The *Xenopus* Xmus101 protein is required for the recruitment of Cdc45 to origins of DNA replication

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The initiation of eukaryotic DNA replication involves origin recruitment and activation of the MCM2-7 complex, the putative replicative helicase. Minichromosome maintenance (MCM)2-7 recruitment to origins in G1 requires origin recognition complex (ORC), Cdt1, and Cdc6, and activation at G1/S requires MCM10 and the protein kinases Cdc7 and S-Cdk, which together recruit Cdc45, a putative MCM2-7 cofactor required for origin unwinding. Here, we show that the *Xenopus* BRCA1 COOH terminus repeat–containing Xmus101 protein is required for loading of Cdc45 onto the origin. Xmus101 chromatin association is dependent on ORC, and independent of S-Cdk and MCM2-7. These results define a new factor that is required for Cdc45 loading. Additionally, these findings indicate that the initiation complex assembly pathway bifurcates early, after ORC association with the origin, and that two parallel pathways, one controlled by MCM2-7, and the other by Xmus101, cooperate to load Cdc45 onto the origin.

Introduction

The initiation of DNA replication is tightly regulated so that origins of replication are normally utilized only once per cell cycle. Many of the factors and regulators that participate in the initiation phase of DNA replication are known, and the order in which these factors interact with one another and load onto origins is emerging. The process begins in late mitosis or G1, when origin recognition complex (ORC)* binds directly to chromosomal origins of replication (for review see Kelly and Brown, 2000; Bell and Dutta, 2002). Once bound, ORC recruits Cdc6 and Cdt1, which in turn allow assembly of the mini-chromosome maintenance (MCM)2-7 complex onto the DNA. Recruitment of MCM2-7, the presumptive replicative helicase (Labib and Diffley, 2001), completes assembly of the prereplication complex (pre-RC). At the G1/S transition, the pre-RC is converted to a preinitiation complex (pre-IC) through the actions of two protein kinases, S phase–specific cyclin dependent kinase (S-Cdk) and the Cdc7-Dbf4 protein kinase, which cooperate to recruit the initiation factor Cdc45 (Mimura and Takisawa, 1998; Zou and Stillman, 1998, 2000; Jares and Blow, 2000; Walter, 2000). In addition to S-Cdk and Cdc7, another factor, MCM10, has been shown in *Xenopus* extracts to act after MCM2-7 to recruit Cdc45 (Wohlschlegel et al., 2002). After Cdc45 binding, the next discernible step in the initiation process is origin unwinding and recruitment of the single-stranded DNA binding protein RPA, followed by DNA polymerase α (pol α; Mimura and Takisawa, 1998; Tanaka and Nasmyth, 1998; Zou and Stillman, 1998, 2000; Mimura et al., 2000; Walter and Newport, 2000). Because Cdc45 loading is the last known step before origin unwinding and the commencement of DNA synthesis, and because it has been shown to be rate limiting for DNA replication in *Xenopus* egg extracts (Edwards et al., 2002), this event is considered to be critical for the regulation of initiation.

The *Drosophila mus101* gene was isolated 25 years ago in a screen for mutants showing hypersensitivity to DNA damaging agents (Boyd et al., 1976). Further genetic analysis revealed that *mus101* is an essential gene. Hypomorphic alleles have been isolated that exhibit defects in the kinetics of progression through S phase, and in chorion gene amplification (Orr et al., 1984). Therefore, these two phenotypes are consistent with a role for *mus101* in DNA replication. Molecular cloning of *mus101* (Yamamoto et al., 2000)

Figure 1. **Xmus101 is required for DNA replication in *Xenopus*.** (A) The Mus101 protein family. Shown are schematic depictions of Mus101-related proteins from divergent organisms. The shaded gray boxes indicate the position of the BRCT domains, and the numbers to the right indicate the size of the protein, in amino acids. For comparison, the domain structure of budding yeast Dpb11 and fission yeast Cut5 is also shown. (B) Immunoblot analysis of *Xenopus* egg extract (XEE), or egg extract that had been immunodepleted of Xmus101 (α-Xmus101), probed with affinity-purified anti-Xmus101 antibodies. The asterisk denotes a background band recognized by the antibody that is not diminished in the depleted extract. The numbers to the left of the gel denote the migration position and molecular mass, in kD, of molecular mass markers. (C) Egg extracts were prepared, and then depleted of Xmus101 protein (α-Xmus101) or mock-depleted (mock). Additionally, Xmus101-depleted extracts were supplemented with in vitro transcription and translation (IVT) reactions programmed by the Xmus101 cDNA. In vitro transcription was performed in either the sense, or anti-sense orientation. Sperm chromatin was then added, along with ^{32}P-dATP, and DNA replication in the given extract was measured at the indicated times. The graph depicts the amount of DNA replication observed after quantification of the dried gels by PhosphoImager analysis. The data shown are from a single experiment, and are representative of four independent trials. The amount of replication observed in the mock-depleted sample at the 90-min time point was set to 100, and all other values adjusted accordingly.
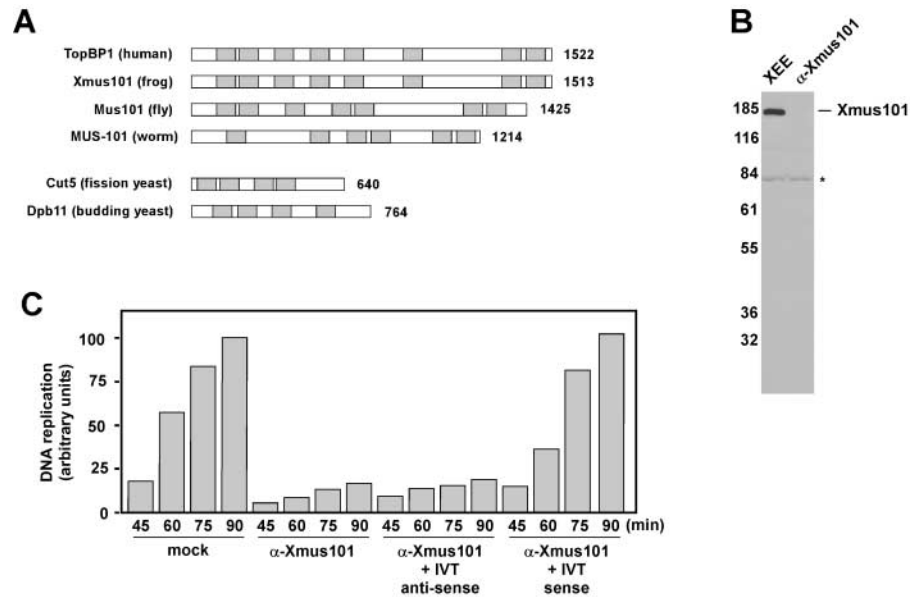
showed that the gene encodes a protein composed of reiterated BRCA1 COOH terminus (BRCT) domains, and that it is highly related to a human protein named TopBP1, a putative DNA damage response protein (Yamane et al., 1997, 2002; Makiniemi et al., 2001). Mus101/TopBP1 is also distantly related to two yeast genes known to be required for DNA replication, budding yeast Dpb11 (Araki et al., 1995) and fission yeast Cut5 (Saka and Yanagida, 1993). In DNA replication, Dpb11 is thought to act after Cdc45 loading, and RPA binding, to recruit pol α and pol ε to the unwound origin (Masumoto et al., 2000). This role for Dpb11 is supported by the findings that Dpb11 requires MCM2-7 and RPA to associate with the origin, that Dpb11 is required for origin binding of pol α and pol ε, but not RPA, and that Dpb11 interacts both genetically and physically with pol epsilon (Araki et al., 1995; Masumoto et al., 2000). In addition to their DNA replication functions, both Dpb11 (Araki et al., 1995; Wang and Elledge, 1999) and Cut5 (Saka and Yanagida, 1993; Saka et al., 1997) are required for cell cycle arrest in response to DNA replication blocks. It is not known if Mus101/TopBP1 shares with Dpb11/Cut5 a role in checkpoint control.
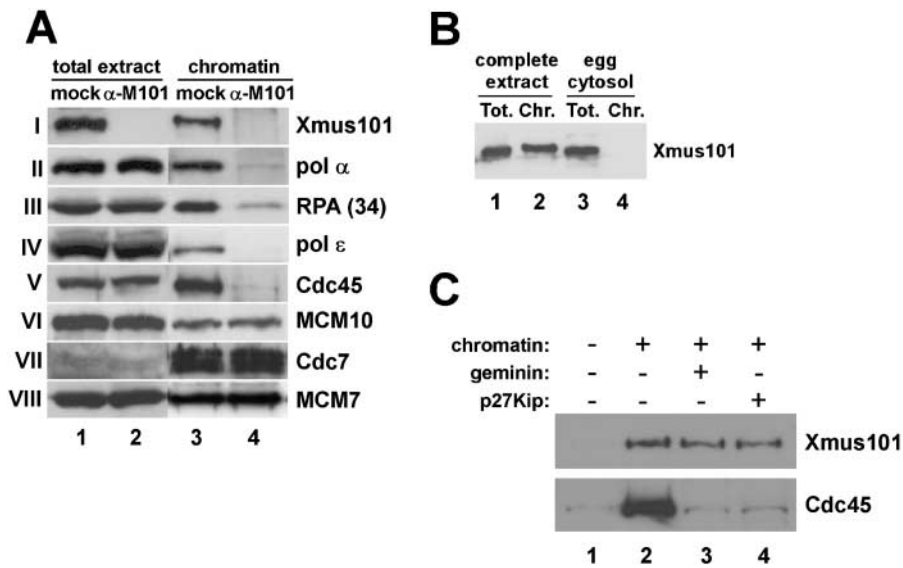
In order to uncover the Mus101 function in DNA replication, we have isolated the *Xenopus* Mus101 protein and used *Xenopus* egg extracts to identify the Mus101-dependent step in DNA replication. The results yield the surprising conclusion that Mus101 functions to load Cdc45 onto replication origins, and that it does so in a manner distinct from the other known Cdc45 loading factors.

Results and discussion

The metazoan Mus101 protein family includes human TopBP1, *Drosophila* Mus101, and an uncharacterized locus

in *C. elegans*, F37D6.1 (Fig. 1 A). To initiate an analysis of this protein family in DNA replication, we isolated a *Xenopus* homologue of Mus101, named Xmus101. The full-length Xmus101 cDNA encodes a 1,513 amino acid protein with significant similarity to TopBP1 (75% amino acid similarity), to *Drosophila* Mus101 (43% similarity), and to *C. elegans* F37D6.1 (39% similarity). The similarity between Xmus101 and Cut5 is restricted to the BRCT domains, whereas no significant sequence similarity between Xmus101 and Dpb11 was detected.

Rabbit polyclonal antibodies were raised against a recombinant protein containing the NH$_2$-terminal 333 amino acids of Xmus101, and affinity purified. These antibodies recognize a 180-kD protein in *Xenopus* egg extract (Fig. 1 B), and can immunoprecipitate the 180 kD in vitro transcription and translation product of the cloned Xmus101 cDNA (unpublished data). To determine if Xmus101 functions in DNA replication in *Xenopus*, the protein was removed from egg extracts, by immunodepletion, and the ability of the depleted extract to replicate sperm chromatin was assessed. As shown in Fig. 1 B, the immunodepletion procedure removed all detectable Xmus101 from the extract. To control for nonspecific effects of immunodepletion, a mock-depleted extract was also prepared, and replication was measured in both the mock- and Xmus101-depleted extracts. DNA replication was severely compromised in the Xmus101-depleted extract, relative to the mock-depleted control extract (Fig. 1 C, compare "mock" sample to "α-Xmus101" sample). Importantly, addition of recombinant Xmus101 protein, produced by transcription and translation in vitro, fully rescued the replication defect in the Mus101-depleted extract (Fig. 1 C, "α-Xmus101+IVT sense" sample). Rescue was not observed when transcription was performed in

Figure 2.   **Xmus101 is required for the loading of Cdc45 onto chromatin during the initiation of DNA replication.** (A) Immunoblot of either total extract samples from mock-depleted (mock, lane 1), or Xmus101-depleted (α-Xmus101, lane 2) extract, or chromatin isolated after a 45-min incubation in either mock-(mock, lane 3), or Xmus101-depleted (α-Xmus101, lane 4) extract. The blots were probed with antibodies against *Xenopus* Xmus101 (panel I), pol α (pol α p60 subunit, panel II), RPA (the 34-kD subunit, panel III), pol ε (pol α p70 subunit, panel IV), Cdc45 (panel V), MCM10 (panel VI), Cdc7 (panel VII), or the MCM7 component of the MCM2-7 complex (panel VIII). (B) Immunoblot of complete egg extract, both total extract (Tot.) and the isolated chromatin fraction (Chr.), and membrane-free egg extract, or egg cytosol, both total extract and the isolated chromatin fraction. (C) Egg extracts were prepared and supplemented with sperm chromatin (lane 2), or sperm chromatin plus recombinant geminin (500 nM, lane 3), or sperm chromatin plus recombinant p27Kip (500 nM, lane 4). After a 45-min incubation, the chromatin was isolated and probed, by immunoblotting, for the presence of Cdc45 and Xmus101. Lane 1 shows a reaction lacking sperm chromatin.

the opposite orientation, to produce the anti-sense Xmus101 mRNA (Fig. 1 C, "α-Xmus101+IVT anti-sense" sample). This rescue by the sense, but not anti-sense Xmus101 mRNA, demonstrates that the Xmus101-depleted extracts failed to replicate their DNA because Xmus101 was removed, and not because of nonspecific inactivation of the extract. These data show that Xmus101 is an essential DNA replication protein in *Xenopus.*

Chromatin immuoprecipitation-based methodologies in budding yeast indicate that Dpb11 acts after RPA has bound to the origin to recruit pol α and pol ε to the assembling replication complex (Masumoto et al., 2000). Because Xmus101 is distantly related to Dpb11, we asked if Xmus101 was required for replication complex assembly in egg extracts. For this, sperm chromatin was added to either mock- or Xmus101-depleted extract, and after 45 min the chromatin was isolated from the extract and probed by immunoblotting for the presence of components of the replication complex. This experiment shows that loading of the MCM2-7 complex onto chromatin was not affected by the absence of Xmus101, as equal amounts of chromatin-associated MCM7 protein were found in the mock-depleted sample relative to the Xmus101-depleted sample (Fig. 2 A, panel VIII, lanes 3 and 4). In contrast, there was a dramatic decrease in the amount of pol α, RPA, pol ε, and, interestingly, Cdc45 associated with chromatin in the Xmus101-depleted sample, compared with the mock-depleted sample (Fig. 2 A, panels II–V, lanes 3 and 4, respectively). This decrease in pol α, RPA, pol ε, and Cdc45 levels was not observed when total extract was blotted (Fig. 2 A, panels II –V, lanes 1 and 2, respectively), indicating that the decrease was not due to quantitative coprecipitation with Xmus101. We also assessed the ability of Cdc7 and MCM10 to bind to chromatin in Xmus101-depleted extract, and found that both of these factors were able to assemble into the initiation complex in the absence of Xmus101 (Fig. 2 A, panels VI and VII, lanes 3 and 4). Based on the data in Fig. 2 A, we con-

clude that Xmus101 is required for chromatin-binding of Cdc45, RPA, pol α, and pol ε, but not for MCM2-7, Cdc7, or MCM10. Thus, unlike budding yeast Dpb11, Xmus101 is a Cdc45 loading factor that acts prior to origin unwinding during initiation.

To better understand how Xmus101 participates in Cdc45 recruitment, we characterized its chromatin-binding properties. We found that Xmus101 did not load onto chromatin in membrane-free egg extracts (egg cytosol) that are competent to form pre-RCs, but incompetent to form nuclei or execute subsequent initiation events (Fig. 2 B, lane 4). In contrast, in nuclear assembly extracts that undergo initiation of replication, Xmus101 bound to chromatin (Fig. 2 B, lane 2). The inability to bind to chromatin in egg cytosol indicates that Xmus101 is not a constituent of the pre-RC. To ask if Xmus101 chromatin binding is dependent upon an intact pre-RC once nuclear formation occurs, we assessed Xmus101 chromatin-association in extracts treated with geminin, an inhibitor of the Cdt1 protein (Wohlschlegel et al., 2000; Tada et al., 2001). Interestingly, we found that Xmus101 chromatin binding was insensitive to geminin (Fig. 2 C, lane 3). To ensure that the geminin was active in this experiment, we also probed the purified chromatin fraction for the presence of Cdc45, and found, as expected (Jares and Blow, 2000), that geminin blocked Cdc45 association with chromatin (Fig. 2 C, lane 3). Next, we determined if Xmus101 chromatin association required S-Cdk activity. For this we used p27Kip (Toyoshima and Hunter, 1994), an S-Cdk inhibitor that has previously been shown to block DNA replication in frog egg extracts (Michael et al., 2000). We found that p27Kip prevented Cdc45 from loading onto chromatin, as expected, but that it could not prevent loading of Xmus101 (Fig. 2 C, lane 4).

The data in Fig. 2 (B and C) demonstrate that Xmus101 requires nuclear formation to bind to chromatin, but that it binds in a S-Cdk– and pre-RC–independent manner. Therefore, there was a possibility that Xmus101 bound to chroma-

Figure 3. **Xmus101 requires ORC for association with sperm chromatin.** In lanes 1–4, egg cytosol (EC) that either contained or lacked ORC, as indicated, was mixed with sperm chromatin and incubated for 30 min. The chromatin was then isolated and probed by immunoblotting for the presence of Xmus101, MCM10, MCM7, or ORC2. In lanes 5–8, egg cytosol that either contained or lacked ORC, as indicated, was mixed with sperm chromatin and incubated for 30 min. After incubation, NPE that either contained or lacked ORC, as indicated, was then added and incubation continued for an additional 30 min before isolation of the chromatin and immunoblotting for the indicated factor. The NPE contained the replication inhibitor aphidicolin, to trap assembled replication complexes and prevent disassembly following the completion of DNA replication.
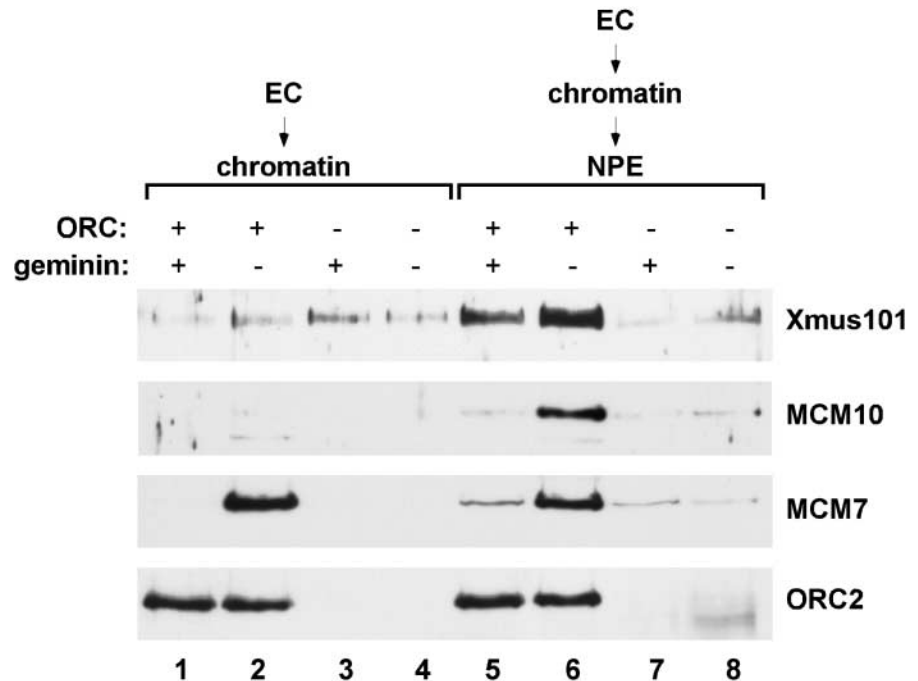
tin independently of replication initiation. To test this, we asked whether its loading was ORC-dependent. We have found that removal of ORC from conventional nuclear assembly extracts by immunodepletion is difficult. Therefore, we turned to a nucleus-free replication system derived from *Xenopus* egg extracts (Walter et al., 1998). In this system, sperm chromatin is first mixed with membrane-free egg cytosol to stimulate pre-RC assembly. Subsequently, a highly concentrated nucleoplasmic extract (NPE) is added to promote pre-IC assembly and drive the initiation of DNA replication. In this way, pre-RC assembly can be experimentally separated from pre-IC assembly and initiation. As reported previously (Walter et al., 1998; Wohlschlegel et al., 2002), when sperm chromatin was incubated in egg cytosol alone, the pre-RC components ORC2 and MCM7 bound to the chromatin, but MCM10 did not (Fig. 3 A, lane 2). Like MCM10, Xmus101 did not bind to sperm chromatin in egg cytosol (Fig. 3 A, lane 2). Upon addition of NPE, both MCM10 and Xmus101 were recruited to the chromatin (Fig. 3 A, lane 6). To determine whether Xmus101 binding to chromatin required ORC, we used ORC2 antibodies to deplete the ORC complex from egg cytosol and NPE. Under these conditions, Xmus101 binding was reduced to background levels (Fig. 3 A, lane 8). As seen in the nuclear assembly system, Xmus101 binding in the nucleus-free system was largely independent of chromatin binding by MCM2-7 (Fig. 3 A, lane 5). We conclude that Xmus101 is recruited to replication origins in an ORC-dependent manner, which links Xmus101 chromatin association to the initiation of DNA replication.

Having established that Xmus101 is essential for initiation, we next asked if the protein is required during the elongation phase of replication. For this, conventional egg extracts were prepared and incubated with sperm chromatin for 30 min to allow replication complex assembly and initiation to occur. After the 30-min incubation, the chromatin was isolated using isolation buffer containing 100 mM KCl,

which removes residual Xmus101 protein from the isolated chromatin (unpublished data). The salt extracted chromatin was then transferred to either a mock- or Xmus101-depleted extract, and incubation was continued in the presence of radio-labeled dATP to measure DNA replication (experimental scheme shown in Fig. 4 A). When DNA replication was measured in this experiment, no significant difference between the mock- and Xmus101-depleted extract was observed (Fig. 4 B). It was important to confirm that the Xmus101-depleted extract was devoid of Xmus101 protein, and that no contaminating Xmus101 was transferred along with the sperm chromatin. For this, we probed samples of the mock- and Xmus101-depleted extracts after transfer of the salt-extracted chromatin to the given extract, and found no detectable Xmus101 in the Xmus101-depleted sample (Fig. 4 C). Thus, despite the lack of detectable Xmus101 in the Xmus101-depleted extract containing the salt-extracted chromatin, DNA replication was not affected. Taken together, the data in Figs. 1 and 4 show that when Xmus101 is removed before initiation, DNA replication fails to occur; however, when it is removed after the initiation phase has passed, there is little effect on DNA replication. These data indicate that Xmus101 is an initiation factor, and that it does not function during elongation.

In this report, we have shown that Mus101 is required for the initiation of DNA replication, and that it is likely dispensable for the elongation phase of replication. We have also pinpointed the Mus101-dependent step in initiation, by showing that Cdc45 fails to load onto chromatin in Xmus101-depleted frog egg extracts. Thus, Xmus101 exerts at least one of its functions in the initiation of DNA replication upstream of the Cdc45 loading step. Consistent with the early execution point of Xmus101, we find that it loads onto chromatin, in an ORC-dependent manner, at the equivalent of the G1/S boundary in our extracts. That is, its loading does not occur in cytosolic extracts which sup-
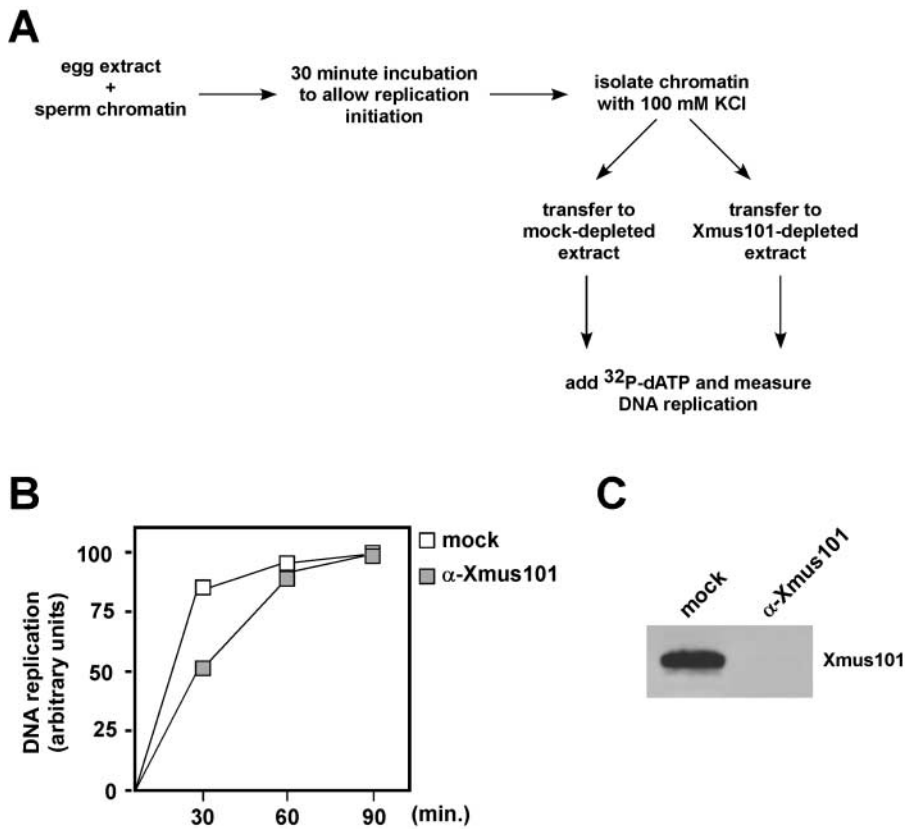
Figure 4. **Xmus101 is dispensable for DNA replication after initiation is complete.** (A) Experimental scheme, see text for details. (B) DNA replication was measured in the given extract at the given time. (C) Immunoblotting of either mock-depleted (mock), or Xmus101-depleted (α-Xmus101) extract after transfer of the chromatin, showing that no detectable Xmus101 is transferred along with the chromatin.

port pre-RC formation, but only after chromatin has been exposed to an initiation-competent nuclear environment. These data imply that, besides ORC, Xmus101 chromatin association also requires an unknown condition that is only met inside the nucleus. This could result from loss of a cytosolic inhibitor of Xmus101 chromatin association, or be due to a positive influence of the nucleus, such as an unknown bridging factor, or a nucleus-specific alteration of Xmus101 activity. Alternatively, because the concentration of DNA replication factors is much higher within nuclei (or in NPE), relative to cytosol, the nuclear-dependence of Xmus101 chromatin association might simply result from a concentration effect. Consistent with this, we find that the concentration of Xmus101 in NPE is at least 20-fold higher than in egg cytosol (unpublished data).

Although origin binding of Xmus101 requires ORC, it is independent of MCM2-7 and S-Cdk. This MCM2-7 independence distinguishes Mus101 from the other known Cdc45 loading factors, Cdc7 and MCM10, both of which require a fully intact pre-RC for chromatin loading (Jares and Blow, 2000; Walter, 2000; Wohlschlegel et al., 2002). Furthermore, we have shown that MCM2-7, Cdc7, and MCM10 all load onto chromatin independent of Xmus101. Thus, the mutual independence of Xmus101 and MCM2-7/Cdc7/MCM10 origin association strongly suggests that the assembly pathway that ultimately culminates in origin loading of Cdc45 bifurcates at a step soon after chromatin-loading of ORC (Fig. 5). One pathway leads via MCM2-7 to the recruitment of Cdc7 and MCM10, and the other pathway works through Xmus101. Besides MCM2-7, MCM10, Cdc7, and Mus101, the other major requirement for Cdc45

loading is S-Cdk activity. The model in Fig. 5 depicts two pathways emanating from ORC, and it is not yet clear which pathway requires S-Cdk. We have found that Xmus101 loads onto chromatin in the absence of S-Cdk activity, suggesting that if S-Cdk acts in the Mus101 branch, then it would be downstream of Mus101. If S-Cdk does act in the Mus101 branch of the initiation pathway, then both branches would be under the control of protein kinases, as the Cdc7 kinase functions in the parallel, MCM2-7 dependent branch. Having each branch of the initiation pathway under the control of a protein kinase would thus afford tight regulation and coordination of the two branches, and perhaps explain why initiation requires two protein kinase activities.

Budding yeast Dpb11 and fission yeast Cut5 are considered homologues of one another because they are both required for DNA replication, they are both required for replication checkpoint control, and they are both composed of reiterated BRCT domains. These arguments have also been
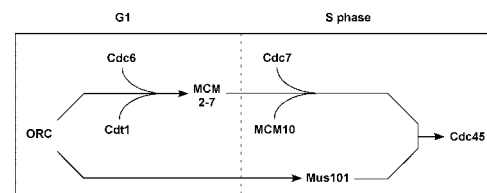

Figure 5. **A model for the replication initiation pathway.** The data presented here suggest two parallel pathways, one MCM2-7 dependent, the other Mus101 dependent, which cooperate to load Cdc45 at the G1/S transition. See Discussion for details.

extended to TopBP1/Mus101, as it has been suggested that Mus101 represents the metazoan homolog of Dpb11/Cut5 (Yamamoto et al., 2000; Makiniemi et al., 2001; Wang and Elledge, 2002). The data reported here suggest a reconsideration of this designation, as we have shown that Mus101 acts in a fundamentally different capacity than does Dpb11 during the initiation of DNA replication. Mus101 is required for Cdc45 loading, whereas Dpb11 has been reported to act much later, after Cdc45 has been loaded and, indeed, after the helicase has been activated and RPA has been recruited to the origin (Masumoto et al., 2000). Another difference is that Dpb11 function during initiation is tightly connected to pol ε, as Dpb11 was initially isolated as in a synthetic lethal screen for genes interacting with pol ε, and Dpb11 has since been shown to physically interact with the pol ε complex. Such interaction with pol epsilon is not likely to be the case for Xmus101, as it has been shown that Cdc45 is required for pol ε chromatin association in frog egg extracts (Mimura et al., 2000); therefore, our data place Xmus101 minimally two steps upstream of pol ε in the initiation pathway. Thus, it is likely that Mus101 is not the metazoan orthologue of Dpb11/Cut5, and that the Dpb11/Cut5 orthologue still awaits identification.

Materials and methods

Cloning of the *Xenopus* Xmus101 cDNA
BLAST searching of NCBI databases using *Drosophila mus101* sequences revealed several overlapping *Xenopus* ESTs containing significant sequence homology to the fly *mus101* gene. Based on the sequence of the frog ESTs, primers were designed to amplify a 1,000 nucleotide fragment corresponding to nucleotides 3,566–4,566 of the Xmus101 open reading frame. The PCR fragment was then used as a probe to screen, by hybridization, a *Xenopus* ovary cDNA library. Library screening resulted in the isolation of a full-length Xmus101 cDNA, which was then sequenced on both strands according to standard methods.

Antibody production
PCR primers were designed to amplify fragments corresponding to the COOH-terminal 333 amino acids of Xmus101. The fragment was subcloned into the pET24a bacterial expression vector (Novagen) and used to produce recombinant protein. Six histidine-tagged recombinant protein was purified on a nickel agarose column, under native conditions. Purified protein was then used as antigen to immunize rabbits. Polyclonal antibodies were obtained, and affinity purified according to standard procedures (Harlow and Lane, 1988). Antibodies against *Xenopus* Cdc45, RPA, MCM10, and Cdc7 have been described (Walter and Newport, 2000; Walter, 2000; Wohlschlegel et al., 2002). Additionally, antibodies against *Xenopus* MCM7 were obtained from John Newport (University of California San Diego, San Diego, CA), and against *Xenopus* pol α and pol ε from Shou Waga (Osaka University, Osaka, Japan).

Xenopus egg extract methodologies
Preparation of frog egg extracts, preparation of sperm chromatin, and DNA replication analysis was all performed according to Walter and Newport (1999). NPE was prepared as described (Walter et al., 1998). For immunodepletion, anti-Xmus101 antibodies were coupled to 50 μL of protein A Sepharose beads, and then incubated with 200 μL of frog egg extract on ice for 1 h. After incubation, the beads were pelleted, and the extract collected for an additional round of depletion. After the second round of depletion, the extract was used for the given experiment. For mock depletion, the procedure was identical except that anti-Xmus101 antibodies were replaced by phosphate-buffered saline. ORC depletion of NPE was performed as described (Walter and Newport, 2000).

Chromatin isolations
For the experiments shown in Fig. 2, chromatin was isolated as described (Michael et al., 2000). For the experiments shown in Fig. 3, chromatin isolation from NPE was performed as described (Wohlschlegel et al., 2002).

For the experiment shown in Fig. 4, the chromatin was isolated according to Jares and Blow (2000), using Nuclear Isolation Buffer containing 100 mM KCl.

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