Involvement of Cyclin K Posttranscriptional Regulation in the Formation of Artemia Diapause Cysts

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Abstract

Background: Artemia eggs tend to develop ovoviviparously to yield nauplius larvae in good rearing conditions; while under adverse situations, they tend to develop oviparously and encysted diapause embryos are formed instead. However, the intrinsic mechanisms regulating this process are not well understood.

Principal Finding: This study has characterized the function of cyclin K, a regulatory subunit of the positive transcription elongation factor b (P-TEFb) in the two different developmental pathways of Artemia. In the diapause-destined embryo, Western blots showed that the cyclin K protein was down-regulated as the embryo entered dormancy and reverted to relatively high levels of expression once development resumed, consistent with the fluctuations in phosphorylation of position 2 serines (Ser2) in the C-terminal domain (CTD) of the largest subunit (Rpb1) of RNA polymerase II (RNAP II). Interestingly, the cyclin K transcript levels remained constant during this process. In vitro translation data indicated that the template activity of cyclin K mRNA stored in the postdiapause cyst was repressed. In addition, in vivo knockdown of cyclin K in developing embryos by RNA interference eliminated phosphorylation of the CTD Ser2 of RNAP II and induced apoptosis by inhibiting the extracellular signal-regulated kinase (ERK) survival signaling pathway.

Conclusions/Significance: Taken together, these findings reveal a role for cyclin K in regulating RNAP II activity during diapause embryo development, which involves the post-transcriptional regulation of cyclin K. In addition, a further role was identified for cyclin K in regulating the control of cell survival during embryogenesis through ERK signaling pathways.

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Introduction

RNA polymerase II (RNAP II) is a key enzyme involved in the synthesis of mRNA, and interruption of its function triggers apoptosis in human cells and induces abnormality in developing embryos [1,2]. Its activation largely depends on phosphorylation of the C-terminal domain (CTD) of its largest subunit (Rpb1) during transcription [3]. The CTD contains repeats of a seven amino acid motif (heptapeptide repeats), which is conserved from yeasts to mammals, although the number of repeats varies [3–5]. Serine residues in the consensus motif are phosphorylated by diverse kinases during the processes of transcription [6] and pre-mRNA processing [7]. Positive transcription elongation factor b (P-TEFb) is of great significance in the transcription process, since it facilitates the transition from abortive to productive elongation by phosphorylating position 2 serines (Ser2) on the heptapeptide repeats [4,5,8–11].

P-TEFb, which comprises a kinase subunit, CDK9, and its cyclin partner, cyclin T, has attracted much attention because of roles in diverse biological processes such as embryonic development, cell differentiation and HIV-1 replication in humans [2,12,13]. Cyclin K, the latest member discovered to be associated with CDK9, is less well studied. Although one report showed that a CDK9-cyclin K complex participates directly in the DNA damage response [14], its role as a component of P-TEFb in vivo is uncertain. An in vitro kinase assay proved that the CDK9-cyclin K complex could functionally substitute the CDK9-cyclin T complex to phosphorylate CTD on Rpb1 of RNAP II without regard to the lower activity [15,16]; however, other research suggested that cyclin K is not involved in DNA transcription in vivo [17,18].

The brine shrimp, genus Artemia, evolved the extraordinary ability to reproduce via encysted gastrula embryos (cysts), which are able to cope with harsh environments including anoxia, high salinity, high pH, and major changes in the ionic composition and temperature of the surrounding environment. The cyst, which has a barely detectable metabolic rate and exists in a state of obligate dormancy called diapause, can survive for extremely long periods [19]. This diapause status can be terminated by exposure to specific environmental stimuli and the embryo then undergoes postdiapause development, eventually emerging as a fully formed nauplius larva in a suitable environment. This development process is very complicated and involves a large number of internal events, all of which are coupled to the expression of a large number of different genes.
To understand the mechanism of diapause completely, it is necessary to investigate the action of RNAP II, since transcription is inhibited in diapause embryos [19]. RNAP II has been purified at different stages of *Artemia* development [20–22], and although a detectable level of RNAP II is present in the diapause embryo, its activity is less than 10% of that in nauplii. Most of it is present in a free form which is not bound to chromatin and becomes actively engaged in transcription upon development [19]. Multiple factors are involved in this transition phase, and the enzyme has a complex composition and is regulated by multiple mechanisms. Its largest subunit, Rpb1, with a molecular mass of Mr 205,000 in developing cysts, is converted into a polypeptide of Mr 172,000 in larvae. This proteolytic modification is thought to be the mechanism involved in regulating RNAP II activity upon larval development [23].

A more direct regulator of RNAP II activity, known as the S protein, has been isolated from the cytosol of dormant and developing cysts. It is known to activate RNAP II through its action on the enzyme rather than on the DNA template and decreases in the period of pre-emergence and early larval development. However, the mechanism of activation is unknown [24].

In the present study, we identified a cyclin K homolog from an *Artemia parthenogenetica* cDNA library and explored its functions in the two different developmental pathways of *Artemia*. Transcription levels, tested by semiquantitative reverse transcription-PCR, showed that cyclin K is most abundant in the postdiapause developmental embryos, suggesting that it plays a specific role during embryonic development. In addition, cyclin K was studied further in the oviparous developmental pathway that includes a long period of diapause. Repression of cyclin K in diapause embryos was related to a specific mechanism that reduces the template activity of mRNA and, hence, inhibits the phosphorylation of RNAP II. Western blot analysis and immunofluorescence staining of nuclei indicated that increasing levels of cyclin K play an essential role in postdiapause development by regulating the phosphorylation of CTD Ser2 of RNAP II. Additionally, RNA interference (RNAi) in *vivo* knockdown of cyclin K in early embryos provided direct evidence that phosphorylation of CTD Ser2 was cyclin K-dependent and showed that a lack of cyclin K induced apoptosis by inhibiting ERK-mediated survival signaling.

**Materials and Methods**

**Animal Culture and Sample Collection**

*A. parthenogenetica* from Gahai Lake, China, were gifted by Prof. Feng-Qi Liu (Nankai University, Tianjin, China). Specimens are separated into two groups and cultured in different conditions. One group was cultured in 8% artificial seawater (Blue Starfish, Hangzhou, China) with a 5-h light cycle per day. Under these conditions, the majority reproduced oviparously and released encysted diapause embryos. The other group was reared in 4% artificial seawater with a 16-h light cycle per day and almost all specimens reproduced ovoviviparously and yielded swimming nauplii. Both groups were reared at 28°C and fed with *Chlorella* powder (Fuding King Dnarmsa Spirulina Co. Ltd., Fuding, China) every 2 days.

*Artemia* with oviparously or ovoviviparously developing embryos were classified by observing the shell gland morphology as described by Liang and MacRae [25]. Samples of the reproductive tract were collected at each developmental stage, and ovissac isolation performed according to the method of Liu et al [26].

Encysted embryos were collected and stored dry at 25°C as examples of diapause embryos, and were used within 2 weeks. The hatchability was below 10% in artificial sea-water (3%) under continuous light at 25°C. Other gathered diapause embryos were dehydrated in saturated sodium chloride solution for 24 h and then stored at −20°C to prepare a sample of postdiapause embryos. Postdiapause cysts were hydrated at 4°C for 5 h and then incubated in artificial sea-water (3%) under continuous light at 25°C. Samples were taken at 0, 2, 4, 8, 10, 12 and 14 h, and at the free-swimming nauplius stage. All samples were snap-frozen in liquid nitrogen and stored at −80°C until required for RNA and protein preparation.

**Semiquantitative Reverse Transcription-PCR**

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. First-strand cDNAs were synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in a 12.5-μl reaction. Cyclin K (GenBank accession number JQ085432), Rpb1 (GenBank accession number U10331) and α-tubulin cDNA fragments (GenBank accession number AF247598) were amplified separately in 25-μl reactions using 1 μl of each reverse transcription product as a template. The primers used are shown in Table 1 (cycKF and cycKR for cyclin K; Rpb1F and Rpb1R for Rpb1; TubF and TubR for α-tubulin).

**Western Blotting**

Proteins were extracted from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s

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**Table 1. Nucleotide sequences and positions of primers used in polymerase chain reactions.**

| Primer | Length (bp) | Position | Direction | Sequence (5’-3’) |
|--------|-------------|----------|-----------|-----------------|
| cycKF  | 20          | 41–60    | F         | TACGAAAAGCCCCCATCGACA |
| cycKR  | 20          | 235–254  | R         | AGAAAGAGGCAACAAAGAC |
| cycKF  | 22          | 10–31    | R         | ATGCGCTGCTGTATAGATA |
| cycKR  | 22          | 1068–1089| R         | ATATGGCGGTCTTGGTGGTTAA |
| cycKF  | 28          | 550–569  | F         | GCTCTAGATGGGAGCCTAAGG |
| cycKR  | 29          | 1032–1051| R         | CCGGAAATTCTGGGTTAAGAAAGCAGTG |
| TubF   | 20          | 532–551  | F         | TCTACTGCGGCTGTGTAGCC |
| TubR   | 20          | 694–713  | R         | ATGCGAGAAGACCTTGGAC |
| Rpb1F  | 20          | 821–840  | F         | CACCGCGGACTGCTATTACC |
| Rpb1R  | 20          | 1135–1154| R         | CTGTGGCCCATCATAAGACA |

F and R indicate the forward and reverse directions, respectively. The underlined regions represent the adscititious recognition sequences of restriction endonucleases.

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instructions and quantified using the Bradford method [27]. From each sample, 25 μg of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with a primary antibody overnight at 4°C and detection was performed using the BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany). A peptide, located at the C-terminus of cyclin K (amino acids [aa] 346 to 359), was chemically synthesized and used to immunize rabbits to obtain the antibody (Hangzhou HuaAn Biotechnology Company, Hangzhou, China). Anti-RNA polymerase II CTD repeat YSPTSPS (phospho S2) [H5] antibody (ab24758) and anti-phospho-RSK antibody (ab10695) were purchased from Abcam (Cambridge, UK); anti-phospho-ERK1/2 antibody (9101) and anti-ERK1/2 antibody (9102) were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-phospho-histone H3 antibody (1173-1) and anti-α-tubulin (1878-1) antibodies were purchased from Epitomics, Inc (Burlingame, CA, USA).

Colocalization of Cyclin K with Phosphorylated RNAPII

Embryos incubated for 14 h were homogenized using Dounce homogenizers in buffer K (10 mM Hepes pH 7.2, 1 mM EDTA, 50 mM NaCl, pH 6.0 or 8.0) and cell fractionation performed as previously described [28]. Cyclin K and phosphorylated CTD Ser2 of RNAPII were detected in both supernatants and pellets as described above. H3 and α-tubulin (Epitomics, Inc, Burlingame, CA, USA) were detected simultaneously to determine the purity of the fractions.

Nuclei from the 14-h incubated embryos were prepared as described [29] and fixed in 4% (w/v) paraformaldehyde. Immunofluorescence staining was performed as described [30]. Cyclin K (1:100) and H5 (1:100) were used as the primary antibodies. The secondary antibodies were FITC-conjugated goat anti-mouse IgG (1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and TRITC-conjugated goat anti-rabbit IgG (1:200; Hangzhou HuaAn Biotechnology Company, Hangzhou, China). After incubation with the secondary antibody and a rinse in PBS, the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China). Slides were examined with a Nikon ECLIPSE TE200-S microscope (Nikon, Tokyo, Japan).

14-h incubated embryos were decapsulated using antiformin [28], and homogenized using Dounce homogenizers in precooled FA lysis buffer ((50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and protease inhibitors) on ice. Cell lysates were clarified by centrifugation and precleared by incubation for 1–2 h with Protein A Sepharose beads (Invitrogen, Carlsbad, CA, USA). The precleared supernatants were then incubated overnight at 4°C with anti-cyclin K antibody or anti-SGEG2a (which has been identified as one component of the cyclin K antibody [31] as a negative control. The proteins were immunoprecipitated using protein A Sepharose beads (Invitrogen, Carlsbad, CA, USA) and then analysed by western blot.

Oligo(dT)-Cellulose Affinity Chromatography and In Vitro Translation

Postdiapause cysts (10 g dry weight) and 8-h incubated embryos were decapsulated using antiformin [28] and ground to a fine powder in liquid nitrogen after being thoroughly washed in distilled water. The powder was transferred to precooled buffer J (10 mM Hepes pH 7.2, 5 mM MgCl2, 50 mM NaCl) [32] containing 0.3 mg/ml heparin and 150 mM sucrose. The postmitochondrial supernatant was prepared as previously described [32–34]. These supernatants were adjusted to 250 mM NaCl and then separated on an oligo(dT)-cellulose chromatography column (Sigma-Aldrich, St. Louis, MO, USA). After extensive washing with buffer L (10 mM Hepes pH 7.2, 250 mM NaCl) [31], the cellulose was eluted with 10 mM Hepes (pH 7.2) at room temperature and concentrated by ultrafiltration [32]. The eluate was used as a template for protein synthesis and for RNA extraction to do northern blot. The in vitro translation assay was performed using the RTS 100 Wheat Germ CECF Kit (3PRIME, Gaithersburg, MD, USA).

Northern Blotting

For postdiapause and 8-h incubated cysts, 8 μg mRNA were separated by 1.0% agarose gel electrophoresis and then transferred to a positively-charged nylon membrane (Millipore, Bedford, MA, USA). After pre-hybridization at 42°C for 1 h, the membrane was hybridized at 42°C overnight with a DIG-labeled probe to detect cyclin K (amplified using the primers cycKF and cycKR [Table 1]) or a DIG-labeled probe to detect tubulin (amplified using the primers TubF and TubR [Table 1]). After extensive washing, hybridized probes were visualized using a DIG chemiluminescent detection system (Roche, Mannheim, Germany).

RNA Interference

A fragment of cyclin K, amplified using the primers, cycKF and cycKR (Table 1), was subcloned into the plasmid pET-T7 [35,36] between the XbaI and EcoRI sites. Plasmids expressing green fluorescent protein (GFP) dsRNA as a negative control were constructed as described [26]. The recombinant plasmids were transformed into Escherichia coli HT115 and the dsRNAs were produced and purified as described [36]. Cyclin K and GFP dsRNAs (500 ng) were injected separately into the reproductive segments of Artemia at stage instar XII (before ovarian development) using the UltraMicroPump II (World Precision Instruments Inc, Sarasota, FL, USA) equipped with a Micro4™ microsyringe pump controller (World Precision Instruments Inc, Sarasota, FL, USA). The injected individuals were cultured under the conditions already described for rearing ovoviviparous Artemia.

TUNEL Assay

Adult Artemia with embryos of each stage were anesthetized on ice, snap-freezing in liquid nitrogen, and embedded in Tissue Tek (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Frozen sections, each 5-μm thick, were prepared using a frozen ultra-microtome. The terminal deoxynucleotidyl transferase dUTP nick-end-labeling (TUNEL) assay was performed using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Results

Characterization and mRNA Expression Pattern of Cyclin K and Rpb1 in the two Developmental Pathways of Artemia

An A. parthenogenetica cDNA library was constructed from both oviparous and ovoviviparous whole animals [37] and sequencing performed. One transcript containing a full-length open reading frame encoding a 359-aa protein was identified. Sequence analysis showed it contained two typical cyclin boxes (aa 29 to 128 and aa 136 to 254, Fig. 1) and the deduced amino acid sequence comparison results showed that Artemia cyclin K had 52.9%, 54.0%, 53.6%, 52.1% and 52.4% sequence identity with cyclin K of human, mouse, Xenopus, zebrafish, and Drosophila respectively.
Confirming that the transcript identified was an Artemia cyclin K ortholog. The nucleotide sequence of this cyclin K encoding cDNA was submitted to GenBank under the accession number JQ085432.

Cyclin K mRNA was detected in different tissues and developmental stages of Artemia. Semiquantitative RT-PCR showed that the cyclin K transcript was detectable throughout the life-cycle of Artemia, and was most abundant during embryonic development (Fig. 2A). In addition, it showed a higher level of expression in the ovisac (Fig. 2B), consistent with the fact that cyclin K is abundantly expressed in developing germ cells of mouse [15]. However, no significant differences in expression were detected between embryos in the two developmental pathways or during the hatching process of the encysted embryo (Fig. 2C and 2D).

Primers were designed according to the sequence of A. salina Rpb1 and semiquantitative RT-PCR was also performed to examine the Rpb1 gene expression level in the embryos of the two developmental pathways and during the hatching process of the encysted embryo. The results showed that there were no significant differences in Rpb1 expression between the two developmental pathways; however, Rpb1 was expressed at relatively low levels in...
diapause and postdiapause embryos, and its expression was restored as development was initiated (Fig. 2C and 2D).

Expression and Localization of Cyclin K Protein and Activated RNAP II in the Development of Diapause-Destined Embryos

Before postdiapause development resumes, RNA synthesis ceases totally in encysted embryos [19]. Considering that cyclin K can activate transcription through RNAP II in vitro, cyclin K and the phosphorylation of CTD Ser2 of RNAP II were investigated simultaneously in the two developmental pathways and in the hatching process of encysted embryos. A rabbit polyclonal antibody was generated against a synthetic peptide based on the partial sequence of A. parthenogenetica cyclin K, and Western blotting revealed that the cyclin K protein was hardly detected in oocytes and then, it accumulated along the embryo developing in both two developmental pathways. Moreover, it was specifically down-regulated in the oviparous embryo one day before release to the environment and reverted to a relatively high level when development resumed (Fig. 3A and 3B). The RNAP II phosphorylation during the development of diapause-destined embryos was examined using an H5 mouse monoclonal antibody. It has been used to detect CTD Ser2 phosphorylation in many species ranging from yeast to humans [4], although a previous study reports that H5 also shows some cross-reactivity with CTD phosphate at Ser5 [38]. As speculated, Western blot analysis showed that CTD Ser2 phosphorylation appeared when cyclin K was expressed and that the phosphorylation level altered along with cyclin K expression levels in both developmental pathways, except that it had high phosphorylation level in the ovoviviparous late oocytes whereas cyclin K had little expression in this sample (Fig. 3A and 3B).

Next, we extracted pellet and supernatant proteins of 14-h-incubated cysts according to the method previously established [28]. The pellet fraction contains nuclei and yolk platelets, while the supernatant contains cytoplasmic proteins. As shown in Fig. 3C, cyclin K and CTD Ser2 phosphorylation of RNAP II were mainly detected in the pellet extracts, with little or no expression or phosphorylation in the supernatant extracts. Immunofluorescence staining of nuclei further confirmed that cyclin K and CTD Ser2 phosphorylated RNAP II were co-localized in the nucleus. However, the cyclin K staining degree were not proportional to the staining of phospho-RNAP II (Fig. 3D). Moreover, the phospho-RNAP II could not be detected in the immunoprecipitates by cyclin K antibody (Fig. 3E). All of these results suggested that cyclin K is a possible key factor involved in regulating the activity of RNAP II during diapause-destined embryo development, even if cyclin K could not bound to CTD Ser2 phosphorylated RNAP II.

Cyclin K is regulated at the Post-transcriptional Level in Artemia Diapause Embryos

Previous research reports that the amount of poly(A)-containing mRNAs are associated with one translational inhibitor RNA or protein p38 in dormant embryos and present in a repressed form [32,39]. Since we observed that the cyclin K protein declined in encysted embryos (Fig. 3A and 3B) whereas mRNA remained at a constant level (Fig. 2C and 2D), the template activity of cyclin K mRNA was tested. Total poly(A)-containing mRNA was purified from postdiapause and 8-h incubated embryos using oligo(dT)-cellulose columns and in vitro translation was performed. Northern blotting detected cyclin K in both postdiapause and 8-h incubated embryos purified mRNA by oligo(dT)-cellulose chromatography (Fig. 4A). However, the cyclin K protein was not detected following in vitro translation of the purified mRNA from diapause embryos, but was detectable in 8-h incubated embryos (Fig. 4B). This indicates that cyclin K was repressed in postdiapause cysts and is somehow reactivated after the initiation of development.
Figure 3. Cyclin K protein expression and phosphorylation of RNAP II in different developmental phases and their subcellular localization in *Artemia* embryos. (A) Cyclin K protein expression and CTD Ser2 phosphorylation of RNAP II in the two developmental pathways. EO, early oocytes; LO, late oocytes; 1dE, 3dE and 4dE represent embryos entering uterus for 1 day, 3 days and 4 days, respectively. (B) Cyclin K protein expression and CTD Ser2 phosphorylation of RNAP II during the hatching process of diapause embryos (includes diapause embryo, postdiapause embryo, 0- to 14-h incubated embryos and nauplius); α-tubulin was used as a loading control. (C) Supernatant (S) and pellet (P) fractions were prepared using buffer K (pH 6.0 or 8.0) from 14-h incubated embryos. Cyclin K and CTD Ser2 phosphorylation of RNAP II were detected by Western blotting. Tubulin and H3 were also examined to indicate the purity of the different extracts. (D) Immunofluorescence staining of nuclei from 14-h incubated embryos confirmed that cyclin K co-localizes with phosphorylated RNAP II in nuclei. a, cyclin K; b, phosphorylation of CTD Ser2; c, DAPI stain. The bars represent 10 μm. (E) Cyclin K and its associated factors (anti-cyclin K immunoprecipitates) were affinity purified from the 14-h incubated embryos and analysed by western blot. Another polyclonal antibody produced in rabbit (anti-SGEG2a) was used in a parallel procedure for control (con). The input loading quantity was 1/100 of the total supernatants.

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Figure 4. The template activity of cyclin K mRNA is repressed in encysted embryos. Poly(A)-containing mRNA was purified from postdiapause and 8-h incubated (8 h) embryos using oligo(dT)-Cellulose, and *in vitro* translation was performed using each purified mRNA as a template. (A) Cyclin K was detected in each purified mRNA sample by Northern blotting. (B) Detection of cyclin K *in vitro* translation product by Western blotting. NS: nonspecific bands. CK−, DEPC-treated water was used as template for the *in vitro* translation control. The molecular weight was shown on the left.

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Lack of Cyclin K Inhibits RNAP II Activity and Induces Apoptosis Mediated by ERK Pathways in Early Embryonic Development of Artemia

P-TEFb (CDK9/cyclin T) is required for the transcription of early embryonic genes [2,12,13]. Considering that cyclin K can form a complex with CDK9 to function as P-TEFb in vitro, whether it is involved in early embryo development was investigated. Double-stranded RNA (dsRNA), based on the cyclin K sequence, was injected into Artemia at instar XII stage as previously described [40], and GFP dsRNA was injected as a control. Both mRNA and protein levels of cyclin K were substantially depleted (Fig. 5A and 5B), suggesting a severe reduction in cyclin K expression in vivo. The morphology of adult and embryonic Artemia were examined, and although the cyclin K RNAi maternal Artemia showed no significant abnormal phenotypes compared with the control group (Fig. 5C, a and b), they released embryos that could not develop into nauplii (Fig. 5C, c and d), and Trypan Blue staining of the embryos entering uterus for four days confirmed that cells in RNAi embryos had lost viability (Fig. 5C, e and f). DAPI staining showed that the cyclin K RNAi embryo was normal for the first 24 h after it entered the uterus, whereas the nuclei were disorganized at the blastula stage (32 h after embryos entered the uterus) (Fig. 5D). A TUNEL assay confirmed that the cells in the RNAi embryos were apoptotic (Fig. 5E).

To study the mechanism of apoptosis triggered by the loss of cyclin K further, the signaling pathway was investigated. As speculated, phosphorylation of the RNAP II CTD Ser2 diminished after cyclin K knockdown, indicating an indispensable role for cyclin K in regulating RNAP II activity. The phosphorylation of ERK was also clearly down-regulated in cyclin K RNAi embryos, whereas the phosphorylation of RSK, its downstream kinase, was not affected. The phospho-H3 level was not reduced, indicating that cell division was not influenced by the depletion of cyclin K (Fig. 6). These results indicated that knockdown of cyclin K induced apoptosis by repressing the ERK pathway, although the downstream effector was not RSK.

Discussion

Cyclin K was first identified by its ability to rescue a lethal deletion of the G1 cyclin genes in S. cerevisiae [15]. Subsequent studies focused on its CTD kinase activity with CDK9 in vitro [16,17], while a recent study reported a direct role for the cyclin K-CDK9 complex in maintaining genome integrity in response to replication stress [14]. The results of the present study show that post-transcriptional regulation of cyclin K is involved in the regulation of RNAP II in Artemia diapause embryo development, and that cyclin K is involved in cell survival controlled by ERK signaling pathways during early development of Artemia embryos.

Phosphorylation of RNAP II CTD Ser2 In Vivo is Cyclin K-Dependent

Previous studies showed that the cyclin K-CDK9 complex phosphorylates the CTD of RNAP II in vitro. In the current study, the in vivo cyclin K knockdown provided direct evidence that phosphorylation of Ser2 in the RNAP II CTD repeats is cyclin K-dependent.

A study on the structure-function relationship of CDK9 cyclin partners reported that cyclin K lacks the histidine-rich stretch present in the C-terminal domain of cyclin T1, which is regarded as the unphosphorylated CTD binding site [41]. Another report revealed that cyclin K could only activate transcription via RNAP II when tethered to RNA but not to DNA [17]. By contrast, a co-immunoprecipitation assay performed by another group showed that cyclin K is associated with a hypophosphorylated form of RNAP II (IIa) [15]. In our study, Western blot analysis of different portions of the cyst extracts confirmed that cyclin K and the hyperphosphorylated form of RNAP II (IIo), detected by the H5 antibody, were both located in the nuclei of 14-h incubated embryos (Fig. 3C), but immunofluorescence staining of nuclei showed that the cyclin K staining degree were not proportional to the staining of IIo (Fig. 3D), and IIo was not detected in cyclin K immunoprecipitates (IPs) from 14-h incubated embryo extracts (Fig. 3E). From
show differential expression between the two developmental pathways at the oocyte stage [43,44]. However, RNAP II activity at this stage is not cyclin K-dependent.

**In Vivo Knockdown of Cyclin K in Artemia Early Embryos Induces Apoptosis at the Blastula Stage**

Metazoan early embryonic development is controlled initially by maternal mRNAs and the onset of embryonic transcription occurs at a later developmental stage, ranging from the two-cell stage in mice to cell-cycle 12 to 14 in flies [44]. In the present study, CTD Ser2 phosphorylation of RNAP II was monitored during early embryonic development and the results showed that transcription activity of RNAP II appears around 24 h after the embryo enters the uterus (early blastula stage, GFP control group in Fig. 6).

Previous studies suggest that impairment of RNAP II activity affects normal embryonic development. Loss of cyclin H function, a subunit of the general transcription factor complex, TFIID, delays the onset of transcription in early zebrafish embryos and induces apoptosis 3 h post-fertilization [45]. In addition, depletion of CDK9 or cyclin T seems to have more severe effects on embryos during early development leading to loss of viability during metamorphosis in flies and at the 100-cell stage in *C. elegans* [2]. In this study, the *in vivo* knockdown of cyclin K in embryos eliminated the CTD Ser2 phosphorylation of RNAP II, which prevented the embryos from developing into nauplius larvae (Fig. 5C). This suggests that cyclin K participates in the activation of embryonic gene transcription by RNAP II and plays an essential role in early embryonic development. Adult *Artemia* with cyclin K knockdown showed no abnormal phenotypes (Fig. 5C, a and b). One explanation for this is that cyclin K is not essential for the activation of RNAP II transcription in the adult, which is supported by the fact that CDK9 can activate RNAP II transcription in combination with other cyclin partners [46].

Previous studies report that inhibition of ERK survival signaling leads to apoptosis in many cell types [47–50]. In our knockdown experiments, the ERK phosphorylation was reduced markedly after depletion of cyclin K and, subsequently, apoptosis was induced in developing embryos (Fig. 5E and 6). These data suggest that apoptosis may possibly have been induced through the ERK pathway. This signaling pathway is not RSK-dependent, as RSK knockdown showed normal phenotypes (Fig. 5E, a and b). A subsequent study supported the fact that CDK9 can activate RNAP II transcription in combination with other cyclin partners [46].

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Metazoan early embryonic development is controlled initially by maternal mRNAs and the onset of embryonic transcription occurs at a later developmental stage, ranging from the two-cell stage in mice to cell-cycle 12 to 14 in flies [44]. In the present study, CTD Ser2 phosphorylation of RNAP II was monitored during early embryonic development and the results showed that transcription activity of RNAP II appears around 24 h after the embryo enters the uterus (early blastula stage, GFP control group in Fig. 6).

Previous studies suggest that impairment of RNAP II activity affects normal embryonic development. Loss of cyclin H function, a subunit of the general transcription factor complex, TFIID, delays the onset of transcription in early zebrafish embryos and induces apoptosis 3 h post-fertilization [45]. In addition, depletion of CDK9 or cyclin T seems to have more severe effects on embryos during early development leading to loss of viability during metamorphosis in flies and at the 100-cell stage in *C. elegans* [2]. In this study, the *in vivo* knockdown of cyclin K in embryos eliminated the CTD Ser2 phosphorylation of RNAP II, which prevented the embryos from developing into nauplius larvae (Fig. 5C). This suggests that cyclin K participates in the activation of embryonic gene transcription by RNAP II and plays an essential role in early embryonic development. Adult *Artemia* with cyclin K knockdown showed normal phenotypes (Fig. 5C, a and b). One explanation for this is that cyclin K is not essential for the activation of RNAP II transcription in the adult, which is supported by the fact that CDK9 can activate RNAP II transcription in combination with other cyclin partners [46].

Previous studies report that inhibition of ERK survival signaling leads to apoptosis in many cell types [47–50]. In our knockdown experiments, the ERK phosphorylation was reduced markedly after depletion of cyclin K and, subsequently, apoptosis was induced in developing embryos (Fig. 5E and 6). These data suggest that apoptosis may possibly have been induced through the ERK pathway. This signaling pathway is not RSK-dependent, as RSK knockdown showed no abnormal phenotypes (Fig. 5E, a and b). One explanation for this is that cyclin K is not essential for the activation of RNAP II transcription in the adult, which is supported by the fact that CDK9 can activate RNAP II transcription in combination with other cyclin partners [46].

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In conclusion, the findings reported in this study illustrate the post-transcriptional regulation of cyclin K and its potential role in regulating RNAP II activity in *Artemia* diapause embryo development. Furthermore, we have identified a further role for cyclin K in regulating the control of cell survival through the ERK pathway during *Artemia* development.

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**Author Contributions**

Conceived and designed the experiments: WJY YZ. Performed the experiments: YZ XD XY ZMD. Analyzed the data: WJY YZ. Contributed reagents/materials/analysis tools: JSY XY. Wrote the paper: WJY YZ,JSY.
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