

Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts

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Tipin and its interacting partner Tim1 (Timeless) form a complex at replication forks that plays an important role in the DNA damage checkpoint response. Here we identify *Xenopus laevis* Tipin as a substrate for cyclin E/cyclin-dependent kinases 2 that is phosphorylated in interphase and undergoes further phosphorylation upon entry into mitosis. During unperturbed DNA replication, the Tipin/Tim1 complex is bound to chromatin, and we were able to detect interactions between Tipin and the MCM helicase. Depletion of Tipin from *Xenopus* extracts did not significantly impair normal replication but substantially blocked the ability of stalled replication forks to recover after removal of a block imposed by aphidicolin. Tipin-depleted extracts also showed defects in the activation of Chk1 in response to aphidicolin, probably because of a failure to load the checkpoint mediator protein Claspin onto chromatin.

cell cycle | checkpoint | cyclin | DNA damage

Eukaryotic cells have evolved multiple mechanisms that promote the fidelity of DNA replication. Stalled replication forks arising at sites of DNA damage or after nucleotide depletion are sensed and repaired by surveillance mechanisms called checkpoints. Once activated, the checkpoint machinery triggers a number of events that activate DNA repair pathways to coordinate a delay in cell cycle progression with repair of damage, ensuring the integrity of the genome (1–4). The importance of the checkpoint pathways is underlined by the conservation of the proteins involved from yeast to humans (5, 6) and by the fact that mutations in genes encoding checkpoint proteins tend to predispose affected individuals to cancer (7).

Different types of DNA damage trigger specific checkpoint pathways; UV light and stalled replication forks activate the ATR-Chk1 pathway, whereas DNA double-strand breaks activate the ATM-Chk2 pathway. The key function of the ATR-Chk1 pathway is thought to be to stabilize and preserve the stalled replication forks until the damage is repaired, permitting replication to resume (8–12).

Recent studies in yeast and mammalian cells have identified three proteins, Timeless (Tim1), Tipin, and Claspin, important for the repair of stalled DNA replication forks. The budding and fission yeast homolog of Tim1 are, respectively, Tof1 and Swi1. Tof1 (13, 14) is part of the Mrc1 (Claspin) pathway that is required for replication forks to pause when barriers to replication are encountered (13, 15–17). Swi1, a functional homolog of Tof1 (18), is essential for full activation of Cds1 and stabilization of replication forks (17, 19, 20). Recently, human Tim1 was reported to be a mediator of the intra S-phase checkpoint (21).

Tipin was originally identified as a Tim1 interacting protein by a yeast two-hybrid screen (22). Tipin homologs have been identified in budding (Csm3) and in fission yeast (Swi3) (18, 23). Csm3 and Swi3 interact with Tof1 and Swi1, respectively, and are required for checkpoint responses to replication stress (24–29). Recent data in mammalian cells showed that Tipin is required for efficient cell cycle arrest in response to DNA damage (30). Reduction in the levels of Tipin or Tim1 by siRNA renders cells sensitive to ionizing radiation (31) and results in reduced level of

Chk1 phosphorylation upon hydroxyurea or ultraviolet radiation (100–280 nm) treatment. These findings indicate that the Tipin/Tim1 complex is a mediator of the UV-induced intra S-phase checkpoint (30–33). Tim1 is also important for replication fork progression in the absence of damage whereas Tipin slows DNA chain elongation in active replicons in UV-damaged cells (32).

In this work we focused our attention on the *Xenopus laevis* homolog of Tipin and studied its role during S phase progression. We demonstrate that depletion of Tipin from *Xenopus* extracts does not alter the overall efficiency of DNA replication. However, Tipin is necessary for the intra S-phase checkpoint response to replication block, which may result from a failure to load Claspin onto chromatin when Tipin is absent. Furthermore, we find that Tipin associates with replication forks and ensures fork stability by coupling the replicative polymerase and helicase activities, thereby preventing the helicase from excessively unwinding the DNA when the polymerase has stalled. Our data support a model in which Tipin mediates replication fork pausing in response to replication stress and thus participates in the DNA damage checkpoint response.

Results

Tipin Is a Cyclin E/Cyclin-Dependent Kinase (CDK) 2 Substrate. In a screen for cyclin E/CDK2 substrates (unpublished data), we identified Tipin as a protein that undergoes a mobility shift upon phosphorylation by CDK2 (Fig. 1A). To confirm that Tipin is phosphorylated by CDK *in vivo* we added ³⁵S-labeled protein to an interphase extract and observed retardation in electrophoretic mobility, which was abolished if the extract was pre-treated with roscovitine to inhibit CDK activity (Fig. 1A) (34).

Tipin is a 386-residue protein that contains six S/T-P motifs and one complete CDK consensus site, (S/T)PXX/R (35, 36), at Ser-326. We generated a S326A mutant and tested the ability of this mutant to be phosphorylated by cyclin E/CDK2 or cyclin A/CDK2. For both kinases the mobility shift was abolished by the S326A mutation (Fig. 1B). However, when the phosphorylation status of the S326A mutant was assessed by ³²PO₄ incorporation, cyclin A/CDK2 was still able to phosphorylate the S326A mutant, whereas cyclin E/CDK2 did not (Fig. 1C). This suggests that there are other CDK phosphorylation sites on the protein. We therefore analyzed the behavior of Tipin in different stages of the cell cycle. Addition of ³⁵S-labeled Tipin to an extract in interphase induced a rapid mobility shift, which presumably reflects the phosphorylation by cyclin E/CDK2. A further mobility shift is induced at 40 min, when the extract enters mitosis

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Abbreviations: CDK, cyclin-dependent kinase; RPA, replication protein A.

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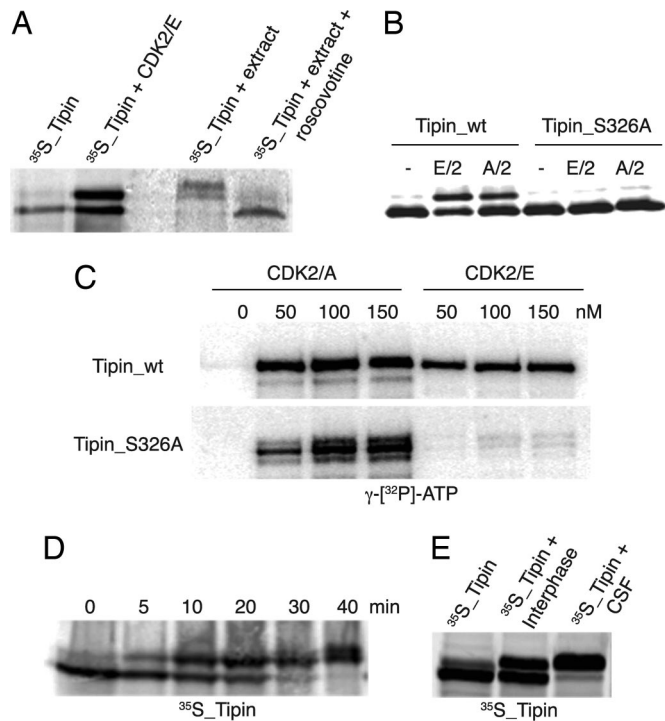


Fig. 1. Tipin is a substrate for cyclin E/CDK2. (A) *In vitro*-translated Tipin was supplemented or not with recombinant cyclin E/CDK2 and ATP. Alternatively, ^{35}S -labeled protein was added to a *Xenopus* extract in the presence or absence of roscovitine. (B) Cyclin E- or cyclin A-CDK2 and ATP were added to *in vitro*-translated Tipin (wild type or S326A mutant). (C) Recombinant GST-Tipin (wild type or S326A) was incubated with increasing concentrations of cyclin E- or cyclin A-CDK2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. (D) ^{35}S -labeled Tipin was added to a *Xenopus* cycling extract, and samples were collected from 0 to 40 min. (E) Wild-type ^{35}S -labeled Tipin was added to an interphase or metaphase arrested extract (CSF, cytostatic factor-mediated metaphase II arrested extract). Samples were analyzed by SDS/PAGE and autoradiography.

(data not shown), suggesting the existence of additional phosphorylation sites in Tipin (Fig. 1D). To further confirm this, the mobility of Tipin was compared between interphase extracts and a metaphase arrested egg extract, revealing an additional retardation in the mobility of Tipin in the M-phase environment (Fig. 1E).

Tipin Is a Phosphoprotein in *Xenopus* Extracts. To study the function of Tipin in *Xenopus* extracts, we generated polyclonal antibodies against the full-length recombinant protein expressed in bacteria. Although Tipin has a calculated molecular mass of 42 kDa, the antibody recognizes a specific band ≈ 60 kDa in *Xenopus* extract, which disappeared after Tipin depletion. Because our data indicate that Tipin is a substrate for cyclin E/CDK2 and this kinase is always present in early *Xenopus* embryos (37), we treated the extract with λ phosphatase in the presence or absence of sodium vanadate, a phosphatase inhibitor. The band corresponding to endogenous Tipin is shifted down (Fig. 2A) upon phosphatase treatment showing that Tipin is constitutively phosphorylated in an interphase extract.

Tipin Associates with Chromatin and Forms a Stable Complex with Tim1. To determine whether Tipin interacts with Tim1 in *Xenopus*, we performed coimmunoprecipitation experiments. Tipin and Tim1 coimmunoprecipitated from *Xenopus* extracts, demonstrating that these two proteins form a complex *in vivo* (Fig. 2B). Because Tipin is phosphorylated in *Xenopus* extracts, we asked whether the Tipin/Tim1 interaction depended on phos-

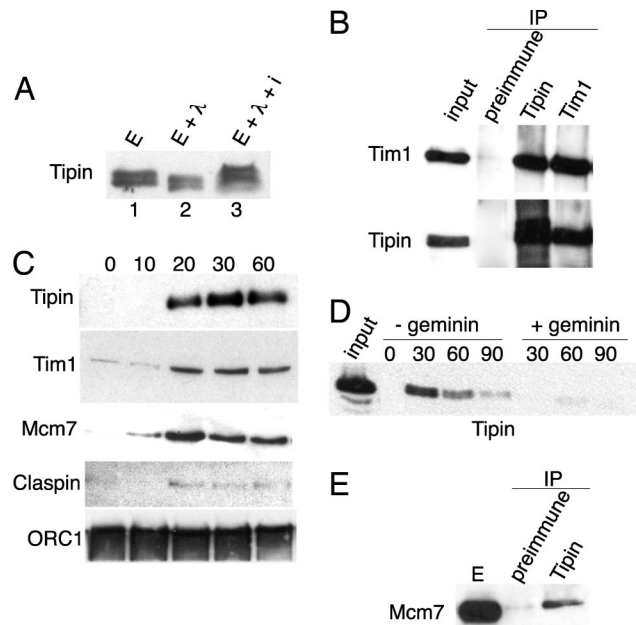


Fig. 2. Endogenous Tipin is a chromatin-associated phosphoprotein that interacts with Tim1 and MCM7. (A) *Xenopus* extract was treated (lane 2) or not (lane 1) with λ -phosphatase in the presence (lane 3) or absence of sodium vanadate. Endogenous Tipin was detected by immunoblotting. (B) Equal amounts of extract were immunoprecipitated with anti-Tipin and anti-Tim1 antibodies or preimmune serum. Purified proteins were immunoblotted as indicated. (C) Sperm nuclei (3,000 nuclei per microliter) were added to *Xenopus* extract, and chromatin was harvested at different times. Chromatin-bound proteins were analyzed by immunoblotting. (D) Sperm nuclei were added to *Xenopus* extract, and chromatin was harvested in the presence or absence of geminin (5 ng/ μl). Samples were probed with anti-Tipin antibody. (E) *Xenopus* extract was immunoprecipitated with either anti-Tipin or preimmune serum. Samples were probed with anti-MCM7 antibody.

phorylation, but treatment with λ -phosphatase had no effect on the interaction of Tipin and Tim1 (data not shown).

Previous work in both yeast and human cells demonstrated that the Tipin/Tim1 complex is associated with the replication fork (33), is required for efficient S-phase progression (30), and is important for the intra S-phase checkpoint (30–33). We therefore investigated the role of Tipin in DNA replication in *Xenopus* extracts. To determine whether Tipin associated with chromatin in *Xenopus* extracts, we added demembrated sperm nuclei to an interphase extract and purified chromatin at different times. Members of the prereplication complex, such as ORC1, are rapidly loaded on to the chromatin when nuclei are added to the extract (Fig. 2C); other proteins required for replication initiation are found in the chromatin at later time points. MCM7 is loaded onto the chromatin 20 min after nuclei addition. Fig. 2C shows that Tipin, Tim1, and Claspin, a mediator of the intra S-phase checkpoint response, appeared in the chromatin fraction with the same kinetics as MCM7 (Fig. 2C). In the absence of deliberate DNA damage, very little Claspin loads onto chromatin.

To determine whether the association of Tipin with chromatin required DNA replication, we added geminin to prevent loading of the MCM proteins onto chromatin (38–40). Fig. 2D shows that addition of geminin inhibited the loading of Tipin onto chromatin. We next asked whether Tipin was directly associated with the replication machinery. Even without adding DNA to the extract, Tipin immunoprecipitates were found to contain MCM7, a member of the putative replicative helicase (Fig. 2E). The idea that Tipin and Tim1 are members of a multiprotein complex at the replication fork is strengthened by the fact that they migrate as a high-molecular-mass complex of ≈ 600 –850

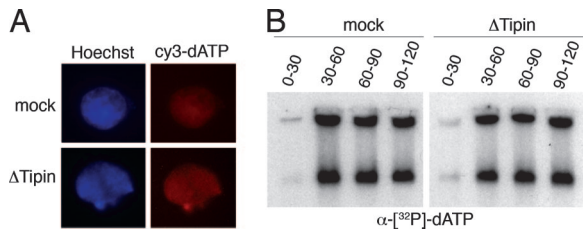


Fig. 3. Tipin is not essential for replication. (A) Mock- or Tipin-depleted extracts were supplemented with sperm nuclei (3,000 nuclei per microliter) and cy3-dATP (Amersham). After 30 min, 3 μ l was spotted on a microscope slide and mixed with a fixative containing Hoechst 33258 (blue fluorescence). Red fluorescence due to cy3-dATP incorporation indicates a replicating nucleus. (B) Sperm nuclei (1,000 nuclei per microliter) were added to an interphase *Xenopus* extract (mock- or Tipin-depleted). Replication was labeled with [α - 32 P]dATP from 0 to 30 min, from 30 to 60 min, from 60 to 90 min, or from 90 to 120 min from nuclei addition. Replicated DNA was purified, loaded on agarose gel, and analyzed by autoradiography.

kDa and coelute with Claspin and MCM7 upon gel filtration (data not shown).

Tipin Is Not Essential for Replication. Although Tipin is associated with the replication fork, its role during DNA replication is controversial. Reduction of Tipin levels in human cells by siRNA resulted in a delay in S-phase progression (30–32). To gain further insight into the role of Tipin during DNA replication, we assessed the impact of Tipin depletion from *Xenopus* extracts on DNA replication. Perhaps surprisingly, both mock-depleted and Tipin-depleted extracts formed nuclei that incorporated cy3-dATP at the same rate (Fig. 3A). Similarly, there was no reproducible difference in the labeling of the newly replicated DNA with [α - 32 P]dATP between extracts containing or lacking Tipin (Fig. 3B).

Tipin Is Important for the Checkpoint Response to Replication Block. Budding and fission yeast orthologues of Tipin and Tim1 are involved in DNA replication checkpoint responses (13, 17, 28). Recent data in mammalian cells showed that down-regulation of Tipin or Tim1 by siRNA resulted in reduced levels of Chk1 phosphorylation (21, 30–33). Therefore, we examined the role of Tipin in *Xenopus* S-phase checkpoint responses by monitoring the phosphorylation of Chk1 in response to a replication block. Sperm nuclei were added to an interphase *Xenopus* extract treated or not with aphidicolin, a DNA replication inhibitor that induces replication fork stalling and triggers the activation of Chk1 (41, 42). After 70 min of aphidicolin treatment, nuclei were isolated and the level of Chk1 phosphorylation assessed by using a phosphoantibody that recognizes P-Ser-344 in Chk1 (Fig. 4A). In Tipin-depleted extracts, the phosphorylation of Chk1 was barely detectable compared with the control, although the level of Chk1 in the depleted extract was unaffected (Fig. 4B). Phosphorylation of Chk1 was partially rescued by adding back recombinant Tipin to the depleted extract (Fig. 4A), but, compared with the mock-depleted extract, the level of Chk1 phosphorylation was considerably reduced. In all Tipin depletion experiments, however, Tim1 levels were also greatly reduced. Thus, by adding back Tipin alone, rather than the Tipin/Tim1 complex, the residual Tim1 remaining in the extract may be limiting and not sufficient to completely restore Chk1 phosphorylation. On the other hand, we observed a similarly low level of rescue when 1 μ l of mock-depleted extract [low-speed supernatant (LSS)] was added back to the depleted extract, suggesting that the process of depletion has compromised something else, which can only be partially rescued even when complete extract is added. There does seem to be an excess of Tim1 over Tipin in

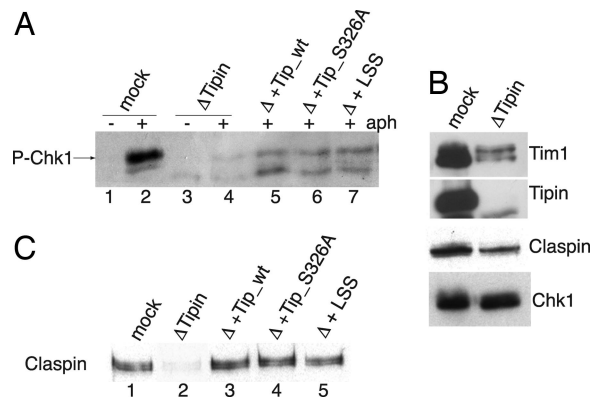


Fig. 4. Tipin is required for Chk1 phosphorylation and Claspin loading on chromatin. (A) Sperm nuclei were added to mock-depleted (lanes 1 and 2) or Tipin-depleted (lanes 3 and 4) extract in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of aphidicolin and incubated at 23°C for 70 min. Nuclei were purified, and proteins were analyzed by immunoblot with anti-phospho-Chk1 antibody. Recombinant Tipin (wild type, lane 5; S326A, lane 6) or 1 μ l of mock-depleted extract (LSS, lane 7) were added to the depleted extract, and the experiment was performed as described above. (B) Mock- or Tipin-depleted extract (1 μ l) was analyzed by immunoblotting as indicated. (C) Chromatin was purified from mock-depleted (lane 1) or Tipin-depleted (lanes 2–5) extract supplemented with aphidicolin and recombinant wild-type Tipin (lane 3) or S326A Tipin (lane 4) or 1 μ l of extract (LSS, lane 5). Samples were probed with anti-Claspin antibody.

the extract, so that when Tipin is added back, it can find its partner and form an active complex, but this may be at too low a concentration to perform a complete rescue.

When replication is blocked, the *Xenopus* homologue of ATR is responsible for the activation of Chk1 (43, 44). This process requires the mediator protein Claspin (45–47), which also associates with the replication fork (48, 49). Because Claspin is an essential mediator of the checkpoint response, we analyzed its behavior in Tipin-depleted extracts. Although Claspin levels were not significantly reduced in Tipin-depleted extracts (Fig. 4B), Claspin was not loaded onto the chromatin in the absence of Tipin (Fig. 4C). The loading of Claspin onto the chromatin was rescued by the addition of wild-type recombinant Tipin to the depleted extract (Fig. 4C). The defect in Claspin loading onto the chromatin and the phosphorylation of Chk1 was also rescued by adding back recombinant Tipin with the S326 mutated to either Ala or Glu (Fig. 4A and data not shown), indicating that Tipin phosphorylation is not important for its checkpoint function.

Tipin Is Required for Stabilizing the Replication Fork During DNA Damage. The Human Tipin/Tim1 complex associates with replication protein A (RPA)34, Claspin, MCM helicase, and DNA polymerase, and it has been suggested that a possible role for the Tipin/Tim1 complex at the DNA replication fork is to couple the MCM-mediated unwinding activity with the polymerase-mediated DNA synthesis, stabilizing the replisome (33). To address the role of Tipin at the replication fork, we depleted Tipin from the extract and monitored the accumulation of RPA on chromatin at different times after blocking replication with aphidicolin. Fig. 5A shows that RPA rapidly accumulated to high levels on chromatin in Tipin-depleted extract compared with the mock-depleted control, suggesting that single-stranded DNA accumulates at blocked replication forks in the absence of Tipin. The quantitation shown in the accompanying bar graph (Fig. 5A Right) was performed in three independent experiments using the Li-Cor system (Lincoln, NE) for the analysis of the immunoblots.

We next examined the role of Tipin during the recovery of

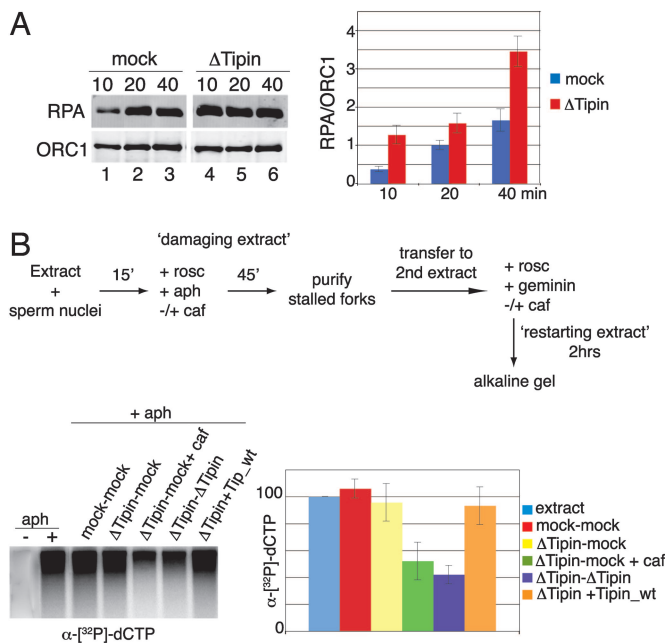


Fig. 5. Tipin depletion induces accumulation of ssDNA and impairs recovery from stalled replication forks. (A) Mock-depleted (lanes 1–3) or Tipin-depleted (lanes 4–6) extract was incubated with 20 μ M aphidicolin. Chromatin was harvested at 10, 20, and 40 min and analyzed by immunoblotting with anti-RPA and anti-ORC1 antibodies. The amount of RPA loaded on the chromatin was determined with respect to ORC1. Three independent experiments are averaged in the bar graphs; mock-depleted extracts is represented by blue bars, and Tipin-depleted extracts are represented by red bars. (B) Chromatin replication was initiated in an interphase extract (damaging extract). Sperm nuclei were added to the extract and incubated at 23°C to allow origins to fire and replication to start. After 15 min, fork progression was blocked by adding 40 μ M aphidicolin and 0.5 mM roscovitine. Damaged forks were isolated at 60 min and added to a second extract (restarting extract) made incompetent for origin firing and origin assembly by addition of 0.5 mM roscovitine and 5 ng/ μ l geminin. Different damaging and restarting (mock- or Tipin-depleted) extracts were used as stated in the different lanes. Lanes 1 and 2, *Xenopus* extract either with (lane 2) or without (lane 1) aphidicolin in the first incubation; lanes 3–7, all had aphidicolin in the first incubation; lane 3, both damaging and restarting extract were mock-depleted; lanes 4 and 5, the damaging extract was Tipin-depleted, and forks were restarted in a mock-depleted extract; lane 5, caffeine was added to the second incubation; lane 6, both damaging and restarting extract were Tipin-depleted; lane 7, the same as lane 6 except that recombinant Tipin was added in the second incubation. In all cases, [α -³²P]dCTP was added in the second incubation to monitor replication. Incorporation was analyzed by alkaline agarose gel electrophoresis and autoradiography. Three independent experiments are averaged in the bar graphs, taking the amount of replication of the positive control (e.g., lane 2) as 100%. The error bars represent standard deviations from the mean values.

stalled replication forks using a chromatin transfer assay (Fig. 5B) (50). First, sperm nuclei were added to an extract (“damaging extract”) to allow origin firing and the initiation of DNA replication. After 15 min, aphidicolin was added to stall replication forks. Roscovitine was added at the same time to inhibit CDK activity and block further origin firing (where indicated, caffeine was added to inhibit ATM and ATR activity). After a further 45 min, chromatin containing the stalled replication forks was isolated and transferred to a second extract (“restarting extract”) rendered incompetent for origin firing or assembly of new origins by addition of both roscovitine and geminin. Aphidicolin treatment led to replication arrest in the damaging extracts. Fig. 5B shows that, when the chromatin from the first incubation was added to the restarting extract, significant incorporation of [α -³²P]dCTP into DNA occurred if the damaging extract contained aphidicolin (Fig. 5B, lane 2), but not if

aphidicolin was omitted and no damage was inflicted (Fig. 5B, lane 1). When both the damaging extract and the restarting extract were depleted of Tipin, DNA synthesis was reduced to \approx 50% of the control (Fig. 5B, lane 6). Tipin must be present in the restarting extract (Fig. 5B, lane 4). To test whether Tipin was directly involved in restart of damaged replication forks, Tipin-depleted damaging and restarting extracts were supplemented with recombinant Tipin. This restored normal DNA replication (Fig. 5B, lane 7). Trezn *et al.* (50) have shown that ATR-ATM-dependent checkpoint is required for restarting of collapsed fork, but not of stalled forks, and we found that addition of caffeine to both damaging and restarting extracts prevented restart (Fig. 5B, lanes 4 and 5), suggesting that restart of forks impaired by Tipin depletion requires ATM-ATR activity. In a Tipin-depleted extract, loss of Chk1 activation in response to DNA damage indicates that the ATR checkpoint is defective, but ATM is presumably still active, which helps to prevent collapse of replication forks. When both ATR and ATM are inhibited by caffeine, however, the checkpoint is completely inactive, and forks collapse in the absence of Tipin. Once collapsed, replication forks are not able to restart.

Discussion

The DNA damage checkpoint network is extremely important for the faithful completion of DNA replication during S-phase. When a replication fork is stalled, checkpoint signaling pathways are activated and DNA replication is temporarily inhibited to prevent the irreversible breakdown of replication forks (51). A replication-pausing complex is thought to assemble at arrested forks to activate the checkpoint, allowing repair of the damaged DNA and resumption of replication (16–18, 28). In budding yeast, the Mec1/Rad53 (ATR/Chk2) checkpoint response prevents the collapse of stalled replication forks and allows the restart of DNA replication after recovery (52). This response requires both Tof1 (Tim1) and Mrc1 (Claspin) proteins (16, 48). Nedelcheva *et al.* (27) suggested a mechanism where Tof1-Csm3-Mrc1 (Tim1-Tipin-Claspin) contributes to a “pausing complex” that prevents uncoupling of the replication machinery from DNA unwinding upon DNA damage. After pausing, there is limited accumulation of ssDNA to which RPA binds and promotes the recruitment of the Mec1-Ddc2 (ATR-ATRIP) complex, which activates the checkpoint cascade (53). A similar model has been suggested for the human S-phase checkpoint, where the Tim/Tipin/Claspin complex is believed to be present at the replisome (6).

In mammals, Tim1, Tipin, and Claspin are considered checkpoint mediators, because loss of any one of these proteins results in reduced phosphorylation of Chk1 and a somewhat inefficient progression through S-phase (30). In *Xenopus*, Claspin associates directly with Chk1, an interaction that strongly enhances the ability of ATR to phosphorylate Chk1 (54, 55). We have shown that immunodepletion of Tipin from *Xenopus* extract also results in a substantial removal of Tim1, whereas Claspin and Chk1 levels are unaffected. In these Tipin (and Tim1)-depleted extracts Chk1 phosphorylation in response to aphidicolin treatment is considerably reduced, consistent with the postulated mediator functions of Tipin. We also found that Claspin is not loaded onto chromatin in Tipin-depleted extracts, in agreement with recent experiments in human cells (30). It is likely that the failure to load Claspin is responsible for the failure to phosphorylate Chk1 in the absence of Tipin after replication stress. Both the loading of Claspin onto the chromatin and Chk1 phosphorylation are rescued by adding back recombinant Tipin. Truncated forms of Claspin lacking several hundred residues from the N terminus show reduced ability to bind chromatin but can still activate Chk1 to some extent, indicating that stable association of Claspin with chromatin is not absolutely essential

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