

# *Xenopus* Cut5 is essential for a CDK-dependent process in the initiation of DNA replication

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**Fission yeast Cut5/Rad4 and its budding yeast homolog Dpb11 are required for both DNA replication and the S-phase checkpoint. Here, we have investigated the role of the *Xenopus* homolog of Cut5 in the initiation of DNA replication using *Xenopus* egg extracts. *Xenopus* Cut5, which shows sequence similarity to DmMus101 and HsTopBP1, is essential for DNA replication in the egg extracts. It is required for the chromatin binding of Cdc45 and DNA polymerases, but not for the formation of pre-replicative complexes or the elongation stage of DNA replication. The chromatin binding of Cut5 consists of two distinct modes. S-phase cyclin-dependent kinase (S-CDK)-independent binding is sufficient for DNA replication while S-CDK-dependent binding is dispensable. Further, S-CDK acts after the chromatin binding of Cut5 and before the binding of Cdc45. These results demonstrate that the chromatin binding of Cut5 is required for the action of S-CDK, which in turn triggers the formation of pre-initiation complexes of DNA replication.**

**Keywords:** Cdc45/Cut5/DNA replication/Dpb11/S-CDK

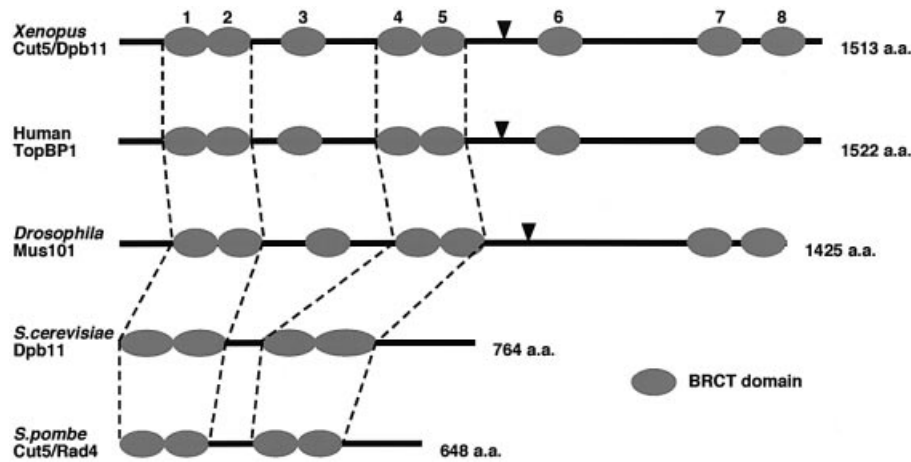
## Introduction

In eukaryotic cells, the regulated assembly of replication factors onto origins is crucial for the control of the initiation of DNA replication. At the end of M-phase and during G<sub>1</sub>-phase of the cell cycle, origin recognition complex (ORC), Cdc6, Cdt1 and minichromosome maintenance (MCM) proteins are sequentially assembled onto each replication origin, leading to the formation of pre-replicative complexes (pre-RCs) (reviewed in Nishitani and Lygerou, 2002). At the onset of S-phase, pre-RCs are activated through the action of two kinds of protein kinase, S-phase cyclin-dependent kinase (S-CDK) and Dbf4-dependent Cdc7 kinase (DDK). This converts the pre-RCs into pre-initiation complexes (pre-ICs) (Zou and Stillman, 2000). The exact roles of S-CDK and DDK are not fully understood but accumulating evidence suggests that S-CDK and DDK target specific replication proteins such as Sld2/Drc1 (Masumoto *et al.*, 2002; Noguchi *et al.*, 2002) and MCM proteins (Jiang *et al.*, 1999). Upon the phosphorylation of target proteins, Cdc45 becomes associated with MCM proteins in pre-RCs, leading to the formation of pre-ICs and the unwinding of origins

(Mimura and Takisawa, 1998; Zou and Stillman, 1998; Walter and Newport, 2000). Replication protein A (RPA) and DNA polymerase  $\alpha$  are sequentially recruited (Tanaka and Nasmyth, 1998; Mimura *et al.*, 2000; Walter and Newport, 2000), and DNA polymerase  $\epsilon$  is independently recruited (Mimura *et al.*, 2000) onto the unwound DNA, forming initiation complexes on the origins. After initiation, S-CDK is no longer required for the elongation of DNA replication (Strausfeld *et al.*, 1994; Walter and Newport, 1997) but it serves to inhibit re-initiation during S phase through multiple mechanisms (Nguyen *et al.*, 2001), ensuring that DNA replication occurs only once in each cell cycle.

In addition to the known components of pre-RCs, recent studies have revealed additional factors necessary for the initiation of DNA replication (Takisawa *et al.*, 2000; Bell and Dutta, 2002). In budding yeast, Dpb11 has been shown to play a pivotal role in the loading of DNA polymerases onto replication origins (Masumoto *et al.*, 2000). Dpb11 was originally identified as a multicopy suppressor of the *pol2-12* and *dpb2-1* mutations, which affect the catalytic and the second largest subunits of DNA polymerase  $\epsilon$ , respectively (Araki *et al.*, 1995). The identification of five essential genes called *SLD2–5* (synthetically lethal with *dpb11-1*) further supports the importance of Dpb11 in DNA replication (Kamimura *et al.*, 1998). The fission yeast protein Cut5/Rad4 is a functional homolog of Dpb11 and both mutants display similar phenotypes of abnormal cell division with incomplete DNA replication, suggesting that Cut5/Rad4 and Dpb11 are required for DNA replication itself as well as for the replication checkpoint (Saka and Yanagida, 1993; Saka *et al.*, 1994; Araki *et al.*, 1995). Further, the association between Dpb11/Cut5 and Sld2/Drc1 is required for DNA replication (Kamimura *et al.*, 1998; Wang and Elledge, 1999) and is under the control of CDK (Masumoto *et al.*, 2002; Noguchi *et al.*, 2002). Here, we will use the single name Cut5 to refer to Cut5/Rad4/Dpb11 because Cut5 was the first factor identified as an essential replication protein (Saka and Yanagida, 1993). Human TopBP1 and *Drosophila* Mus101 share sequence similarities with Cut5 (Yamane *et al.*, 1997; Yamamoto *et al.*, 2000) and TopBP1 is also involved in DNA replication and the DNA damage response (Mäkinen *et al.*, 2001; Yamane *et al.*, 2002). Some mutants of Mus101 display mutagen sensitivities, defects in DNA synthesis and chromosome instability (Yamamoto *et al.*, 2000). These results strongly suggest that TopBP1 and Mus101 are the functional homologs of yeast Cut5 but the role of Cut5 in the initiation of DNA replication is only poorly understood in higher eukaryotes.

In this study, we have identified and characterized a *Xenopus* homolog of Cut5 using the cell-free system of *Xenopus* egg extracts. We found that Cut5 is required for the association of Cdc45 with chromatin. Furthermore,



**Fig. 1.** Schematic comparison of Cut5/Dpb11 homologs. Elliptical symbols represent BRCT domains. Dotted lines indicate highly conserved BRCT domains from yeast to human. BRCT domains 1–2 of the *Xenopus* protein share sequence similarity with the homologs in human (98%), *Drosophila* (54%), *S. cerevisiae* (30%) and *S. pombe* (47%), and BRCT domains 4–5 of the *Xenopus* protein share similarity with BRCT domains 4–5 of human (91%) and *Drosophila* (31%), and with BRCT domains 3–4 of *S. cerevisiae* (34%) and *S. pombe* (37%). Arrowheads indicate putative target sites of CDK conserved among *Xenopus*, human and *Drosophila*. Total numbers of amino acid residues are indicated at the right side.

Cut5 is required for S-CDK to function in the initiation reaction. These results suggest that Cut5 plays an essential role in the formation of pre-ICs upon the action of S-CDK.

## Results

### Identification of *Xenopus* Cut5

To isolate *Xenopus* Cut5, we searched the EST database for a *Xenopus* homolog of human TopBP1, a putative human Cut5, and obtained a *Xenopus* EST sequence with high homology (accession No. AW635109). Using the EST sequence as a probe, we screened a *Xenopus* oocyte cDNA library and isolated a *Xenopus* homolog of TopBP1 (accession No. AB091779). Figure 1 compares the primary structure of the predicted *Xenopus* protein with those of human TopBP1, *Drosophila* Mus101 and yeast Cut5/Dpb11. The *Xenopus* protein consists of 1513 amino acids and contains eight copies of BRCA1 C-terminus (BRCT) domains, which are thought to be involved in protein–protein interactions. Four of the BRCT domains, indicated by dotted lines, are highly conserved from yeast to human while the others are unique to higher eukaryotes. The overall identities between *Xenopus* and *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Drosophila* and human are 15%, 16%, 24% and 63%, respectively. These structural features show that the isolated clone is a *Xenopus* homolog of TopBP1 and Mus101.

A previous study showed that TopBP1 is unable to suppress temperature-sensitive mutants of either budding yeast Dpb11 or fission yeast Cut5 (Mäkinen *et al.*, 2001). We have examined the suppression of these yeast mutants with the full-length and N-terminal half (amino acids 1–761, a conserved part of Cut5 homologs) of *Xenopus* Cut5. The overexpression of either protein failed to suppress these yeast mutants (data not shown; see also Materials and methods). This failure in functional complementation is not surprising if we consider the sequence differences between these gene products. Nevertheless, functional similarity among Cut5 homologs

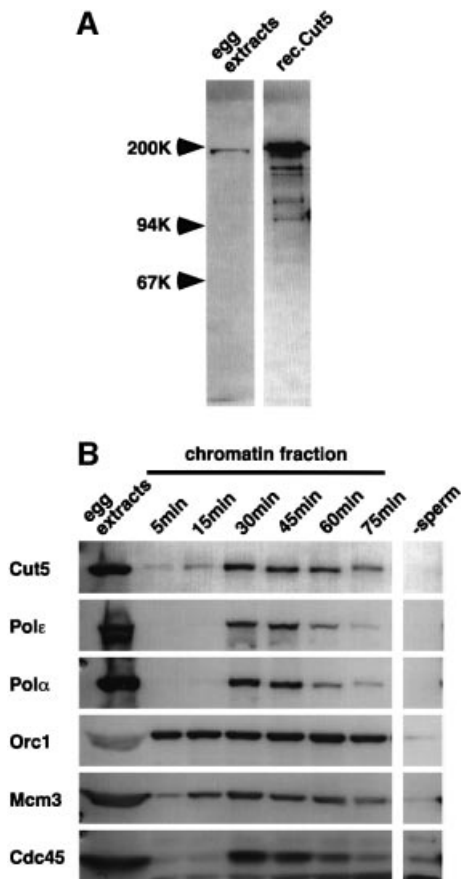
supports the view that TopBP1-related proteins in higher eukaryotes are the counterparts of yeast Cut5/Dpb11 (see also Discussion) and we therefore designate the *Xenopus* homolog of TopBP1, *Xenopus* Cut5.

### Role of *Xenopus* Cut5 in DNA replication

To investigate the role of *Xenopus* Cut5 in DNA replication, we expressed His-tagged *Xenopus* Cut5 in insect cells and produced antibodies against the recombinant protein. The affinity-purified antibody recognized a single polypeptide with an apparent molecular mass of 180 kDa in interphase egg extracts, and also recognized recombinant Cut5 with a similar mobility on SDS–PAGE (Figure 2A).

Using this antibody, we first examined the time-course of Cut5 association with chromatin during DNA replication in egg extracts. Upon addition of sperm chromatin to the extracts, components of pre-RCs such as Orc1 and Mcm3 were assembled onto chromatin within 15 min (Figure 2B) and nuclear structures were formed around the chromatin within 30 min, allowing replication initiation. Various replication proteins involved in the formation of initiation complexes, such as Cdc45 and DNA polymerases  $\alpha$  and  $\epsilon$ , were bound to chromatin upon initiation (30 min) and were gradually dissociated from the chromatin during the progression of DNA replication, while Orc1 binding remained constant throughout the incubation. Cut5 showed essentially the same behavior as the replication proteins involved in the initiation complex (Figure 2B) but we also detected a low level of chromatin binding before initiation (15 min). These results show that *Xenopus* Cut5 is a chromatin binding protein, possibly involved in DNA replication.

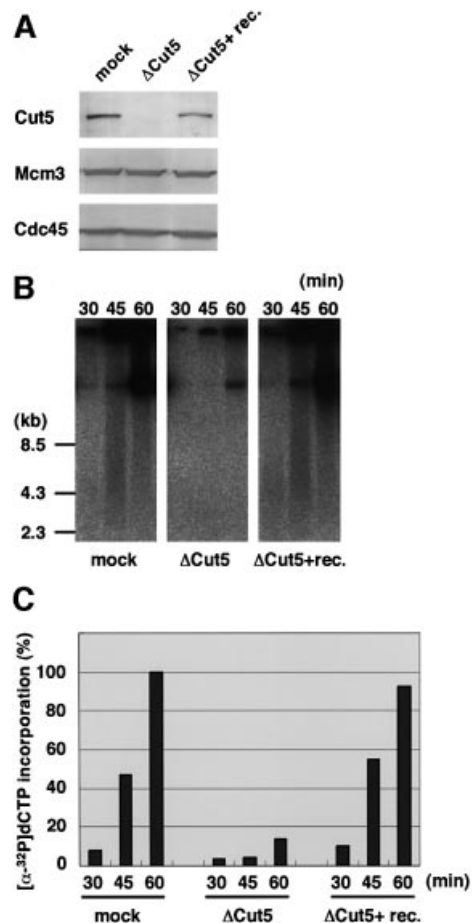
To determine whether or not Cut5 is required for DNA replication, we first depleted Cut5 from the extracts using anti-*Xenopus* Cut5 antibody (Figure 3A). Endogenous Cut5 could be almost completely eliminated from the egg extracts with the antibody treatment but other replication proteins such as Mcm3 and Cdc45 remained in the depleted extracts (Figure 3A). Addition of 5 ng of



**Fig. 2.** Time-courses of the chromatin binding of Cut5 and other replication proteins. (A) Specificity of anti-Cut5 antibody. S-phase egg extracts (1.5  $\mu$ l) and 125 ng of recombinant *Xenopus* Cut5 protein (rec.Cut5) were resolved with SDS-PAGE and immunoblotted with anti-*Xenopus* Cut5 antibody. (B) Sperm nuclei were incubated in 50  $\mu$ l of S-phase egg extract for the times indicated at 23°C. Chromatin fractions were isolated by centrifugation through a 10% sucrose layer and the isolated chromatin fractions and 1.5  $\mu$ l of the extracts were resolved by SDS-PAGE, then immunoblotted with antibodies against the various replication proteins indicated.

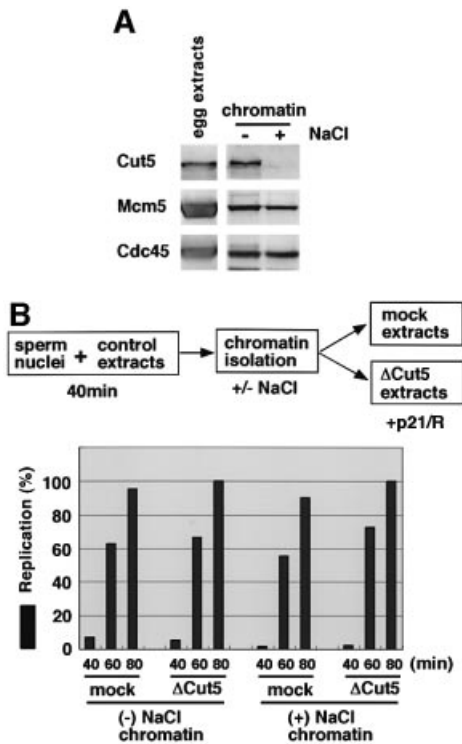
recombinant Cut5 to 1  $\mu$ l of the depleted extracts gave an immunostaining signal similar to that of mock-depleted extracts, indicating that the concentration of endogenous Cut5 is  $\sim$ 5  $\mu$ g/ml. We next examined the replication activity of Cut5-depleted extracts (Figure 3B and C). DNA replication activity decreased markedly upon the depletion of Cut5, to  $\sim$ 14% of the activity of mock-depleted extract (60 min). Recombinant Cut5 could rescue the decreased activity and restore it to  $>90\%$  of control levels. These results therefore show that the replication defect in the Cut5-depleted extract is due to the selective removal of Cut5, and that no additional factor is required for its restoration.

Analysis of replication products by agarose gel electrophoresis further suggests that Cut5 is required for the initiation stage of DNA replication (Figure 3B). In mock-depleted extracts, replication products consisted mainly of high-molecular-weight DNA (Blow and Laskey, 1986). With Cut5-depleted extracts, similar sizes but much smaller amounts of the products were observed and low-molecular-weight replication products of  $<10$  kb did not accumulate, suggesting that the elongation reaction was



**Fig. 3.** Requirement of Cut5 for DNA replication. (A) Immunodepletion of Cut5 from S-phase egg extracts. The egg extracts were treated with control and anti-Cut5 antibodies bound to a protein A matrix. Aliquots of mock-depleted (mock) and Cut5-depleted extracts with ( $\Delta$ Cut5+rec.Cut5) or without ( $\Delta$ Cut5) 5 ng/ $\mu$ l of recombinant Cut5 were resolved by SDS-PAGE, then immunoblotted with antibodies against the proteins indicated in the figure. (B) Replication activity of Cut5-depleted extracts. Sperm nuclei were incubated in the treated egg extracts for the indicated times at 23°C. Replication activity was monitored as the incorporation of [ $\alpha$ - $^{32}$ P]dCTP into sperm DNA. Replication products were subjected to agarose gel electrophoresis followed by autoradiography. (C) Relative intensities of autoradiography were analyzed and plotted against time, taking the highest value of the mock-depleted extracts as 100%.

not affected in the depleted extracts (Waga *et al.*, 2001). To test further whether Cut5 is required for the elongation stage of DNA replication, we examined directly the elongation activity of isolated replicating chromatin fractions in the presence of CDK inhibitors, which inhibit initiation but not elongation reactions (Strausfeld *et al.*, 1994; Walter and Newport, 1997). Replicating chromatin fractions were isolated from the extracts by first incubating sperm chromatin for 40 min to allow maximal loading of DNA polymerases onto chromatin (see Figure 2B). When the chromatin fractions were then washed with a buffer containing 0.1 M NaCl and NP-40, almost all Cut5 bound to the chromatin was removed but MCM proteins and Cdc45, both of which are required for the elongation stage of DNA replication, remained on the chromatin (Figure 4A). NaCl treatment did not affect the replication activity of the chromatin in mock-depleted extracts, indicating that elongation is not affected by NaCl

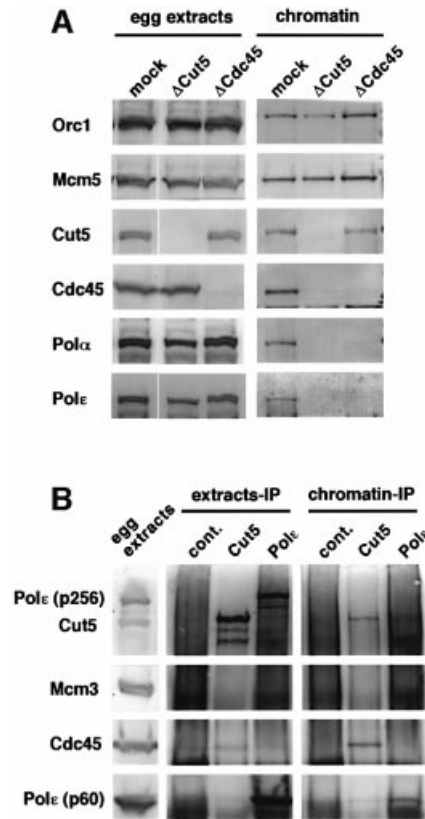


**Fig. 4.** Requirement of Cut5 for elongation stages of DNA replication. (A) Selective removal of Cut5 from chromatin. Sperm nuclei were incubated in 50  $\mu$ l of control egg extracts for 40 min at 23°C. The egg extracts (1.5  $\mu$ l) and the chromatin fractions, isolated and washed with EB containing 0.0025% NP-40 in the absence (-) or presence (+) of 0.1 M NaCl, were subjected to immunoblotting. (B) Elongation activity of the isolated replicating chromatin fractions. The chromatin fractions prepared as in (A) were resuspended and incubated in mock- and Cut5-depleted egg extracts in the presence of 50  $\mu$ g/ml GST-p21 and 100  $\mu$ M roscovitine (+p21/R). Elongation activity was detected as the incorporation of Cy3-dCTP into chromatin DNA. The fluorescence intensities of 40 nuclei were analyzed. Relative average intensities per nucleus were then plotted against time, taking the maximal value as 100%.

treatment (Figure 4B, mock). Furthermore, we found that the elongation activity of Cut5-depleted chromatin fractions in Cut5-depleted extracts (+NaCl chromatin) was essentially the same as in mock-depleted extracts (Figure 4B, right panel). In contrast, the replication of sperm chromatin in Cut5-depleted extracts in the absence of inhibitors but without 40 min pre-incubation in control extracts was <20% of that of replicating chromatin (data not shown, see also Figure 3C). These results suggest that Cut5 is not required for the elongation activity of replicating chromatin.

#### Role of Cut5 in the formation of the initiation complex

In budding yeast, Cdc45 and Dpb11/Cut5 play essential roles in the loading of DNA polymerases onto chromatin (see Introduction). However, the precise roles of Dpb11 and Cdc45 in replication initiation have not yet been clarified. We therefore examined the role of *Xenopus* Cut5 in the loading of DNA polymerases and the mutual dependence of Cut5 and Cdc45 for their chromatin binding. Upon depleting Cut5 and Cdc45 from the extracts, each protein was specifically eliminated while



**Fig. 5.** Requirement of Cut5 for the loading of Cdc45 onto chromatin. (A) Sperm nuclei were incubated in mock-, Cut5- and Cdc45-depleted egg extracts for 40 min at 23°C. For each extract, 1.5  $\mu$ l depleted extract and the isolated chromatin fractions (chromatin) from 50  $\mu$ l extracts were resolved with SDS-PAGE and immunoblotted. (B) Co-immunoprecipitation of Cdc45 with Cut5 on replicating chromatin. Sperm nuclei were incubated in S-phase egg extract in the presence of 50  $\mu$ g/ml aphidicolin for 50 min at 23°C. Chromatin DNA was fragmented by sonication and the fragmented chromatin fractions were clarified by centrifugation. The egg extracts and the fragmented chromatin fractions (chromatin-IP) were immunoprecipitated with antibodies as indicated, then the immunoprecipitates were resolved with SDS-PAGE and immunoblotted.

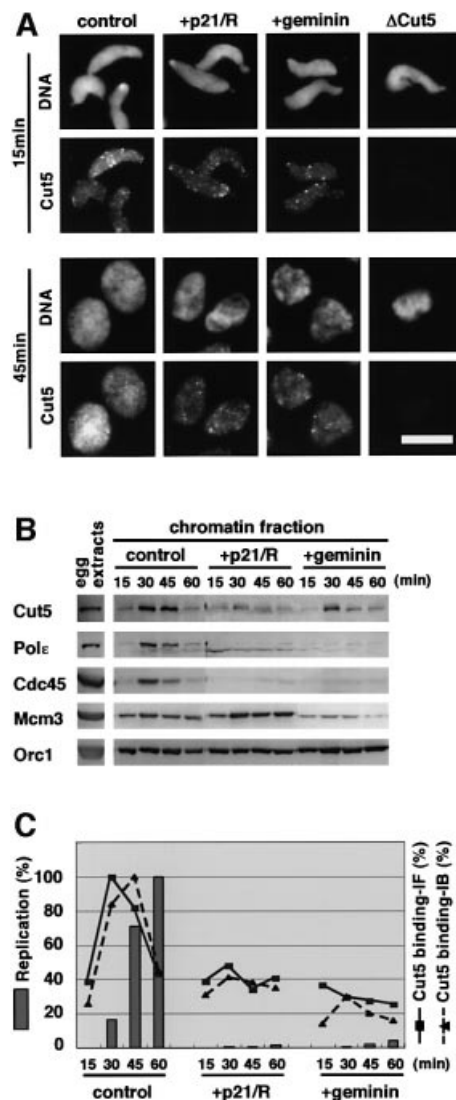
other replication proteins involved in the initiation complex remained in the extracts (Figure 5A). Using these extracts, sperm nuclei were incubated for 40 min and the isolated chromatin fractions were subsequently examined by immunoblotting (Figure 5A, chromatin). After 40 min incubation, DNA replication had been initiated in the mock-depleted extracts and all of the replication proteins so far examined were found to be associated with the chromatin. In the Cut5-depleted extract, ORC and MCM proteins were bound to chromatin at a similar level but neither Cdc45 nor DNA polymerases were detected in the chromatin. In Cdc45-depleted extracts, the binding of DNA polymerases  $\alpha$  and  $\epsilon$  was suppressed without affecting the formation of pre-RCs. In addition, Cut5 was bound to chromatin at a similar level to that of the mock-depleted extracts. These results show that Cut5 is indispensable for the chromatin binding of Cdc45 and DNA polymerases, but is dispensable for the assembly of pre-RCs, as monitored by the chromatin binding of ORC and MCM proteins.

The sequential binding of Cut5 and Cdc45 suggests a possible interaction between these two proteins. In addition, previous studies suggest an interaction between Dpb11/TopBP1 and DNA polymerase  $\epsilon$  (Masumoto *et al.*, 2000; Mäkinen *et al.*, 2001). We therefore examined physical interactions between Cut5 and other replication proteins. When the egg extracts were immunoprecipitated with anti-Cut5 antibody, we could detect Cut5 and a small amount of Cdc45 in the immunoprecipitates but not other replication proteins, including Mcm3 and DNA polymerase  $\epsilon$  (Figure 5B). With antibodies against the 60 kDa subunit of the polymerase, the co-immunoprecipitation of the largest subunit of the polymerase (p256) was detected but not the other proteins, including Cut5. To examine the interaction after the assembly onto chromatin, sperm nuclei were incubated for 50 min in the presence of aphidicolin, an inhibitor of DNA polymerases  $\alpha$ ,  $\delta$  and  $\epsilon$ , which allowed the maximal binding of Cut5, Cdc45, DNA polymerases  $\alpha$  and  $\epsilon$  onto chromatin (Mimura *et al.*, 2000). When the fragmented chromatin fractions were then immunoprecipitated with anti-Cut5 antibody, Cdc45 and a very small amount of the 60 kDa subunit of the polymerase were co-precipitated, but not the largest subunit of the polymerase (Figure 5B, chromatin-IP). Using a parallel assay with anti-60 kDa antibody, we could likewise detect only the 60 kDa subunit, suggesting that the largest subunit is rapidly degraded during the isolation. These results show, however, that Cut5 interacts with Cdc45.

### S-CDK-dependent and -independent binding of Cut5 to chromatin

To further investigate the role of Cut5, we examined the time-course of the chromatin binding of Cut5 under various conditions using immunofluorescence microscopy (Figure 6A). Before nuclear formation, which was confirmed by the absence of nuclear structures around chromatin DNA under phase contrast microscopy, weak signals of Cut5 were detected on chromatin DNA and focus structures were also observed on some fractions of chromatin (Figure 6A, control 15 min). Only a background signal was detected on DNA from Cut5-depleted extracts (Figure 6A,  $\Delta$ Cut5). The chromatin binding of Cut5 before initiation was not affected by either addition of p21 and roscovitine or geminin, both of which inhibit the replication activity of the extracts completely (Figure 6C). Upon initiation of DNA replication, the chromatin binding of Cut5 was greatly increased but it was almost completely suppressed by addition of p21 and roscovitine or geminin (Figure 6A). These results show that Cut5 binds to chromatin in two distinct modes. The first mode of binding was identified as weak Cut5 signals associated with chromatin even in the absence of S-CDK activity, or in the presence of geminin, which inhibits the formation of pre-RCs through its interaction with Cdt1 (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001). The second mode of binding was identified as strong signals dependent on pre-RC formation and S-CDK activity.

To compare the two modes of Cut5 binding more quantitatively, the amounts of Cut5 bound to chromatin were estimated based on immunofluorescence intensities (Figure 6C). In the control egg extract, the amount of Cut5 binding at 30 min had increased ~3-fold from the initial

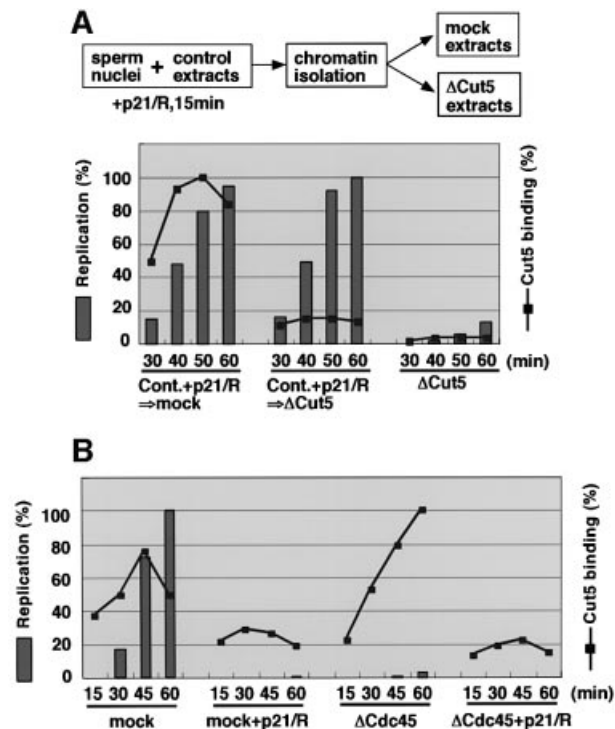


**Fig. 6.** CDK-dependent and -independent binding of Cut5 to chromatin. (A) Immunofluorescent detection of Cut5 binding. Sperm nuclei were incubated in S-phase egg extracts in the absence (control) or in the presence of 50  $\mu$ g/ml GST-p21 and 100  $\mu$ M roscovitine (+p21/rosc.) or 15  $\mu$ g/ml GST-geminin (+geminin) for the times indicated at 23°C. Cut5-depleted egg extracts ( $\Delta$ Cut5) were used as a negative control of immunofluorescence. Samples were treated with 0.25% NP-40 and then fixed with 3.7% formaldehyde. Nuclear localization of Cut5 was visualized with rabbit anti-Cut5 antibody followed by Alexa 488-labelled anti-rabbit IgG. DNA was visualized with Hoechst 33258. Scale bar 10  $\mu$ m. (B) Time-course of chromatin binding of various replication proteins. Sperm nuclei were incubated in 50  $\mu$ l of the extracts for the times indicated in the absence (control) or presence of p21 and roscovitine (+p21/rosc.) or 15  $\mu$ g/ml GST-geminin (+geminin). One microliter of egg extract and the isolated chromatin fractions were resolved by SDS-PAGE, then immunoblotted with antibodies against various replication proteins. (C) Time-courses of DNA replication and chromatin binding of Cut5. Fluorescence images captured as in (A) and the immunoblotted bands in (B) were quantified in order to estimate the amounts of chromatin-bound Cut5. Replication activity was detected as the incorporation of Cy3-dCTP into DNA. The fluorescence intensities of 40 nuclei were analyzed using NIH Image software. Relative average intensities per nucleus were then plotted against time, taking the maximal value as 100% (Replication and Cut5 binding-IF). For immunoblotting, the intensities of immunoblotted bands of Cut5 were normalized against those of Orc1, a loading control for chromatin fractions, and ratios of the amounts of Cut5 to Orc1 were then plotted against time, taking the maximal value as 100% (Cut5 binding-IB).

level (15 min) and it then decreased back to the initial level at 60 min, at which point replication was almost complete. In contrast, the amount of Cut5 remained at the initial level throughout the incubation with p21 and roscovitine or geminin. Similar results were also obtained from an immunoblotting analysis of chromatin fractions (Figure 6B, Cut5) and the amounts of chromatin-bound Cut5 estimated from the blotting data fit well with those from the immunofluorescence data (Figure 6C). In accordance with the observation of DNA replication activity by immunofluorescence, both DNA polymerase  $\epsilon$  and Cdc45 bound to chromatin had returned to nearly basal levels at 60 min, when replication was almost complete. In the presence of geminin, the chromatin binding of Mcm3 was inhibited but Orc1 and Cut5 were not affected (Figure 6B). These results show that the low level of Cut5 binding is independent of S-CDK and pre-RC formation.

Thus, Cut5 binds to chromatin in both S-CDK-independent and S-CDK-dependent modes but it is not known which mode is required for DNA replication. We first examined whether or not S-CDK-independent binding of Cut5 plays any role in DNA replication. In order to restrict Cut5 to its first, S-CDK-independent, binding mode, sperm nuclei were incubated in egg extract with p21 and roscovitine for 15 min and the chromatin fractions were then isolated from the extract. The isolated chromatin fractions were transferred to and incubated in mock- and Cut5-depleted egg extracts, and DNA replication activities were measured (Figure 7A). As shown in the figure, both extracts supported DNA replication to a similar level but Cut5 binding remained at a constant low level in the Cut5-depleted extract (Figure 7A, compare left and middle). As a negative control, sperm nuclei incubated in the Cut5-depleted extract throughout showed only marginal replication activity and the level of Cut5 binding was as low as 20% of the control level of CDK-independent binding (Figure 7A, right), indicating that Cut5 was almost completely depleted from this extract. These results show that the quantity of Cut5 bound in the absence of S-CDK is sufficient to support full DNA replication.

Cut5 binding to chromatin increased only after the action of S-CDK (Figure 6). Since S-CDK activity is required for the binding of Cdc45 to chromatin, a question remains as to whether S-CDK-dependent binding of Cut5 requires Cdc45 or not. We therefore examined the binding of Cut5 in the absence and presence of Cdc45. With mock-depleted extracts, Cut5 binding increased in an S-CDK-dependent manner (Figure 7B, compare mock and mock+p21/R). Similar results were obtained with Cdc45-depleted extracts (Figure 7B,  $\Delta$ Cdc45 and  $\Delta$ Cdc45+p21/R). The Cut5 binding in Cdc45-depleted extracts increased with incubation time and reached a higher level than in the mock-depleted extracts (Figure 7B, mock and  $\Delta$ Cdc45). These results show that Cdc45 is dispensable for the S-CDK-dependent binding of Cut5. Taken together, the modes of Cut5 binding to chromatin are summarized as follows. Initially, small but significant amounts of Cut5 bind to chromatin before the activation of S-CDK and this is sufficient for DNA replication. The majority of Cut5 binding occurs after the activation of S-CDK, independently of Cdc45 and DNA replication, and this

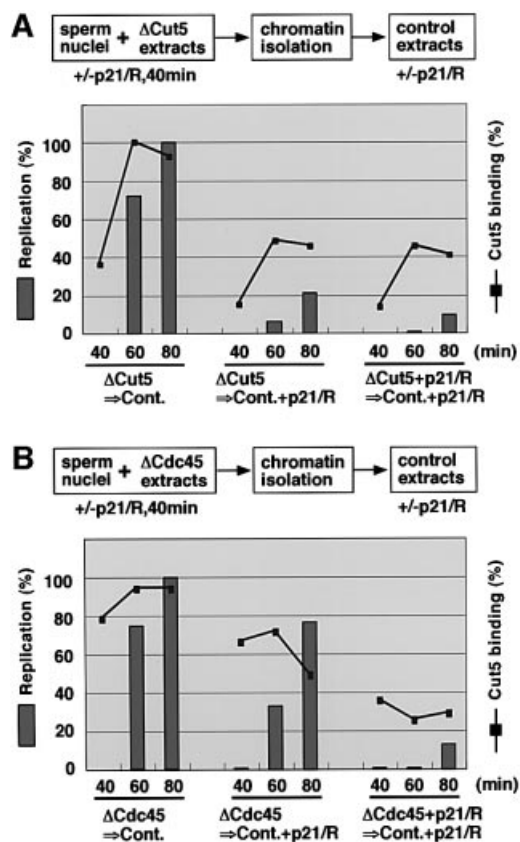


**Fig. 7.** Requirement of chromatin binding of Cut5 for DNA replication. (A) Sperm nuclei were pre-incubated in S-phase egg extract in the presence of 50  $\mu$ g/ml p21 and 100  $\mu$ M roscovitine (p21/R) for 15 min at 23°C. The chromatin fractions were then isolated and incubated in mock- and Cut5-depleted egg extracts, and DNA replication activities were measured (Figure 7A). As shown in the figure, both extracts supported DNA replication to a similar level but Cut5 binding remained at a constant low level in the Cut5-depleted extract (Figure 7A, compare left and middle). As a negative control, sperm nuclei incubated in the Cut5-depleted extract throughout showed only marginal replication activity and the level of Cut5 binding was as low as 20% of the control level of CDK-independent binding (Figure 7A, right), indicating that Cut5 was almost completely depleted from this extract. These results show that the quantity of Cut5 bound in the absence of S-CDK is sufficient to support full DNA replication. (B) S-CDK-dependent binding of Cut5 to chromatin in the absence of Cdc45. Sperm nuclei were incubated in mock- and Cdc45-depleted egg extracts with or without p21/roscovitine at 23°C. After incubation for the times indicated, samples were analyzed as in (A).

increased amount of binding is dispensable for DNA replication.

#### **Determination of the order of reaction of S-CDK, Cut5 and Cdc45 in the initiation of DNA replication**

Both Cut5 and S-CDK are required for the initiation of DNA replication, and S-CDK-independent binding of Cut5 is fully responsible for this initiation. This raises two possibilities as to the function of Cut5: one is that both Cut5-dependent and S-CDK-dependent processes are separately required for initiation, the other is that Cut5 binding is required for the action of S-CDK in initiation. In order to distinguish these two possibilities, we examined whether or not S-CDK could act in the absence of Cut5. For this purpose, we first incubated sperm nuclei in Cut5-depleted extracts in the absence of CDK inhibitors for 40 min. Since the addition of CDK inhibitors after 40 min incubation could only slightly inhibit the replication activity of mock-depleted extracts (data not shown, see also Strausfeld *et al.*, 1994), it is reasonable to assume that S-CDK function has been almost executed after 40 min incubation. Next, the nuclear fractions were isolated and transferred to egg extracts in the presence or absence of



**Fig. 8.** Requirement of Cut5 and Cdc45 for the action of S-CDK. (A) Sperm nuclei were pre-incubated in Cut5-depleted egg extracts in the presence or absence of 50  $\mu\text{g/ml}$  p21 and 100  $\mu\text{M}$  roscovitine (p21/R) for 40 min at 23°C. The chromatin fractions were isolated and incubated in egg extracts in the absence (Cont.) or presence of p21/roscovitine (Cont.+p21/R) at 23°C. After incubation for the times indicated, which include 40 min pre-incubation time, samples were treated with NP-40 and fixed with formaldehyde. The amount of Cut5 bound to chromatin and the incorporation of Cy3-dCTP were analyzed as described in the legend for Figure 6. (B) Similar experiments to those in (A) were performed, using Cdc45-depleted extracts instead of Cut5-depleted extracts.

S-CDK inhibitors, and DNA replication activity was examined (Figure 8A). In the absence of the inhibitors, we could detect as much DNA replication activity as was observed in the control extracts (data not shown), and the chromatin association of Cut5 also increased with time (Figure 8A,  $\Delta\text{Cut5} \rightarrow \text{Cont.}$ ). On the other hand, in the presence of CDK inhibitors during the incubation, DNA replication was reduced to as little as 20% and Cut5 binding to <50% of the levels observed without the inhibitors (compare Figure 8A,  $\Delta\text{Cut5} \rightarrow \text{Cont.}$  and  $\Delta\text{Cut5} \rightarrow \text{Cont.} + \text{p21/R}$ ). A similarly low level of association of Cut5 was observed when the inhibitors were included in the extracts throughout the experiment (Figure 8A,  $\Delta\text{Cut5} + \text{p21/R} \rightarrow \text{Cont.} + \text{p21/R}$ ). These results show that S-CDK activity is still required even after pre-incubating chromatin in Cut5-depleted extracts in the presence of CDK activity.

Both S-CDK and Cut5 are required for the loading of Cdc45 but it is not known whether S-CDK function is required simultaneously with Cdc45 or not. In order to clarify this point, sperm nuclei were incubated in Cdc45-

depleted extracts for 40 min. The chromatin fractions were then isolated, transferred to egg extracts in the presence or absence of the CDK inhibitors, and DNA replication activities were measured (Figure 8B). In the control experiment without the inhibitors, we found high activity of DNA replication and high levels of Cut5 binding to chromatin during the incubation (Figure 8B,  $\Delta\text{Cdc45} \rightarrow \text{Cont.}$ ). Surprisingly, even in the presence of the inhibitors, we could detect DNA replication activity as high as 70% of control levels (Figure 8B,  $\Delta\text{Cdc45} \rightarrow \text{Cont.}$  and  $\Delta\text{Cdc45} \rightarrow \text{Cont.} + \text{p21/R}$ ). However, DNA replication was almost completely inhibited when CDK activity was inhibited throughout the experiment (Figure 8B,  $\Delta\text{Cdc45} + \text{p21/R} \rightarrow \text{Cont.} + \text{p21/R}$ ). These results show that S-CDK could act in the absence of Cdc45. In other words, Cdc45 functions after the action of S-CDK.

## Discussion

In this paper, we have identified *Xenopus* Cut5 and characterized its function using the cell-free replication system of *Xenopus* egg extracts. Major features of the present findings are as follows. First, we found that Cut5 is required for the loading of Cdc45 onto chromatin, which eventually leads to the loading of replicative DNA polymerases, but not for the elongation stages of DNA replication. Secondly, we found that Cut5 binds to chromatin in two distinct modes—one is S-CDK-independent and the other S-CDK-dependent—and that the quantity of Cut5 bound in the former mode is sufficient to support full replication. Thirdly, we found that S-CDK acts after the binding of Cut5 and before the binding of Cdc45 to chromatin. These results demonstrate that Cut5 plays a crucial role in S-CDK-dependent formation of the pre-initiation complex of DNA replication.

### Essential role of Cut5 in the initiation of DNA replication

Previous studies have shown that Cut5 homologs, including SpCut5, ScDpb11 and HsTopBP1, are implicated in DNA replication (Saka and Yanagida, 1993; Araki *et al.*, 1995; Mäkinen *et al.*, 2001). In addition, a recent report on the budding yeast *dpb11-1* mutant suggests that Dpb11 plays an important role in the loading of polymerases onto replication origins (Masumoto *et al.*, 2000). Here, we found that *Xenopus* Cut5 is essential for DNA replication in *Xenopus* egg extracts (Figure 3), and that it is required for the loading of polymerases  $\alpha$  and  $\epsilon$  (Figure 5A). These results confirm the universal role of Cut5 in replication initiation. We also found that Cut5 is required for the binding of Cdc45 to chromatin, while Cdc45 is not required for Cut5 binding. Since Cdc45 is required for polymerase loading (Mimura and Takisawa, 1998; Aparicio *et al.*, 1999; Mimura *et al.*, 2000), these results suggest the sequential binding of Cut5, Cdc45 and DNA polymerases onto chromatin. In contrast, a chromatin immunoprecipitation assay with budding yeast suggested that Cdc45 associates with some early origins during G<sub>1</sub> phase (Aparicio *et al.*, 1999) and that Dpb11 becomes associated with origins only at the onset of S-phase (Masumoto *et al.*, 2000), thus supporting the idea that Dpb11 functions after the binding of Cdc45. However, the amount of Cdc45 bound to chromatin increases only at the

onset of S-phase (Aparicio *et al.*, 1999; Zou and Stillman, 2000) and Dpb11 is required for the chromatin binding of Cdc45 (Y.-S.Tak and H.Araki, personal communication). This would suggest that Cut5 is required for functional chromatin binding of Cdc45. In accordance with this notion, *Xenopus* Cut5 binds to chromatin before nuclear formation and in the absence of S-CDK activity, while the binding of Cdc45 is dependent on both these events (Mimura and Takisawa, 1998).

A recent study suggests that Mcm10 is also required for the binding of Cdc45 to chromatin in a nucleus-free system derived from *Xenopus* egg extracts (Wohlschlegel *et al.*, 2002). In this system, the recruitment of Mcm10 onto chromatin is independent of S-CDK and Cdc7 activities but it is dependent on MCM proteins and occurs only after the addition of nucleoplasmic extract, which may mimic the nuclear formation in our system. It is not known whether Cut5 binding is dependent on Mcm10 or not but since it is apparently independent of pre-RC formation, it is reasonable to assume that Cut5 binding is independent of Mcm10. The genetic interactions between Dpb11 and Mcm10 (Kawasaki *et al.*, 2000) may suggest that Cut5/Dpb11 is required for the loading of Cdc45 in cooperation with Mcm10.

Physical interaction of Dpb11/TopBP1 with DNA polymerase  $\epsilon$  suggests that Dpb11/TopBP1 has a direct role in the loading of the polymerase onto unwound origins (Masumoto *et al.*, 2000; Mäkinen *et al.*, 2001). We found that only a small amount of the 60 kDa subunit of polymerase  $\epsilon$  could be co-immunoprecipitated with *Xenopus* Cut5 in fragmented chromatin fractions, and that the co-immunoprecipitation of the polymerase with Cut5 could not be detected using the egg extracts (Figure 5B). These results suggest that a physical interaction between Cut5 and DNA polymerase  $\epsilon$  is weak and may be transient. Actually, a significant interaction between Dpb11 and the polymerase was observed only after the chemical cross-linking of the proteins bound to chromatin fractions (Masumoto *et al.*, 2000). More importantly, we found the co-immunoprecipitation of Cdc45 and Cut5 from egg extracts as well as chromatin fractions (Figure 5B). Such a physical interaction between Cut5 and Cdc45 suggests that Cut5 plays a direct role in the loading of Cdc45 onto chromatin.

### Two modes of Cut5 binding to chromatin

Here we found that Cut5 binds to chromatin in both an S-CDK-dependent and an S-CDK-independent manner (Figure 6). To obtain further insights into the function of Cut5, we estimated the amount of Cut5 bound to chromatin independently of S-CDK. The concentration of Cut5 in the extracts was estimated as 5 ng/ $\mu$ l based on immunoblotting (Figure 3A), and the amount of Cut5 on chromatin (control 15 min) from 50  $\mu$ l of the egg extracts (4000 nuclei/ $\mu$ l) was calculated to be roughly equal to the amount in 0.3  $\mu$ l of the extracts (Figure 6B). Given that the *Xenopus* genome contains  $2.9 \times 10^9$  bp of DNA (Wohlschlegel *et al.*, 2002), Cut5 binds to chromatin at one molecule/ $\sim 10^4$  bp, which is close to the average replicon size ( $\sim 10$  kb) in *Xenopus* egg extract (Walter and Newport, 1997; Blow *et al.*, 2001). This result may infer that S-CDK-independent binding is dependent on the ORC. Since recombinant TopBP1 binds to DNA *in vitro*

(Yamane and Tsuruo, 1999), it is now important to clarify whether Cut5 also has the ability to bind to chromatin independently of ORC.

The S-CDK-dependent binding of Cut5 is dispensable for replication activity (Figure 7A) and does not require Cdc45 (Figure 7B), showing that it does not depend on origin firing events, such as the loading of DNA polymerases. Previous studies have shown that Cut5 has dual functions, one for DNA replication and the other for the S-phase checkpoint (Saka and Yanagida, 1993; Saka *et al.*, 1994; Araki *et al.*, 1995; Wang and Elledge, 1999). It is tempting to speculate that the CDK-dependent Cut5 binding may be required for the checkpoint function. Since a defect in Cut5 leads to premature mitosis without DNA replication (Saka and Yanagida, 1993; Araki *et al.*, 1995), Cut5 may monitor entry into S-phase and send signals to prevent premature mitosis.

### Sequential action of Cut5, S-CDK and Cdc45 in the formation of pre-ICs

Two kinase activities, DDK and S-CDK, are required for the initiation of DNA replication (Takisawa *et al.*, 2000; Bell and Dutta, 2002). In the present study, we did not examine the role of DDK in the chromatin binding of Cut5. However, DDK associates with chromatin at about the time of nuclear assembly (Jares and Blow, 2000), suggesting that S-CDK-independent binding does not require DDK. Since DDK acts before S-CDK in the *Xenopus* system (Jares and Blow, 2000; Walter, 2000), our results suggest that DDK and S-CDK both act sequentially after the binding of Cut5. Our results further show that Cut5 but not Cdc45 is required for S-CDK to function in initiation (Figure 8). Since the S-CDK-independent binding of Cut5 is sufficient for initiation (Figure 7A), it is reasonable to assume that Cut5 binds first, then S-CDK functions and finally Cdc45 joins in the formation of pre-ICs. This scenario suggests that the target of S-CDK may be Cut5 itself. Cut5 homologs in metazoans have a conserved CDK target site (see Figure 1), and *Xenopus* Cut5 is phosphorylated by cyclin A/Cdk2 *in vitro* (our unpublished observation). However, the mutant *Xenopus* protein obtained by converting threonine into alanine in the CDK site (amino acid 814) still supported the replication activity of Cut5-depleted extracts (Supplementary data). In addition, yeast Cut5/Dpb11 does not contain such a CDK target site and no evidence has been obtained for its phosphorylation. Recent studies with yeast demonstrate that Sld2 is a novel target of S-CDK, and that S-CDK-dependent phosphorylation of Sld2/Drc1 positively regulates DNA replication through the association of Sld2/Drc1 with Dpb11/Cut5 (Masumoto *et al.*, 2002; Noguchi *et al.*, 2002). A homolog of Sld2/Drc1 has not been identified in other organisms but the present data suggest that such a homolog is also functioning in higher eukaryotes. Further study of the function of Cut5 will clarify the molecular mechanisms for the action of S-CDK in pre-RC activation in higher eukaryotes.

## Materials and methods

### Cloning and sequencing of *Xenopus* Cut5

To clone *Xenopus* Cut5, we obtained a partial sequence of a potential *Xenopus* Cut5 (accession No. AW635109) from the EST database by



homology searching with human TopBP1. The sequence was amplified from *Xenopus* oocyte cDNA by PCR using 5'-primer (ATTATACAC-CAGCAAAGGAATC) and 3'-primer (ATGTTGGTCACAGCACT-CAG). The amplified PCR fragment was used as a probe to screen a  $\lambda$ ZAPII-derived cDNA library of *Xenopus* oocyte mRNA. From  $5 \times 10^5$  plaques, we isolated four positive clones for *Xenopus* Cut5 with insert sizes of ~1.5 kb, 2 kb, 3 kb and 4.7 kb. The largest clone, of 4.7 kb, was sequenced with an automatic DNA sequencer (ABI, 377). The sequence thus determined encoded a protein lacking the N-terminus so the 5' region was further expanded using the 5'RACE system (Gibco BRL) from total *Xenopus* oocyte RNA. The full-length *Xenopus* Cut5 gene (accession No. AB091779) consisted of 5078 bp containing a 5'UTR of 103 bp, an ORF of 4542 bp and a 3'UTR of 433 bp.

#### Protein expression and antibody production

A full-length ORF of *Xenopus* Cut5 was prepared as follows. The plasmid (pBluescript) carrying the cDNA of 4.7 kb (corresponding to amino acids 86–1513) was digested with *EcoRI* and *XhoI* and a 4.7 kb fragment was subcloned into 6 $\times$  His-tag vector pFastBac HTb (Gibco BRL). The plasmid obtained was further digested with *NcoI* and *ApaI* and the digested plasmid containing the ORF, corresponding to amino acids 310–1513, was used for the following ligation reaction. The N-terminal coding region (corresponding to amino acids 1–309) was amplified by RT-PCR from total *Xenopus* oocyte RNA using 5' primer (TACCATGG-GTTTCGAGTGAACG) and 3' primer (AAGGGCCCCGACTATTTG-GTTTGC). The PCR product was digested with *NcoI* and *ApaI*, whose recognition sites were included in the primer ends (indicated by underlining). The digested PCR product and the digested plasmid were ligated to construct the full-length ORF (amino acids 1–1513). A recombinant baculovirus 6 $\times$  His-*Xenopus* Cut5 was obtained using BAC-TO-BAC HT recombinant baculovirus expression system (Gibco BRL). The recombinant protein was expressed in Sf9 cells and purified on a Ni-NTA column (Qiagen) following the instructions of the supplier. Polyclonal rabbit antiserum was raised against the recombinant protein (Hokudo Inc., Japan) and further affinity-purified with the recombinant protein immobilized on Affi-Gel 10 (Bio-Rad).

#### Complementation in yeast mutant strains

A full-length coding region of *Xenopus* Cut5 (corresponding to amino acids 1–1513) and an N-terminal coding region of *Xenopus* Cut5 (corresponding to amino acids 1–761) were cloned into the pREP expression vector (Maundrell, 1993) at the *NdeI* and *SalI* sites for *S.pombe*, and into the pKT10 expression vector (Tanaka *et al.*, 1990) at the *KpnI* and *XhoI* sites for *S.cerevisiae*. *S.pombe* cut5-T401 and *S.cerevisiae* dpb11-1 mutants were transformed with these vectors and transformants were plated at restrictive, semi-permissive or permissive temperature.

#### Preparation of *Xenopus* egg extract and chromatin fractions

S-phase egg extract and demembrated sperm nuclei were prepared as described (Kubota and Takisawa, 1993). Aphidicolin was added to the egg extracts at a concentration of 40  $\mu$ g/ml to inhibit DNA polymerases. GST-p21 (Mimura and Takisawa, 1998) and roscovitine (Sigma) were added to the extracts at 50  $\mu$ g/ml and 100  $\mu$ M, respectively, to inhibit S-CDK. GST-geminin (Mimura *et al.*, 2000) was added at 15  $\mu$ g/ml to inhibit the licensing reaction. To isolate chromatin fractions, sperm nuclei were incubated in 50–100  $\mu$ l of the extracts (4000 nuclei/1  $\mu$ l extracts) for appropriate times at 23°C. The samples were diluted with 10 vol of EB (100 mM KCl, 2.5 mM MgCl<sub>2</sub> and 50 mM HEPES-KOH pH 7.5) containing 0.25% NP-40, then centrifuged through a 10% sucrose layer at 10 000 g and the pellets were washed with EB. The pellets were treated as follows. For immunoblotting, the chromatin-associated proteins were extracted with SDS-PAGE sample buffer and separated from DNA by passage through a 0.45  $\mu$ m filter. Quantification of the immunoblots was performed with NIH Image software. For immunoprecipitation, the pellets were resuspended in 1 vol of EB with protease inhibitors (1  $\mu$ g/ml each of leupeptin, pepstatin and aprotinin). The suspensions were then sonicated with a sonicator (U50; IKA Labortechnik) until DNA was sheared into fragments with an average size of 700 bp and the soluble fractions were recovered by centrifugation at 10 000 g. For the chromatin transfer experiments, the chromatin fractions were prepared as described above except that NP-40 was omitted from the buffers, a 20% sucrose layer was used and the isolated chromatin fractions were resuspended in egg extracts.

#### Assay for DNA replication activity

The replication activities of the egg extracts were measured as the incorporation of [ $\alpha$ -<sup>32</sup>P]dCTP or Cy3-dCTP into sperm DNA. [ $\alpha$ -<sup>32</sup>P]dCTP incorporation was carried out as described (Mimura and Takisawa, 1998) except that the autoradiography was quantified by Image Gauge software (Fuji Film). For Cy3-dCTP incorporation, the method was as described below in fluorescence microscopy.

#### Immunodepletion and immunoprecipitation

Immunodepletion of *Xenopus* proteins was carried out as described (Mimura and Takisawa, 1998) except that rProtein A Sepharose Fast Flow (Amersham Biosciences) was used instead of Affi-Prep protein A matrix (Bio-Rad). The immunoprecipitations of egg extracts and fragmented chromatin fractions were performed as described (Mimura and Takisawa, 1998; Mimura *et al.*, 2000).

#### Fluorescence microscopy

Sperm nuclei (4000/ $\mu$ l) were incubated in 10  $\mu$ l of the egg extract containing 10  $\mu$ M Cy3-dCTP (Amersham Biosciences) for appropriate times at 23°C. The samples were prepared as described (Mimura *et al.*, 2000) and fluorescence images were captured with the OpenLab imaging program (Improvision) and analyzed with NIH Image software (Kubota *et al.*, 1997) to estimate the amounts of chromatin-bound Cut5 and the replication activities of the egg extracts.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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