

# Direct requirement for Xmus101 in ATR-mediated phosphorylation of Claspin bound Chk1 during checkpoint signaling

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**T**opBP1-like proteins, which include *Xenopus laevis* Xmus101, are required for DNA replication and have been linked to replication checkpoint control. A direct role for TopBP1/Mus101 in checkpoint control has been difficult to prove, however, because of the requirement for replication in generating the DNA structures that activate the checkpoint. Checkpoint activation occurs in *X. laevis* egg extracts upon addition of an oligonucleotide duplex (AT70). We show that AT70 bypasses the requirement for replication in checkpoint activation. We take advantage of this replication-independent check-

point system to determine the role of Xmus101 in the checkpoint. We find that Xmus101 is essential for AT70-mediated checkpoint signaling and that it functions to promote phosphorylation of Claspin bound Chk1 by the ataxia-telangiectasia and Rad-3-related (ATR) protein kinase. We also identify a separation-of-function mutant of Xmus101. In extracts expressing this mutant, replication of sperm chromatin occurs normally; however, the checkpoint response to stalled replication forks fails. These data demonstrate that Xmus101 functions directly during signal relay from ATR to Chk1.

## Introduction

Replication checkpoint signaling initiates when the DNA structures that form at stalled replication forks activate the ataxia-telangiectasia and Rad-3-related (ATR) protein kinase (for review see Sancar et al., 2004). ATR then phosphorylates and activates the Chk1 kinase. The pathway leading from activated ATR to Chk1 is complex and involves numerous intermediaries. In fission yeast, activation of Chk1 by the ATR homologue Rad3 requires, amongst other factors, Rad9, Crb2, and Cut5. Cut5 plays a central role in transducing the checkpoint signal from activated Rad3 to Chk1, as it forms complexes with both Crb2 and Rad9 (for review see Garcia et al., 2005). In metazoans, Chk1 activation also requires ATR, Rad9, and the Cut5 homologue TopBP1/Mus101. Despite this conservation, there is an important difference between the fission yeast and metazoan Chk1 activation pathways. In metazoans, the Claspin protein plays an essential role in Chk1 activation (Kumagai and Dunphy, 2000), whereas in fission yeast the Claspin homologue Mrc1 is not

involved (Tanaka and Russell, 2001). Likewise, in fission yeast, Crb2 is essential for Chk1 activation (Saka et al., 1997), whereas in metazoans the Crb2 homologue 53BP1 is not known to be involved. Because Crb2/53BP1 is not required for Chk1 activation in metazoans, it is unclear what role, if any, TopBP1/Mus101 plays in promoting activation of Chk1 by ATR.

Cut5/TopBP1 is unique amongst Chk1 activators in that it is also essential for DNA replication (Garcia et al., 2005). This is an important feature of Cut5/TopBP1, as replication is required to generate the DNA structures that activate ATR and initiate the checkpoint response (Michael et al., 2000). In fission yeast, temperature-sensitive alleles of Cut5 have allowed a separation of the replication and checkpoint functions of the protein (Garcia et al., 2005); however, it is not yet known if the replication function can be uncoupled from checkpoint function for TopBP1. One recent study of the *Xenopus laevis* TopBP1 homologue Xmus101 (also known as Xcut5) showed that Xmus101 is required to recruit both ATR and DNA polymerase  $\alpha$  (pol  $\alpha$ ) to chromatin during a checkpoint response (Parrilla-Castellar and Karnitz, 2003). Pol  $\alpha$  is required to generate the DNA structures that activate ATR (Michael et al., 2000), and it is therefore possible that the role of Xmus101 in Chk1 activation is limited to this early phase of the process.

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Abbreviations used in this paper: ATR, ataxia-telangiectasia and Rad-3-related; CKBD, Chk1 binding domain; MCM, minichromosome maintenance; NTA, nitrilotriacetic acid.

The online version of this article contains supplemental material.

Alternatively, Xmus101 could also have a later function, during relay of the checkpoint signal from activated ATR to Chk1. The differences in the mechanism of this signal relay between fission yeast and metazoans preclude clear predictions about what role, if any, Xmus101 might play in promoting phosphorylation of Chk1 by ATR. To address this important issue, we have used conditions in *X. laevis* egg extracts that bypass the requirement for pol  $\alpha$  and for generating DNA structures in activating Chk1. We report on the role that Xmus101 plays in Chk1 activation under these bypass conditions. Our results demonstrate that Xmus101 has a late checkpoint function, to promote phosphorylation of Chk1 by activated ATR.

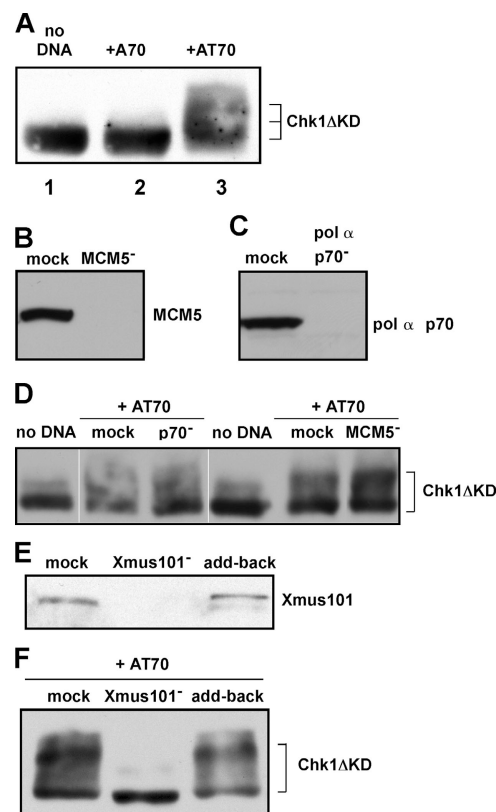
## Results and discussion

### AT70-mediated Chk1 activation

To study Chk1 activation, we used the AT70 system in *X. laevis* egg extracts (Kumagai and Dunphy, 2000). In this system, two short oligonucleotides, A70 and T70, are annealed to one another and then added to extracts. Addition of the duplex, but not either single oligonucleotide alone, triggers robust Chk1 phosphorylation. Chk1 activation in this system is dependent on ATR, Claspin, and other checkpoint proteins (Kumagai and Dunphy, 2000, 2003; Jeong et al., 2003). To monitor checkpoint activation, we used a previously established assay based on a fragment of the *X. laevis* Chk1 protein, Chk1 $\Delta$ KD, that is phosphorylated in an ATR- and Claspin-dependent manner in egg extracts (Michael et al., 2000; Jeong et al., 2003). Phosphorylation of Chk1 $\Delta$ KD results in an easily detectable mobility shift on SDS-PAGE gels. An example of the AT70 checkpoint system is shown in Fig. 1 A. Egg extracts were supplemented with Chk1 $\Delta$ KD and either the single A70 oligonucleotide, the AT70 duplex, or no DNA at all. After a 100-min incubation, samples were taken and probed by immunoblotting for Chk1 $\Delta$ KD. A Chk1 $\Delta$ KD mobility shift was observed in the samples containing AT70 (Fig. 1 A, lane 3) but not in the sample containing A70 (lane 2) or no DNA (lane 1). This demonstrates that AT70 specifically triggers Chk1 $\Delta$ KD phosphorylation. Control experiments, detailed in the supplemental text (available at <http://www.jcb.org/cgi/content/full/jcb.200601076/DC1>), showed conclusively that Chk1 $\Delta$ KD is a reliable surrogate for the endogenous Chk1 in these assays (Fig. S1).

### Xmus101, but not minichromosome maintenance (MCM) complex or pol $\alpha$ , is required for Chk1 activation in the AT70 system

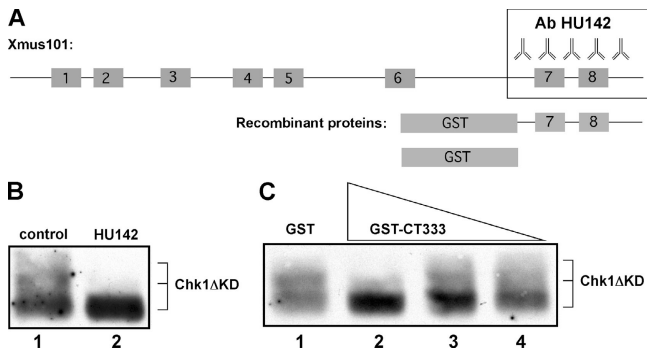
Previous work has shown that when sperm chromatin and the DNA replication inhibitor aphidicolin are used to activate the checkpoint in egg extracts, both the MCM complex and pol  $\alpha$  are required for Chk1 activation (Michael et al., 2000). Both of these proteins promote replication (for review see Bell and Dutta, 2002). The MCM complex is a hexameric assembly that unwinds DNA during replication, whereas pol  $\alpha$  synthesizes the primers that initiate replication. To determine whether these proteins are required for Chk1 activation in the AT70 system, we removed MCM and pol  $\alpha$  by immunodepletion. Fig. 1 B



**Figure 1. Xmus101 is required, but replication proteins are not, for Chk1 activation in the AT70 system.** (A) Recombinant Chk1 $\Delta$ KD was added to extract, along with no DNA (lane 1), the A70 oligonucleotide (lane 2), or the preannealed AT70 oligonucleotide (lane 3). After a 100-min incubation, samples were taken and the phosphorylation status of Chk1 $\Delta$ KD was assessed by SDS-PAGE and immunoblotting with the T7 monoclonal antibody (Novagen), which recognizes the T7 epitope tag present on recombinant Chk1 $\Delta$ KD. The retarded mobility of Chk1 $\Delta$ KD in lane 3 is due to phosphorylation (Michael et al., 2000). (B) MCM5 was immunodepleted from egg extract. The depleted extract was probed for MCM5 by immunoblotting and compared with a mock-depleted extract. (C) Same as B except that the p70 subunit of pol  $\alpha$  was immunodepleted. (D) Mock-depleted and depleted extracts were supplemented with recombinant Chk1 $\Delta$ KD and AT70. After a 100-min incubation, samples were taken and processed as in A. The lanes labeled "no DNA" refer to extracts incubated without AT70 but with recombinant Chk1 $\Delta$ KD. (E) Xmus101 was immunodepleted from egg extracts. The depleted extracts were probed for Xmus101 by immunoblotting and compared with a mock-depleted extract. Add-back refers to Xmus101-depleted extracts supplemented with recombinant Xmus101. (F) The extracts depicted in E were assayed for Chk1 $\Delta$ KD phosphorylation as in A.

shows that all detectable MCM5 protein was removed from the extract, and Fig. 1 C shows that same was true of the p70 subunit of pol  $\alpha$ . Despite the loss of MCM and pol  $\alpha$  in these extracts, addition of AT70 still activated Chk1, as shown by Chk1 $\Delta$ KD mobility shift (Fig. 1 D). MCM and pol  $\alpha$  are thus dispensable for Chk1 activation in the AT70 system. We conclude that the AT70 system bypasses the requirement for replication proteins in Chk1 activation.

If the role of Xmus101 in Chk1 activation was restricted to generating the checkpoint-activating DNA structure, then it, like MCM and pol  $\alpha$ , should be dispensable for Chk1 activation in the AT70 system. To address this, Xmus101 was removed from extract by immunodepletion (Fig. 1 E) and the samples

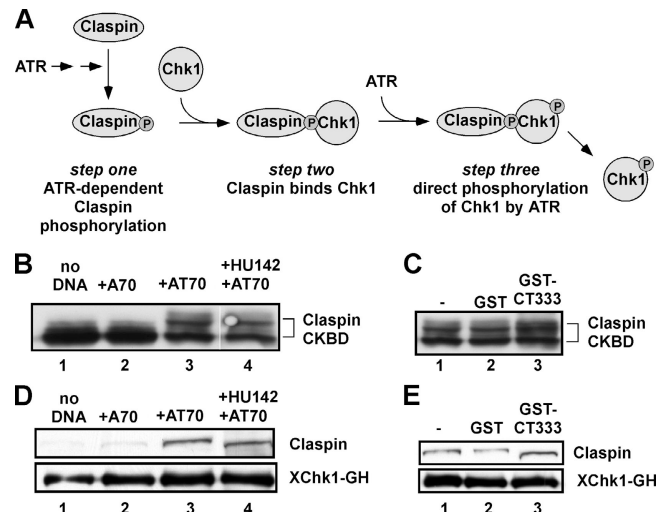


**Figure 2. Two inhibitors of the Xmus101 Chk1 activation function.** (A) A cartoon of Xmus101 showing the region of the protein that was used as antigen to produce the HU142 antibody as well as recombinant proteins used in this study. The numbered boxes refer to BRCT domains (Garcia et al., 2005). (B) Extracts were supplemented with either nonspecific IgG (lane 1) or HU142 (lane 2; final concentration: 50 ng/ $\mu$ l). Both samples were then further supplemented with recombinant Chk1 $\Delta$ KD and AT70. After a 100-min incubation, samples were taken and probed for Chk1 $\Delta$ KD as in Fig. 1 A. (C) Either GST (15  $\mu$ M; lane 1) or GST-CT333 (806 nM, lane 2; 322 nM, lane 3; 161 nM, lane 4) was added to egg extract along with AT70 and Chk1 $\Delta$ KD. After a 100-min incubation, samples were taken and probed for Chk1 $\Delta$ KD as in Fig. 1 A.

were assayed for Chk1 activation after addition of AT70. As shown in Fig. 1 F, and in contrast to MCM and pol  $\alpha$ , removal of Xmus101 prevented Chk1 $\Delta$ KD phosphorylation. Importantly, Chk1 activation was restored in Xmus101-depleted extract after supplementation of the extract with recombinant Xmus101 that had been produced in rabbit reticulocyte lysates (Fig. 1 F). Supplementation of Xmus101-depleted extract with unprogrammed reticulocyte lysates did not rescue Chk1 $\Delta$ KD phosphorylation (unpublished data). We conclude that Xmus101 is essential for Chk1 activation in the AT70 system. This result thus distinguishes Xmus101 from the replication fork components MCM and pol  $\alpha$  and demonstrates that the role of Xmus101 in Chk1 activation extends beyond generating the checkpoint-activating DNA structure.

#### A neutralizing antibody and a dominant-negative fragment inhibit Xmus101 function during Chk1 activation

We considered the possibility that our anti-Xmus101 antibody, HU142, might inhibit Xmus101 function when added to extract. To test this, we added purified HU142 antibody directly to extract along with AT70. Addition of HU142 prevented AT70-induced phosphorylation of Chk1 $\Delta$ KD, whereas nonspecific IgG did not (Fig. 2 B). This result demonstrates that HU142, which recognizes the COOH-terminal 333 amino acids of Xmus101 (Fig. 2 A), blocks AT70-mediated checkpoint signaling. One explanation for this is that binding of HU142 to the COOH-terminal 333 amino acids of Xmus101 prevents an interaction between this region and a factor that is required for checkpoint signaling. If so, then we might expect overexpression of the isolated 333 amino acid domain to also inhibit Chk1 activation through sequestration of this presumptive factor away from the full-length endogenous Xmus101. To test this, we titrated a recombinant protein consisting of GST fused to the 333 COOH-terminal amino acids of Xmus101 (GST-CT333; Fig. 2 A) into



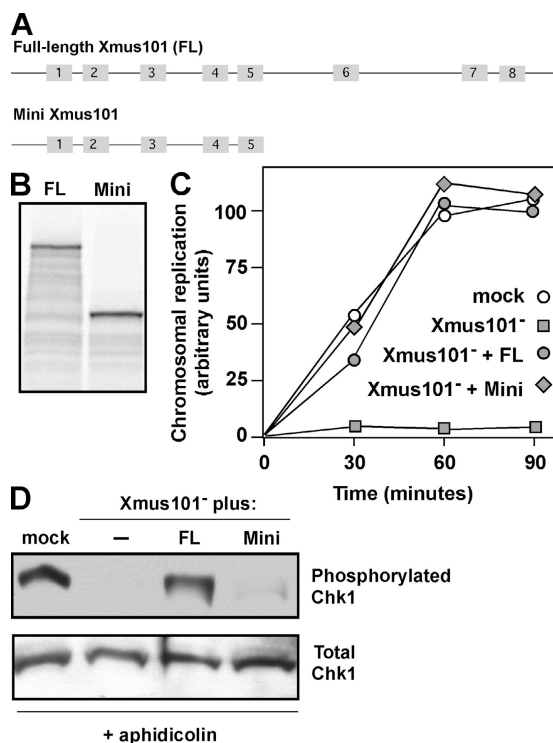
**Figure 3. Xmus101 functions after assembly of the Claspin-Chk1 complex during AT70-mediated Chk1 activation.** (A) A depiction of the Chk1 activation pathway. (B) Extracts were supplemented with recombinant GST-Claspin CKBD and the following additional components: buffer (lane 1), the A70 oligonucleotide (lane 2), preannealed AT70 oligonucleotides (lane 3), or preannealed AT70 oligonucleotides plus HU142 (final concentration: 50 ng/ $\mu$ l; lane 4). GST-Claspin CKBD phosphorylation was assessed after a 100-min incubation, according to published procedures (Kumagai and Dunphy, 2003). (C) GST-Claspin CKBD shift assay in extracts containing AT70 and buffer (lane 1), GST (15  $\mu$ M; lane 2), or GST-CT333 (806 nM; lane 3). (D) Extracts were supplemented with bead bound recombinant Xchk1-GH (Kumagai and Dunphy, 2000) and the following additional components: buffer (lane 1), the A70 oligonucleotide (lane 2), preannealed AT70 oligonucleotides (lane 3), or preannealed AT70 oligonucleotides plus HU142 (final concentration: 50 ng/ $\mu$ l; lane 4). After a 100-min incubation, the beads were isolated and washed, and bound proteins were eluted with 2 $\times$  SDS-PAGE sample buffer. The presence of Claspin and Xchk1-GH in the eluate was then determined by immunoblotting with antibodies against Claspin and GST, respectively (Jeong et al., 2003). (E) Claspin-Chk1 binding assay in extracts containing AT70 and buffer (lane 1), GST (15  $\mu$ M; lane 2), or GST-CT333 (806 nM; lane 3).

extracts and then added AT70 to activate Chk1. As shown in Fig. 2 C, addition of GST-CT333, but not GST alone, inhibited Chk1 $\Delta$ KD phosphorylation in a dose-dependent manner. We conclude that when the function of the extreme COOH terminus of endogenous Xmus101 is antagonized, through either binding of HU142 or overexpression of the isolated domain, then Chk1 activation is blocked.

#### Identification of the Xmus101-dependent step in Chk1 activation

The results in Figs. 1 and 2 show that loss of Xmus101 blocks AT70-mediated Chk1 activation and that two independent inhibitors of Xmus101, a neutralizing antibody and a dominant-negative fragment, do the same. We next used these inhibitors to define the Xmus101-dependent step in Chk1 activation. In *X. laevis*, Chk1 activation is (minimally) a three-step process (Fig. 3 A). Upon ATR activation, the first identifiable step is phosphorylation of the Claspin Chk1 binding domain (CKBD; Kumagai and Dunphy, 2003). This phosphorylation is ATR dependent and thus also serves as an indicator of ATR activation. After Claspin CKBD phosphorylation, the next step (step 2) is assembly of a Claspin-Chk1 complex. Claspin-Chk1 complex assembly is dependent on step 1 and is required for step 3 in the





**Figure 4. A separation-of-function mutant of Xmus101.** (A) Schematic representations of full-length Xmus101 and Mini. (B) Constructs encoding either full-length Xmus101 (FL) or Mini were transcribed and translated in vitro in rabbit reticulocyte lysates in the presence of [<sup>35</sup>S]methionine. The radio-labeled proteins were then run out on SDS-PAGE and visualized after autoradiography. (C) Xmus101 was depleted from egg extract. Depleted extract was then supplemented (0.1 vol) with either unprogrammed reticulocyte lysates (Xmus101<sup>-</sup>) or lysates expressing either FL or Mini Xmus101. A mock-depleted sample was also prepared. The reconstituted extracts were then combined with sperm chromatin and  $\alpha$ -[<sup>32</sup>P]dATP, and DNA replication was measured according to standard procedures (Walter and Newport, 1999). Samples were taken at 30, 60, and 90 min for analysis. (D) The reconstituted extracts described in C were supplemented with sperm chromatin and the DNA replication inhibitor aphidicolin (100  $\mu$ g/ml). After a 60-min incubation, the samples were probed by immunoblotting for both activated and total Chk1.

process, direct phosphorylation of Chk1 by ATR (Kumagai and Dunphy, 2003). To assay for completion of step 1, CKBD phosphorylation, we used a fragment of Claspin corresponding to the CKBD and assessed its phosphorylation by mobility shift on SDS-PAGE according to published procedures (Kumagai and Dunphy, 2003). The GST-Claspin CKBD did not undergo a mobility shift in extracts that lacked DNA, nor did it shift in extracts containing just the A70 oligonucleotide; however, we did detect a shift in extracts containing AT70 (Fig. 3 B, lanes 1–3). Interestingly, the GST-Claspin CKBD mobility shift occurred normally in extracts containing both AT70 and HU142 (Fig. 3 B, lane 4). This result shows that although HU142 prevents AT70-mediated Chk1  $\Delta$ KD phosphorylation, it has no effect on CKBD phosphorylation.

We next determined whether step 2, assembly of a Claspin–Chk1 complex, occurs in extracts containing Xmus101 inhibitors. For this, we used a previously published Claspin Chk1 binding assay (Jeong et al., 2003; Kumagai and Dunphy, 2003). A full-length GST- and His-tagged Chk1 protein (Xchk1-GH)

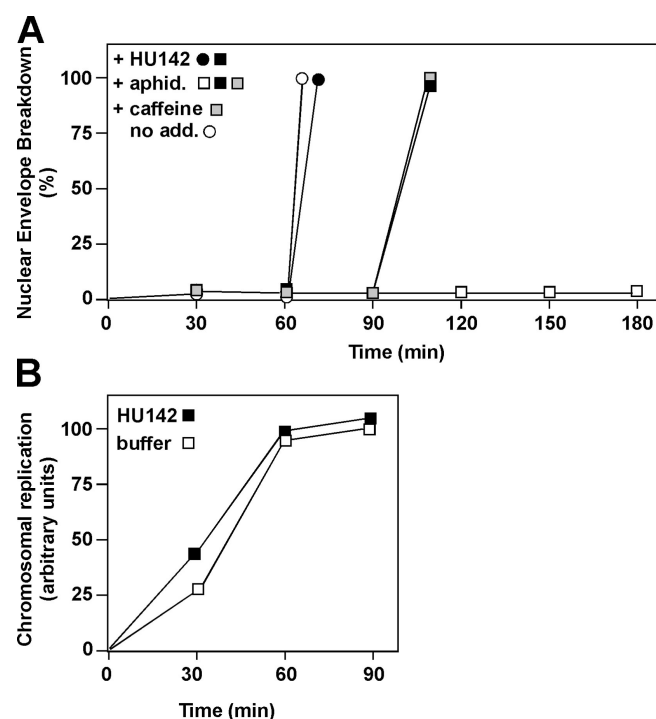
was coupled to Ni–nitrilotriacetic acid (NTA) agarose beads, and the beads were then added to extracts. After a 100-min incubation, the beads were recovered, washed, and probed for the presence of Claspin by immunoblotting. A Claspin–Chk1 complex could be detected specifically in extracts that had activated the checkpoint through addition of AT70 (Fig. 3 D, lane 3) and was not detected in extracts lacking DNA or containing A70 (Fig. 3 D, lanes 1 and 2). Importantly, the Claspin–Chk1 complex readily formed in extracts containing both AT70 and HU142 (Fig. 3 D, lane 4). These data demonstrate that HU142 inhibits the ATR-dependent phosphorylation of Chk1 but not assembly of a Claspin–Chk1 complex.

To confirm the results obtained with HU142, we repeated the experiments with an independent inhibitor of Xmus101 function, the recombinant GST-CT333 protein. Despite the ability of GST-CT333 to prevent phosphorylation of Chk1 (Fig. 2 C), it had no effect on either mobility shift of GST-Claspin CKBD or formation of a Claspin–Chk1 complex (Fig. 3, C and E). These results are fully consistent with those obtained with HU142 and demonstrate that although the COOH terminus of Xmus101 is required for Chk1 phosphorylation, it is not required for the ATR-dependent assembly of a Claspin–Chk1 complex. We conclude that one position of Xmus101 in the checkpoint activation pathway is after assembly of the Claspin–Chk1 complex and before phosphorylation of Chk1 by ATR.

#### A separation-of-function mutant of Xmus101

The results presented thus far show that Xmus101 plays a direct role in AT70-mediated Chk1 activation. To see if this is also true when checkpoint activation occurs under more physiological conditions, we examined the role of Xmus101 in Chk1 activation by replication-blocked sperm chromatin templates. Under these conditions, Xmus101 is required for replication fork assembly; thus, depletion of Xmus101 would indirectly affect Chk1 activation by virtue of a failure to generate DNA structures. To get around this, we asked if we could separate the replication and checkpoint functions of Xmus101 mutationally. We constructed a deletion mutant of Xmus101 named Mini Xmus101 or Mini (Fig. 4 A). Mini corresponds to the first 759 amino acids of the protein and thus lacks the COOH-terminal 333 amino acids that have been implicated in Chk1 activation. Endogenous Xmus101 was immunodepleted, and the depleted extracts were supplemented with rabbit reticulocyte lysates that had been programmed for in vitro transcription/translation reactions using constructs encoding either full-length Xmus101 or Mini. Fig. 4 B shows that the full-length Xmus101 and Mini were produced to an equivalent extent in the rabbit reticulocyte lysates. The reconstituted extracts were supplemented with sperm chromatin and  $\alpha$ -[<sup>32</sup>P]dATP, and replication of the sperm chromatin was assessed. Supplementation of depleted extract with either full-length Xmus101 or Mini rescued the replication defect completely, whereas add-back of unprogrammed reticulocyte lysates did not promote replication (Fig. 4 C). We conclude that all of the replication functions of Xmus101 are contained within Mini.

Because Mini was competent for DNA replication, we asked whether it was also sufficient for Chk1 activation.



**Figure 5. HU142 allows mitosis in aphidicolin-treated cycling extracts.** (A) Cycling extracts were prepared and supplemented with sperm chromatin (1,000/ $\mu$ l). Extracts were further supplemented, where indicated, with aphidicolin, HU142, or 5 mM of caffeine. Entry into mitosis was determined by nuclear envelope breakdown. (B) Egg extracts were prepared and supplemented with sperm chromatin and HU142, as indicated. DNA replication was then assessed as in Fig. 4 C.

To examine this, we added sperm chromatin and the DNA polymerase inhibitor aphidicolin to extracts that had been immunodepleted of Xmus101 and supplemented with either full-length Xmus101 or Mini. Chk1 activation was then monitored by immunoblotting of the extracts with antibodies specifically recognizing Chk1 that had been phosphorylated on serine 344. Serine 344-phosphorylated Chk1 represents the activated form (Guo et al., 2000). As shown in Fig. 4 D, depletion of Xmus101 prevented Chk1 activation, and this was restored by add-back of full-length Xmus101. Importantly, add-back of Mini did not rescue Chk1 activation (Fig. 4 D). These data show that Mini is replication competent and Chk1 activation deficient. Based on the ability of Mini to separate the replication and Chk1 functions of Xmus101, we conclude that Xmus101 functions directly in Chk1 activation, independent of its role in replication fork assembly.

In a final experiment, we sought to connect the function of Xmus101 in Chk1 activation to the checkpoint response that prevents mitosis when replication is blocked. For this, we used cycling egg extracts, which when treated with aphidicolin are prevented from entering mitosis in a checkpoint-dependent manner (Dasso and Newport, 1990). Mitosis was assessed by examining sperm nuclei for nuclear envelope breakdown, as described previously (Murray, 1991). Addition of aphidicolin delayed mitosis, as expected (Fig. 5 A). Importantly, this delay was reversed by addition of the Xmus101 inhibitor HU142 (Fig. 5 A). HU142 was as effective as the known checkpoint inhibitor caffeine in releasing the checkpoint-mediated arrest. Addition of

HU142 to interphase extracts had no adverse effect on DNA replication (Fig. 5 B). We conclude that Xmus101 is required for both Chk1 activation and for the checkpoint-mediated delay in entrance into mitosis when replication is blocked. Together with previous work (Parrilla-Castellar and Karnitz, 2003), the results presented here demonstrate that Xmus101 has at least two functions during a checkpoint response: it acts early to recruit ATR and pol  $\alpha$  to damaged DNA and it functions later to promote phosphorylation of Claspin bound Chk1 by activated ATR. The challenge for future studies will be to determine the exact mechanism whereby Xmus101 performs this late function.

## Materials and methods

### Extracts and extract procedures

*X. laevis* egg extract preparation and DNA replication analysis were performed as described previously (Walter and Newport, 1999). For the experiment shown in Fig. 5 A, cycling extracts were used, and they were prepared as described previously (Murray, 1991). For immunodepletion of Xmus101, affinity-purified HU142 antibody was cross-linked to protein A-Sepharose beads (GE Healthcare) using the dimethylpimelidate direct-coupling method, as described previously (Harlow and Lane, 1988). Immunodepletion was then performed as described previously (Van Hatten et al., 2002). Immunodepletion of pol  $\alpha$  has been described (Stokes and Michael, 2003), and immunodepletion of MCM5 was performed in an analogous manner. A70 and AT70 oligonucleotides were added to 50 ng/ $\mu$ l of extract, and the phosphatase inhibitor tautomycin was included, at 3  $\mu$ M, as previously described (Kumagai and Dunphy, 2000). For Chk1 $\Delta$ KD shift assays, a bacterially expressed recombinant Chk1 $\Delta$ KD was added, to 400 nM, to extracts and then visualized on immunoblots using a T7 monoclonal antibody (Novagen). GST-Claspin CKBD shift assays were performed by adding bacterially expressed GST-Claspin CKBD (final concentration: 50 ng/ $\mu$ l) to extract followed by visualization with anti-GST antibody (GE Healthcare; Kumagai and Dunphy, 2003). The Claspin-Chk1 interaction assay was performed exactly as described previously (Jeong et al., 2003).

### Expression vectors

Xmus101 BRCT 1–8 (Fl) corresponds to full-length Xmus101 (nucleotides 1–4542) subcloned into pCS2 + MT. Mini corresponds to Xmus101 nucleotides 1–2277 subcloned into pCS2 + MT. For transcription/translation in vitro, a TNT SP6 Quick Master Mix kit (Promega) was used in all cases. Chk1 $\Delta$ KD has been described (Michael et al., 2000). GST-Claspin CKBD was produced by subcloning *X. laevis* Claspin nucleotides 2609–2779 into pGEX-4T-1. Xchk1-GH has been described previously (Kumagai and Dunphy, 2000). GST-CT333 was produced by subcloning Xmus101 nucleotides 3540–4542 into pGEX-4T-1.

### Recombinant proteins

Chk1 $\Delta$ KD was expressed as a His-tagged fusion protein in *Escherichia coli* and purified over nickel NTA agarose according to standard procedures. Recombinant Xchk1-GH was produced via infection of Sf9 insect cells. Purification of Xchk1-GH over a nickel NTA agarose column was performed as described previously (Kumagai and Dunphy, 2000). GST, GST-Claspin CKBD, and GST-CT333 were expressed in *E. coli* and purified over glutathione agarose according to standard procedures.

### Antibodies

The anti-Xmus101 HU142 antibody and its affinity purification have been described (Van Hatten et al., 2002). Antibodies against the p70 subunit of pol  $\alpha$  have been described (Stokes and Michael, 2003). Antibodies against Claspin were produced by W. Dunphy (California Institute of Technology, Pasadena, CA). Antibodies against Chk1 and serine 344-phosphorylated Chk1 were obtained from Santa Cruz Biotechnology, Inc., and Cell Signaling Technology, respectively. Antibodies against MCM5 were obtained from Bethyl Laboratories.

### Online supplemental material

Figure S1 shows that Chk1 $\Delta$ KD is a reliable surrogate for endogenous Chk1 in the AT70 checkpoint system. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200601076/DC1>.

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