the transcript level of *As–RAD9* and delay the embryonic development process of *A. sinica*. These results suggested that *As–RAD9* plays an essential role in the early development of *A. sinica*.

In conclusion, the *As–RAD9* protein may be involved in different signaling pathways during diapause occurred and post-diapause development in *A. sinica*. The stalled DNA replication fork, caused by environmental stress or genotoxic insult, may invoke the checkpoint signaling pathway (Figure 10A), in which RAD9, HUS1, and RAD1 can form a complex (9–1–1) and load onto the damage sites by RAD17. Then, the RAD9 would be phosphorylated and recruited to DPB11, the new 9–1–1–DPB11 complexes activate the MEC1 bound to RPA-coated ssDNA [36], further phosphorylate and remodel both RAD9 and CHK1, which in turn facilitate in cis autophosphorylation of CHK1, subsequently releasing the RAD9/CHK1 complex from the injured ssDNA. Phosphorylation of CHK1 can lead to cell cycle arrest, which may be involved in embryonic diapause, although the mechanism is still obscure. The RAD9/CHK1 complex is constitutive and exits in cycling cells (Figure 10B), also participating in DNA damage recovery and recruited by other RAD9 complex [26]. The immunofluorescence assay indicated that *As—RAD9* was distributed more extensively in the cytoplasm than in nucleus, which may be due to the translocation of shuttling proteins like 14–3–3 and TLK1B [25,26]. Besides this, the *As–RAD9* protein may function as monomer or oligomer in the cytoplasm (Figure 10C). Actually, there are a lot of proteins that interact with RAD9 directly in diverse physiological functions, like P53, TRF2 (telomere integrity), CAD (ribonucleotide synthesis), Bcl–2 (apoptosis), and so forth [12]. In one word, the mechanism of diapause occurred and broken is complicated and may be involved with multiple genes and signaling pathways. Our discoveries are the tip of the iceberg and need further research in the future.

4. Materials and Methods

4.1. Preparation of Animal

The collection and preparation of *A. sinica* samples were carried out according to our previous studies [28,37].
4.2. Cloning the Full-Length cDNA of As–RAD9

Total RNAs were isolated from the *A. sinica* 0 h cyst by a Trizol kit (Thermo Fisher, Shanghai, China) and reverse transcribed into cDNA according to the supplier’s instructions. Primers were designed based on the *Artemia franciscana* EST sequence in GenBank, and synthesized by Sangon (Shanghai, China) (Table 2). The RT–PCR condition was 94 °C for five minutes, followed 36 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s), and a final extension at 72 °C for 10 min. The PCR product was isolated, purified, and cloned into the pMD–19T vector and then sequenced by Takara (Dalian, China). Therefore, the 473 bp EST sequence of As–RAD9 was obtained.

Table 2. Oligonucleotide primers used in this study.

| Primer       | Sequence(5′–3′)          |
|--------------|-------------------------|
| RAD9F        | TGGTGATTACATTTACTTTG    |
| RAD9R        | CGGCACATCAACTACATCAC    |
| 3′RAD9       | AGATTGGGCCTTGAGTGCTTAC  |
| 5′RAD9       | GGAAGTGAGCCGAAAACAGTCAG |
| RT-rad9F     | CTAAACCCGAATTGGATGCTCT  |
| RT-rad9R     | CAGATGGACTTGTTTGCTGCC   |
| β–actinF     | GGTGACGATGATTTGGC      |
| β–actinR     | GCTGTCCTTTTGACCAATTCC   |
| ORF-rad9F    | CCGGAATTCATGGGGGAGCGCAAATTTT |
| ORF-rad9R    | ACGCGTCGACTTAACTCTCATCTGAATCAA |
| SiRNA A1     | CCTACGAGCAAGAACAAT      |

The full-length sequence of As–RAD9 was cloned according to the protocol of the SMART RACE cDNA kit (Clontech, Chicago, IL, USA). The gene-specific primers are shown in Table 2. The RACE–PCR product was purified and sequenced by Takara. The sequencing fragments were assembled by DNAMAN 7.1.0 (Lynnon Biosoft, San Ramon, CA, USA); and the resulting full-length cDNA sequence of As–RAD9 was submitted to GenBank (accession number: MH_797557).

4.3. Bioinformatic and Biostatistics Analyses

The identity and similarity of the nucleotide sequences of As–RAD9 were analyzed by BLASTX in the National Center for Biotechnology Information (NCBI) [38]. The ORF was identified using the ORF Finder [39]. The PROSITE tool [40] and SMART [41] were used to predict the structure and functional domains of As–RAD9 protein. The molecular weight of the protein and the theoretical isoelectric point (PI) were analyzed by ExPASy’s ProtParam tool [42], and the PSORT and iPSORT service [43] were used to predict the protein subcellular localization. Prediction of transmembrane helices and hydrophilicity were conducted by the Tmpred program [44] and the TMHMM Server 2.0.; Homology analysis of the RAD9 amino acid sequences was performed by the ClustalX 2.0, and MEGA 4.1 was used to further construct the Adjacency (NJ) phylogenetic tree (bootstrapping = 1000). Significant test was analyzed by *t* test using SPSS 18.0 software, and the significance was set to *P* < 0.05.

4.4. Expression Pattern of As–RAD9 by qPCR

4.4.1. Expression of As–RAD9 in Early Embryo Development

The cDNA templates were prepared at the same concentration using the method mentioned above. The qPCR primers of As–RAD9 and β–actin (inner reference) are listed in Table 2. The qPCR was performed in triplicate for each sample using TB Green Premix Ex Taq (Takara, Dalian, China) and Takara detection system TaKaRa TP800 (Takara, Dalian, China). The reaction procedures were initial denaturation at 95 °C for 30 s, then 38 cycles (95 °C for five seconds, 58 °C for 30 s, 95 °C for 15 s, 60 °C for 30 s) [28]. Based on the Ct values of As–RAD9 and β–actin, the data were analyzed using the
comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$ method) to calculate the fold increase. Data obtained from real-time qPCR analysis were analyzed by least-squares difference (LSD).

4.4.2. Temperature and Salinity Stress Assays

The cDNA templates were prepared from *A. sinica* (20 h) in the Nauplius stage, which was incubated at the different gradients of temperatures and salinity as described in our previous studies [4,28], and qPCR was performed to examine relative expression values.

4.5. Purification of Recombinant As–RAD9 Protein

The complete ORF of As–RAD9 was obtained using primers containing specific enzyme sites of EcoRI and Sall (Table 2). The purified PCR product and pET–28a vectors were cut by the two enzymes of EcoRI and Sall, and ligated overnight at 16 °C by T4 DNA ligase (Takara, Dalian, China). After sequencing, the expression vector pET–28a–RAD9 was confirmed inerrably and transferred into competent cell BL21 (DE3) for further induction. Expression of the As–RAD9 fusion protein was induced for three hours by four different conditions: 0.25 mM or 1 mM IPTG at 37 °C; 0.25 mM or 1 mM IPTG at 30 °C. Cells were harvested and washed three times with PBS, then collected by centrifugation and sonication. Purification of the As–RAD9 fusion protein was performed by Ni–NTA Resin column (Transgen, Beijing, China) according to the supplier’s protocol. Different imidazole concentrations were tested, and 60 mM was selected as the optimal concentration for purification. Proteins were then dialyzed in 20 mM Tris–HCl and fractions were collected and detected by SDS–PAGE and his–Tag western blotting.

4.6. Production of Polyclonal Antibodies

Polyclonal antibodies against As–RAD9 fusion protein were prepared in rabbits, as described in our previous study [45]. The specificity of the antibody for the purified protein was verified using western blotting.

4.7. Western Blotting

Animal samples were prepared from different developmental stages, different temperatures, and salt concentrations. Total proteins were extracted from each sample using RIPA lysis buffer and quantified by the BCA protein assay kit. Protein samples (80 μg each) were subjected to fractionation by SDS–PAGE and transferred to Polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was blocked with 5% skim milk powder for two hours at room temperature and allowed to incubate with the original antibody overnight at 4 °C. Rabbit anti–As–RAD9 polyclonal antibody and GAPDH antibody were diluted 1:500 and 1:1000 with PBST, respectively. The membrane was washed three times with PBST and then incubated with HRP-conjugated secondary antibody for one hour at 37 °C and then washed three times with PBST and once with PBS. Reactive protein bands on the membrane were visualized using ECL reagents (Transgen, Beijing, China) in a dark room. Image grayscale analysis in Image J software was used to compare the density (corresponding to intensity) of the bands on the Western blot, and the resulting data were used to construct a histogram. The intensity of expression of a particular protein band is normalized to the GAPDH band. Other antibodies, such as RAD17, RAD1, CHK1, and HUS1 were purchased from BOSTER (Wuhan, China) according to sequence homology, with all homology exceeding 80%.

4.8. Immunofluorescence (IF)

Paraffin sections of different developmental stages tissues (0 h, 15 h, 7 days, and 10 h) were cut at 8 μm thickness. After being dewaxed and hydrated, they were fixed for 10 min in 4% paraformaldehyde, washed by PBS with 0.2% TritonX-100 for 10 min. Rabbit anti–Ac–RAD9 antibody was added and diluted 1:20 with PBST and then incubated overnight at 4 °C. On the next day, sections were incubated
in Cy3-conjugated goat anti-rabbit IgG (1:30 dilution; Proteintech) for one hour at 37°C in the dark, followed by washing with PBST. The samples were then stored in a mounting medium containing DAPI (4′, 6-diamidino-2-phenylindole; ZSGB-BIO, Beijing, China) and examined under a confocal laser microscope.

4.9. RNA Interference

According to the cloned A. sinica rad9 gene full-length, RNA probes (Sense siRNA, AntiRNAi) were designed online, and synthesized by Takara. The 0 h cyst was deshelled with 50% NaClO. Before the electroporation experiment, the cysts were placed in an electroporation buffer with 400 V shock for 1 s using an EC100 electroporator. The cysts containing 4 mM double-stranded RNA in the electroporation buffer was used as the experimental group, and the control group was not included. Subsequently, the shock Artemia cyst was added to seawater, and cultured in a constant temperature incubator every 5 h. The time points were 5 h, 10 h, 15 h, and 20 h, respectively. Animals were collected for qPCR and photographed every 5 h.

5. Conclusions

A 1238 bp full-length cDNA of As–RAD9 (GenBank accession number: MH797557) was obtained, which contained an 1131 bp ORF, encoding a 376-amino-acid protein. We determined that the changed mRNA and protein expression level of As–RAD9 is related to the cell cycle reactivation during diapause embryo restarting. To further understand the functions of the 9–1–1 complex and the involved signaling pathway, expression levels of As–RAD9, As–RAD1, As–HUS1, As–RAD17, and As–CHK1 were analyzed by Western blotting. Results showed that the expression levels of these proteins increased significantly at 0–10h, probably due to the rapid embryo reactivation, accelerated mitosis, expanded DNA replication, and RNA transcript, leading to an increase in the expression of cell cycle related proteins. After 15h, the expression levels of these proteins were decreased. This may be due to the mitosis process going stable, and the DNA damage or mispairing may be weakened. The transcriptional and protein expressions of As–RAD9 were highly upregulated when the temperature was lowered, and downregulated when the salinity rose up, which indicates that As–RAD9 may be involved in the different signaling pathway under different stresses. The RNA interference assay further suggested that As–RAD9 is necessary for diapause embryo restarting and early embryo development in A. sinica. Our current study has provided a new reference for further study of RAD9 in invertebrates.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/10/10/768/s1, Figure S1: (A) Nucleotide sequence and deduced amino acid sequence of Rad9 gene in A. sinica. (B) Results of domain analysis of putative As-RAD9 protein. Figure S2: Multiple sequence alignment of As-RAD9 protein. Sequence alignment of known RAD9 sequences from 13 species. The sequences and their accession numbers are as follows: the mouse Mus musculus RAD9 (MmRAD9), NP_035367.1; Cricetulus griseus RAD9 (CgRAD9), XP_003509986.1; human Homo sapiens RAD9 (HsRAD9), NP_004575.1; Pan troglodytes RAD9 (PtRAD9), XP_016776852.1; chicken Gallus gallus RAD9,GgRAD9, NP_998748.1; Aquila chrysaetos canadensis RAD9 (AccRAD9), XP_011599156.1; Xenopus tropicalis RAD9 (XtRAD9), NP_001005810.1; Danio rerio RAD9 (DrRAD9), NP_956501.2; Oryzias latipes RAD9 (OIRAD9), XP_004073282.1; silkworm Bombyx mori RAD9 (BmRAD9), XP_004926904.1; Bombus terrestris RAD9 (BrRAD9), XP_020723628.1; A.sinica RAD9 (AsRAD9), MH_797557; Daphnia magna RAD9 (DmRAD9), KZS14537.1. The sequence of the RAD9 domain is shown in red. Figure S3: Phylogenetic tree constructed by RAD9 proteins. The sequence and registration number of the RAD9 are the same as in the legend of Figure S2. The red diamond indicates As-RAD9 from A. sinica.

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Abbreviations

As-rad9 rad9 mRNA from Artemia sinica
As-RAD9 RAD9 Cell cycle checkpoint control protein from Artemia sinica
As-RAD17 RAD17 checkpoint clamp loader component protein from Artemia sinica
As-RAD1 RAD1 checkpoint DNA exonuclease protein from Artemia sinica
As-CHK1 serine/threonine protein kinase CHK1 from Artemia sinica
UTR untranslated region
ATR ataxia-telangiectasia
TOPBP1 DNA topoisomerase II binding protein 1
ATRIP ATR interacting protein.
Dpb11 DNA replication regulator DPB11

References

1. Qin, T.; Zhao, X.; Luan, H.; Ba, H.; Yang, L.; Li, Z.; Hou, L.; Zou, X. Identification, expression pattern and functional characterization of As-MyD88 in bacteria challenge and during different developmental stages of Artemia sinica. Dev. Comp. Immunol. 2015, 50, 9–18. [CrossRef] [PubMed]

2. Zhang, M.; Yao, F.; Qin, T.; Hou, L.; Zou, X. Identification, expression pattern and functional characterization of As-kip2 in diapause embryo restarting process of Artemia sinica. Gene 2017, 608, 28–40. [CrossRef] [PubMed]

3. Zhang, W.; Yao, F.; Zhang, H.; Li, N.; Zou, X.; Sui, L.; Hou, L. The Potential Roles of the Apoptosis-Related Protein PDRG1 in Diapause Embryo Restarting of Artemia sinica. Int. J. Mol. Sci. 2018, 19, 126. [CrossRef] [PubMed]

4. Wang, X.; Yao, F.; Liang, X.; Zhu, X.; Zheng, R.; Jia, B.; Hou, L.; Zou, X. Cloning and expression of retinoblastoma-binding protein 4 gene in embryo diapause termination and in response to salinity stress from brine shrimp Artemia sinica. Gene 2016, 591, 351–361. [CrossRef]

5. MacRae, T.H. Stress tolerance during diapause and quiescence of the brine shrimp, Artemia. Cell Stress Chaperones 2016, 21, 9–18. [CrossRef]

6. Yasutis, K.M.; Kozminski, K.G. Cell cycle checkpoint regulators reach a zillion. Cell Cycle 2013, 12, 1501–1509. [CrossRef] [PubMed]

7. Siefert, J.C.; Clowdus, E.A.; Sansam, C.L. Cell cycle control in the early embryonic development of aquatic animal species. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 2015, 178, 8–15. [CrossRef]

8. Wang, G.; Tong, X.; Weng, S.; Zhou, H. Multiple phosphorylation of Rad9 by CDK is required for DNA damage checkpoint activation. Cell Cycle 2012, 11, 3792–3800. [CrossRef]

9. Siede, W.; Friedberg, A.S.; Friedberg, E.C. RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 1993, 90, 7985–7989. [CrossRef]

10. Hopkins, K.M.; Auerbach, W.; Wang, X.Y.; Hande, M.P.; Hang, H.; Wolgemuth, D.J.; Joyner, A.L.; Lieberman, H.B. Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality. Mol. Cell. Biol. 2004, 24, 7235–7248. [CrossRef]

11. Broustas, C.G.; Lieberman, H.B. Contributions of Rad9 to tumorigenesis. J. Cell Biochem. 2012, 113, 742–751. [CrossRef] [PubMed]

12. Lieberman, H.B.; Bernstock, J.D.; Broustas, C.G.; Hopkins, K.M.; Leloup, C.; Zhu, A. The role of RAD9 in tumorigenesis. J. Mol. Cell Biol. 2011, 3, 39–43. [CrossRef] [PubMed]

13. Cotta-Ramusino, C.; McDonald, E.R., 3rd; Hurov, K.; Sowa, M.E.; Harper, J.W.; Elledge, S.J. A DNA damage response screen identifies RHINO, a 9-1-1 and TopBP1 interacting protein required for ATR signaling. Science 2011, 332, 1313–1317. [CrossRef] [PubMed]

14. Lindsey-Boltz, L.A.; Kemp, M.G.; Capp, C.; Sancar, A. RHINO forms a stoichiometric complex with the 9-1-1 checkpoint clamp and mediates ATR-Chk1 signaling. Cell Cycle 2015, 14, 99–108. [CrossRef] [PubMed]

15. Delacroix, S.; Wagner, J.M.; Kobayashi, M.; Yamamoto, K.; Karnitz, L.M. The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. Genes Dev. 2007, 21, 1472–1477. [CrossRef] [PubMed]

16. Burrows, A.E.; Elledge, S.J. How ATR turns on: TopBP1 goes on ATRIP with ATR. Genes Dev. 2008, 22, 1416–1421. [CrossRef] [PubMed]
17. Perry, J.A.; Kornbluth, S. Cdc25 and Wee1: Analogous opposites? Cell Div. 2007, 2, 12. [CrossRef] [PubMed]
18. Ueda, S.; Takeishi, Y.; Ohashi, E.; Tsurimoto, T. Two serine phosphorylation sites in the C-terminus of Rad9 are critical for 9-1-1 binding to TopBP1 and activation of the DNA damage checkpoint response in HeLa cells. Genes Cells 2012, 17, 807–816. [CrossRef] [PubMed]
19. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 2007, 24, 1596–1599. [CrossRef] [PubMed]
20. Dang, T.; Bao, S.; Wang, X.F. Human Rad9 is required for the activation of S-phase checkpoint and the maintenance of chromosomal stability. Genes Cells 2005, 10, 287–295. [CrossRef]
21. Wysocki, R.; Javaheri, A.; Allard, S.; Sha, F.; Cote, J.; Kron, S.J. Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol. Cell Biol. 2005, 25, 8430–8443. [CrossRef] [PubMed]
22. Kelly, R.; Davey, S.K. Tousled-like kinase-dependent phosphorylation of Rad9 plays a role in cell cycle progression and G2/M checkpoint exit. PLoS ONE 2013, 8, e85859. [CrossRef] [PubMed]
23. Xu, M.; Bai, L.; Gong, Y.; Xie, W.; Hang, H.; Jiang. Structure and functional implications of the human rad9-hus1-rad1 cell cycle checkpoint complex. J. Biol. Chem. 2009, 284, 20457–20461. [CrossRef] [PubMed]
24. Sohn, S.Y.; Cho, Y. Crystal structure of the human rad9-hus1-rad1 clamp. J. Mol. Biol. 2009, 390, 490–502. [CrossRef] [PubMed]
25. Awate, S.; De Benedetti, A. TLK1B mediated phosphorylation of Rad9 regulates its nuclear/cytoplasmic localization and cell cycle checkpoint. BMC Mol. Biol. 2016, 17, 3. [CrossRef] [PubMed]
26. Hustedt, N.; Gasser, S.M.; Shimada, K. Replication checkpoint: Tuning and coordination of replication forks in s phase. Genes 2013, 4, 388–434. [CrossRef]
27. Hand, S.C.; Denlinger, D.L.; Podrabsky, J.E.; Roy, R. Mechanisms of animal diapause: Recent developments from nematodes, crustaceans, insects, and fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2016, 310, 1193–1211. [CrossRef]
28. Li, N.; Yao, F.; Huang, H.; Zhang, H.; Zhang, W.; Zou, X.; Sui, L.; Hou, L. The potential role of Annexin 3 in diapause embryo restart of Artemia sinica and in response to stress of low temperature. Mol. Reprod. Dev. 2019, 86, 530–542. [CrossRef]
29. Zhao, W.; Yao, F.; Zhang, M.; Jing, T.; Zhang, S.; Hou, L.; Zou, X. The Potential Roles of the G1LEA and G3LEA Proteins in Early Embryo Development and in Response to Low Temperature and High Salinity in Artemia sinica. PLoS ONE 2016, 11, e0162272. [CrossRef]
30. Xu, X.; Shi, R.; Zheng, L.; Guo, Z.; Wang, L.; Zhou, M.; Zhao, Y.; Tian, B.; Truong, K.; Chen, Y.; et al. SUMO-1 modification of FEN1 facilitates its interaction with Rad9-Rad1-Hus1 to counteract DNA replication stress. J. Mol. Cell Biol. 2018, 10, 460–474. [CrossRef]
31. Liu, S.; Song, N.; Zou, L. The conserved C terminus of Claspin interacts with Rad9 and promotes rapid activation of Chk1. Cell Cycle 2012, 11, 2711–2716. [CrossRef] [PubMed]
32. Zhang, G.; Ma, F.; Li, L.; Li, J.; Li, P.; Zeng, S.; Sun, H.; Li, E. Palbociclib triggers apoptosis in bladder cancer cells by Cdk2-induced Rad9-mediated reorganization of the Bak.Bcl-xl complex. Biochem. Pharmacol. 2019, 163, 133–141. [CrossRef] [PubMed]
33. Lieberman, H.B.; Panigrahi, S.K.; Hopkins, K.M.; Wang, L.; Broustas, C.G. p53 and RAD9, the DNA Damage Response, and Regulation of Transcription Networks. Radiat. Res. 2017, 187, 424–432. [CrossRef] [PubMed]
34. Ghandhi, S.A.; Panigrahi, S.K.; Hopkins, K.M.; Cui, Q.; Hei, T.K.; Amundson, S.A.; Lieberman, H.B. RAD9 deficiency enhances radiation induced bystander DNA damage and transcriptional response. Radiat. Oncol. 2014, 9, 206. [CrossRef] [PubMed]
35. Damasceno, J.D.; Obonaga, R.; Santos, E.V.; Scott, A.; McCulloch, R.; Tosi, L.R. Functional compartmentalization of Rad9 and Hus1 reveals diverse assembly of the 9-1-1 complex components during the DNA damage response in Leishmania. Mol. Microbiol. 2016, 101, 1054–1068. [CrossRef] [PubMed]
36. Abreu, C.M.; Kumar, R.; Hamilton, D.; Dawdy, A.W.; Creavin, K.; Eivers, S.; Finn, K.; Balsbaugh, J.L.; O’Connor, R.; Kiely, P.A.; et al. Site-specific phosphorylation of the DNA damage response mediator rad9 by cyclin-dependent kinases regulates activation of checkpoint kinase 1. PLoS Genet. 2013, 9, e1003310. [CrossRef] [PubMed]
37. Zhang, S.; Yao, F.; Jing, T.; Zhang, M.; Zhao, W.; Zou, X.; Sui, L.; Hou, L. Cloning, expression pattern, and potential role of apoptosis inhibitor 5 in the termination of embryonic diapause and early embryo development of Artemia sinica. Gene 2017, 628, 170–179. [CrossRef] [PubMed]
38. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
39. Rombel, I.T.; Sykes, K.F.; Rayner, S.; Johnston, S.A. ORF-FINDER: A vector for high-throughput gene identification. *Gene* **2002**, *282*, 33–41. [CrossRef]
40. Sigrist, C.J.; Cerutti, L.; Hulo, N.; Gattiker, A.; Falquet, L.; Pagni, M.; Bairoch, A.; Bucher, P. PROSITE: A documented database using patterns and profiles as motif descriptors. *Brief. Bioinform.* **2002**, *3*, 265–274. [CrossRef]
41. Letunic, I.; Bork, P. 20 years of the SMART protein domain annotation resource. *Nucleic Acids Res.* **2018**, *46*, 493–496. [CrossRef] [PubMed]
42. Wilkins, M.R.; Gasteiger, E.; Bairoch, A.; Sanchez, J.C.; Williams, K.L.; Appel, R.D.; Hochstrasser, D.F. Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* **1999**, *112*, 531–552. [PubMed]
43. Bannai, H.; Tamada, Y.; Maruyama, O.; Nakai, K.; Miyano, S. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* **2002**, *18*, 298–305. [CrossRef] [PubMed]
44. Hofmann, K.; Stoffel, W. TMbase-A database of membrane spanning proteins segments. *Biol. Chem. Hoppe Seyler* **1993**, *374*, 166.
45. Zhang, M.; Yao, F.; Luan, H.; Zhao, W.; Jing, T.; Zhang, S.; Hou, L.; Zou, X. APC/C(CDC20) and APC/C play pivotal roles in the process of embryonic development in *Artemia sinica*. *Sci. Rep.* **2016**, *6*, 39047. [CrossRef] [PubMed]

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