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Characterization of a *Drosophila* Ortholog of the Cdc7 Kinase

*A ROLE FOR Cdc7 IN ENDOREPLICATION INDEPENDENT OF CHIFFON**

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Background: Cdc7 is a kinase that, together with its activator Dbf4, regulates the initiation of DNA replication.
Results: *Drosophila* Cdc7 is a functional ortholog of Cdc7 that is required for endoreplication and gene amplification, although Chiffon/Dbf4 is not essential for endoreplication.
Conclusion: Cdc7 can function during endoreplication independently of Chiffon.
Significance: Either Cdc7 functions alone during endocycling or undiscovered Cdc7 activator(s) exist in *Drosophila.*

Cdc7 is a serine-threonine kinase that phosphorylates components of the pre-replication complex during DNA replication initiation. Cdc7 is highly conserved, and Cdc7 orthologs have been characterized in organisms ranging from yeast to humans. Cdc7 is activated specifically during late G_1/S phase by binding to its regulatory subunit, Dbf4. *Drosophila melanogaster* contains a Dbf4 ortholog, Chiffon, which is essential for chorion amplification in *Drosophila* egg chambers. However, no *Drosophila* ortholog of Cdc7 has yet been characterized. Here, we report the functional and biochemical characterization of a *Drosophila* ortholog of Cdc7. Co-expression of *Drosophila* Cdc7 and Chiffon is able to complement a growth defect in yeast containing a temperature-sensitive Cdc7 mutant. Cdc7 and Chiffon physically interact and can be co-purified from insect cells. Cdc7 phosphorylates the known Cdc7 substrates Mcm2 and histone H3 *in vitro*, and Cdc7 kinase activity is stimulated by Chiffon and inhibited by the Cdc7-specific inhibitor XL413. *Drosophila* egg chamber follicle cells deficient for Cdc7 have a defect in two types of DNA replication, endoreplication and chorion gene amplification. However, follicle cells deficient for Chiffon have a defect in chorion gene amplification but still undergo endocycling. Our results show that Cdc7 interacts with Chiffon to form a functional Dbf4-dependent kinase complex and that Cdc7 is necessary for DNA replication in *Drosophila* egg chamber follicle cells. Additionally, we show that Chiffon is a member of an expanding subset of DNA replication initiation factors that are not strictly required for endoreplication in *Drosophila*.

DNA replication initiates from many genomic loci, termed origins of replication, during S phase of the cell cycle. To ensure that replication initiates only once from each origin per cell cycle during normal mitosis, replication initiation is divided into two distinct temporal phases as follows: origin licensing in late M/G_1 phase and origin firing during S phase (1). During origin licensing, the pre-replicative complex (pre-RC),[2] consisting of the origin recognition complex (ORC), Cdt1, and Cdc6, assembles on origins where it functions to load the Mcm2-7 DNA helicase onto chromatin in an inactive state. The subsequent activation of the Mcm2-7 DNA helicase is required for origin firing and the initiation of DNA replication, and this activation is controlled by a series of phosphorylation events catalyzed by the cell cycle kinases cyclin-dependent kinase and Cdc7 (2, 3). Together, cyclin-dependent kinase and Cdc7 phosphorylate components of the pre-RC and other replication initiation factors, leading to the recruitment of additional replisome components to the pre-RC, the formation of an active Cdc45·Mcm2-7·GINS (CMG) helicase complex, and the bidirectional progression of active replication forks away from the origin. The timing of both origin licensing and origin firing is tightly regulated by cell cycle-dependent oscillations of cyclin-dependent kinase and Cdc7 kinase activity (4, 5).

Whereas origins fire only once per cell cycle during normal mitosis, specialized tissues in both plants and animals can undergo a form of genome replication termed endoreplication, which consists of alternating G/S phases without any intervening mitotic division. Endoreplication in terminally differentiated cells such as ovarian nurse and follicle cells, and larval tissues in *Drosophila*, has been proposed to support growth and development by increasing metabolic capacity (6). Additionally, endoreplication may play a role in continued cell growth and repair in cells that are aging or undergoing other forms of genotoxic stress (7, 8). In humans, polyploidization is commonly observed in many types of cancer (8, 9), and endoreplication may be a contributing factor to both cancer development and resistance to chemotherapeutic agents (7, 10). During endoreplication in *Drosophila*, alternating G and S phases are controlled by oscillations in Cdk2-cyclin E activity (11–14). Because late replication patterns of DNA synthesis, including replication of heterochromatic sequences, are not observed in endoreplicating egg chamber cells, the *Drosophila* endocycle is assumed to have no S phase checkpoint ensuring that genomic replication is complete (12). In addition, an increasing body of

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[2] The abbreviations used are: pre-RC, pre-replicative complex; DDK, Dbf4-dependent kinase; Ni-NTA, nickel-nitrilotriacetic acid; ORC, origin recognition complex.

evidence suggests that the initiation of DNA replication may be fundamentally different between mitotically proliferating and endocycling cells, as several of the *Drosophila* licensing factors necessary for mitotic proliferation, including several ORC proteins (15, 16), Mcm2 (17), and Mcm4/dpa (18), are not strictly required for endocycling. For example, endoreplication continues in the absence of *Drosophila* ORC proteins, although the resulting genomic ploidy is reduced, and the pattern of genomic regions fully replicated during endocycling is altered (15, 16). This suggests that disruption of these pre-RC components leads to alteration, but not complete loss, of endocycling. In contrast, other pre-RC components, such as Cdt1/dup and Mcm6, are required for both endocycling and mitotic DNA replication (15, 19). Although Cdc7 plays a critical role in regulating the timing of origin firing during normal mitotic proliferation, its role in regulating endoreplication has not been characterized.

During mitosis, Cdc7 kinase activity peaks during the late G_1/S phase of the cell cycle due to its association with a cyclin-like regulatory subunit, Dbf4 (20). The Cdc7-Dbf4 (Dbf4-dependent kinase (DDK)) complex is capable of phosphorylating every component of the Mcm2-7 helicase *in vitro*, with the exception of Mcm5 (21, 22). Additionally, DDK-mediated phosphorylation of Mcm4 stimulates association of Cdc45 with Mcm2-7 (23, 24) and relieves an inhibitory activity within the Mcm4 N-terminal serine/threonine-rich domain (25). Thus, DDK-mediated phosphorylation of the Mcm2-7 helicase induces both direct conformational changes and creates new binding sites for regulatory factors, which together result in activation of the helicase and the initiation of DNA replication. In addition to Mcm2-7, DDK phosphorylates other substrates that might also regulate efficient origin firing. In yeast, DDK has been shown to phosphorylate Thr-45 of histone H3, which is a histone modification associated with proper DNA replication (26). Because two of the major targets of DDK, Mcm2 and Mcm4, are not required for endocycling in *Drosophila* (17, 18), it has been unclear as to whether DDK would be required for origin firing during this specialized form of DNA replication. Prior to this study, it was not possible to examine the role for DDK in endoreplication because although Cdc7 orthologs had been identified in other eukaryotes ranging from yeast to humans (27–36), and a Dbf4 subunit had been identified in *Drosophila* (37), the *Drosophila* ortholog of Cdc7 had not been experimentally identified.

Here, we identify the previously uncharacterized kinase lethal(1)G0148 as the *Drosophila* ortholog of Cdc7. We show that *Drosophila* DDK, composed of l(1)G0148 (Cdc7) and Chiffon, is capable of functionally complementing a Cdc7 deficiency in *Saccharomyces cerevisiae*. We further show that Cdc7 and Chiffon physically associate and that recombinant DDK phosphorylates the known Cdc7 substrates Mcm2 and histone H3 *in vitro*. Finally, we demonstrate that Cdc7 is necessary for both chorion gene amplification and endoreplication. Surprisingly, however, we find that although Chiffon is necessary for gene amplification, endoreplication in *Drosophila* continues in the absence of Chiffon, albeit with altered timing. We conclude that *Drosophila* Cdc7 is an ortholog of Cdc7 that is necessary for multiple forms of DNA replication in *Drosophila* and that although Chiffon/Dbf4 may play some role in the *Drosophila* endocycle, it is not essential for endoreplication to occur.

EXPERIMENTAL PROCEDURES

Construction of Alignment and Phylogenetic Tree—Cdc7 amino acid sequences were aligned using ClustalOmega, and a phylogenetic tree was constructed from the aligned sequences using the neighbor-joining method (MAFFT version 7). Bootstrap analysis of the predicted tree was performed, and confidence values were calculated.

Yeast Strains and Manipulation—The *Drosophila* Gene Collection (DGC) clone AT30978 was used as a template for PCR cloning of *Drosophila cdc7* (*l(1)G0148*, *CG32742*; FBgn0028360). *CG5790* (FBgn0032677) was amplified from OregonR genomic DNA. The cDNA for *chiffon* (*CG5813*; FBgn0000307) was amplified from OregonR 0–12-h embryo cDNA. Coding sequences for *S. cerevisiae CDC7, Drosophila cdc7,* and *CG5790* were cloned into the galactose-inducible yeast expression vector pRS313gal (38). Full-length *chiffon* and *chiffonN* (N-terminal 1–400 amino acids) coding sequences were cloned into pRS316gal (38). Constructs were co-transformed into *cdc7-90 S. cerevisiae* (AKY1664 *MAT**a** HMR**ae**** cdc7-90 ade2-1 his3 leu2-3,112 ura3 can1-100*) (39). Assays were performed to assess the growth of yeast strains at permissive and restrictive temperatures. Briefly, yeast strains were grown overnight in selective media with 2% glucose, subcultured to $A_{600} = 0.5$, and grown to $A_{600} \sim 1.0$. Cells were then diluted to $A_{600} = 1.0$, spotted on pre-warmed selective media containing 2% galactose and 1% raffinose as a 10-fold serial dilution, and incubated at permissive (23 °C) or restrictive (30 °C) temperatures. For Western analysis of ectopic protein expression, $\sim 3.6 \times 10^8$ cells were harvested from liquid cultures induced with 2% galactose. For co-immunoprecipitation, $\sim 1.5 \times 10^{10}$ cells were harvested from liquid cultures induced with 2% galactose, washed in PBS, and resuspended in Yeast Lysis Buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 5 mM EDTA, supplemented with protease and phosphatase inhibitors). Cells were mechanically lysed in the presence of 0.5-mm diameter zirconia/silica beads, and lysates were clarified by centrifugation at 17,500 × *g* at 4 °C. 3 mg of each lysate was incubated with 25 μl of anti-HA affinity gel (Sigma) for 2 h at 4 °C with rotation, washed four times with Yeast Lysis Buffer, and analyzed by SDS-PAGE and Western blotting.

Western Blots—The following antibodies were used: HA-HRP (Roche Applied Science); FLAG-HRP (Sigma); rabbit histone H3 (Active Motif). Western blots were developed using Luminata Crescendo HRP substrate (Millipore), imaged on a Bio-Rad ChemiDocXRS, and analyzed using ImageLab (version 5.0) software.

Mutagenesis of cdc7—A D269N substitution mutant of *cdc7* was generated using the QuikChange system (Stratagene). pRS313gal-Cdc7 was used as a template for mutagenesis PCR with the following primers: 5′ CGG CGA GAG TTT CTC CTC GTT AAC TTC GGT CTG GCC CAG CAT GTG 3′ and 5′ CAC ATG CTG GGC CAG ACC GAA GTT AAC GAG GAG AAA CTC TCG CCG 3′. Mutagenesis products were verified by sequencing.

Cloning and Purification of Mcm2-GST and Mcm4-GST—The following DGC clones were used as templates for PCR cloning: LD47441 for *Mcm2* (*CG7538*, FBgn0014861) and RE04051 for *Mcm4* (disc proliferation abnormal, *CG1616*, FBgn0015929). The cDNA regions encoding the N-terminal 1–279 amino acids of *Mcm2* and the N-terminal 1–233 amino acids of Mcm4 were cloned into pGex6P1, expressed, and purified from *Escherichia coli*, and dialyzed into Elution Buffer II (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol).

Cloning and Purification of Recombinant Cdc7 and DDK from Insect Cells—Coding sequences for *Cdc7* and *ChiffonN* (N-terminal 1–400 amino acids) were cloned into pBACPAK8 vectors with N-terminal His-FLAG or 2×HA epitope tags, respectively. pBACPAK8-HisFLAG-Cdc7 and pBACPAK8-HA-ChiffonN were transfected into Sf21 cells, and viral supernatants were generated as described previously (40). Sf21 cells were infected with HisFLAG-Cdc7 alone to purify Cdc7 or with HisFLAG-Cdc7 and HA-ChiffonN to purify DDK, and recombinant protein or complex was purified using tandem affinity chromatography. Briefly, cells were lysed in Lysis Buffer (50 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 10% glycerol, 0.2% Triton X-100, 20 mM imidazole, supplemented with complete protease inhibitor mixture (Roche Applied Science)) containing either 500 mM (Cdc7) or 300 mM (DDK) NaCl, and the soluble supernatant was incubated with Ni-NTA-agarose (Cdc7 and DDK), washed in lysis buffer, and eluted in Ni-NTA Elution Buffer I (50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 250 mM imidazole). Ni-NTA elutions were pooled and bound to either anti-FLAG affinity gel (Sigma; Cdc7) or anti-HA affinity gel (Sigma; DDK). Beads were washed twice with High Salt Wash Buffer (50 mM HEPES, pH 7.5, 10% glycerol, 300 mM NaCl) and once with Low Salt Wash Buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol), and Cdc7 or DDK was eluted in Elution Buffer II (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, supplemented with complete protease inhibitor mixture (Roche Applied Science)) containing either 0.5 mg/ml 3×FLAG peptide or 0.2 mg/ml 3×HA peptide.

Kinase Assays—In vitro kinase assays were performed as described previously (41) with the following modifications. Briefly, 25-μl reaction volumes containing a final concentration of 40 mM HEPES-NaOH, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT, 10 mM magnesium acetate, 0.1 mM unlabeled ATP, and 2 μCi of [^{32}P]ATP (PerkinElmer Life Sciences, catalog no. BLU502A250UC), substrate (0.5 μg of Mcm4-GST or Mcm2-GST or 2 μg of purified HeLa core histones), and Cdc7 (6, 12, or 24 ng) or DDK (6, 12, 24 ng Cdc7 with co-purified ChiffonN) were incubated at 30 °C for 30 min (Mcm2/4 as substrate) or 60 min (core histones as substrate). The XL413 inhibitor was synthesized by us as described previously (42) and solubilized in DMSO. Reaction mixtures were separated by SDS-PAGE (10% gel, Mcm2/4; 15% gel, HeLa core histones), stained with Coomassie, imaged, dried, and exposed to a phosphor screen (GE Healthcare). Screens were scanned on a Typhoon phosphorimager, and images were analyzed using ImageQuant TL software.

Drosophila Stocks—cdc7^{G0148} flies (*w^{67c23} P{w^{+mC} = lacW}cdc7^{G0148}/FM7c*) were obtained from the Bloomington *Drosophila* Stock Center (stock number 11937). *chif^{ETBE3}* flies (*chif^{ETBE3}/CyO*) were kindly provided by John Towe, and contain an ~6-kb genomic deletion in *chiffon* together with a mutation in a second gene, *cactus* (37). For the generation of Cdc7 rescue flies, the genomic region containing the coding sequence and regulatory elements for *cdc7* (chromosome X, 6548176-6554728) consisting of −1007 bp from the start codon (ATG, +1) of *cdc7* (within the adjacent gene *CG3226*) to +5546 bp (within the adjacent gene *CG3224*) was cloned into the pCa4B vector and integrated into the *attP40* site using site-directed transgenesis (43, 44) to generate *w^{1118}*; *P{w^{+mC} = Cdc7}attP40* flies. The Cdc7 rescue stock was crossed with *w^{67c23} P{w^{+mC} = lacW}cdc7^{G0148}/FM7c, P{w^{+mC} = GAL4-twi.G}108.4, P{UAS-2×EGFP}AX* flies using standard genetic techniques to generate *w^{67c23} P{w^{+mC} = lacW}cdc7^{G0148}*; *P{w^{+mC} = Cdc7}attP40* flies, which are homozygous viable (Table 1).

Immunohistochemistry—Somatic clones were induced in egg chambers from *w^{67c23} P{w^{+mC} = lacW}cdc7^{G0148}, P{w$^{+mW.hs}$ = FRT(w^{hs})}101/y^{1} w^{67c23}, P{w^{+mC} = Ubi-GFP.D}ID-1, P{w$^{+mW.hs}$ = FRT(w^{hs})}101*; *P{w$^{+mW.hs}$ = en2.4-GAL4}e22c, P{w^{+mC} = UAS-FLP1.D}JD1* or *y^{1} w^{1118}, P{ry$^{+t7.2}$ = 70FLP}3F; chif^{ETBE3}, P{ry$^{+t7.2}$ = neoFRT}40A/P{w^{+mC} = Ubi-GFP(S65T)nls}2L, P{ry$^{+t7.2}$ = neoFRT}40A* adult females. As a control, wild-type clones were induced in egg chambers from *y^{1} w* v^{24} P{w$^{+mW.hs}$ = FRT$^{w(hs)}$}101/y^{1} w^{67c23}, P{w^{+mC} = Ubi-GFP.D}ID-1, P{w$^{+mW.hs}$ = FRT(w^{hs})}101*; *P{w$^{+mW.hs}$ = en2.4-GAL4}e22c, P{w^{+mC} = UAS-FLP1.D}JD1* adult females. Clones were induced without heat shock for *cdc7^{G0148}* and wild-type *FRT101* controls and with 40 min of heat shock at 37 °C on the day prior to ovary dissection for *chif^{ETBE3}* females. Ovaries were dissected from adult females at 3 days post-eclosion, labeled with 5-bromo-2-deoxyuridine (BrdU), fixed, and immunostained as described previously (45). BrdU-labeled egg chambers were immunostained with mouse anti-BrdU antibody (Pharmingen) followed by anti-mouse Alexa568 antibody (Invitrogen) and stained with DAPI. A Nikon A1R-MP microscope was used to image immunostained egg chambers, and images were analyzed using NIS-Elements software. Confocal images are presented as reconstructed maximum intensity projections of multiple *z*-stack planes for each egg chamber.

RESULTS

Drosophila Contains Two Cdc7 Homologs—Cdc7 orthologs have been identified and characterized from numerous model organisms (27–36), but no characterization of a *Drosophila* Cdc7 ortholog had been reported prior to this study. However, because the Cdc7-Dbf4 (DDK) kinase plays a highly conserved role in the initiation of DNA replication, and because a Dbf4 ortholog, Chiffon, has previously been identified in *Drosophila* (37), we predicted that the *Drosophila* genome would also encode a homolog of Cdc7. We identified two potential candidates for *Drosophila* Cdc7 by BLAST search, lethal(1)G0148 and CG5790, and compared these proteins to known Cdc7 orthologs using phylogenetic analysis (Fig. 1*A*). l(1)G0148 and CG5790 share 26–29% overall amino acid identity with *S. cerevisiae* Cdc7, *S. pombe* Hsk1 and Spo4, and 35–36% amino acid identity with *Xenopus*, mouse, and human Cdc7 orthologs. In addition, l(1)G0148 and CG5790 are 44% identical overall

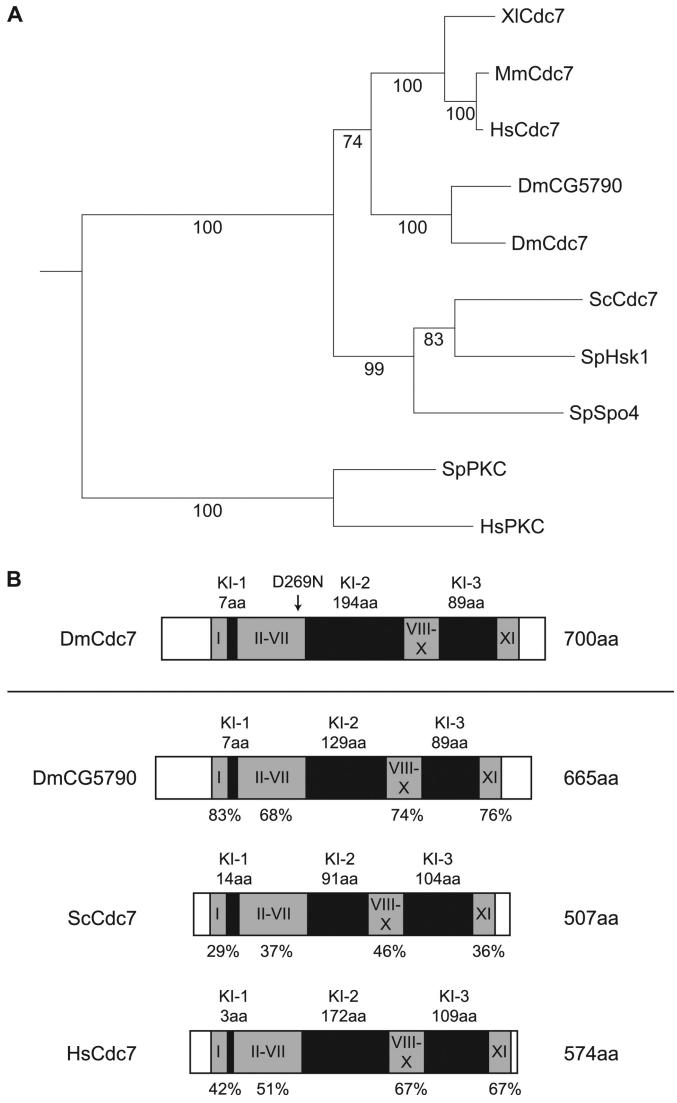

FIGURE 1. **Phylogenetic and schematic comparison of *Drosophila* Cdc7 orthologs.** *A,* phylogenetic tree comparing Cdc7 orthologs and two putative *Drosophila melanogaster* Cdc7 orthologs, l(1)G0148 (Cdc7) and CG5790. The phylogenetic tree was constructed from aligned protein sequences using the neighbor-joining method, and confidence values from bootstrap analysis of the predicted tree are indicated on *tree branches*. *S. pombe* protein kinase C and *Homo sapiens* protein kinase Cϵ type are included as an out-group. Accession numbers are as follows; *Xenopus laevis* Cdc7, AAD21532.1; *Mus musculus* Cdc7, AAH80702.1; *H. sapiens* Cdc7, AAC52080.1; *D. melanogaster* CG5790, NP_609876.2; *D. melanogaster* lethal(1)G0148 (Cdc7), AAF46180.2; *S. cerevisiae* Cdc7, CAA98574.1; *S. pombe* Hsk1, NP_596328.1; *S. pombe* Spo4, NP_596598; *S. pombe* PKC, AAA35323.1; *H. sapiens* PKCϵ type, NP_005391.1. *B*, schematic comparison of *D. melanogaster* Cdc7 with *D. melanogaster* CG5790, *S. cerevisiae* Cdc7, and *H. sapiens* Cdc7. The locations of conserved kinase domains I to XI are shown in *gray*, and the relative positions and lengths of Cdc7-specific kinase insert (*KI*) domains 1–3 are indicated in *black*. The percent identity of conserved kinase domains in each Cdc7 ortholog relative to the corresponding domains in DmCdc7 is indicated *below* each domain. The location of a mutation in a conserved kinase motif, D269N, is indicated with an *arrow* above *Drosophila* Cdc7. *aa,* amino acids.

and are more closely related to each other than to any other Cdc7 ortholog.

Cdc7 is known to contain characteristic kinase insert domains, which are large sequence inserts between the conserved kinase catalytic subdomains VII and VIII (kinase insert 2) and X and XI (kinase insert 3) (28–30, 32–34, 36, 46). To further characterize l(1)G0148 and CG5790, we compared the amino acid sequences of l(1)G0148 and CG5790 with *S. cerevisiae* Cdc7 and human Cdc7, and we found that l(1)G0148 and CG5790 both contain kinase insert domains at the same positions relative to the catalytic subdomains (Figs. 1*B* and 2). These results strongly suggest that l(1)G0148 and CG5790 are homologous to Cdc7, and thus, we conclude that l(1)G0148 and CG5790 are paralogs of Cdc7.

Because there are two paralogs of Cdc7 in *Drosophila*, we reasoned that one of these proteins might have developed an evolutionarily diverged function relative to other Cdc7 homologs. To determine which *Drosophila* Cdc7 paralog was most likely to function in mitotic proliferation, we examined the expression pattern of l(1)G0148 and CG5790. The *lethal(1)G0148* (FBgn0028360) gene encodes a 700-amino acid protein with a predicted mass of 79 kDa, whereas *CG5790* (FBgn0032677) encodes a 665-amino acid protein with a predicted mass of 75 kDa. High throughput tissue-specific transcript expression data shows that *l(1)G0148* is highly expressed in adult *Drosophila* ovaries and testes and has low to moderate expression in most other tissues throughout development (47). In contrast, *CG5790* is expressed almost exclusively in adult male testes, with moderate expression in imaginal discs (47). Two DDK complexes have previously been reported in another species. In *S. pombe*, Hsk1-Him1/Dfp1 controls the initiation of DNA replication during the S phase of mitosis (33, 48, 49), whereas Spo4-Spo6 executes a function unrelated to DNA replication during meiosis (34). Because *l(1)G0148* is expressed ubiquitously, and *CG5790* is testis-specific, CG5790 is unlikely to be required for DNA replication in all tissues. Furthermore, a large scale RNA-mediated interference screen in cultured *Drosophila* cells showed that knockdown of *l(1)G0148* resulted in delays in S phase progression (50), consistent with a potential role for l(1)G0148 in origin firing during mitotic proliferation. We have refrained from naming CG5790, despite its homology to Cdc7, because the function of this protein in DNA replication or meiosis is unclear. However, for the sake of clarity, we will hereafter refer to the second more widely expressed Cdc7 paralog, l(1)G0148, as Cdc7 based on the results presented below.

Expression of Drosophila DDK Restores Growth in Yeast Containing the Temperature-sensitive cdc7-90 Allele—It has been previously been shown that the human DDK complex is capable of functionally complementing a yeast strain deficient for DDK (51). We therefore asked whether the expression of *Drosophila* Cdc7 could similarly complement a yeast strain lacking functional DDK. We expressed *Drosophila* Cdc7 alone, or in combination with Chiffon, in a strain of *S. cerevisiae* that contains the temperature-sensitive *cdc7-90* allele (39). At the restrictive temperature, *cdc7-90* yeast fail to grow (Fig. 3*A*, *vector/vector control*), but growth is restored by the expression of *S. cerevisiae* Cdc7 (Fig. 3*A*, *ScCdc7/vector*). Notably, we observed that whereas expression of *Drosophila* Cdc7 alone could not restore growth at the restrictive temperature, co-expression of DmCdc7 and Chiffon complemented the temperature-sensitive growth defect (Fig. 3*A*, *DmCdc7/Chiffon*). These results are similar to those previously observed by Davey *et al.* (51) in which complementation of a nonfunctional yeast DDK required the expression of both the Cdc7 and Dbf4 (ASK) sub-

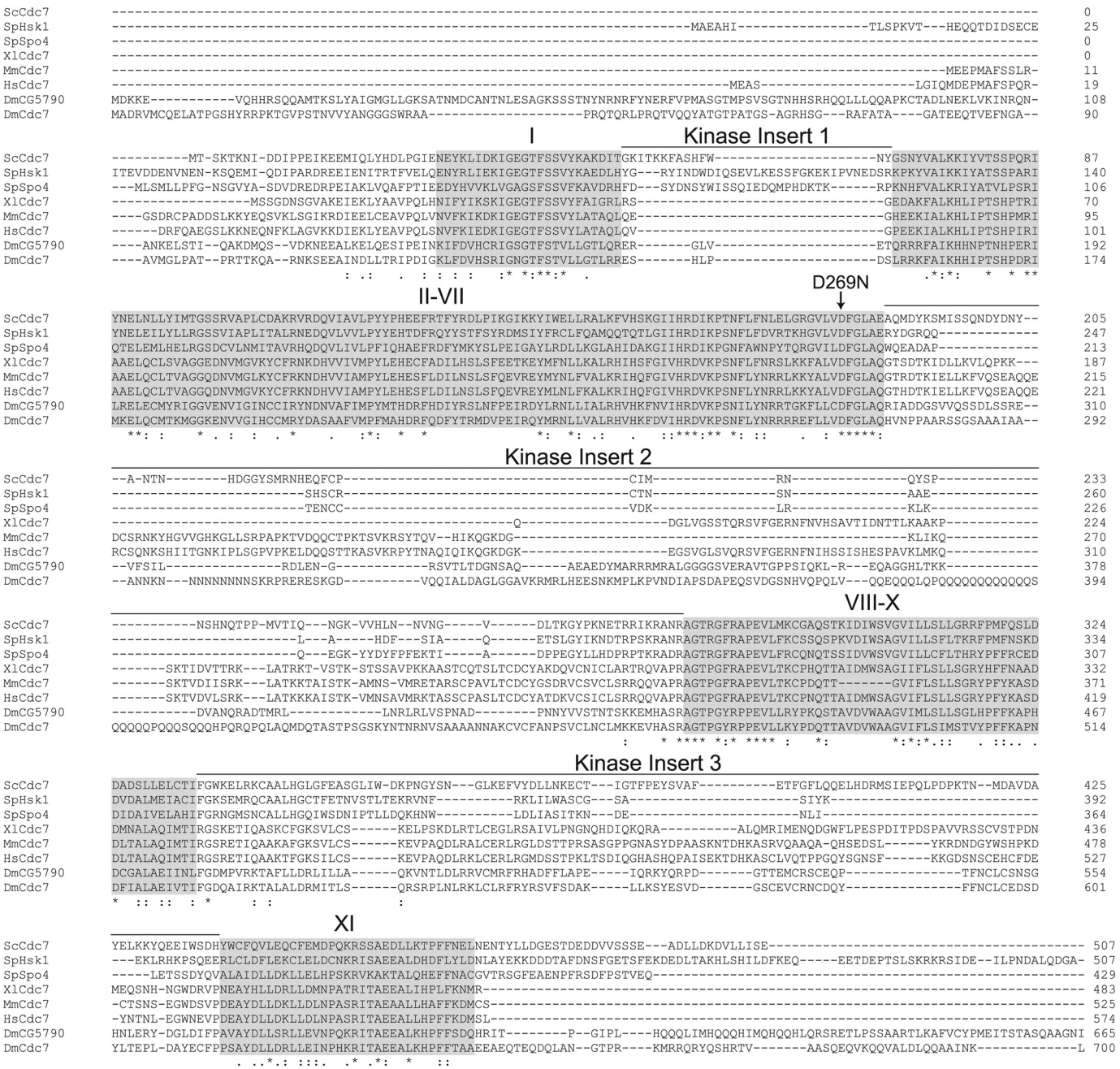

FIGURE 2. **Protein alignment of Cdc7 orthologs.** Alignment of *Drosophila* Cdc7 with Cdc7 orthologs as follows: *S. cerevisiae* Cdc7, CAA98574.1; *S. pombe* Hsk1, NP_596328.1; *S. pombe* Spo4, NP_596598; *X. laevis* Cdc7, AAD21532.1; *M. musculus* Cdc7, AAH80702.1; *H. sapiens* Cdc7, AAC52080.1; *D. melanogaster* CG5790, NP_609876.2; and *D. melanogaster* lethal(1)G0148 (Cdc7), AAF46180.2. The locations of conserved kinase subdomains *I* to *XI* are noted, along with the locations of Cdc7-specific kinase insert domains 1–3. Identical residues are indicated by an *asterisk*, and highly similar residues are indicated by a *colon*. An *arrow* indicates the location of a substitution mutation, D269N, in a conserved kinase motif within Cdc7.

units of the human DDK complex. Importantly, a Cdc7 substitution mutant, DmCdc7 D269N, which contains a mutation in a conserved DFG motif critical for phosphorylation (52), failed to complement the *cdc7-90* temperature-sensitive growth defect when co-expressed with Chiffon (Fig. 3*A*, *DmD269Cdc7/Chiffon*), even though its expression was detectable at similar levels as wild-type DmCdc7 (Fig. 3*B*). This finding indicates that the complementation of the *cdc7-90* growth defect is dependent on the kinase activity of *Drosophila* DDK. Notably, we failed to detect expression of full-length Chiffon in rescue strains at its expected size of 188 kDa; however, we consistently detected an N-terminal ~50-kDa fragment of Chiffon in these strains (Fig. 3*B*). This finding indicates that full-length Chiffon may be subject to proteolytic cleavage but that the N-terminal region of Chiffon is sufficient for both the interaction with and activation of Cdc7. We have also observed a similar truncation product when full-length Chiffon is expressed in Sf21 cells and in *Drosophila* S2 cells, indicating that Chiffon might also be subject to cleavage *in vivo* (data not shown).

We next asked whether CG5790 could also complement the mitotic growth defect in *cdc7-90* yeast. We expressed CG5790 alone, or in combination with Chiffon, in *cdc7-90* yeast as

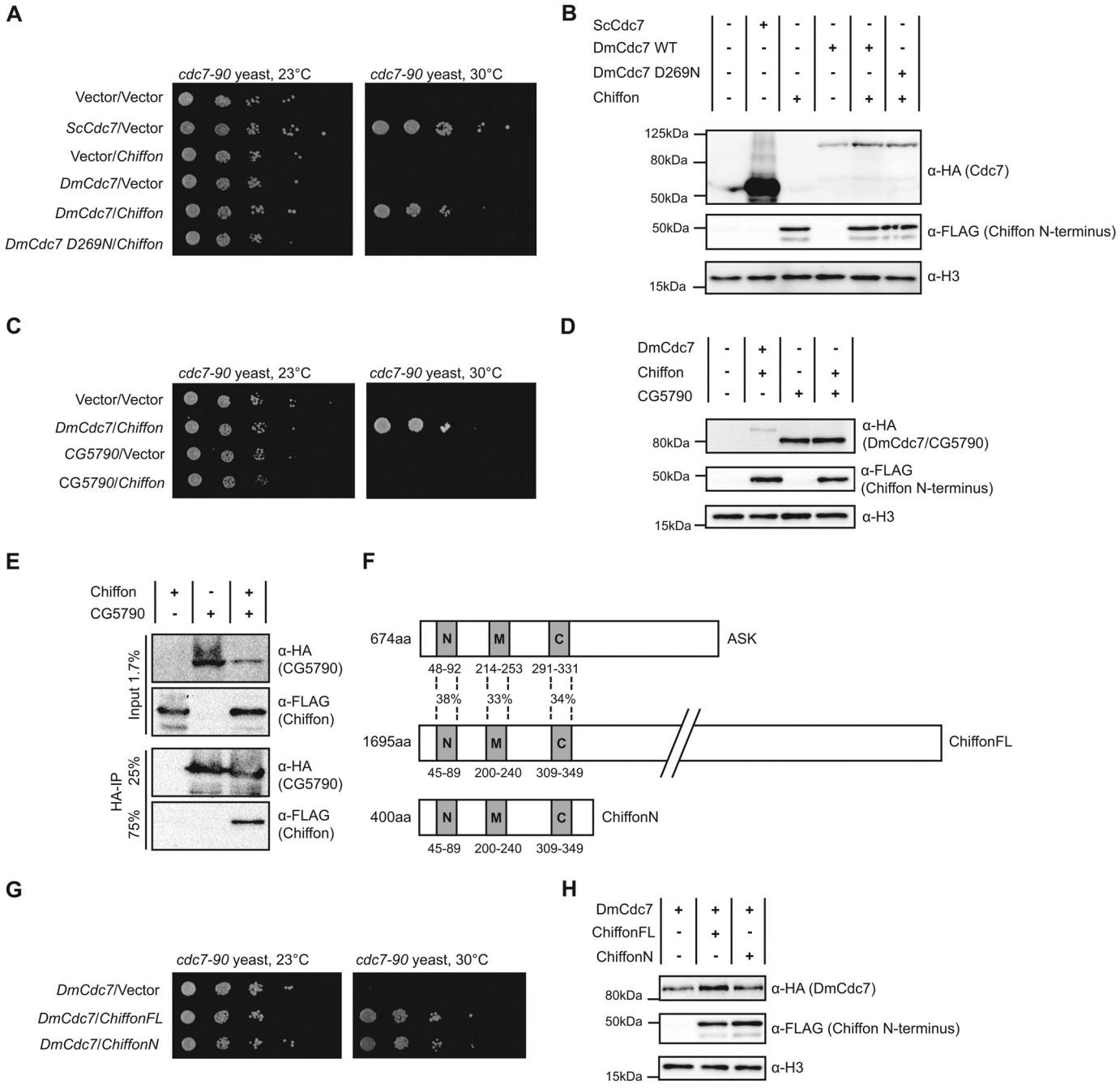

FIGURE 3. ***Drosophila* DDK complex can rescue a temperature-sensitive growth defect in *cdc7-90* yeast.** *A,* growth assay showing that co-expression of *D. melanogaster* Cdc7 and Chiffon restores growth at the restrictive temperature (30 °C) in yeast carrying the temperature-sensitive *cdc7-90* allele, whereas the expression of DmCdc7 with a mutation in a conserved kinase motif (D269N) does not. *cdc7-90* yeast were co-transformed with the indicated plasmid combinations, and yeast strains were plated on selective media and incubated at permissive (23 °C) or restrictive (30 °C) temperatures. *B,* Western blots verifying the expression of Cdc7 and Chiffon in the *cdc7-90* rescue strains shown in *A*. Cdc7 and Chiffon were expressed as fusion proteins with N-terminal epitope tags as follows: HA, Cdc7; FLAG, Chiffon. Histone H3 was analyzed as a loading control. *C,* growth assay showing that co-expression of *D. melanogaster* CG5790 and Chiffon does not restore growth of yeast carrying the temperature-sensitive *cdc7-90* allele at the restrictive temperature (30 °C), as described in *A*. Plasmid combinations contained within each yeast strain are as indicated. *D,* Western blots verifying the expression of Cdc7, CG5790, and Chiffon in the *cdc7-90* rescue strains shown in *C*, as described in *B*. CG5790 was expressed as a fusion protein with an N-terminal HA epitope tag. *E,* HA-CG5790 co-immunoprecipitates Chiffon in lysates from *cdc7-90* yeast strains ectopically expressing epitope-tagged CG5790 and Chiffon as described in *C*. Lysates were incubated with anti-HA affinity resin, and co-immunoprecipitating proteins were analyzed by SDS-PAGE and Western blotting. *F,* schematic comparing the locations of conserved N, M, and C domains in the Dbf4 orthologs ASK, Chiffon, and an N-terminal Chiffon truncation, ChiffonN (amino acids 1–400). The percent identity of the N, M, and C domains in human ASK and Chiffon is indicated. *G,* growth assay showing that co-expression of DmCdc7 with either full-length Chiffon (ChiffonFL) or ChiffonN restores growth at the restrictive temperature (30 °C) in yeast carrying the temperature-sensitive *cdc7-90* allele as described in *A*. *H,* Western blots verifying the expression of Cdc7 and full-length and truncated Chiffon in the *cdc7-90* rescue strains shown in *G*, as described in *B*.

described above. However, in contrast to Cdc7, co-expression of CG5790 and Chiffon failed to rescue the *cdc7-90* temperature-sensitive growth defect (Fig. 3*C*, *CG5790/chiffon*). To address the possibility that Chiffon may not interact with and activate CG5790, we examined whether CG5790 and Chiffon interact in *cdc7-90* yeast strains expressing both *Drosophila*

proteins. To do this, we immunoprecipitated HA-tagged CG5790 from yeast lysates using antibodies against HA, and we examined the co-immunoprecipitating proteins for the presence of FLAG-tagged Chiffon by Western blotting. We observe that the N-terminal portion of Chiffon co-immunoprecipitates with CG5790 (Fig. 3*E*), indicating that, although CG5790 cannot complement the mitotic growth defect of *cdc7-90* yeast, Chiffon does interact with CG5790 in *cdc7-90* yeast strains. Thus, we conclude that CG5790 does not functionally complement the mitotic growth requirement for *S. cerevisiae* Cdc7, suggesting that this Cdc7 paralog may have an alternative function in *Drosophila*. Together, these findings support our conclusion that l(1)G0148 is the *Drosophila* ortholog of Cdc7 that is involved in DNA replication initiation during mitotic proliferation and that CG5790 might have a non-DNA replication function specific to highly meiotic tissues, such as the testis. Additionally, our results suggest that CG5790 function in *Drosophila* testis could be regulated by Chiffon.

N-terminal Region of Chiffon Is Sufficient for the Interaction with and Activation of Cdc7—Because the N-terminal ~50-kDa degradation fragment of Chiffon appeared to be sufficient to activate *Drosophila* Cdc7 activity in the yeast rescue experiment (Fig. 3, *A* and *B*), we sought to determine whether the N-terminal region of Chiffon was indeed sufficient to interact with and activate Cdc7. The primary sequence of Dbf4 orthologs are generally poorly conserved, with the exception of three short regions as follows: the N, M, and C motifs (53, 54). Motifs M and C have been previously identified as being necessary for the binding and activation of Cdc7 by Dbf4 (55–57). We aligned Chiffon with previously characterized Dbf4 orthologs (20, 37, 48, 49, 58–66) to identify the N, M, and C motifs (Figs. 3*F* and 4). Motifs N and C in Chiffon were previously described as the CDDN2 and CDDN1 domains, respectively (37). Because the M and C motifs are contained within the N-terminal first 400 amino acids of Chiffon, we co-expressed a truncated version of Chiffon, ChiffonN, that consists of the first 400 amino acids, together with Cdc7 in *cdc7-90* yeast, and examined growth at the restrictive temperature. Co-expression of Cdc7 with the N-terminal truncation of Chiffon, ChiffonN, restored growth at the restrictive temperature, similar to expression of full-length Chiffon (Fig. 3*G*). Furthermore, the same ~50-kDa degradation fragment of Chiffon was detected in yeast expressing full-length Chiffon or ChiffonN (Fig. 3*H*), indicating that ChiffonN contains all the sequence present in the full-length Chiffon-presumed proteolytic cleavage product. We therefore conclude that the N-terminal 400 amino acids of Chiffon containing the N, M, and C motifs are sufficient for the ability of Cdc7 to rescue the mitotic growth defect of *cdc7-90* yeast *in vivo*.

We next sought to determine whether the N-terminal region of Chiffon was sufficient to interact with Cdc7 *in vitro*. To accomplish this, we co-expressed epitope-tagged Cdc7 and ChiffonN in Sf21 insect cells, and we purified the DDK complex using tandem affinity chromatography against both epitope tags. Using this approach, we purified Cdc7 alone (Fig. 5*A, lane 1*) or bound to ChiffonN (Fig. 5*A, DDK, lane 2*). These results indicate that Cdc7 and ChiffonN interact directly to form the DDK complex and that the N-terminal region of Chiffon is sufficient for this interaction. We note that ChiffonN co-purified with Cdc7 appears as a diffuse smear when separated by SDS-PAGE (Fig. 5, *A* and *B*). This result is consistent with observations of the human DDK complex, in which ASK appears as a smear on SDS-polyacrylamide gels due to autophosphorylation of DDK (41). Taken together, our results indicate that the N-terminal region of Chiffon is sufficient for both its interaction with Cdc7 and for rescue of the mitotic growth defect of *cdc7-90* yeast *in vivo*.

Chiffon Stimulates Cdc7 Kinase Activity, Which Is Inhibited by XL413—The DDK complex is proposed to regulate the initiation of DNA replication through phosphorylation of the Mcm2-7 helicase (2, 3, 67, 68). Because Dbf4 is required to activate Cdc7 kinase activity, the activity of DDK varies throughout the cell cycle because of oscillating Dbf4 protein levels, resulting in the activation of Cdc7 kinase activity specifically at late G_1/S phase (20, 21, 58, 69–71). Because Chiffon interacts with *Drosophila* Cdc7 *in vitro*, and is able to rescue the growth defect of *cdc7-90* yeast when co-expressed with Cdc7, we next sought to determine whether Chiffon activates the kinase activity of Cdc7. To accomplish this, we performed *in vitro* kinase assays with recombinant DDK (Cdc7 with co-purified ChiffonN) or Cdc7 alone on N-terminal fragments of *Drosophila* Mcm2 and Mcm4, which are known substrates of Cdc7 (24, 36, 41, 72, 73). In contrast to previous *in vitro* studies with human Cdc7 (41, 64), we found that *Drosophila* Cdc7 was capable of phosphorylating Mcm2 in a dose-dependent manner in the absence of Chiffon (Fig. 5*C*). However, incubation of Mcm2 with DDK yielded substantially increased phosphorylation of Mcm2 (Fig. 5*C*), even though Cdc7 was present in DDK at similar amounts as compared with those lanes containing Cdc7 alone (Fig. 5*B*). These results indicate that Cdc7 alone is capable of phosphorylating Mcm2 but show that Chiffon stimulates Cdc7 kinase activity *in vitro*. We did not detect phosphorylation of Mcm4 in the presence of Cdc7 alone, and we detected only very weak phosphorylation of Mcm4 in the presence of the highest amounts of DDK (Fig. 5*C*). These results are consistent with previous *in vitro* kinase assays performed with the human DDK complex, which showed that Mcm2 is a better *in vitro* substrate for DDK phosphorylation than Mcm4 (41).

Recently, a small molecule inhibitor of Cdc7, XL413 (Fig. 5*D*), was synthesized, tested for inhibition of human Cdc7 *in vitro* and *in vivo*, and advanced into clinical trials as a potential chemotherapeutic agent (42). To determine whether *Drosophila* Cdc7 is relevant as a model for human Cdc7 function, we tested whether XL413 could inhibit the kinase activity of *Drosophila* Cdc7 *in vitro*. To accomplish this, we performed *in vitro* kinase assays using either Cdc7 or DDK with Mcm2 as substrate in the presence of increasing concentrations of XL413, relative to vehicle alone (DMSO). We observed a dose-dependent inhibition of Mcm2 phosphorylation by both Cdc7 and DDK in the presence of increasing concentrations of XL413 (Fig. 5*E*). We therefore conclude that XL413, a selective inhibitor of human Cdc7, inhibits the kinase activity of *Drosophila* Cdc7.

Drosophila DDK Phosphorylates Histones H3 and H2B—In addition to the Mcm2-7 complex, Thr-45 of histone H3 has been identified as a substrate for Cdc7 phosphorylation in *S. cerevisiae* (26). We therefore asked whether *Drosophila* DDK

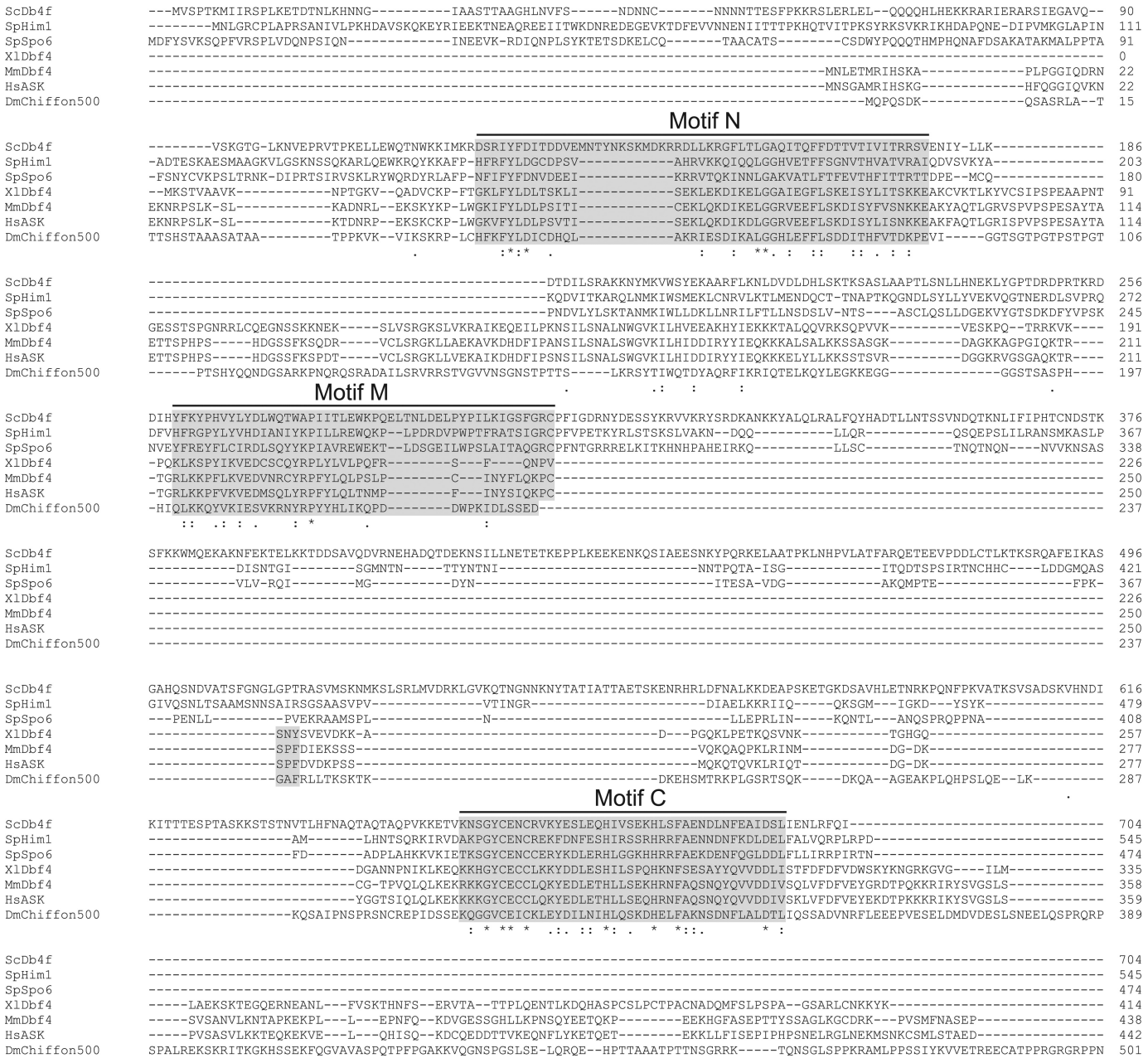

FIGURE 4. **Protein alignment of Dbf4 orthologs.** Alignment of the first 500 amino acids of *D. melanogaster* Chiffon with Dbf4 orthologs as follows: *S. cerevisiae* Dbf4, CAA98869.1; *S. pombe* Him1, O59836.1; *X. laevis* Dbf4, BAC76421.1; *M. musculus* Dbf4, Q9QZ41.1; *H. sapiens* ASK, Q9UBU7.1; *D. melanogaster* Chiffon, AAD48779.1. The locations of the conserved Dbf4 N, M, and C motifs are indicated. Identical residues are indicated by an *asterisk*, and highly similar residues are indicated by a *colon*.

could also phosphorylate histone H3. We performed kinase assays in which DDK was incubated with purified HeLa core histones as substrate. We observed substantial phosphorylation of histone H3 in the presence of increasing amounts of DDK *in vitro*, and unexpectedly, we also observed lower levels of phosphorylation of histone H2B (Fig. 5*F*). Phosphorylation of histone H2B was not observed by Baker *et al.* (26). Thus, this activity could reflect either the expanded activity of DDK *in vitro* on core histones relative to nucleosomes or a novel biological target in *Drosophila* relative to yeast.

Cdc7 Is Required for DNA Replication in Drosophila Egg Chamber Follicle Cells—Depletion or disruption of DDK has been shown to inhibit DNA replication in a variety of metazoan systems (61, 64, 74–78). Thus, we sought to examine the effect of disrupting Cdc7 activity on DNA replication in *Drosophila*. We identified a fly line containing a *P*-element transposon insertion in the fourth intron of the X-linked *cdc7* gene, $cdc7^{G0148}$ (79), which is lethal in the homozygous state such that no $cdc7^{G0148}$ male flies are observed (Table 1). This lethality is rescued by expression of *cdc7* from a genomic rescue construct inserted on another chromosome, such that viable $cdc7^{G0148}$; $P\{w^{+mC} = Cdc7\}attP40$ adult progeny can be obtained in the expected ratios in both male and female adult progeny (Table 1). This finding indicates that proper Cdc7 function is required for viability in *Drosophila*. However, we note that $cdc7^{G0148}$ is unlikely to be a null allele because of the

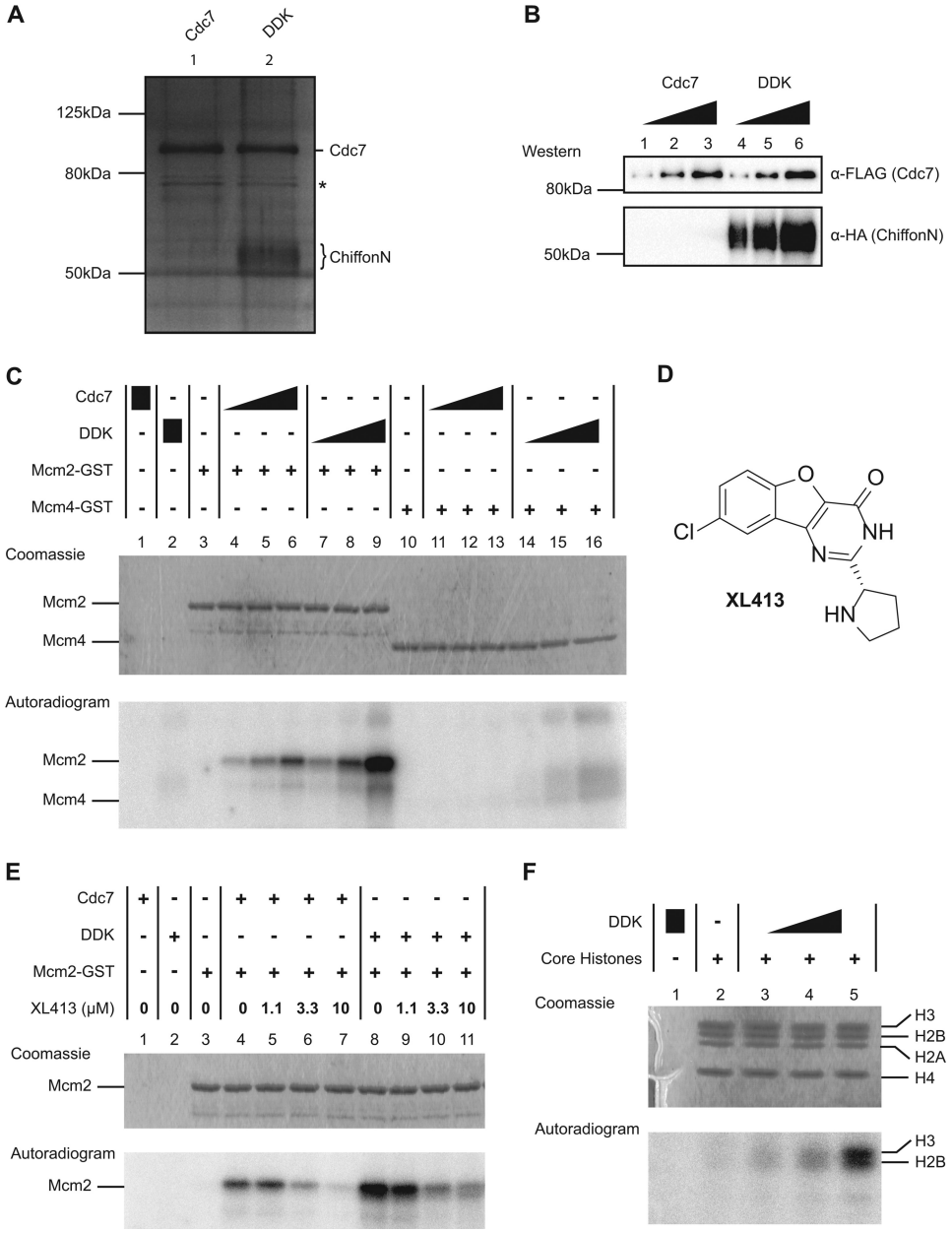

FIGURE 5. ***Drosophila* Cdc7 phosphorylates Mcm2 and histone H3, Chiffon stimulates Cdc7 kinase activity, and XL413 inhibits Cdc7 kinase activity.** *A*, silver-stained SDS-polyacrylamide gel showing purified recombinant *Drosophila* Cdc7 or DDK complex. Cdc7 and ChiffonN were expressed in Sf21 cells as fusion proteins with N-terminal epitope tags as follows: His-FLAG, Cdc7; HA, ChiffonN. Cdc7 was expressed alone or co-expressed with ChiffonN. Cdc7 was purified by sequential Ni-NTA-agarose chromatography followed by FLAG-agarose chromatography, and DDK was purified using sequential nickel-agarose chromatography, followed by HA-agarose chromatography. *Lane 1*, Cdc7 purification; *lane 2*, Cdc7 + ChiffonN (DDK) co-purification. ChiffonN appears as a diffuse band (*brace*) due to phosphorylation by Cdc7. A nonspecific contaminant present at low levels in Cdc7 and DDK purifications is marked by an *asterisk*. *B,* Western analysis of Cdc7 and DDK used for kinase assay in *C*. Increasing amounts of Cdc7 (6, 12, or 24 ng) or DDK (6, 12, 24 ng of Cdc7, with co-purified ChiffonN) were resolved by SDS-PAGE and detected by Western blotting with antibodies against FLAG (Cdc7) and HA (ChiffonN). *C,* Chiffon stimulates Cdc7 kinase activity. *In vitro* kinase assays were performed in the presence of [γ-^{32}P]ATP at 30 °C for 30 min. Increasing amounts of Cdc7 (6, 12, and 24 ng) or DDK complex (6, 12, and 24 ng of Cdc7, with co-purified ChiffonN) were incubated with 0.5 μg of Mcm2(N1–279)-GST or Mcm4(N1–233)-GST. Reactions were separated by SDS-PAGE, stained with Coomassie (*upper panel*), and exposed to a phosphor screen (*lower panel*). *D,* chemical structure of the Cdc7-specific inhibitor XL413. *E,* Cdc7 kinase activity is inhibited by the Cdc7-specific inhibitor XL413. Kinase assays were performed as in *C*. Cdc7 (12 ng) or DDK (12 ng Cdc7) was incubated with [γ-^{32}P]ATP and 0.5 μg of Mcm2-GST in the presence of either vehicle (DMSO) or increasing concentrations of XL413 (1.1, 3.3, and 10 μM, respectively). *F*, DDK phosphorylates histones H3 and H2B. Increasing amounts of DDK (6, 12, and 24 ng of Cdc7) were incubated with 2 μg of core histones in the presence of [γ-^{32}P]ATP at 30 °C for 60 min. Reactions were separated and analyzed as in *C*.

intronic position of the transposon insertion and because we can obtain, albeit rarely, somatic clones of this allele, suggesting that some low level of Cdc7 protein expression remains in this mutant genotype.

To determine whether Cdc7 is required for DNA replication, we examined the egg chamber follicle cells of the ovary, which are a well characterized system for studying DNA replication in *Drosophila* (45, 80). Follicle cells in *Drosophila* egg chambers undergo mitotic divisions early in egg chamber development, followed by three endocycles in which the entire genome is replicated without any intervening cell division, leading to polyploidy. These endocycles are then followed by an amplifi-

TABLE 1

Lethality of *cdc7* flies is rescued by expression of Cdc7

Male $cdc7^{G0148}$ flies carrying the *Cdc7* genomic rescue construct on the second chromosome or male *FM7c* flies without the genomic rescue construct were crossed with female $cdc7^{G0148}/FM7c$ flies, and the surviving adult progeny was scored for presence of the *FM7c* balancer chromosome using the visible Bar eye marker. The percentage of flies corresponding to each genotype and the total number of scored progeny for each cross are indicated.

males	X females	Surviving adult progeny and corresponding genotype					Total scored progeny
		$\frac{cdc7^{G0148}}{Y}$	$\frac{FM7c}{Y}$	$\frac{cdc7^{G0148}}{cdc7^{G0148}}$	$\frac{cdc7^{G0148}}{FM7c}$	$\frac{FM7c}{FM7c}$	
$\frac{cdc7^{G0148}; Cdc7}{Y \quad Cdc7}$	$\frac{cdc7^{G0148}}{FM7c}$	24%	19%	29%	28%	0%	655
$\frac{FM7c}{Y}$	$\frac{cdc7^{G0148}}{FM7c}$	0%	24%	0%	50%	26%	422

cation phase, during which the genomic loci encoding the chorion eggshell protein are over-replicated relative to the rest of the genome. Importantly, chorion loci amplification requires all known components of the DNA replication machinery and is recognized as a sensitive model system for identifying factors that are required for proper DNA replication (45). We used the FLP/FRT genetic mosaic system to generate somatic clones that were homozygous mutant for Cdc7 in *Drosophila* egg chambers (81, 82). Homozygous $cdc7^{G0148}/cdc7^{G0148}$ egg chamber clones were generated in females that were otherwise heterozygous for the strong hypomorphic $cdc7^{G0148}$ allele. DNA replication in egg chambers was then visualized through incorporation of BrdU followed by immunostaining with antibodies against BrdU. Whereas BrdU incorporation in wild-type (GFP-positive) follicle cells is observed in both the endocycle and amplification stage, we do not observe BrdU incorporation in homozygous $cdc7^{G0148}/cdc7^{G0148}$ egg chamber clones (GFP-negative, outlined in *white*) in either the endocycle (Fig. 6*A*, *panels a–d*) or amplification stage follicle cells (Fig. 6*A*, *panels e–h*). We also observe smaller, condensed DAPI-positive nuclei in homozygous $cdc7^{G0148}/cdc7^{G0148}$ amplification stage clones, which together with the lack of BrdU incorporation is consistent with a failure of these cells to undergo proper DNA endoreplication. The more intense DAPI staining in these small $cdc7^{G0148}$ nuclei is indicative of increased chromatin density, possibly due to chromatin condensation, but does not represent canonical apoptosis as determined by immunostaining with antibodies against activated caspase-3 (data not shown). We quantified these data by determining the percentage of BrdU-labeled, DAPI-positive nuclei in $cdc7^{G0148}$ clones relative to control wild-type *FRT101* clones (Fig. 6*C*). Multiple clones were analyzed from individual animals (*n*) for each genotype where possible. However, we note that very few clones (~1 clone per two animals examined) were able to be identified for $cdc7^{G0148}$ relative to both *chiffon* and the wild-type control. This observation is consistent with the hypomorphic nature of the $cdc7^{G0148}$ allele and indicates that Cdc7 function is usually required for the mitotic proliferation necessary for clone formation. Thus, we conclude that Cdc7 is required for DNA replication both in endocycling and in amplification stage cells. These observations suggest that Cdc7 is generally required for DNA replication in *Drosophila* and is likely also to be required for mitotic proliferation.

Chiffon Is Essential for Amplification Stage, but Not Endocycle Stage, of DNA Replication—Because Dbf4 is required for the activity of Cdc7 during late G_1 and S phase, we next sought to determine whether Chiffon is required for DNA replication by Cdc7 in both the endocycle and amplification stage egg chambers. Mutations in *chiffon* result in a thin and fragile chorion and reduce amplification of chorion loci DNA, consistent with a requirement for Chiffon in DNA replication in developing egg chambers (37, 83). However, the direct effect of *chiffon* mutations on DNA replication in endocycling follicle cells had not previously been examined. Thus, we used the FLP/FRT system to generate somatic clones homozygous for the $chif^{ETBE3}$ null allele (37), and we examined BrdU incorporation in *Drosophila* egg chambers. Whereas BrdU incorporation in wild-type (GFP-positive) follicle cells is observed in both the endocycle and amplification stage, we found that chorion amplification was eliminated in clones homozygous null for *chiffon* (Fig. 6*B*, *panels m–p*), consistent with previous findings (37, 83). However, nuclei within the homozygous $chif^{ETBE3}/chif^{ETBE3}$ clones appear to be the same size as nuclei in follicle cells outside of the clone (Fig. 6*B*, *panel o*). We therefore examined BrdU incorporation in endocycle stage clones homozygous for the null *chiffon* allele. Unexpectedly, examination of $chif^{ETBE3}/chif^{ETBE3}$ clones in endocycle stage egg chambers revealed that endocycle stage DNA replication continued in clones lacking Chiffon (Fig. 6*B*, *panels i–l*). We quantified these data by determining the percentage of BrdU-labeled nuclei in *chiffon* clones relative to control wild-type clones (Fig. 6*C*). These data show that whereas BrdU incorporation is never observed in *chiffon* amplification stage follicle cells, endocycling *chiffon* clones exhibit a similar fraction of nuclei incorporating BrdU to that observed in wild-type control clones. Although DNA replication is not eliminated in $chif^{ETBE3}/chif^{ETBE3}$ endocycling cells, we did observe differences in the timing of endocycling in the *chiffon* clones relative to the surrounding wild-type cells. Notably, BrdU incorporation is still observed in several of the *chiffon* clones after endoreplication in nonclonal regions of the egg chamber has largely ceased (Fig. 6*D*, representative images from three individual animals shown). Our data do not support the conclusion that *chiffon* clones undergo an extra endocycle, because post-endocycle nuclei within *chiffon* clones appear to be the same size as nuclei in the rest of the egg chamber in amplification stage egg chambers (Fig. 6*B*, *panel o*). In addition, we do not observe endocycling patterns of BrdU incorporation in amplification stage egg chambers in the *chiffon* clones, suggesting that the developmental transition from endocycling to amplification is not affected by loss of Chiffon. Rather, the continued BrdU incorporation in *chiffon* mutant clones in stage 8 egg chambers is most consistent with a delayed exit of the *chiffon* cells from the endocycle S phase relative to their wild-type counterparts. This observation indicates that although Chiffon is not necessary for endoreplication within egg chamber follicle cells, Chiffon might still function in the proper timing or progression of endoreplication. Thus, together our findings suggest that the DDK complex containing Cdc7 and Chiffon is required for the initiation of DNA replication during chorion gene amplification, although Cdc7 can function independently of Chiffon during the *Drosophila* endocycle.

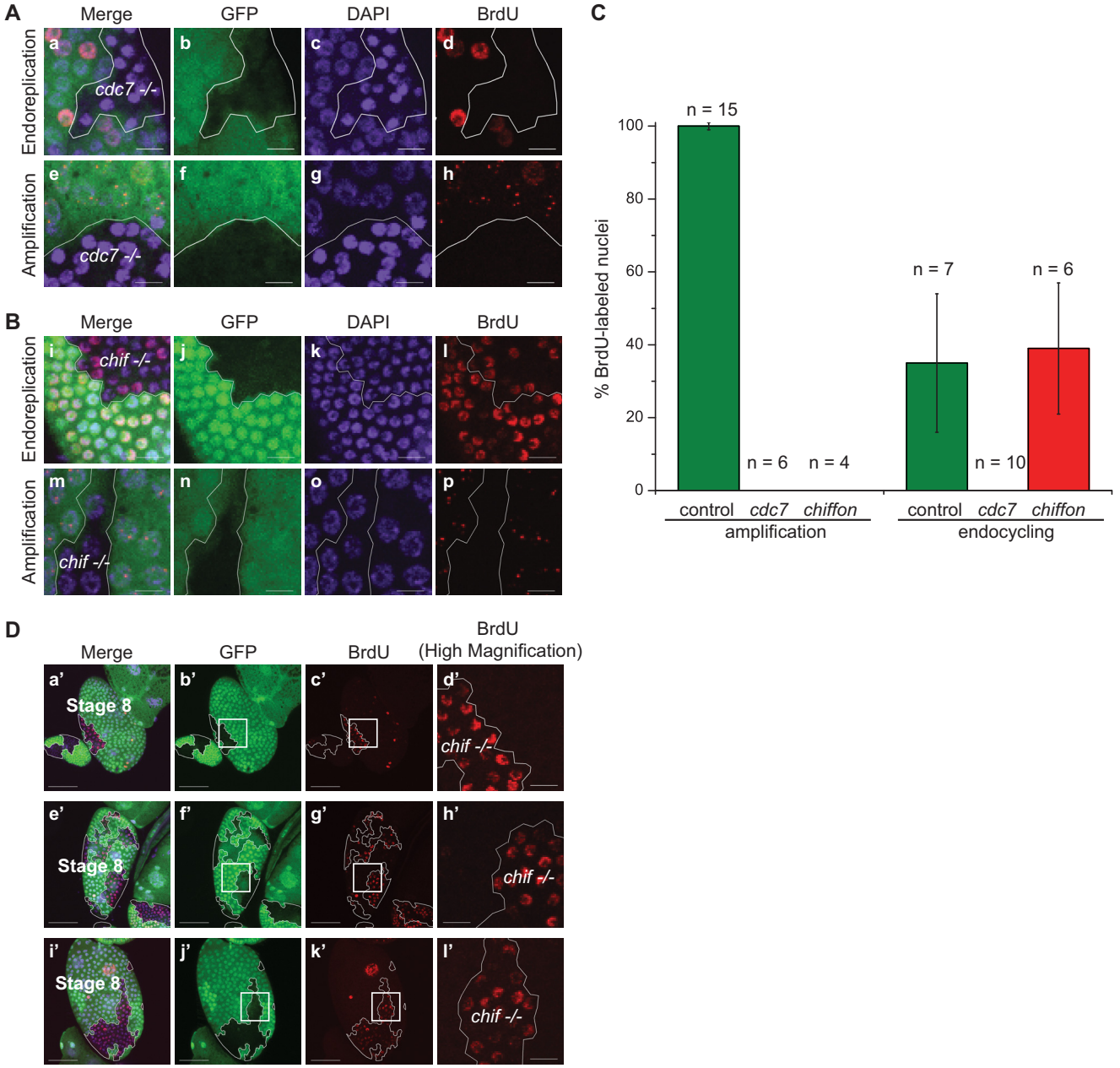

FIGURE 6. **Mutations in DDK differentially disrupt DNA replication in *Drosophila* egg chamber follicle cells.** *A,* endocycle and amplification stage DNA replication is eliminated in egg chamber follicle cells lacking Cdc7. Mosaic egg chambers were generated using the FLP/FRT system. BrdU incorporation is shown in egg chamber follicle cells, and DAPI staining is shown for comparison. Somatic *cdc7*^{G0148} clones are marked by the absence of GFP (outlined in *white*). A representative endocycle stage egg chamber (*panels a–d*) and representative amplification stage egg chamber (*panels e–h*) are shown. *Scale,* 10 μm. *B,* amplification stage DNA replication is eliminated in egg chamber follicle cells lacking Chiffon, but the endocycle stage replication persists. Egg chambers carrying somatic *chif*^{ETBE3} clones were generated using the FLP/FRT system and BrdU and DAPI analyzed as in *A*. A representative endocycle stage egg chamber (*panels i–l*) and a representative amplification stage egg chamber (*panels m–p*) are shown. *Scale,* 10 μm. *C,* quantification of DNA replication defects observed in *cdc7*^{G0148} and *chif*^{ETBE3} somatic clones shown in *A* and *B*. The percentage of BrdU-labeled nuclei in clones deficient for *cdc7* or *chiffon* was compared with control somatic clones (*FRT101*), in which the FLP/FRT system was used to generate egg chambers mosaic for GFP expression in an otherwise wild-type genetic background. Mean percentages ± S.D. are shown for the number of animals (n) examined for each genotype and developmental stage. *D, chif*^{ETBE3} somatic clones continue endocycling after endoreplication in nonclonal regions of the egg chamber has ceased. Egg chambers carrying somatic *chif*^{ETBE3} clones were generated using the FLP/FRT system, and BrdU and DAPI were analyzed as in *A*. Representative images from three different animals (*panels a′–d′*, *panels e′–h′*, and *panels i′–l′*) are shown. *Scale,* 50 μm (*Merge, GFP, BrdU*) or 10 μm (*BrdU high magnification*).

DISCUSSION

Drosophila Contains Two Cdc7 Homologs—We have identified two *Drosophila* genes that encode proteins with homology to Cdc7 as follows: the ubiquitously expressed gene *l(1)G0148* (*cdc7*), and the testis-specific gene *CG5790*. The only other organism in which two Cdc7 homologs have been identified is *S. pombe*, which encodes two DDK complexes with distinct functions and regulatory subunits, Hsk1-Him1/Dfp1, which regulate S phase DNA replication initiation during mitotic proliferation (33, 48, 49), and Spo4-Spo6, which is dispensable for mitosis but essential for the progression of meiosis II (34, 66). *S. cerevisiae* DDK has also been found to regulate several aspects of meiosis (84), suggesting that DDK-type complexes might play a general role in regulating meiosis in multiple organisms.

The expression patterns of *Drosophila cdc7* and *CG5790,* and the inability of CG5790 to complement the *cdc7-90* temperature-sensitive growth defect in *S. cerevisiae*, suggest that, of the two paralogs, Cdc7 is more likely to play a general role in S phase DNA replication during mitotic proliferation. Furthermore, the testis-specific expression pattern of *CG5790* would be most consistent with a role for this putative kinase in meiosis. We predict that Chiffon could act as the regulatory subunit for CG5790 because Chiffon is the only protein encoded by the *Drosophila* genome that has any significant homology to known Dbf4 orthologs and because Chiffon has been identified in an enhancer-trap screen for genes that are strongly expressed in post-mitotic spermatogonia and spermatocytes in *Drosophila* testis (85). Additionally, our observation that the N terminus of Chiffon, which contains the canonical Cdc7-interacting motifs, co-immunoprecipitates CG5790 from yeast lysates, indicates that Chiffon and CG5790 are capable of interacting. This supports a potential role for CG5790 and Chiffon in *Drosophila* meiosis. However, because *cdc7* and *CG5790* are both expressed in *Drosophila* testis, the meiotic role of each Cdc7 paralog and the identity of the *in vivo* regulatory Dbf4-like subunit for CG5790 remain to be determined.

Cdc7 and Chiffon Are Differentially Required for Endocycling—The results from our analysis of somatic mosaic egg chambers show that although Cdc7 is required for both endocycling and follicle cell amplification, Chiffon is required for amplification but is not essential for endocycling. Consistent with our observations, Chiffon was previously found to be required for amplification of the chorion loci in egg chamber follicle cells (37, 83), and examination of DAPI-stained *chiffon* null egg chambers suggested that follicle cells could complete endocycling normally in the absence of Chiffon (86). Together, these results indicate that Cdc7 can initiate DNA replication in endocycling cells independently of Chiffon.

How might Cdc7 function independently of Chiffon during the endocycle? One potential explanation is that an alternative Dbf4-like activating subunit exists within *Drosophila* that serves to activate Cdc7 specifically during the endocycle. Multiple Dbf4 paralogs that regulate the same Cdc7 ortholog at distinct developmental stages have been identified in both human and *Xenopus* (87–90). In *Xenopus* egg extracts, depletion of the Cdc7-Drf1 DDK complex severely impairs DNA synthesis, whereas depletion of the alternative Cdc7-Dbf4 DDK complex has no effect on replication (90, 91). Cdc7-Drf1 and Cdc7-Dbf4 are developmentally regulated, as Cdc7-Drf1 is the primary DDK complex in *Xenopus* oocytes and early embryos, and Cdc7-Dbf4 predominates at later stages of embryonic development. It is possible that *Drosophila* Cdc7 is similarly regulated by multiple activating subunits during different developmental stages, with Chiffon being required for the activation and targeting of Cdc7 to replication origins during gene amplification, and an as-of-yet undiscovered Dbf4-like regulator(s) functioning during endoreplication and mitotic proliferation. Although Chiffon is the only protein within *Drosophila* with significant homology to known Dbf4 orthologs, several findings support the possibility that additional Cdc7 activating or targeting subunits could exist in *Drosophila*. First, examination of *Xenopus* egg extracts found that a substantial portion of Cdc7 is not complexed with either Drf1 or Dbf1, and extracts immunodepleted for Drf1 and Dbf1 retained some capacity for supporting DNA replication (90). Second, the observation that *chiffon* appears to be dispensable for mitotic proliferation, because *chiffon* null mutants are viable (37) and we were able to recover large *chiffon* clones in mosaic *Drosophila* egg chambers, suggests that *Drosophila* could contain an undiscovered Cdc7 activator for mitotic proliferation. Together, these observations suggest that an additional Cdc7 regulating subunit, which may potentially bear little similarity to Dbf4, remains to be discovered. Because this putative Cdc7 regulatory subunit may be present in both *Xenopus* and *Drosophila*, it is intriguing to speculate that it could also function in humans to target or activate Cdc7 in a Dbf4-independent manner.

Another possibility is that Cdc7 can phosphorylate and activate the pre-RC during endocycling in the absence of any regulatory subunit. Although there are few reported cases of Cdc7 functioning independently of a Dbf4 subunit, our *in vitro* kinase results, which are consistent with observations of yeast Cdc7 activity (72), show that *Drosophila* Cdc7 does possess some ability to phosphorylate Mcm2 in the absence of Chiffon. However, it is possible that Cdc7 can phosphorylate its substrates independently of Chiffon *in vitro* but that Chiffon or another protein is required to target its activity *in vivo*. This explanation would be consistent with the inability of Cdc7 to rescue the mitotic growth defect of *cdc7-90* yeast in the absence of Chiffon. A recent study has shown that Dbf4 knockdown has no effect on Cdc7-dependent induction of smooth muscle cell differentiation in murine cell lines, which is instead dependent on interactions between Cdc7 and the SMAD3 transcriptional modulator (92). Thus, there is a precedent for the ability of Cdc7 to function in specific developmental processes independently of Dbf4, potentially through interactions with other unrelated factors.

An additional possibility is that the unique nature of the replication initiation machinery during the endocycle might lead to a partial bypass of the requirement for DDK for the initiation of DNA replication. Previously, endoreplication in *Drosophila* has been shown to continue in the absence of several pre-RC components that are otherwise essential for mitotic proliferation, including *dpa*/Mcm4 (18), ORC proteins 1, 2, and 5 (15), Mcm2 (17), and Mcm5, although this last factor is disputed (15, 93). We note that although endoreplication continues in these mutants, there is evidence that the pattern and level of replication is altered from that of the wild type (15, 16). Two distinct Mcm complexes are present in *Drosophila* that contain either Mcm2 and Mcm4 or Mcm5 (94). The key substrate of DDK for mitotic replication initiation in *S. cerevisiae* is Mcm4, and deletion of an inhibitory domain in the Mcm4 N-terminal tail bypasses the cell cycle requirement for DDK (25). Because *dpa*/Mcm4 and Mcm2 may not strictly be required for endocycling, and because Mcm complexes exist that lack Mcm4, this key mitotic target of DDK might not be required for the initiation of DNA replication in endocycling cells.

What then might be the primary target of Cdc7 in endocycling cells? Because Cdc7 interacts directly with Mcm5 (95), and because both yeast Cdc7 (72) and *Drosophila* Cdc7 exhibit kinase activity toward Mcm2 in the absence of Dbf4, it is plau-

sible that other subunits of the Mcm2-7 helicase might be the primary targets of Cdc7 in endocycling cells that are critical for DNA replication. Mcm6 could be a potential target for Cdc7 phosphorylation during endocycling because Mcm6 is phosphorylated by DDK *in vitro* and *in vivo*, and there is evidence that the phosphorylation of Mcm2, Mcm4, and Mcm6 serves a redundant purpose during proliferation (23, 96, 97). Consistent with this idea, loss of Mcm6 (19) or knockdown of the Mcm2-7 loading factor Cdt1 (15) severely impairs endocycling, suggesting that the Mcm2-7 helicase, in one form or another, does play a critical role in endoreplication.

Prolonged Endocycling Occurs in the Absence of Chiffon—Although our analysis shows that Chiffon is not necessary for endocycling, we did observe prolonged BrdU labeling in *chiffon* mutant endocycle stage clones, which appears to be most consistent with increased length of the endocycle S phase in these cells. Prolonged endocycling due to increased time in S phase has been previously observed in egg chamber follicle cells deficient for Dacapo (98), which is a cyclin-dependent kinase inhibitor of cyclin E/cdk2 that promotes Mcm2 association with chromatin during endocycling (99). Similarly, it has been hypothesized that the direct disruption of cyclin E levels also leads to an elongated endocycle S phase, because the replication of late-replicating heterochromatic DNA sequences that usually become under-represented during endoreplication is restored in a *cycE* hypomorph (12). It is possible that although Chiffon is not necessary for Cdc7 activity in endocycling cells, it still plays a role in efficient origin licensing or firing, and therefore S phase timing, during endoreplication.

Examining the replication of heterochromatic sequences in *chiffon* mutants may be helpful in future studies to determine whether Chiffon, similar to cyclin E (12) and ORC (16), although not necessary for endoreplication, does influence S phase length and the overall pattern of genomic replication during endoreplication.

C-terminal Extension of Chiffon Is Not Required for DDK Function—Although full-length Chiffon consists of 1695 amino acids, we found that the N-terminal 400 amino acids of Chiffon were sufficient to activate Cdc7 in the *cdc7-90* yeast strain, interact with Cdc7 *in vitro*, and stimulate Cdc7 kinase activity. This raises the question as to the function of the large C-terminal extension present in *Drosophila* Chiffon, which does not appear to be present in the mammalian and yeast Dbf4 orthologs. Mammalian ASK does contain a modest C-terminal extension that is not present in yeast Dbf4 (Figs. 3 and 4) and that is implicated in an autoinhibition of Cdc7 that is relieved through interactions with LEDGF (100, 101). However, there is no detectable sequence similarity between the C-terminal portions of Chiffon and ASK. Instead, the large C-terminal extension of Chiffon appears to be an insect-specific variant of the Dbf4 protein (86). Our results indicate that full-length Chiffon might be regulated by proteolytic cleavage because both full-length recombinant Chiffon and Chiffon expressed in yeast appear as an N-terminal 50-kDa truncation fragment that is sufficient to interact with Cdc7. Additionally, an ~50-kDa N-terminal truncation product of Chiffon was also observed when epitope-tagged Chiffon was transiently expressed in cultured S2 cells (data not shown). Dbf4 is known to be a substrate of the anaphase-promoting complex (21), and it is possible that Chiffon is regulated by the anaphase-promoting complex during the cell cycle. However, the conservation of the C-terminal extension of Chiffon in insect species indicates that the C-terminal region of Chiffon, although not required for DDK activity, might be required for some unknown function that could potentially be distinct from DDK activity.

Conclusion—In summary, we report the characterization of *Drosophila* Cdc7, a functional homolog of Cdc7 that is necessary for multiple forms of DNA replication in *Drosophila*. Surprisingly, although Cdc7 is essential for endoreplication in ovarian follicle cells, its activating subunit Chiffon is not. Although not necessary for endoreplication, Chiffon might still play a role in the timing and progression of S phase during the endocycle. The involvement of Cdc7 in polyploidization, which may be a route to aneuploidy and drug resistance in cancer (7), may increase its importance as a target of novel chemotherapeutic agents. Additionally, because Cdc7 appears to function during endocycling in the absence of Chiffon, future studies on the role of Cdc7 in endocycling cells might yield the identification of novel activators of Cdc7 and provide further insight into the specialized mode of replication initiation that occurs during endoreplication.

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