Juvenile Hormone Activates the Transcription of Cell-division-cycle 6 (Cdc6) for Polyploidy-dependent Insect Vitellogenesis and Oogenesis

Zhongxia Wu¹, Wei Guo², Yingtian Xie³, Shutang Zhou⁴*

¹School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China
²State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China
³College of Life Sciences, Jilin University, Changchun, Jilin 30012, China
⁴State Key laboratory of Cotton Biology, Institute of Plant Stress Biology, School of Life Sciences, Henan University, Kaifeng, Henan 475004, China

Running title: Cdc6 and JH-modulated polyploidy and reproduction

* Correspondence to: Shutang Zhou, State Key laboratory of Cotton Biology, Institute of Plant Stress Biology, School of Life Sciences, Henan University, Kaifeng, Henan 475004, China. Tel: 86-0371-63886272; Email: szhou@henu.edu.cn

Key words: Cdc6; juvenile hormone; polyploidy; vitellogenin; oocyte maturation; ovarian development

Abstract

Although juvenile hormone (JH) is known to prevent insect larval metamorphosis and stimulate adult reproduction, the molecular mechanisms of JH action in insect reproduction remain largely unknown. Earlier, we have reported that the JH-receptor complex comprised of Methoprene-tolerant (Met) and steroid receptor co-activator acts on mini-chromosome maintenance (Mcm) genes, Mcm4 and Mcm7 to promote DNA replication and polyploidy for the massive vitellogenin (Vg) synthesis required for egg production in the migratory locust (1). In this study, we have investigated the involvement of cell-division-cycle 6 (Cdc6) in JH-dependent vitellogenesis and oogenesis, as Cdc6 is essential for the formation of pre-replication complex. We demonstrate here that Cdc6 is expressed in response to JH and Met, and Cdc6 transcription is directly regulated by the JH-receptor complex. Knockdown of Cdc6 inhibits polyploidization of fat body and follicle cells, resulting in the substantial reduction of Vg expression in the fat body, as well as severely impaired oocyte maturation and ovarian growth. Our data indicate the involvement of Cdc6 in JH pathway and a pivotal role of Cdc6 in JH-mediated polyploidization, vitellogenesis and oogenesis.

In addition to repressing insect larval metamorphosis, juvenile hormone (JH) has an essential role in stimulating adult reproduction (2, 3). During the larval stages, JH maintains the larval characteristics of insects by modulating the cellular responses to 20-hydroxyecdysone (20E) during each molting. In the final-instar larvae, the very low titer or the absence of JH leads to 20E-induced metamorphosis (2, 4). In adult insects, newly synthesized JH stimulates many aspects of reproduction, including the
previtellogenic development, vitellogenesis and oogenesis (3, 5). Cumulative studies have demonstrated that JH exerts the genomic actions through its receptor, Methoprene-tolerant (Met) (6). JH induces the heterodimerization of Met with steroid receptor co-activator (SRC) (also known as Taiman in Drosophila or FISC in the mosquito, Aedes aegypti) to form a transcriptionally active complex to regulate the transcription of target genes in several insect systems, including the beetle Tribolium castaneum, silkworm Bombyx mori, migratory locust Locusta migratoria, and mosquito A. aegypti (1, 7-11).

Vitellogenesis, vitellogenin (Vg) synthesized in the fat body of many insects and taken up by maturing oocytes, plays a critical role in egg production. In Drosophila melanogaster, both JH and 20E are involved in vitellogenesis though 20E is responsible for the high rate of Vg synthesis in the fat body (4, 12, 13). In the mosquito A. aegypti, JH controls the previtellogenic development of fat body competent for Vg synthesis in the fat body (4, 12, 13). In the red flour beetle T. castaneum, JH regulates Vg synthesis in the fat body while 20E affects Vg synthesis through oocyte maturation (16-18). In many other insect species including the linden bug Pyrrhocoris apterus and the German cockroach Blattella germanica as well as L. migratoria, JH acts independently of 20E to stimulate vitellogenesis and oocyte maturation (3, 5, 19-21). Despite this understanding of JH regulation, the molecular mechanisms of JH action in insect vitellogenesis and oocyte maturation remain poorly understood.

Polyploidy, the existence of more than two genome copies in a cell, is found in highly metabolically active cells and tissues like fat body, follicular epithelium, nurse cells, midgut, salivary gland, and wing imaginal discs of insects (22-25). Polyploidy is generated by repeated G/S cycles and enhanced DNA replication (26, 27). It has been reported that 20E regulates DNA replication and polyploidy during insect metamorphosis, but the underlying mechanisms have yet been defined (3, 28-31). In locusts, polyploidization in the fat body and follicle cells of adult females during vitellogenesis and oocyte maturation is dependent on JH (1, 32, 33). In a previous report, we have shown that JH acts through its receptor complex Met/SRC on two mini-chromosome maintenance (Mcm) genes, Mcm4 and Mcm7 to promote DNA replication and polyploidization for the massive Vg synthesis required for egg production in locusts (1).

At the onset of the G1 phase, recruitment of the replicative helicase Mcm2-7 onto origins of DNA replication to form the pre-replication complex (pre-RC) requires the loading factor cell-division-cycle 6 (Cdc6), a member of AAA+ ATPase family (34-38). Along with the origin recognition complex and Cdt1 (Cdc10 protein-dependent transcript 1), Cdc6 loads Mcm proteins onto origins of replication to facilitate the formation of stable pre-RC in G1 phase thereby licensing these sites to initiate DNA replication in S phase (39-41). The loading of Mcm helicase complex onto DNA at the origins of replication is central to DNA replication (42-44). In our RNA-seq-based gene expression profiling, Cdc6 together with Mcm2-7 were identified as the up-regulated genes in the fat body of JH-deprived adult female locusts further treated with methoprene (1). Given the functional
importance of Cdc6 in loading Mcm2-7, we speculated that Cdc6, like Mcm, is a regulatory target of JH pathway. We therefore wondered if JH and its receptor complex target Cdc6 for transcriptional regulation, which consequently modulates locust polyploidy, vitellogenesis and oogenesis. We found that JH stimulates the expression of Cdc6, and that JH-induced Met/SRC complex directly activates Cdc6 transcription by binding to the upstream consensus sequence with the E-box-like motif. We observed that depletion of Cdc6 results in substantial reduction of Vg expression, arrested oocyte maturation and blocked ovarian growth. This work implicates a crucial role of Cdc6 in JH-dependent polyploidy, vitellogenesis and oogenesis, which provides new insight into the mechanisms of JH regulation in insect reproduction.

Experimental Procedures

Insects—The gregarious colony of migratory locust L. migratoria was maintained at a density of ~300 locusts per cage (25 cm × 25 cm × 25 cm) under a photoperiod of 14L:10D and at 30 ± 2°C. Locusts were fed with wheat bran supplied continuously and wheat seedlings provided once daily. The wheat seedlings were grown 5-7 days on the soil-less culture under a photoperiod of 14L:10D and at 22 ± 2°C (45).

Hormone Treatment—JH-deprived female adult locusts were obtained by inactivation of the corpora allata via the topical application of 500 µg (100 µg/µl dissolved in acetone) precocene III (Sigma-Aldrich) to the dorsal neck membrane of locust within 12 h after eclosion (46). To restore the JH activity, an active JH analog, s-(+)-methoprene was topically applied at 150 µg (30 µg/µl dissolved in acetone) per locust 10 d post precocene treatment (46). Topical application of acetone (5 µl per locust) alone was used as the solvent control.

RNA Isolation and qRT-PCR—Total RNA from locust tissues and Drosophila Schneider 2 cells (S2 cells) were extracted using TRNzol reagent (Tiangen) following the manufacturer’s instruction. First-strand cDNA was reverse-transcribed with 2 µg total RNA using FastQuant RT kit with gDNase (Tiangen). qRT-PCR was performed using Mx3005P detection system (Agilent) and RealMasterMix SYBR Green kit (Tiangen), initiated at 95°C for 15 min, then 40 cycles at 95°C for 10 s followed by 58°C for 20 s and 72°C for 30 s. Melting curve analysis was conducted to confirm the specificity of amplification. The 2ΔΔCt method was used for calculating the relative gene expression levels, normalized by β-actin. Primers for qRT-PCR are listed in Table 1. The specificity of primers was confirmed by BLAST in the NCBI database and the locust genome (47). The qRT-PCR products were sequenced for further confirmation of specificity.

RNA Interference (RNAi)—cDNA templates were amplified by PCR, cloned into pGM-T easy vector (Tiangen) and confirmed by sequencing. Double-stranded RNA (dsRNA) was then synthesized by in vitro transcription with T7 RiboMAX Express RNAi system (Promega) according to the manufacturer’s manual. For RNAi in locusts, female adults within 12 h after eclosion were intra-abdominally injected with 8 µg (in a volume of 8 µl) of Met (GenBank: KF471131) or Cdc6 (GenBank: KT692979) dsRNA dissolved in a mixture of acetone and H2O (2:1 ratio) and boosted
Cdc6 and JH-modulated polyploidy and reproduction

on day 5. In JH rescue experiments, methoprene or acetone (solvent control) was applied on day 6, and the effects were examined on day 8. For RNAi in S2 cells, *Drosophila Met* (FlyBase: FBpp0073368) and *Taiman* (FlyBase: FBpp0292873) dsRNA (38 nM) were transfected into S2 cells using Lipofectamine 2000 (Invitrogen) for 48 h, prior to transfection of the recombinant vectors. In all RNAi experiments, dsGFP was used as the control. Primers used for dsRNA synthesis are included in Table 1. The specificity of primers was confirmed by BLAST in the NCBI database and locust genome (47).

**Tissue Imaging and Confocal Microscopy**—Ovaries and ovarioles were imaged with a Nikon D7000 camera and an Olympus CKX-41 microscope, respectively. The length and width of primary oocytes were measured using Leica M205C microscope. For cell staining, fat bodies and ovarioles were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton X-100 for additional 15 min. F-actin was stained with 0.165 μM Alexa-Fluor 488 Phalloidin (excitation wavelength 488 nm) (Invitrogen). Nuclei were stained with 5 μM Hoechst 33342 (excitation wavelength 350 nm) (Sigma-Aldrich). The images were captured by ZEISS LSM 710 confocal microscopy and analyzed with ZEN2010 software (Carl Zeiss).

**Flow Cytometry**—The fat body, follicle epithelium and brain were separately homogenized in Dounce homogenizer. Cells were collected by centrifugation (800 ×g), fixed in 70% ethanol overnight, and further incubated with PBS buffer containing 50 μg/ml propidium iodide (Sigma), 100 μg/ml RNaseA (Promega) and 0.2% Triton X-100 for 2 h at 4°C. The cells were then filtered by 300-mesh cell strainers (BD Falcon) and analyzed using a BD FACSCalibur Flow Cytometry System with Flowjo 7.6.1 software (BD Biosciences). Brain nuclei were used as a diploid control.

**Western Blot and Immunoprecipitation**—The procedures were described previously (1). Briefly, locust *Met* (nt 1–3108) and *SRC* (GenBank: KF471132; nt 1-1786) cDNA were cloned into pAc5.1/Flag and pAc5.1/V5 vectors (Invitrogen), respectively. The constructs of pAc5.1/Flag-Met (N-terminally tagged) and/or pAc5.1/SRC-V5 (C-terminally tagged) were transfected into *Drosophila* S2 cells using Lipofectamine 2000 (Invitrogen). Ten μM JH III or methoprene was used for cell treatment. Cells were lysed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% NP-40, 1 mM PMSF, 1 mM NaF and a protease inhibitor cocktail (Roche). After centrifugation at 14,000 ×g for 10 min, lysates were fractionated on 8% SDS-PAGE and transferred to PVDF membranes (Millipore). Western blots were carried out using anti-Flag and anti-V5 antibodies (MBL), with anti-actin antibody (Abmart) as the loading control. For immunoprecipitation, the precleared lysates were incubated with anti-V5 antibody for 60 min at 4°C, and the immunocomplexes were captured with protein A agarose (Sigma-Aldrich) and eluted in Laemmli sample buffer, followed by Western blotting with anti-Flag antibody.

**Luciferase Reporter Assay**—The upstream promoter region (nt -1253 to -60) of Cdc6 gene was cloned into pGL4.10 vector (Promega), and confirmed by sequencing. After pre-treatment with *Drosophila Met* and *Taiman* dsRNA for 48 h,
S2 cells were transfected with pAc5.1/Flag-Met and/or pAc5.1/ SRC-V5 plus pGL4.10-Cdc6$^{1253}$ to $^{60}$ using Lipofectamine 2000 (Invitrogen). After 48 h, 10 µM methoprene was applied for 6 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System and GloMax 96 Microplate Luminometer (Promega).

Electrophoresis Mobility Shift Assay (EMSA)–Nuclear extracts from locust fat bodies and S2 cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific). Cdc6 probe (5’-CGAGAAACACGCGAAAAATA-3’) was end-labeled with $\gamma$-32P-ATP by T4 DNA kinase (New England Biolabs), purified by Sephadex G-25 column (GE Healthcare), and incubated with nuclear protein extracts in the binding buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM MgCl$_2$, 1 mM DTT, 1 mM EDTA, 10% glycerol, and 50 ng/µl poly(dI/dC). In the competition assays, 50× molar excess of unlabeled Cdc6 probe or non-specific AP2 oligonucleotide (Promega) (48) was added into the binding reaction. In the supershift assays, anti-Flag antibody (Sigma-Aldrich), anti-V5 (Invitrogen) antibody or the control IgG (Sigma-Aldrich) was pre-incubated with the nuclear extracts at 4°C for 1 h prior to the addition of labeled probes. The DNA-protein complex was resolved in 5% native polyacrylamide gels and visualized using X-ray film (Kodak). The band intensity was quantified by ImageJ.

Statistical Analysis–Student’s t-test by SPSS 20.0 software (SPSS) was used for statistical analyses. Significant difference was considered at $P<0.05$. Values were reported as mean ± SE.
Cdc6 and JH-modulated polyploidy and reproduction

the vitellogenic phase (50, 51), an increase of Cdc6 mRNA levels in both fat body and ovary appeared to correlate with the phase of elevated JH titers.

To explore the dynamics of JH-stimulated Cdc6 expression, qRT-PCR was conducted using total RNA from the fat body and ovary of precocene-treated female adults for 10 d as well as those further treated with methoprene for 6, 12, 24 and 48 h. As shown in Fig. 1C and D, chemical allatostomy by precocene treatment resulted in 59% and 91% reduction of Cdc6 mRNA levels in the fat body and ovary, respectively. We next assessed the effects of JH application on Cdc6 expression using JH-deprived adults further treated with methoprene for 6-48 h. Compared to JH-deprived fat bodies, Cdc6 mRNA levels were significantly increased by 2.1-fold at 6 h, and then continually elevated by 6.1- to 13.8-fold at 12-48 h post methoprene treatment (Fig. 1C). In the ovary, Cdc6 mRNA levels were increased by 2.3- to 2.8-fold at 6-24 h post methoprene application, but declined at 48 h (Fig. 1D). In the parallel experiment of solvent controls, acetone treatment had no significant effect on Cdc6 expression in fat bodies or ovaries (Fig. 1C and D). The data indicate that JH stimulates the expression of Cdc6 in both the fat body and ovary of locusts.

**Cdc6 Is Transcriptionally Regulated by the JH-receptor Complex**—As an initial step, Met RNAi was carried out to determine the requirement of Met for JH-dependent Cdc6 expression, using fat bodies as the representative tissue. qRT-PCR demonstrated that 61-85% of Met RNAi efficiency was obtained in the fat body of dsMet-injected adult females at 4-8 days PAE (Fig. 2A). Correspondingly, Cdc6 mRNA levels were reduced by 61%, 46% and 36% at 4, 6 and 8 days PAE, respectively (Fig. 2B), indicating the dependence of Cdc6 expression on Met. Analysis of the upstream sequence of locust Cdc6 gene revealed an E-box-like motif (CACGCG, nt -1063 to -1058) that has been previously reported for Met binding as a JH-response element (1, 8, 48, 52, 53). To test the recognition of Cdc6 DNA element by Met, EMSA was conducted using nuclear extracts from dsMet- vs. dsGFP-treated fat bodies. A 20-mer nucleotide probe corresponding to the sequence containing E-box-like motif in the upstream of locust Cdc6 was used (Fig. 2C). Two bands were visualized when the 32P-Cdc6 probe and nuclear extracts derived from dsGFP-treated fat bodies were incubated, but only the faster moving band was abolished with 50× molar excess of unlabeled Cdc6 probe (Fig. 2D). This faster moving band showed 34% reduction in intensity when nuclear extracts from Met-depleted fat bodies were used (Fig. 2D and E). It suggests the possible involvement of endogenous Met in the Cdc6 probe-binding complex.

As locust cell line and Met antibody are unavailable, we performed luciferase reporter assays and EMSA using S2 cells to further confirm Met binding to the Cdc6 promoter. We cloned cDNAs of locust Met and SRC into pAc5.1/Flag and pAc5.1/V5 vectors, respectively to express the Flag-Met and SRC-V5 fusion proteins in S2 cells. To diminish the effect of endogenous Met, Gce (Germ cell-expressed; the paralog of Met) and Taiman (Tai), S2 cells were subjected to RNAi using Drosophila Met and Tai dsRNA (1), prior to transfection of the recombinant vectors. It is noted that the sequence of Drosophila Met dsRNA shares about 40% identity to that of Drosophila Gce (FlyBase: FBpp0292296). Pretreatment
of S2 cells by *Drosophila Met* and *Tai* dsRNA resulted in ~80% reduction of endogenous *Met, Gce* and *Tai* expression (Fig. 3A), but had no significant effect on transfected *Flag-Met* and *SRC-V5* (Fig. 3B). Immunoprecipitation and Western blot demonstrated that addition of JH III or methoprene induced the interaction of expressed Flag-Met and SRC-V5 (Fig. 3C), indicating the dependence of JH on the formation of locust Met and SRC heterodimer. It has been previously reported that *Cdc6* is transcriptionally regulated in an E2F-dependent manner (54, 55). Two putative E2F-binding sites were found in the proximal region (nt -59 to -1) of locust *Cdc6* gene. To eliminate the possible interference by endogenous E2F, the *Cdc6* upstream sequence from nt -1253 to -60 containing the E-box-like motif was cloned into the pGL4.10 vector and co-transfected with pAc5.1/Flag-Met and/or pAc5.1/SRC-V5 for luciferase assays. In the absence of methoprene, the co-expression of Flag-Met and SRC-V5 slightly induced *Cdc6* reporter activity, similar to that of Flag-Met or SRC-V5 expression alone (Fig. 3D). However, after methoprene treatment, Flag-Met plus SRC-V5 led to 5.2-fold increase in *Cdc6* reporter activity compared to the control (Fig. 3D). The data indicate that JH-induced Met-SRC complex activates *Cdc6* transcription.

In EMSA using methoprene-treated nuclear extracts from S2 cells with expressed Flag-Met and SRC-V5, a band comprised with the \(^{32}\text{P}-\text{Cdc6}\) probe was eliminated by 50× molar excess of the unlabeled Cdc6 probe (Fig. 3E). When 50× molar excess of unlabeled AP2 oligonucleotide was used, the specific binding was not competed (Fig. 3E). This specific band was diminished when cell nuclear extracts were pre-incubated with anti-Flag or anti-V5 antibody (Fig. 3E). When IgG was pre-incubated with the cell nuclear extracts, this band was reduced in intensity but not abolished (Fig. 3E). These results confirm that the JH-receptor complex binds to the 20-mer DNA sequence of *Cdc6* promoter with specificity.

**Cdc6 Knockdown Inhibits Polyploidization in the Fat Body and Follicle Cells**—In the fat body of adult female locusts, 76% knockdown efficiency of *Cdc6* RNAi was obtained at 4 days PAE (Fig. 4A). On day 6 and 8, *Cdc6* mRNA levels in the fat body were reduced by 74% and 62%, respectively (Fig. 4A). It has been previously reported that efficient gene knockdown in locust ovary is unachievable via intra-abdominal injection of dsRNA dissolved in H\(_2\)O due to inefficient dsRNA uptake into follicle cells and oocytes (56). In the present study, dsRNA was dissolved in a mixture of acetone and H\(_2\)O at the ratio of 2:1 to facilitate dsRNA uptake. As shown in Fig. 4B, *Cdc6* mRNA levels in the ovary were significantly reduced to 57% and 44% of its normal levels at 6 and 8 days PAE, respectively. However, *Cdc6* expression was not significantly altered on day 4 (Fig. 4B).

To visualize the morphological change of nuclei after *Cdc6* RNAi, F-actin and nuclei were stained with fluorescence-labeled Phalloidin and Hoechst 33342, respectively, followed by confocal microscopy. Knockdown of *Cdc6* resulted in smaller nuclei in both fat body and follicle cells (Fig. 5A and B). Notably, when *Cdc6* was depleted by RNAi, ~15% of fat body cells and ~21% of follicle cells were seen with double nuclei accompanied with insignificant change of cell numbers on day 6-8. The observation suggests that locust Cdc6 has a role in controlling the G2-M transition in addition to the initiation of
DNA replication.

Quantitative analysis of ploidy by flow cytometry showed that Cdc6-depleted fat body and follicle cells had markedly lower DNA contents compared to the dsGFP controls (Fig. 5C and D). At 4 days PAE, Cdc6-knockdown fat bodies showed 2C and 4C peaks as well as 8C populations, whereas dsGFP-treated fat bodies had 2C and 8C peaks and 4C populations. Distinct from peaks at 8C and 16C in dsGFP controls, only 2C and 4C peaks were observed in Cdc6-depleted fat bodies on day 6. At 8 days PAE, dsCdc6-treated fat bodies had dominantly 4C contents compared to the dsGFP control with 8C and 16C peaks (Fig. 5C). With respect to follicle cells, Cdc6-knockdown samples were chiefly at 4C on day 6-8, whereas the dsGFP controls had 8C populations on day 6, and 8C plus 16C populations or peak on day 8 (Fig. 5D).

Cdc6 RNAi Blocks Locust Vitellogenesis and Oogenesis—The migratory locust has two Vg genes, VgA and VgB, which are coordinately induced by JH and expressed in similar patterns (57). VgA (GenBank: KF171066) was selected as the representative to evaluate the effect of Cdc6 knockdown on Vg expression in the fat body (1). Knockdown of Cdc6 reduced VgA mRNA levels to 47%, 34% and 4% of its control levels on day 4, 6 and 8, respectively (Fig. 6A). We next examined the effect of Cdc6 knockdown on oocyte maturation and ovarian growth. Depletion of Cdc6 via RNAi resulted in blocked oocyte maturation and arrested ovarian development (Fig. 6B and C). Consequently, the primary oocytes and ovaries of Cdc6-depleted locusts remained small on day 6-8. Conversely, the primary oocytes and ovaries of dsGFP controls markedly enlarged (Fig. 6B). Statistically, the length*width index of primary oocytes of dsGFP control locusts were increased from 0.9 to 6.3 on day 4 to 8, whereas that of Cdc6 RNAi locusts had no significant change (Fig. 6C). It must be noted that the impaired oocyte maturation and ovarian growth were also seen in Cdc6 RNAi locusts at 4 days PAE, which was presumably due to declined synthesis of Vg and other forms of yolk proteins as the efficient Cdc6 knockdown was achieved in the fat body on day 4 (Fig. 4A).

Next, we treated Cdc6 RNAi locusts with methoprene and examined the effect on ploidy, Vg expression, oocyte maturation and ovarian growth. As shown in Fig. 7A and B, further application of methoprene on Cdc6-depleted locusts did not restore the defective nuclei and decreased ploidy of either fat body or follicle cells to their normal levels. However, after methoprene treatment, Cdc6-RNAi locusts showed a decrease of 2C population and an increase of 8C contents in the fat body and follicle cells (Fig. 7B). These observations suggest that JH regulates other factors involved in the stimulation of polyploidization and that the increase in ploidy may be time sensitive (Fig. 6). The percentage of cells with double nuclei did not change significantly in either fat body or follicle cells of methoprene-treated locusts that were previously subjected to Cdc6 RNAi. As shown in Fig. 7C, the capacity of methoprene to induce VgA expression in the fat body was abrogated by Cdc6 knockdown. Similarly, methoprene application on dsCdc6-treated female locusts was unable to restore the defective oocyte maturation and ovarian growth to the normal levels (Fig. 7D and E). Taken together, these data indicate a crucial role of Cdc6 in polyploidization of fat body and follicle cells, as well as vitellogenesis, oocyte maturation and successful egg production in locusts.
Discussion

Cdc6 and JH-dependent Vitellogenesis and Oogenesis—In the present study, we have demonstrated that depletion of Cdc6 resulted in lower ploidy and significantly reduced Vg expression in the fat body as well as blocked oocyte maturation and arrested ovarian growth, similar to that caused by knockdown of Mcm4 or Mcm7 (1). Moreover, JH treatment of Cdc6-depleted locusts did not restore the defective phenotypes to the normal levels, resembling the failure of JH rescue on Mcm4 or Mcm7 RNAi (1). These data provide the evidence that Cdc6, like Mcm4 and Mcm7, is essential for JH-dependent polyploidization, vitellogenesis and egg production. It has been well established that Cdc6 is a key factor for replication origin licensing late in G1 phase by regulating the formation of pre-replication complexes that initiate DNA synthesis at S phase (37, 43, 58, 59). Polyploidy is known to promote transcriptomic and metabolomic outputs (27, 60). It is likely that JH stimulates the expression of Cdc6, along with Mcm and genes coding for other DNA replication factors, to replicate multiple copies of genome for the need of massive yolk protein synthesis in the fat body required for the production of a number of matured eggs. In human, Cdc6 has been linked to oncogenesis through its role in DNA replication initiation and its interference with the tumor suppressor genes INK4/ARF (61-63). Overexpression of Cdc6 in human primary cells promotes DNA hyperreplication related to oncogene activation, whereas Cdc6 knockdown prevents cell proliferation and promotes apoptosis (62, 64).

Follicle cells, constituting the follicular epithelium that surrounds the oocyte, play important functions in oocyte development (3, 5, 20). The follicular epithelium not only determines the size and shape of ovarian follicle, but also initiates the intercellular spaces, known as patency, to facilitate the transport of Vg to the oocyte membrane where Vg is internalized into maturing oocytes by receptor-mediated endocytosis (3, 14, 65). In this study, efficient RNAi of Cdc6 in locust ovary was achieved using an alternative approach via dissolving dsRNA in the mixture of acetone and H2O. We observed that disruption of Cdc6 in the ovary led to decreased ploidy in follicle cells as well. The impaired polyploidization in follicle cells is likely to restrain the development of follicular epithelium, which in turn blocks patency initiation and consequently suppresses Vg transportation and ovarian growth.

Intriguingly, in synchrony with declined ploidy, about 15% of fat body cells and 21% of follicle cells had double nuclei after Cdc6 knockdown while the cell numbers were not significantly changed. These phenotypes suggest that Cdc6-depleted fat body and follicle cells are missing checkpoints to prevent mitotic entry, in addition to decreased DNA replication. Studies in the yeast, Drosophila, Xenopus and mammalian cells have shown that Cdc6 activates and maintains the checkpoint response to prevent mitosis before DNA replication is completed (34, 62, 66). Cdc6 RNAi in synchronized Drosophila S2 cells results in the entrance of cells into mitosis with incompletely replicated DNA (67). Our data therefore support the dual functions of Cdc6 in initiation of DNA synthesis and in S-M phase transition.

Regulation of Cdc6 Expression by JH—Cdc6 expression is tightly regulated at the level of transcription in a cell...
cycle-dependent manner (43, 62, 63). Androgen can act through its receptor binding to a 15-bp palindromic androgen response element in the Cdc6 promoter on the transcription of Cdc6 gene in prostate cancer cells (55, 68). By qRT-PCR, we showed that Cdc6 expression was significantly increased in the fat body and ovary post adult female eclosion, which are in conformity with the elevated JH titers (50, 51). Methoprene treatment of JH-deprived locusts induced Cdc6 expression, whereas Met RNAi resulted in remarkable decrease of Cdc6 abundance. Since Cdc6 was expressed in response to JH and is Met-dependent in S2 cells, and an E-box-like motif was identified in the upstream promoter region of Cdc6 gene, we reasoned that Met could directly target Cdc6 for transcriptional regulation. Our luciferase reporter assays demonstrated that Cdc6 transcription was activated by the JH-receptor complex comprised of Met and SRC in the presence of methoprene. EMSA using nuclear extracts from locust fat bodies and Drosophila S2 cells with expressed Flag-Met and SRC-V5 documented the specific binding of JH-receptor complex to the DNA sequence containing E-box-like motif in the upstream of Cdc6 gene. Collectively, JH and its receptor appear to directly target the components of DNA replication machinery, including Cdc6, Mcm4 and Mcm7 (1), to coordinately initiate DNA replication for the multiple sets of chromosomes.

In summary, our present study has demonstrated that JH acts through its receptor complex Met/SRC to regulate the transcription of Cdc6. Loss of Cdc6 function can result in significantly decreased ploidy and precocious mitotic entry in both fat body and follicle cells, accompanied by substantial reduction of Vg expression and arrested development of follicular epithelium. Consequently, oocyte maturation and ovarian growth are blocked. Upon JH induction, Cdc6 along with Mcm and possibly other factors involved in DNA replication and cell cycle coordinate to replicate the genome for multiple copies in the fat body and follicle cells for the massive synthesis and efficient uptake of Vg and possibly other macromolecules required for producing a large number of eggs. Further identification and characterization of genes responsible for JH-dependent regulation of polyploidy during vitellogenesis and oogenesis should help decipher the mechanisms of JH-modulated reproduction and high fecundity in insects.

Acknowledgments

This work was supported by National Natural Science Foundation of China grant 31172149 and National Basic Research Program of China grant 2012CB114101.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

SZ conceived the study and wrote the paper. WZ performed and analyzed the experiments and wrote the paper. WG designed and performed the experiments. YX performed the experiments. All authors reviewed the results and approved the final version of the manuscript.
Cdc6 and JH-modulated polyploidy and reproduction

References

1. Guo, W., Wu, Z., Song, J., Jiang, F., Wang, Z., Deng, S., Walker, V. K., and Zhou, S. (2014) Juvenile hormone-receptor complex acts on Mcm4 and Mcm7 to promote polyploidy and vitellogenesis in the migratory locust. PLoS Genet. 10, e1004702
2. Riddiford, L. M. (1994) Cellular and molecular actions of juvenile hormone I. general considerations premetamorphic actions. Adv. In Insect Phys. 24, 213-274
3. Wyatt, G. R., and Davey, K. G. (1996) Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. Adv. In Insect Phys. 26, 1-155
4. Riddiford, L. M. (2012) How does juvenile hormone control insect metamorphosis and reproduction? Gen. Comp. Endocrinol. 179, 477-484
5. Raikhel, A. S., Brown, M. R., and Belles, X. (2005) Hormonal control of reproductive processes. In Comprehensive molecular insect science (Gilbert, L. I., latrou, K., and Gill, S. S. eds.), pp. 433-491, Elsevier, Boston
6. Jindra, M., Palli, S. R., and Riddiford, L. M. (2013) The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181-204
7. Charles, J. P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J., and Jindra, M. (2011) Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc. Natl. Acad. Sci. U.S.A. 108, 21128-21133
8. Li, M., Mead, E. A., and Zhu, J. (2011) Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. Proc. Natl. Acad. Sci. U.S.A. 108, 638-643
9. Zhang, Z., Xu, J., Sheng, Z., Sui, Y., and Palli, S. R. (2011) Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. J. Biol. Chem. 286, 8437-8447
10. Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y., and Shinoda, T. (2012) Transcriptional regulation of juvenile hormone-mediated induction of Kruppel homolog 1, a repressor of insect metamorphosis. Proc. Natl. Acad. Sci. U.S.A. 109, 11729-11734
11. Song, J., Wu, Z., Wang, Z., Deng, S., and Zhou, S. (2014) Kruppel-homolog 1 mediates juvenile hormone action to promote vitellogenesis and oocyte maturation in the migratory locust. Insect Biochem. Mol. Biol. 52, 94-101
12. Bownes, M. (1989) The roles of juvenile hormone, ecdysone and the ovary in the control of Drosophila vitellogenesis. J. Insect Physiol. 35, 409-413
13. Richard, D. S., Jones, J. M., Barbarito, M. R., Cerula, S., Detweiler, J. P., Fisher, S. J., Brannigan, D. M., and Scheswohl, D. M. (2001) Vitellogenesis in diapausing and mutant Drosophila melanogaster: further evidence for the relative roles of ecdysteroids and juvenile hormones. J. Insect Physiol. 47, 905-913
14. Raikhel, A. S., and Dhadialla, T. S. (1992) Accumulation of yolk proteins in insect oocytes. Annu. Rev. Entomol. 37, 217-251
15. Raikhel, A. S., Kokoza, V. A., Zhu, J., Martin, D., Wang, S. F., Li, C., Sun, G., Ahmed, A., Dittmer, N., and Attardo, G. (2002) Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. Insect Biochem. Mol. Biol. 32, 1275-1286
16. Parthasarathy, R., Sheng, Z., Sun, Z., and Palli, S. R. (2010) Ecdysteroid regulation of ovarian growth and oocyte maturation in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **40**, 429-439

17. Parthasarathy, R., Sun, Z., Bai, H., and Palli, S. R. (2010) Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **40**, 405-414

18. Parthasarathy, R., and Palli, S. R. (2011) Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **41**, 294-305

19. Cruz, J., Martin, D., Pascual, N., Maestro, J. L., Piulachs, M. D., and Belles, X. (2003) Quantity does matter. Juvenile hormone and the onset of vitellogenesis in the German cockroach. *Insect Biochem. Mol. Biol.* **33**, 1219-1225

20. Belles, X. (2004) Vitellogenesis directed by juvenile hormone. In *Reproductive biology of invertebrates* (Raikhel, A. S. ed.), pp. 157-197, Science Publisher, Inc., Enfield/Plymouth

21. Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., and Dolezel, D. (2014) Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. *Insect Biochem. Mol. Biol.* **45**, 69-76

22. Lapointe, M. C., Koepple, J. K., and Nair, K. K. (1985) Follicle cell polyploidy in *Leucophaea maderae*: regulation by juvenile hormone. *J. Insect Physiol.* **31**, 187-193

23. Buntrock, L., Marec, F., Krueger, S., and Traut, W. (2012) Organ growth without cell division: somatic polyploidy in a moth, *Ephesia kuehniella*. *Genome* **55**, 755-763

24. Nordman, J., and Orr-Weaver, T. L. (2012) Regulation of DNA replication during development. *Development* **139**, 455-464

25. Jacobson, A. L., Johnston, J. S., Rotenberg, D., Whitfield, A. E., Booth, W., Vargo, E. L., and Kennedy, G. G. (2013) Genome size and ploidy of Thysanoptera. *Insect Mol. Biol.* **22**, 12-17

26. Edgar, B. A., and Orr-Weaver, T. L. (2001) Endoreplication cell cycles: more for less. *Cell* **105**, 297-306

27. Lee, H. O., Davidson, J. M., and Duronio, R. J. (2009) Endoreplication: polyploidy with purpose. *Genes Dev.* **23**, 2461-2477

28. Wielgus, J. J., Bollenbacher, W. E., and Gilbert, L. I. (1979) Correlations between epidermal DNA synthesis and haemolymph ecdysteroid titre during the last larval instar of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* **25**, 9-16

29. Dean, R. L., Bollenbacher, W. E., Locke, M., Smith, S. L., and Gilbert, L. I. (1980) Haemolymph ecdysteroid levels and cellular events in the intermoult/moult sequence of *Calpodes ethlius*. *J. Insect Physiol.* **26**, 267-280

30. Koyama, T., Iwami, M., and Sakurai, S. (2004) Ecdysteroid control of cell cycle and cellular commitment in insect wing imaginal discs. *Mol. Cell Endocrinol.* **213**, 155-166

31. Sun, J., Smith, L., Armento, A., and Deng, W. M. (2008) Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling. *J. Cell Biol.* **182**, 885-896

32. Nair, K. K., Chen, T. T., and Wyatt, G. R. (1981) Juvenile hormone-stimulated polyploidy in adult locust fat body. *Dev. Biol.* **81**, 356-360

33. Oishi, M., Locke, J., and Wyatt, G. R. (1985) The ribosomal RNA genes of *Locusta migratoria*: copy number and evidence for underreproduction in a polyploid tissue. *Can. J. Biochem. Cell Biol.* **63**, 1064-1070
34. Piatti, S., Lengauer, C., and Nasmyth, K. (1995) Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *Embo J.* 14, 3788-3799
35. Perkins, G., and Diffley, J. F. (1998) Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Mol. Cell* 2, 23-32
36. Weinreich, M., Liang, C., and Stillman, B. (1999) The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 96, 441-446
37. Evrin, C., Fernandez-Cid, A., Zech, J., Herrera, M. C., Riera, A., Clarke, P., Brill, S., Lurz, R., and Speck, C. (2013) In the absence of ATPase activity, pre-RC formation is blocked prior to MCM2-7 hexamer dimerization. *Nucleic Acids Res.* 41, 3162-3172
38. Sun, J., Evrin, C., Samel, S. A., Fernandez-Cid, A., Riera, A., Kawakami, H., Stillman, B., Speck, C., and Li, H. (2013) Cryo-EM structure of a helicase loading intermediate containing ORC-Cdc6-Cdt1-MCM2-7 bound to DNA. *Nat. Struct. Mol. Biol.* 20, 944-951
39. Lei, M., and Tye, B. K. (2001) Initiating DNA synthesis: from recruiting to activating the MCM complex. *J. Cell Sci.* 114, 1447-1454
40. Takara, T. J., and Bell, S. P. (2011) Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 helicases. *Embo J.* 30, 4885-4896
41. Riera, A., and Speck, C. (2015) Opening the gate to DNA replication. *Cell Cycle* 14, 6-8
42. Ayad, N. G. (2005) CDKs give Cdc6 a license to drive into S phase. *Cell Cycle* 12, 825-827
43. Riera, A., Li, H., and Speck, C. (2013) Seeing is believing: the MCM2-7 helicase trapped in complex with its DNA loader. *Cell Cycle* 12, 2917-2918
44. Evrin, C., Fernandez-Cid, A., Riera, A., Zech, J., Clarke, P., Herrera, M. C., Tognetti, S., Lurz, R., and Speck, C. (2014) The ORC/Cdc6/MCM2-7 complex facilitates MCM2-7 dimerization during prereplicative complex formation. *Nucleic Acids Res.* 42, 2257-2269
45. Zhou, S., Zhang, J., Fam, M. D., Wyatt, G. R., and Walker, V. K. (2002) Sequences of elongation factors-1 alpha and -1 gamma and stimulation by juvenile hormone in *Locusta migratoria*. *Insect Biochem. Mol. Biol.* 32, 1567-1576
46. Chinzei, Y., White, B. N., and Wyatt, G. R. (1982) Vitellogenin mRNA in locust fat body: identification, isolation, and quantitative changes induced by juvenile hormone. *Can. J. Biochem.* 60, 243-251
47. Wang, X., Fang, X., Yang, P., Jiang, X., Jiang, F., Zhao, D., Li, B., Cui, F., Wei, J., Ma, C., Wang, Y., He, J., Luo, Y., Wang, Z., Guo, X., Guo, W., Wang, X., Zhang, Y., Yang, M., Hao, S., Chen, B., Ma, Z., Yu, D., Xiong, Z., Zhu, Y., Fan, D., Han, L., Wang, B., Chen, Y., Wang, J., Yang, L., Zhao, W., Feng, Y., Chen, G., Lian, J., Li, Q., Huang, Z., Yao, X., Lv, N., Zhang, G., Li, Y., Wang, J., Wang, J., Zhu, B., and Kang, L. (2014) The locust genome provides insight into swarm formation and long-distance flight. *Nat. Commun.* 5, 2957
48. Shin, S. W., Zou, Z., Saha, T. T., and Raikhel, A. S. (2012) bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 16576-16581
49. Chen, S., Yang, P., Jiang, F., Wei, Y., Ma, Z., and Kang, L. (2010) De novo analysis of transcriptome dynamics in the migratory locust during the development of phase traits. *PLoS One* 5, e15633
50. Dale, J. F., and Tobe, S. S. (1986) Biosynthesis and titre of juvenile hormone during the first gonotrophic cycle in isolated and crowded *Locusta migratoria* females. *J. Insect Physiol.* 32,
Cdc6 and JH-modulated polyploidy and reproduction

763-769
51. Glinka, A. V., Braun, R. P., Edwards, J. P., and Wyatt, G. R. (1995) The use of a juvenile hormone binding protein for the quantitative assay of juvenile hormone. *Insect Biochem. Mol. Biol.* **25**, 775-781

52. He, Q., Wen, D., Jia, Q., Cui, C., Wang, J., Palli, S. R., and Li, S. (2014) Heat shock protein 83 (Hsp83) facilitates methoprene-tolerant (Met) nuclear import to modulate juvenile hormone signaling. *J. Biol. Chem.* **289**, 27874-27885

53. Zou, Z., Saha, T. T., Roy, S., Shin, S. W., Backman, T. W., Girke, T., White, K. P., and Raikhel, A. S. (2013) Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E2173-2181

54. Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998) Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. *Mol. Cell Biol.* **18**, 6679-6697

55. Jin, F., and Fondell, J. D. (2009) A novel androgen receptor-binding element modulates Cdc6 transcription in prostate cancer cells during cell-cycle progression. *Nucleic Acids Res.* **37**, 4826-4838

56. Ren, D., Cai, Z., Song, J., Wu, Z., and Zhou, S. (2014) dsRNA uptake and persistence account for tissue-dependent susceptibility to RNA interference in the migratory locust, *Locusta migratoria*. *Insect Mol. Biol.* **23**, 175-184

57. Dhadialla, T. S., Cook, K. E., and Wyatt, G. R. (1987) Vitellogenin mRNA in locust fat body: coordinate induction of two genes by a juvenile hormone analog. *Dev. Biol.* **123**, 108-114

58. Donovan, S., Harwood, J., Drury, L. S., and Diffley, J. F. (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5611-5616

59. Tanaka, T., Knapp, D., and Nasmyth, K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**, 649-660

60. Schoenfelder, K. P., and Fox, D. T. (2015) The expanding implications of polyploidy. *J. Cell Biol.* **209**, 485-491

61. Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F., and Serrano, M. (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature* **440**, 702-706

62. Borlado, L. R., and Mendez, J. (2008) CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis* **29**, 237-243

63. Petrakis, T. G., Vougas, K., and Gorgoulis, V. G. (2012) Cdc6: a multi-functional molecular switch with critical role in carcinogenesis. *Transcription* **3**, 124-129

64. Okayama, H. (2012) Cell cycle control by anchorage signaling. *Cell. Signal.* **24**, 1599-1609

65. Pratt, G. E., and Davey, K. G. (1972) The corpus allatum and oogenesis in *Rhodnius prolixus* (Stal) I. The effects of allatectomy. *J. Exp. Biol.* **56**, 201-214

66. Sclafani, R. A., and Holzen, T. M. (2007) Cell cycle regulation of DNA replication. *Annu. Rev. Genet.* **41**, 237-280

67. Crevel, G., Mathe, E., and Cotterill, S. (2005) The *Drosophila* Cdc6/18 protein has functions in both early and late S phase in S2 cells. *J. Cell Sci.* **118**, 2451-2459

68. Mallik, I., Davila, M., Tapia, T., Schanen, B., and Chakrabarti, R. (2008) Androgen regulates Cdc6 transcription through interactions between androgen receptor and E2F transcription
Cdc6 and JH-modulated polyploidy and reproduction

factor in prostate cancer cells. *Biochim. Biophys. Acta* **1783**, 1737-1744

**Footnote**
The abbreviations used are: Cdc6, cell-division-cycle 6; JH, juvenile hormone; 20E, 20-hydroxyecdysone; Met, methoprene tolerant; SRC, steroid receptor co-activator; Gce, germ cell-expressed; Vg, vitellogenin; Mcm, mini-chromosome maintenance; RNAi, RNA interference; dsRNA, double-stranded RNA; qRT-PCR, quantitative real time RT-PCR; WB, Western blot; IP, immunoprecipitation; EMSA, electrophoresis mobility shift assay.
Figure legends

Figure 1. CDC6 expression and response to juvenile hormone in adult female locusts. (A) Relative mRNA levels of CDC6 in seven selected tissues from adult females at 8 days post adult eclosion (PAE). CDC6 mRNA levels in the fat body were used as the calibrator. Fb, fat body; Ov, ovary; Cu, cuticle; Mg, midgut; Th, thorax muscle; Bl, back leg; He, head. Different letters indicate significant difference at \( P < 0.05 \). \( n = 12-16 \). (B) Developmental profiles of CDC6 abundance in the fat body and ovary of adult females from the day of eclosion (0 PAE) to 8 days PAE. CDC6 mRNA levels at 0 PAE were arbitrarily set to 1.0. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \). \( n = 12-16 \). (C-D) Relative mRNA levels of CDC6 in the fat body (C) and ovary (D) of adult females treated with precocene for 10 days (P) and those further treated with methoprene or acetone (solvent control) for 6-48 h. PAE10, 10-day-old adult females as the positive control. Fb, fat body; Ov, ovary. *, \( P < 0.05 \) and **, \( P < 0.01 \). \( n = 6-8 \).

Figure 2. Responsiveness of CDC6 expression to Met. (A) Met knockdown efficiency in the fat body on day 4-8. **, \( P < 0.01 \) and ***, \( P < 0.001 \) compared to the respective dsGFP controls. \( n = 8 \). (B) Effect of Met knockdown on CDC6 expression in the fat body on day 4-8. *, \( P < 0.05 \) compared to the respective dsGFP controls. \( n = 8 \). (C) Alignment of DNA sequences containing the E-box-like motif in the upstream of Kr-h1, Rps28 and Early trypsin (ET) from the mosquito Aedes aegypti (Aa) (8, 48, 53), Kr-h1 from Drosophila melanogaster (Dm) (52), Mcm4 and CDC6 from the locust Locusta migratoria (Lm) (1). (D) EMSA using the ^32^P-CDC6 probe and fat body nuclear protein extracts from dsMet- or dsGFP- treated adult females on day 8. A representative experiment is shown. Arrow indicates the specific band. FP , free probe. (E) Quantitative analysis of the intensity of specific band by ImageJ. *, \( P < 0.05 \). \( n = 3 \).

Figure 3. Transcriptional regulation of CDC6 by the JH-receptor. (A) RNAi efficiency of Drosophila Met, Gce and Tai in S2 cells treated with Drosophila Met and Tai dsRNA (dsDmMet+dsDmTai), compared to the dsGFP controls. *, \( P < 0.05 \) and **, \( P < 0.01 \). \( n = 3 \). (B) Effect of dsDmMet+dsDmTai treatment on the expression of Flag-Met (Met) and SRC-V5 (SRC) in S2 cells. \( n = 3 \). (C) Western blot (WB) and immunoprecipitation (IP) showing the expression of Flag-Met (second panel from the top) and SRC-V5 (third panel from the top) in S2 cells, and the interaction of Flag-Met and SRC-V5 in the presence of JH III or methoprene (upper panel). α-Flag, anti-Flag antibody; α-V5, anti-V5 antibody; α-Actin, anti-actin antibody. (D) Luciferase reporter assay using S2 cells transfected with pGL4.10/CDC6-1253 to -60 alone (control), pGL4.10/CDC6-1253 to -60 + pAc5.1/Flag-Met (Met), pGL4.10/CDC6-1253 to -60 + pAc5.1/SRC-V5 (SRC), pGL4.10/CDC6-1253 to -60 + pAc5.1/Flag-Met + pAc5.1/SRC-V5 (Met+SRC). Me, methoprene (10 µM). (E) EMSA using the ^32^P-CDC6 probe and nuclear protein extracts from S2 cells with expressed Flag-Met and SRC-V5 and treated with 10 µM methoprene (Me). AP2, a nonspecific oligonucleotide; α-Flag, anti-Flag antibody; α-V5, anti-V5 antibody. Arrow indicates the specific band. FP , free probe.

Figure 4. CDC6 RNAi efficiency in the fat body and ovary. (A) CDC6 RNAi efficiency in the fat body (Fb) of adult females at 4-8 days post adult eclosion. (B) CDC6 RNAi efficiency in
the ovary (Ov) of adult females at 4-8 days post adult eclosion.*, *P < 0.05 compared to the respective dsGFP controls; n.s., no significant difference. n = 8.

**Figure 5.** *Cdc6* knockdown reduces ploidy in the fat body and follicle cells. (A, B) Morphology of fat body (A) and follicle cells (B) of dsCdc6- vs. dsGFP-treated adult females at 4-8 days post adult eclosion. Fb, fat body; Fc, follicle cells; Blue, nuclei; green, F-actin. Yellow arrows indicate cells with double nuclei. White bar, 20 µm. (C, D) Flow cytometry analysis showing DNA contents in the fat body (C) and follicle cells (D) of dsCdc6- vs. dsGFP-treated adult females at 4 to 8 days post adult eclosion. Six locusts were used in each analysis. Fb, fat body; Fc, follicle cells.

**Figure 6.** *Cdc6* knockdown blocks vitellogenesis and oogenesis. (A) VgA expression levels in fat bodies of dsCdc6-treated adult females at 4-8 days post adult eclosion. *, *P < 0.05 compared to the respective dsGFP controls. n = 8. (B) Comparison of primary oocytes (Po), ovarioles (Ol) and ovaries (Ov) of dsCdc6- and dsGFP-treated adult females at 4-8 days post adult eclosion. Scale bars: white, 5 mm; black, 0.5 mm. (C) Statistical analysis for length*width index of primary oocytes of dsCdc6- and dsGFP-treated adult females at 4-8 days post adult eclosion. ***, *P < 0.001 compared to the respective dsGFP controls. n = 25-30.

**Figure 7.** Methoprene treatment is unable to rescue the defective phenotypes resulted from *Cdc6* RNAi. *Cdc6* dsRNA was injected within 12 h post adult eclosion and boosted on day 5. Methoprene or acetone was applied on day 6, and the effects were examined on day 8. (A) Morphology of fat body (Fb) and follicle cells (Fc) of adult females injected with dsGFP further treated with acetone (dsGFP), dsCdc6 further treated with acetone (dsCdc6), or dsCdc6 further treated with methoprene (dsCdc6+Me). Blue, nuclei; green, F-actin. Yellow arrows indicate cells with double nuclei. Scale bar, 20 µm. (B) Flow cytometry analysis showing DNA contents in the fat body (Fb) and follicle cells (Fc) of three experimental groups. Six locusts were used in each analysis. (C) Relative VgA expression in the fat body of three groups. *, *P < 0.05 compared to the dsGFP+acetone control (dsGFP). n.s., no significant difference. n = 8. (D) Comparison of primary oocytes (Po), ovarioles (Ol) and ovaries (Ov) of three groups. Scale bars: white, 5 mm; black, 0.5 mm. (E) Statistical analysis for length*width index of primary oocytes of three groups. ***, *P < 0.001 compared to the dsGFP+acetone control (dsGFP). n.s., no significant difference. n = 25-30.
Table 1. Primers used for qRT-PCR and RNAi

| Gene    | Forward primer          | Reverse primer          | Product (bp) |
|---------|-------------------------|-------------------------|--------------|
| qRT-PCR |                         |                         |              |
| Cdc6    | CGTGCGTTAGACATTTGGGA    | GGTTGGCTGGATAGATTCA     | 109          |
| VgA     | CCCACAAAGACACAGAAACG    | TTGTCGCCATCAAACAGAAG    | 99           |
| Met     | CCACTTACAGGCCCTTGCTA    | GCCCTTCTTCACCTTCCTT    | 144          |
| β-actin | AATTACCATTGGAAACGAGCGATT| TCGTCTCCATACCCAGGAATGA | 73           |
| DmMet   | GTCCCTTAGATTCGCCCAACC   | GAGGCAGACATACCGGTCC     | 108          |
| DmGce   | CTCAATGCCCTTCACCTTCAT  | ACCTGTTCGTCTCTCTGTCC   | 182          |
| DmTaiman| AGCGATGTAAAGCCCGAGA     | AAGCAGACATTCACCCAC     | 127          |
| Dmβ-actin| ACTCCATCTGGAAAGTGAC    | ATCCGCAAGGATCTTGATGC   | 138          |
| RNAi    |                         |                         |              |
| Cdc6    | CATGGACGCGCGTTTCTTGGA  | TGCAGCACTTTCTCTATCC    | 466          |
| GFP     | CAAATCTTCAGCGTGTCGCC   | GTTACCTTTAGGCGTTTC     | 527          |
| Met     | TTAGGCGACATCGAAAGAAG    | TCGTGGAGGAAGTGGAT      | 421          |
| DmMet   | CTGCCAAGTCATCCCCGGATTTGC| CTTCGCGATAGCACTTTG    | 454          |
| DmTaiman| AGCAATCGACACCGACATCA   | GTCGTTCTGATAAGTGTTG    | 508          |

*Dm: Drosophila melanogaster*
Cdc6 and JH-modulated polyploidy and reproduction

Figure 1

A

B

C

D

Figure 2

A

B

C

D

E

Downloaded from http://www.jbc.org/ by guest on March 23, 2020
Figure 3

A

B

C

D

E

Figure 4

A

B
Figure 5

A

B

dsGFP

dsCdc6

C

D

Figure 6

A

B

dsGFP

dsCdc6

C

Downloaded from http://www.jbc.org/ by guest on March 23, 2020
Juvenile Hormone Activates the Transcription of Cell-division-cycle 6 (Cdc6) for Polyploidy-dependent Insect Vitellogenesis and Oogenesis
Zhongxia Wu, Wei Guo, Yingtian Xie and Shutang Zhou

J. Biol. Chem. published online January 4, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M115.698936

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts