Human Timeless and Tipin stabilize replication forks and facilitate sister-chromatid cohesion

Adam R. Leman, Chiaki Noguchi, Candice Y. Lee and Eishi Noguchi*

Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102, USA

*Author for correspondence (enoguchi@drexelmed.edu)

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Summary

The Timeless-Tipin protein complex has been reported to be important for replication checkpoint and normal DNA replication processes. However, the precise mechanisms by which Timeless-Tipin preserves genomic integrity are largely unclear. Here, we describe the roles of Timeless-Tipin in replication fork stabilization and sister chromatid cohesion. We show in human cells that Timeless is recruited to replication origin regions and dissociate from them as replication proceeds. Cdc45, which is known to be required for replication fork progression, shows similar patterns of origin association to those of Timeless. Depletion of Timeless-Tipin causes chromosome fragmentation and defects in damage repair in response to fork collapse, suggesting that it is required for replication fork maintenance under stress. We also demonstrate that depletion of Timeless-Tipin impairs sister chromatid cohesion and causes a defect in mitotic progression. Consistently, Timeless-Tipin co-purifies with cohesin subunits and is required for their stable association with chromatin during S phase. Timeless associates with the cohesion-promoting DNA helicase ChlR1, which, when overexpressed, partially alleviates the cohesion defect of cells depleted of Timeless-Tipin. These results suggest that Timeless-Tipin functions as a replication fork stabilizer that couples DNA replication with sister chromatid cohesion established at replication forks.

Key words: Timeless-Tipin, The fork protection complex, Fork stabilization, Sister chromatid cohesion, ChlR1

Introduction

Environmental toxins or drugs can cause DNA damage or other lesions that lead to arrest of DNA replication forks. Arrested forks are among the most serious threats to genomic integrity because they might collapse, break or rearrange, and are thought to be the cause of many mutations and chromosome rearrangements (Branzei and Foiani, 2007; Heller and Marians, 2006; Lambert et al., 2007). To circumvent these problems, eukaryotic cells are equipped with a quality control system, termed the DNA replication checkpoint, or S-phase checkpoint. In humans, defects in this checkpoint can cause genetic instability, which in turn leads to a strong predisposition to a variety of genetic disorders (Aguilera and Gomez-Gonzalez, 2008; Bartek and Lukas, 2007; Branzei and Foiani, 2008; Lavin, 2008; Paulsen and Cimprich, 2007). The mechanisms by which checkpoint proteins transduce signals to arrest the cell cycle and coordinate with DNA repair pathways in response to stalled replication forks are well characterized. However, how the replication checkpoint actually senses and stabilizes stalled replication forks is rather poorly understood.

Recently, we found that two proteins, Swi1 and Swi3, form the replication fork protection complex in the fission yeast *Schizosaccharomyces pombe* (Noguchi et al., 2004). The Swi1-Swi3 complex has been shown to move with replication forks as a part of the replisome and has a crucial role in the stabilization of replication forks and the activation of Cds1, the master kinase of the replication checkpoint (Lee et al., 2004; Noguchi et al., 2003; Noguchi et al., 2004; Sommariva et al., 2005). Swi1 and Swi3 are evolutionarily conserved. Swi1 is a member of the structurally conserved Timeless protein family that includes *Drosophila melanogaster* Timeless, mammalian Timeless/Tim1, *Xenopus laevis* Tim1, *Caenorhabditis elegans* Tim-1 and budding yeast Tof1 (Chan et al., 2003; Dalgaard and Klar, 2000; Errico et al., 2007; Foss, 2001; Noguchi et al., 2003). The Swi3 protein family includes mammalian Tipin, *Xenopus* Tipin, and budding yeast Csm3. Swi3-like proteins also exist in several other organisms including *Drosophila* and *C. elegans* (Errico et al., 2007; Gotter, 2003; Noguchi et al., 2004). The Timeless protein binds the cryptochrome protein and controls circadian rhythm in *Drosophila* and mammalian cells (Barnes et al., 2003; Ceriani et al., 1999; Unsal-Kacmaz et al., 2005), and the mammalian Timeless-Tipin complex has been shown to interact with replisome components and is involved in DNA replication (Chou and Elledge, 2006; Gotter et al., 2007; Unsal-Kacmaz et al., 2007; Unsal-Kacmaz et al., 2005; Yoshizawa-Sugata and Masai, 2007). Timeless-Tipin also interacts with Chk1 and ATR (ATM and Rad3-related kinase) to control Chk1 activity. Downregulation of Timeless-Tipin in human cells compromises replication and the intra-S-phase checkpoint, suggesting an intimate connection between circadian rhythm and checkpoint mechanisms (Chou and Elledge, 2006; Gotter et al., 2007; Unsal-Kacmaz et al., 2007; Unsal-Kacmaz et al., 2005; Yoshizawa-Sugata and Masai, 2007). *Xenopus* Tipin also forms a complex with Timeless and has an important role in Chk1 activation and resumption of stalled replication forks (Errico et al., 2007). In *C. elegans*, Tim-1 has been suggested to be involved in chromosome cohesion and segregation (Chan et al., 2003). Budding yeast Tof1, the Swi1 homolog, was originally isolated as a topoisomerase-1-interacting protein in a two-hybrid screen (Park and Sternglanz, 1999). Mutations in the budding yeast Swi3 homolog Csm3, cause a mild defect in chromosome cohesion and segregation (Mayer et al., 2004; Rabitsch et al., 2001; Warren et al., 2004), and interaction of Tof1 with Csm3 was also confirmed by coimmunoprecipitation (Mayer et al., 2004). As in the case of Swi1-Swi3, Tof1-Csm3 has been shown to travel with replication forks as a component of the replisome (Calzada et al., 2005; Gambus et al., 2006; Katou et al., 2003) and is involved in

Rad53 (Cds1 homolog) activation (Foss, 2001; Tong et al., 2004). Thus, it appears that the protein complexes related to Swi1-Swi3 are broadly conserved among eukaryotes.

Although previous studies have reported the role of Timeless-Tipin in DNA replication and checkpoint controls, the mechanisms by which Timeless-Tipin preserves genomic integrity in human cells have not been well understood. Furthermore, how this complex affects different cellular mechanisms has not been elucidated. Therefore, in this report, we describe a series of experiments aimed at understanding the role of Timeless-Tipin in genome maintenance. Our results suggest that Timeless and Tipin are components of a mammalian replication fork protection complex that has an important role in sister chromatid cohesion.

Results

Timeless and Cdc45 are recruited to replication origin regions and dissociate as replication progresses

To investigate the role of Timeless and Tipin in mammalian cells, we generated polyclonal antibodies against the human proteins. Our antibodies specifically recognized Timeless and Tipin, respectively, from human cell extracts (supplementary material Fig. S1). Immunoprecipitation confirmed that Timeless and Tipin form a complex in cell extracts prepared from HEK293 cells (supplementary material Fig. S1B) as previously reported (Chou and Elledge, 2006; Gotter et al., 2007; Unsal-Kacmaz et al., 2007; Yoshizawa-Sugata and Masai, 2007). We also used these antibodies to examine the cellular localization of Timeless and Tipin in HeLa

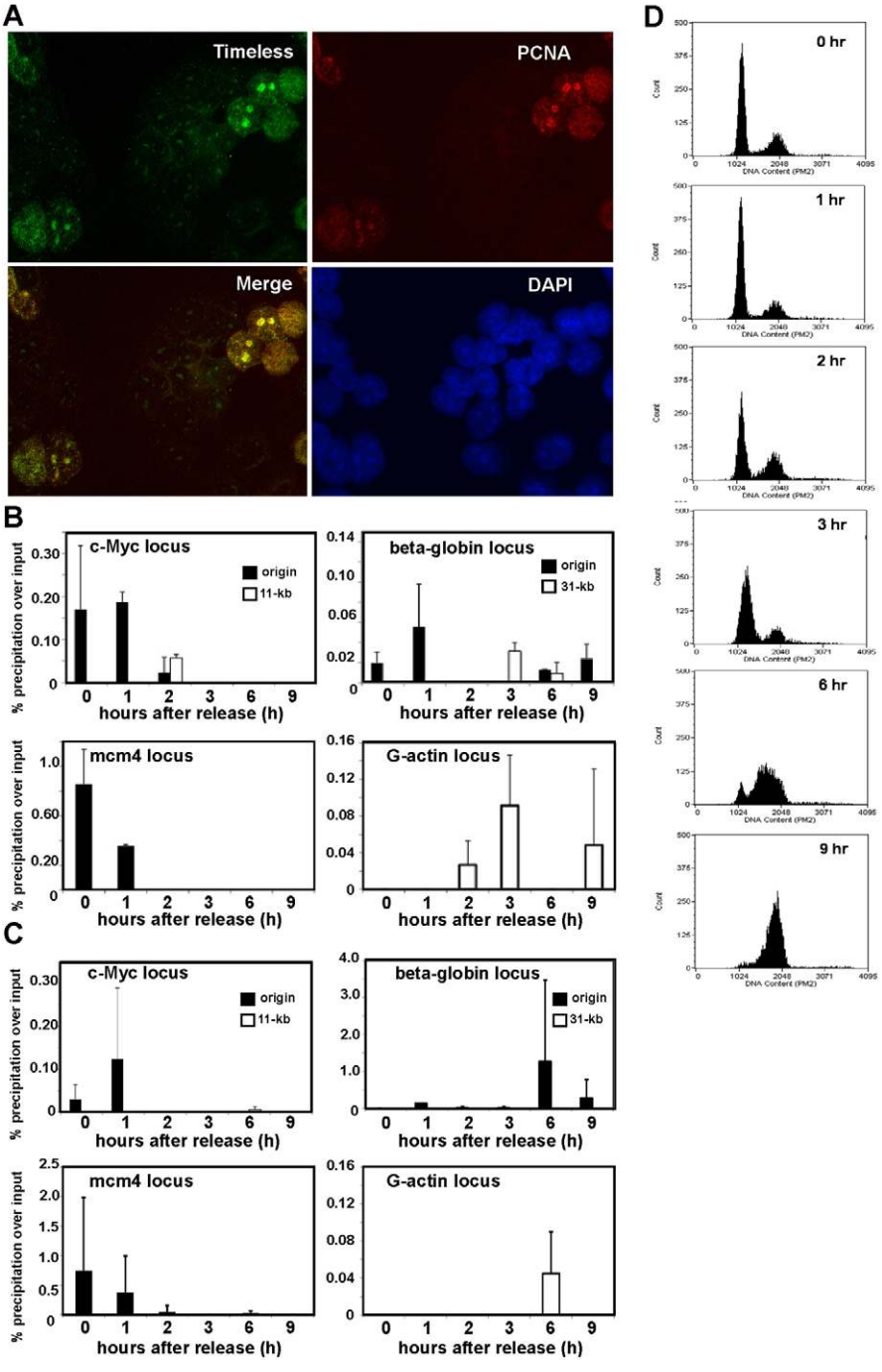


Fig. 1. Timeless is recruited to replication forks. (**A**) HeLa cells were grown overnight on coverslips. In situ extraction of soluble proteins by Triton X-100 was performed before fixation and immunostaining of cells. Timeless and PCNA localization was determined using affinity-purified anti-timeless (Tim, Green) and anti-PCNA (PCNA, Red) antibodies. DNA was costained with DAPI (4′,6′-diamidino-2-phenylindole). The merged image of timeless and PCNA is shown (Merge). Representative images of repeat experiments are shown. (**B**,**C**) HeLa cells were synchronized in very early S phase and released into fresh medium to allow cells to progress through the cell cycle. Cells were collected at the indicated times and processed for ChIP. Precipitated DNA recovered from antibody-containing beads was used to monitor the association of timeless (B) and Cdc45 (C) at *MYC* (c-Myc), *HBB* (β-globin), *MCM4* and *ACTG1* (G-actin) loci. The replication origins (origin: 0 kb) and their proximal positions (11 kb for *MYC*; 31-kb for *HBB*) were analyzed at *MYC* and *HBB* loci. Percentage precipitated DNA over input DNA is shown at each time-point. Data are from at least three independent experiments, and error bars represent s.d. (**D**) DNA content of cells used in ChIP experiments in B determined by flow cytometry analysis.

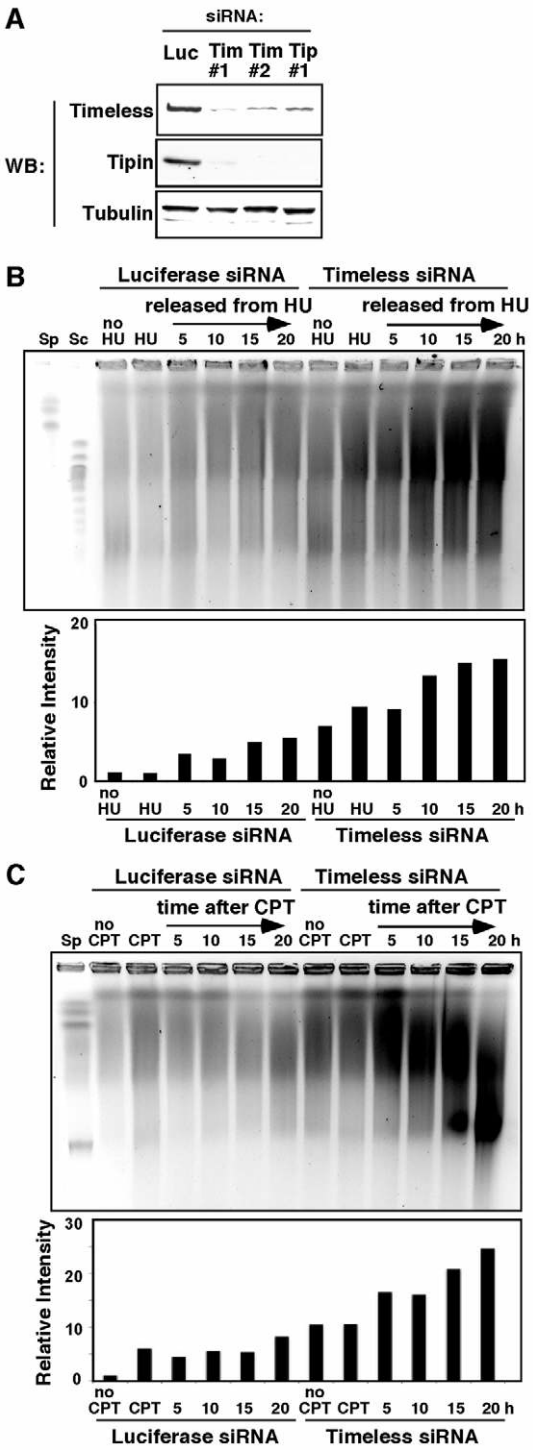
cells treated with Triton X-100, which removes soluble proteins but preserves chromatin-bound proteins in the nucleus (Bravo et al., 1982; Fey et al., 1984; Mirzoeva and Petrini, 2001; Staufenbiel and Deppert, 1984; Toschi and Bravo, 1988). Timeless colocalized with PCNA, a known replication fork protein that associates with chromatin during S phase (Fig. 1A). The Timeless signal strength was severely reduced by siRNA-dependent depletion of Timeless (data not shown). We also obtained similar results with Tipin (data not shown). We noted that Timeless-Tipin also forms foci outside S phase (in cells without PCNA foci) (Fig. 1A). The intensity of these non-S-phase foci was much weaker than the foci in S phase; the physiological importance of these foci is unknown (Fig. 1A). These data confirmed the results of previous reports (Gotter et al., 2007; Yoshizawa-Sugata and Masai, 2007), suggesting that Timeless-Tipin is recruited to the replication fork, although direct evidence is lacking. To address this possibility, we used chromatin immunoprecipitation (ChIP) assays of Timeless on HeLa cell extracts (Fig. 1B). HeLa cells were synchronized in very early S phase by a double-thymidine block and released into fresh medium to allow cells to progress into the cell cycle. Cells were collected at 0, 1, 2, 3, 6 and 9 hours after the release from the beginning of S phase. Flow cytometry analysis confirmed a synchronous cell cycle progression through S and G2-M phases (Fig. 1D). ChIP was performed using the anti-Timeless antibody to monitor the association of Timeless with the *MYC* gene (c-Myc) region that contains a well-characterized early replication origin (Malott and Leffak, 1999; Sibani et al., 2005a; Sibani et al., 2005b). Importantly, Timeless strongly associated with the *MYC* origin region at 0 and 1 hour time points when the majority of cells are at very early S phase, and this association declined during S-phase progression (Fig. 1B,D). By contrast, there was no significant association of Timeless at the proximal position 11 kb away from the *MYC* origin until 2 hours after the release (Fig. 1B), indicating that Timeless is recruited to the *MYC* origin region specifically at the onset of S phase. However, Timeless was detected at 2 hours at the 11 kb position (Fig. 1B), suggesting that Timeless relocates from the *MYC* origin region to this position as cells proceed through S phase. At 3 hours, Timeless dissociated from the 11 kb position (Fig. 1B), indicating that Timeless further moved away from this region. When we examined another active origin at the *MCM4* gene locus that fires early in S phase (Ladenburger et al., 2002; Schaarschmidt et al., 2002), strong association of Timeless was detected specifically at the beginning of S phase. However, we were not able to detect significant Timeless association at regions flanking the *MCM4* origin, possibly because of the fast movement of replication forks. We also monitored a replication origin located at the β-globin gene locus (*HBB*), which is known to fire late in S phase in HeLa cells, but early in S phase in erythroleukemia cells (Dhar et al., 1988). Unexpectedly, Timeless was detected at the β-globin origin region during early S phase (0 and 1 hour), and this association disappeared by 2 hours after the release (Fig. 1B). However, we detected Timeless association at this region again during late S phase (6 and 9 hours) (Fig. 1B), which was expected for HeLa cells. This biphasic association of Timeless with the β-globin origin region might be due to the existence of two distinct populations of cells. Timeless was detected also at the proximal position 31 kb away from the β-globin origin at 3 hours, and this signal diminished at 6 and 9 hours (Fig. 1B). We noticed that the Timeless level at this origin was significantly weaker when compared with the signals at the *MYC* and *MCM4* origins (Fig. 1B). This might suggest that the β-globin origin does not fire efficiently in our cells. Therefore, the Timeless localization at the 31 kb position might represent passive replication originated from another active origin. Next, we examined localization of Timeless at the *ACTG1* (G-actin) gene locus, a region where no active origin is detected (Tan et al., 2006). As expected, there was no detectable Timeless at this locus at the onset of S phase (Fig. 1B). However, there was a weak association of Timeless with the ACTG1 region in mid-S-phase at 2 and 3 hours, which dissociated afterwards (Fig. 1B), probably because of passive replication of this region. There was also an association of Timeless at the *ACTG1* locus at 9 hours. It is possible that there are two distinct populations of cells with differential activation of origins.

These results strongly suggested that Timeless is recruited to replication forks. To confirm this idea, we examined the localization of Cdc45, which is known to be part of the CMG complex required for the progression of replication fork in mammalian cells (Aparicio et al., 2009). In budding yeast, Cdc45 has been shown to move with the fork (Bell and Dutta, 2002; Gambus et al., 2006). ChIP of Cdc45 was performed at the *MYC*, *MCM4*, *HBB* and *ACTG1* loci as described above. Importantly, Cdc45 also associated with the *MYC* and *MCM4* origin regions in early S phase, as was the case for Timeless (Fig. 1C). Similarly to localization of Timeless, Cdc45 also showed biphasic association with the β-globin origin during early and late S phase, although its association at early S phase was weak (Fig. 1C). Cdc45 was only detected at the *ACTG1* locus at 6 hours, probably because of passive replication from another adjacent origin (Fig. 1C). Taken together, our results are consistent with the notion that Timeless and Cdc45 are recruited to replication origin regions and dissociate from them as replication forks progress.

Timeless-Tipin is involved in replication fork stabilization in the presence and absence of genotoxic agents

Depletion of Timeless or Tipin from human cells has been shown to cause accumulation of spontaneous foci containing phosphorylated histone H2AX (γH2AX) (Chou and Elledge, 2006; Urtishak et al., 2009), indicative of DNA damage even in the absence of genotoxic agents. Furthermore, depletion of Timeless causes increased sister chromatid exchange, which is dependent on Brca2 and Rad51 (Urtishak et al., 2009). These results suggest that Timeless-Tipin has a crucial role in preventing DNA damage at replication forks. To examine the effects of Timeless or Tipin depletion on fork stabilization, we used the established siRNA sequences (Tim-#1 and Tim-#2 for Timeless; Tip-#1 for Tipin) that are designed to target Timeless or Tipin (Unsal-Kacmaz et al., 2005; Yoshizawa-Sugata and Masai, 2007) and confirmed that expression of Timeless or Tipin was dramatically reduced (Fig. 2A and supplementary material Fig. S1). As described previously (Chou and Elledge, 2006; Yoshizawa-Sugata and Masai, 2007), we noticed that depletion of Timeless caused a dramatic reduction in the level of Tipin and vice versa (Fig. 2A), indicating that Timeless and Tipin protein levels are mutually dependent. These cells were treated with hydroxyurea (HU), which depletes the nucleotide pool available for DNA replication, resulting in replication fork arrest. Cells were then released into fresh medium to allow cells to recover from replication arrest and processed for chromosome analysis using pulsed-field gel electrophoresis (PFGE) (Fig. 2B). In PFGE, intact human chromosomes are not able to migrate into the gel. However, when chromosomes are fragmented as a result of DNA damage, shorter pieces of chromosomes can enter the gel (Blocher et al., 1989; Joshi and Grant, 2005). Control siRNA-treated cells did not show an increase in fragmented DNA in response to HU (Fig. 2B). After the release from HU arrest, control cells displayed a mild

increase in fragmented DNA at the 5 hour time-point, probably because of minor DNA damage when cells restart replication fork progression. However, the amount of fragmented DNA was not significantly increased later over the course of the experiment (Fig. 2B, 5-20 hours in luciferase siRNA). By contrast, when cells were treated with Timeless siRNA (Tim-#1), cells showed further accumulation of fragmented DNA after release into fresh medium (Fig. 2B, 5-20 hours in Tim siRNA). We obtained similar results with HeLa cells transfected with Tim-#2 or Tip-#1 siRNA (data not shown). These results suggest that HU treatment causes replication fork breakage leading to chromosome fragmentation in the absence of Timeless. They also suggest that cells treated with Timeless siRNA fail to efficiently repair DNA damage caused by HU. Thus, Timeless-Tipin is required for stabilization of stalled replication forks induced by HU treatment.



We also treated control and Timeless siRNA-transfected HeLa cells with camptothecin (CPT), a compound that traps topoisomerase I on DNA and effectively induces chromosome breakage at replication forks during S phase (Pommier, 2006). In control cells, CPT treatment induced a mild increase in subchromosomal DNA as visualized by PFGE (Fig. 2C, no CPT and CPT in luciferase siRNA), indicating that CPT treatment caused fork breakage. However, the amount of subchromosomal DNA did not increase significantly after the removal of CPT from the culture medium (Fig. 2C, 5-20 hours in luciferase siRNA). When cells treated with Timeless siRNA (Tim-#1) were treated with CPT and released into fresh medium, fork breakage was further enhanced as evident from a robust increase in the amount of subchromosomal DNA after the removal of CPT (Fig. 2C, 5-20 hours in Tim siRNA). We obtained similar results with Tim-#2 or Tip-#1 siRNA-treated cells (data not shown), suggesting that broken forks were not efficiently repaired in the absence of Timeless-Tipin. Therefore, these results suggest that Timeless-Tipin is involved in prevention of replication fork breakage and/or subsequent recovery of broken replication forks in human cells.

It should also be noted that Timeless siRNA-treated cells showed a reproducible increase in the amount of subchromosomal DNA in the absence of HU or CPT (Fig. 2B,C; no HU/CPT). This result suggests that Timeless-Tipin is also involved in preventing replication fork collapse in the absence of exogenous DNA damaging agents. Consistently, chromosome spread experiments demonstrated a high incidence of chromosome fragmentation in a metaphase of Timeless or Tipin siRNA cells (Fig. 3A). As shown in Fig. 3B, 34.0±6.4% of Timeless RNAi cells and 29.8±5.2% of Tipin RNAi cells displayed chromosome fragmentation, whereas 9.9±1.9% of control (luciferase) cells showed fragmented chromosomes. These data suggest that Timeless-Tipin is also required for stabilization of replication forks during unperturbed DNA replication.

Timeless is required for proper establishment of sister chromatid cohesion

Studies in *C. elegans* have indicated that the Timeless homolog Tim-1 is associated with the cohesin complex and is involved in chromosome cohesion (Chan et al., 2003). Moreover, in budding and

Fig. 2. Timeless is required for replication fork stabilization. (**A**) HeLa cells were transiently transfected with the indicated siRNAs (Luc, luciferase; Tim, timeless; Tip, tipin). Levels of timeless (Tim), tipin (Tip) and tubulin (Tub), 24 hours after the transfection are shown, as monitored by western blotting (WB). siRNA depletion of timeless also caused a reduction of the tipin level, and vice versa. Representative results of repeat experiments are shown. (**B**,**C**) HeLa cells were transfected with timeless siRNA, then treated with 5 mM HU for 5 hours (B) or 10 μM CPT (C) for 3 hours. Cells were washed and harvested at the indicated times. Cells were counted, and 1×10^6 cells were embedded in an agarose plug for chromosomal DNA preparation. Chromosomal DNA in plugs was separated by PFGE and visualized using ethidium bromide staining. Downregulation of timeless induced strong accumulation of subchromosomal DNA fragments in response to both HU and CPT treatment. Quantification of DNA damage was shown as relative intensity of fragmented DNA by setting the minimum intensity (no drug in control siRNA cells) to 1. Sp and Sc indicate Genomic DNA from *S. pombe* and *S. cerevisiae* as size markers, respectively. Representative results of repeat experiments are shown.

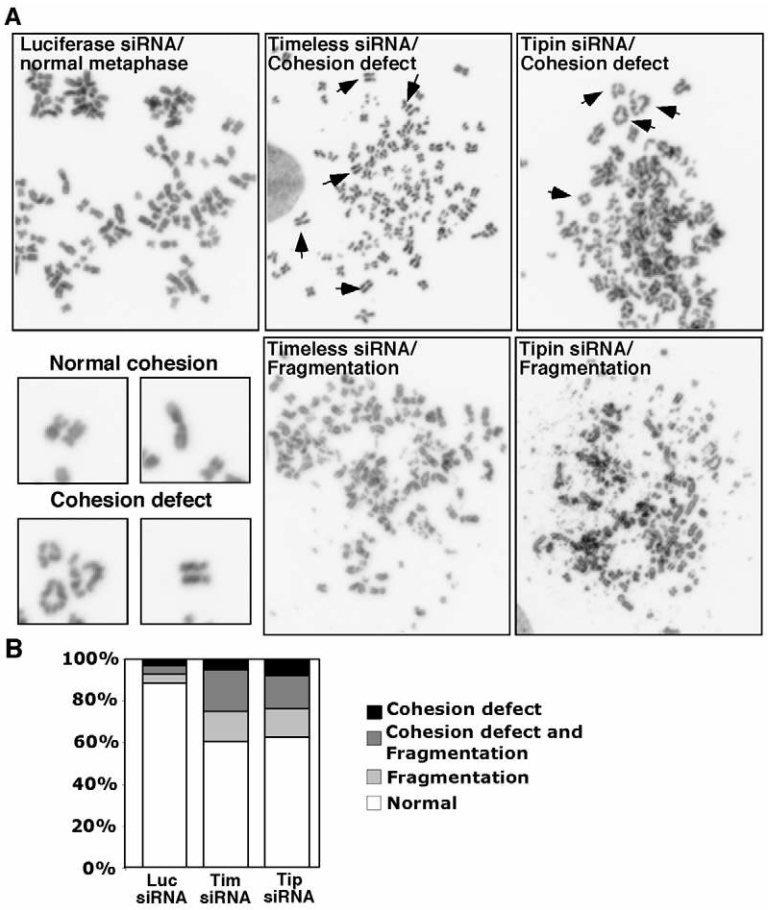


Fig. 3. The timeless-tipin complex is involved in sister chromatid cohesion. (**A**) HeLa cells were transfected with luciferase (Luc), timeless (Tim) or tipin (Tip) siRNA, grown for an additional 30 hours, treated with colcemid for 90 minutes, incubated in hypotonic buffer, and fixed. Fixed cells were dropped onto a glass slide from a height of 0.75 m and stained with DAPI. Representative images of cells with fragmented chromosomes and cells with cohesion defects are shown. (**B**) The frequency of different phenotypes shown in A was determined and expressed as percentage of total metaphase cells. At least 100 metaphase cells were counted for each experiment. Average percentages of three independent experiments are shown.

fission yeast, Timeless-Tipin homologs have been reported to be required for proper establishment of sister chromatid cohesion (Ansbach et al., 2008; Mayer et al., 2004; Warren et al., 2004; Xu et al., 2007). However, the mechanism by which Timeless-Tipin homologs regulate this process is unknown. To determine whether human Timeless-Tipin is involved in chromosome cohesion, we used a chromosome-spread method to visualize metaphase chromosomes of HeLa cells treated with Timeless or Tipin siRNA. Cells were incubated with 0.1 μg/ml colcemid for 1.5 hours to increase the population of metaphase cells. Most control (luciferase) cells showed normal chromatid pairing (Fig. 3A), although a small number of cells displayed cohesion defects in the control experiment as reported previously (Parish et al., 2006; Watrin et al., 2006). When Timeless or Tipin was depleted, cells appeared to display loose pairing of sister chromatids, which is indicative of cohesion defects (Fig. 3A). As shown in Fig. 3A (arrows, panels of cohesion defect), pairing sister chromatids were significantly further apart at the centromeric region. Quantification of these results revealed that 8.8±2.7% of control siRNA-treated cells showed cohesion defects, whereas 27.3±11.0% of Timeless siRNA cells and 20.8±5.8% of Tipin siRNA cells displayed cohesion defects (Fig. 3B); suggesting that Timeless-Tipin is involved in proper establishment of sister chromatid cohesion.

To further understand the role of Timeless-Tipin in sister chromatid cohesion, we investigated whether Timeless-Tipin interacts with components of the cohesin complex. The Timeless-FLAG or Tipin-FLAG fusion proteins were overexpressed in HEK293 cells and immunoprecipitated by anti-FLAG antibodies. As shown in Fig. 4A, endogenous Smc1, Smc3 and SA1, which are subunits of the cohesin complex, copurified with Timeless-FLAG or Tipin-FLAG, whereas actin failed to co-precipitate with Timeless or Tipin. To investigate the possibility that Timeless and Tipin interact with cohesin subunits through DNA, we treated cell extracts with DNaseI before immunoprecipitation. In this condition, Smc1 and SA1 still readily co-precipitated with Timeless (Fig. 4B). However, there was no detectable interaction between Smc3 and Timeless (Fig. 4B). Interestingly, Tipin failed to interact with cohesin subunits in this condition (Fig. 4B). These results suggest that Timeless is able to associate with Smc1 and SA1 independently of DNA. They also suggest that Timeless-Smc3 and Tipin-cohesin interactions are dependent on DNA.

These results suggest that Timeless-Tipin facilitates efficient sister chromatid cohesion by specifically associating with the cohesin complex. Therefore, we investigated whether depletion of Timeless leads to the destabilization of cohesin subunits on chromatin. HEK293 cells treated with control siRNA or Timeless siRNA were synchronized at the G1-S boundary with L-mimosine, released into the cell cycle, and collected at 0, 1, 2, 3, 6 and 9 hours after release (Fig. 4C,D). Cells were fractionated into a Triton-X-100-soluble fraction containing cytosol and nucleoplasm, and a Triton-X-100-insoluble fraction enriched with chromatin- and nuclear matrix-bound proteins as described by Yoshizawa-Sugata and Masai (Yoshizawa-Sugata and Masai, 2007). Actin and CENP-B were exclusively fractionated into Triton-soluble and Triton-insoluble fractions, respectively, indicating that fractionation was successful (Fig. 4C). As shown in Fig. 4C, Smc1, Smc3 and SA1 were recovered in the Triton-insoluble fraction in control siRNA cells.

Interestingly, the level of Smc1 in Triton X-100-insoluble fraction was greatly reduced in G1-S and S phase of Timeless-depleted cells compared with control cells (Fig. 4C). There was also a significant reduction in the levels of Smc3 and SA1 during S phase but not G1-S, when Timeless was downregulated. These results suggest that Timeless is involved in the stable association of the cohesin subunits with chromatin during S phase, which in turn regulates proper establishment of sister chromatid cohesion.

Timeless and ChlR1 cooperate to control sister chromatid cohesion

We have previously shown that overproduction of Chl1, a DNA helicase known to have a role in sister chromatid cohesion in yeast and humans (Parish et al., 2006; Petronczki et al., 2004; Skibbens, 2004), can suppress DNA damage sensitivity of the *swi1* (a Timeless homolog) deletion mutants in fission yeast (Ansbach et al., 2008). However, how these proteins are involved in cohesion establishment was unknown. Therefore, to determine the mechanisms by which Timeless-Tipin depletion causes defective sister chromatid cohesion, we investigated the interaction between Timeless and ChlR1, a human homolog of Chl1 (Parish et al., 2006). Timeless-FLAG was overexpressed in HEK293 cells and immunoprecipitated. As shown in Fig. 5A, endogenous ChlR1 protein from HEK293 cells was consistently co-precipitated with Timeless-FLAG. We also overexpressed ChlR1-FLAG in HEK293 cells and found that endogenous Timeless co-purified with ChlR1-FLAG (Fig. 5A). The

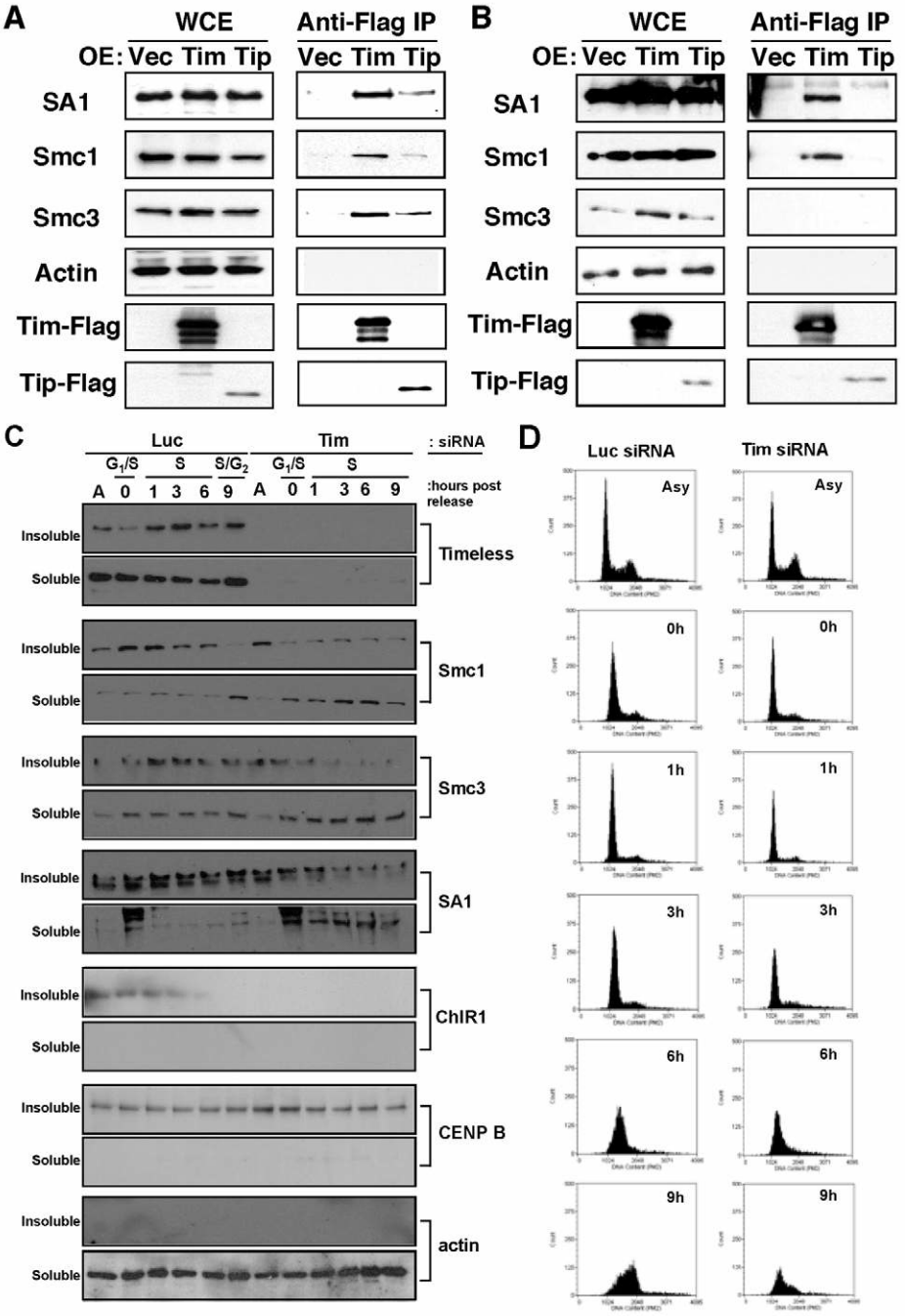


Fig. 4. Timeless is involved in stable maintenance of cohesin subunits on chromatin. (**A**,**B**) HEK293 cells were transfected with pcDNA3-3FLAG (Vec), pcDNA3-timeless-3FLAG (Tim), or pcDNA-tipin-3FLAG (Tip) and grown for 48 hours. Cell extracts were prepared from these cells in the absence (A) or presence (B) of DNaseI. FLAG-tagged proteins were immunoprecipitated from cell extracts with anti-FLAG antibody. Immunoprecipitates were probed with the indicated antibodies to determine interacting proteins. Representative results of repeat experiments are shown. WCE, whole cell extract; anti-FLAG IP, immunoprecipitated fraction; OE, overexpressed protein. (**C**) HEK293 cells were transfected with the indicated siRNA, synchronized at the G1-S transition, and released into the fresh medium to allow cells to progress through the cell cycle. Cells were collected at the indicated times and fractionated into Triton-X-100-soluble and -insoluble fractions for protein analyses. Levels of the indicated proteins were determined by western blotting. Representative results of repeat experiments are shown. A, asynchronous. (**D**) DNA content of cells used in fractionation experiments in C was determined by flow cytometry.

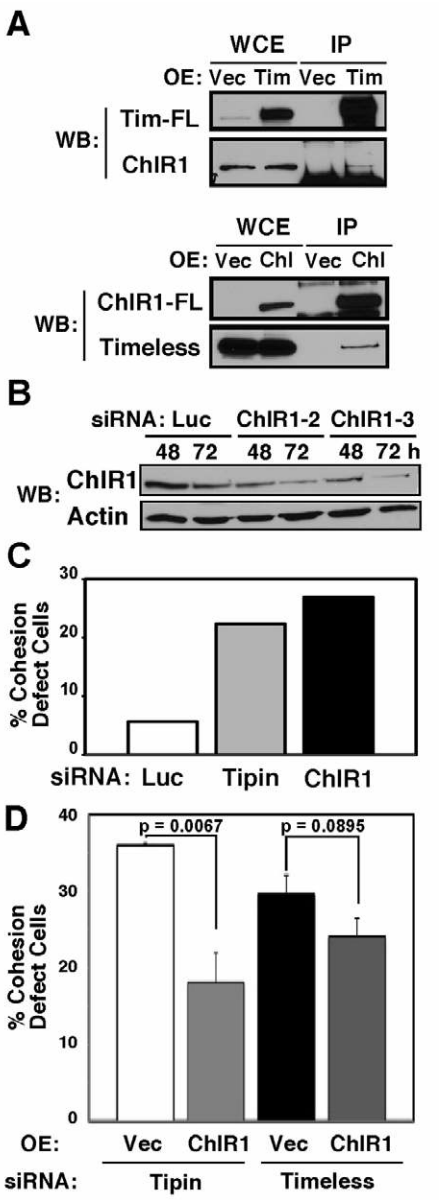


Fig. 5. ChlR1 and timeless-tipin cooperate in establishment of sister chromatid cohesion. (**A**) ChlR1 associates with timeless. HEK293 cells were transfected with pcDNA3-3FLAG (Vec), pcDNA3-timeless-3FLAG (Tim) or pcDNA3-hChlR1-3FLAG (Chl) and grown for 48 hours. Immunoprecipitation (IP) was performed with the anti-FLAG antibody. Associated proteins were examined by western blotting using the indicated antibodies. Representative results of repeat experiments are shown. WCE, whole cell extract; IP, immunoprecipitated fraction; WB, protein detected by western blotting; OE, overexpressed protein. Representative results of repeat experiments are shown. (**B**) HeLa cells were transiently transfected with the indicated siRNAs. Cells were collected 48 or 72 hours after transfection, and levels of ChlR1 were monitored by western blotting, using antibodies against ChlR1. Luc, Luciferase. (**C**) Chromosome spread analysis was performed as described in Fig. 3. Cells treated with tipin siRNA and ChlR1-2 siRNA showed significant cohesion defects. (**D**) ChlR1 overexpression partially suppressed cohesion defects of timeless or tipin-depleted cells. HeLa cells stably overexpressing ChlR1 or vector alone (Vec) were transfected with the indicated siRNA. Cohesion was evaluated by chromosome spread analysis. At least 50 metaphase cells were counted for each experiment. Error bars correspond to the s.d. obtained from three independent experiments. OE, overexpression.

interaction between Timeless and ChlR1 was maintained in DNase-I-treated extracts (data not shown). Thus, Timeless interacts with ChlR1 in human cells. To further examine Timeless and ChlR1 interaction, we fractionated HEK293 cells into Triton-soluble and -insoluble fractions, as described in Fig. 4C. This experiment revealed that ChlR1 was associated with chromatin in control cells (Fig. 4C). However, RNAi-dependent downregulation of Timeless led to the disappearance of ChlR1 (Fig. 4C), suggesting that Timeless is required for stabilization of ChlR1 or maintenance of ChlR1 on chromatin to prevent ChlR1 degradation.

When HeLa cells were transfected with ChlR1 siRNAs, cells showed sister chromatid cohesion defects consistent with the previous reports (Fig. 5B,C) (Farina et al., 2008; Parish et al., 2006) and comparable with the defects observed in Timeless- or Tipin-depleted cells (Fig. 3B, Fig. 5C). To investigate how Timeless-Tipin and ChlR1 participate in the establishment of sister chromatid cohesion, we generated a HeLa cell line stably overexpressing ChlR1-FLAG. We depleted Timeless or Tipin from these cells and performed chromosome-spread analyses. Overexpression of ChlR1 significantly reduced the cohesion defects caused by depletion of Timeless or Tipin by siRNA (Fig. 5D). Cohesion defects in Tipin-depleted cells were reduced from 36.01% to 18.14% when ChlR1 was overexpressed (P=0.0068 by paired Student's t-test). A similar reduction in cohesion defects was also observed in Timeless-depleted cells in the presence of overproduced ChlR1 (from 29.67% to 24.15%; P=0.0895) (Fig. 5D). These data suggest that Timeless-Tipin and ChlR1 cooperate to regulate proper sister chromatid cohesion to preserve genomic integrity.

Depletion of Timeless results in deregulation of mitotic progression

A defect in sister chromatid cohesion in Timeless-depleted cells prompted us to determine the role of Timeless in mitotic progression. HeLa cells were transfected with luciferase or Timeless siRNA and stained with DAPI to visualize chromosomes. When quantified, Timeless-depleted cells showed a slight reduction in the percentage of mitotic cells compared with control cells (3.04±0.84%, Timeless-depleted cells; 4.29±0.65%, control cells) (Fig. 6A). This might be explained by the significant S-phase delay in Timeless-depleted cells (Fig. 4D), causing a longer cell cycle, which in turn leads to the mild reduction in mitotic index. However, there was a strong increase in the number of cells at prophase and prometaphase in Timeless-depleted cells (Fig. 6B). When we quantified percentages of cells in sub-stages of M phase, 31.37±4.3% of mitotic Timeless-depleted cells were in prophase and prometaphase, whereas 16.52±4.8% of mitotic control cells were in these phases (Fig. 6B). There was also a slight increase in cells at metaphase when Timeless was depleted (Fig. 6B). By contrast, cells in anaphase and telophase were significantly reduced in Timeless-depleted cells (Fig. 6B). Thus, these results suggest that Timeless-depleted cells have a delay in mitotic progression during prophase through metaphase, but have shorter anaphase and telophase compared with control cells.

BubR1 has been shown to be involved in the mitotic checkpoint that blocks the activation of anaphase-promoting complex. When the mitotic checkpoint is activated by problems in mitotic spindle tension, BubR1 becomes phosphorylated and shows a slower-migrating species in SDS-PAGE (Ditchfield et al., 2003; Yu, 2002). It has also been shown that mitotic errors cause BubR1-dependent prometaphase delay (Blower et al., 2006). To test whether Timeless depletion causes BubR1 activation, we probed for BubR1 from whole-cell extracts of HeLa cells treated with Timeless or luciferase

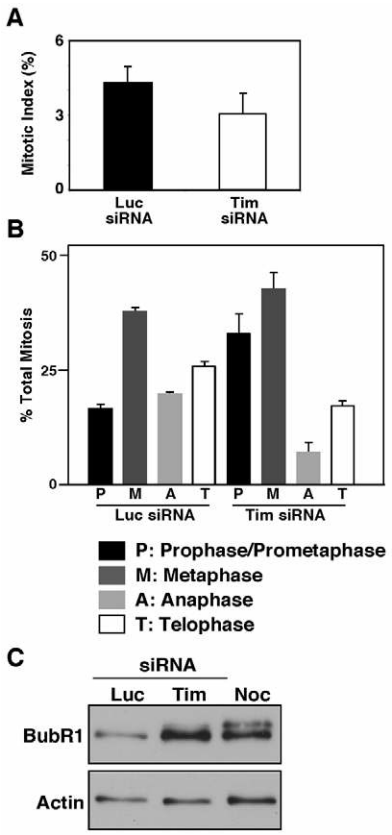


Fig. 6. Depletion of timeless impairs mitotic progression. (**A**) HeLa cells transfected with timeless siRNA showed a slight reduction in the number of mitotic cells compared with cells transfected with luciferase siRNA. At least 300 cells were counted from each experiment. Error bars correspond to the s.d. obtained from three independent experiments. (**B**) Quantification of each mitotic stage from HeLa cells transfected with timeless siRNA or luciferase siRNA. At least 50 mitotic cells were counted from each experiment. Error bars indicate s.d. from three independent experiments. (**C**) BubR1 is activated in HeLa cells transfected with Timeless siRNA. Whole-cell extracts from HeLa cells treated with luciferase (Luc) siRNA, timeless (Tim) siRNA and nocadazole (Noc) were examined by western blotting using the anti-BubR1 antibody. Western blotting of actin is shown as loading control. Representative results of repeat experiments are shown.

siRNA. As a control, HeLa cells treated with nocodazole, which activates the mitotic checkpoint, efficiently induced the slower-migrating form of BubR1, as previously reported (Fig. 6C) (Ditchfield et al., 2003). Luciferase siRNA treatment failed to induce BubR1 activation, as expected (Fig. 6C). However, Timeless-depleted cells displayed significant induction of the slower-migrating BubR1 (Fig. 6C). In addition, the total level of BubR1 was greatly increased in Timeless-depleted cells compared with control cells (Fig. 6C). Therefore, these data are consistent with the notion that Timeless depletion causes activation of BubR1-dependent mitotic checkpoint, leading to a mitotic delay during prophase through metaphase.

Discussion

The role of Timeless-Tipin in fork stabilization and checkpoint signalling

Previous studies have reported that Timeless is associated with replisome components and colocalizes with them. However, there was no direct evidence that Timeless was actually recruited to replication forks. In this study, we have demonstrated using ChIP and quantitative real-time PCR that Timeless and Cdc45 are specifically recruited to replication origin regions and dissociate from them as DNA replication proceeds (Fig. 1B). We were also able to detect Timeless at a non-origin region during mid-S-phase, probably as a result of passive replication from neighboring origins. The levels of Timeless association at active origins were much stronger than those at origin-proximal regions (Fig. 1B). This might suggest that Timeless is localized at the origin during replisome assembly and is stationary at the origin until actual DNA synthesis starts. However, lack of synchrony as replication forks move away from origins could lead to broadening and decrease in amplitude of the Timeless and Cdc45 signal. This effect makes it difficult to ascertain the localization of Timeless or Cdc45 in intergenic regions from ChIP experiments (Fig. 1B,C). Nevertheless, taken together with the fact that Timeless colocalizes with PCNA on chromatin (Fig. 1A) (Yoshizawa-Sugata and Masai, 2007), and that Cdc45 also showed similar origin association patterns in our ChIP analyses (Fig. 1C), our present data suggest that Timeless travels with replication forks as a component of the replisome complex. In support of this idea, human Timeless-Tipin is known to interact with several replisome components, including putative replicative helicase subunits of Mcm (Chou and Elledge, 2006; Errico et al., 2007; Gotter et al., 2007) and ChlR1 (Fig. 5A), which is shown to be involved in lagging strand processing, probably at the replication fork (Farina et al., 2008). In addition, PFGE analyses have revealed that Timeless-depleted cells are not able to recover from DNA damage after hydroxyurea or camptothecin treatment (Fig. 2). Therefore, our results suggest that Timeless is involved in replication fork stabilization when forks collapse, thereby facilitating restart of replication fork progression upon DNA damage. Timeless-Tipin might also be important during unperturbed DNA replication because Timeless or Tipin depletion causes a significant increase in chromosome fragmentation (Figs 2 and 3). Consistently, it has been reported that Timeless is required for efficient progression of replication forks (Unsal-Kacmaz et al., 2007; Yoshizawa-Sugata and Masai, 2007). These data suggest that Timeless is involved in the efficient assembly of replisome components during both perturbed and unperturbed DNA replication.

It is reported that Chk1 activity is decreased in Timeless or Tipin siRNA-treated cells (Unsal-Kacmaz et al., 2007; Unsal-Kacmaz et al., 2005; Yoshizawa-Sugata and Masai, 2007). Consistently, Chk1 is co-immunoprecipitated with Timeless from cell extracts (Unsal-Kacmaz et al., 2005), and Errico and co-workers showed that Tipin is involved in the loading of claspin, an activator of Chk1, onto chromatin (Errico et al., 2007). Based on these observations, it has been suggested that Timeless-Tipin is a mediator of the Chk1-dependent checkpoint. However, our data suggest that the primary function of Timeless-Tipin is to maintain replication fork integrity because DNA damage increases in Timeless- and/or Tipin-depleted cells even in the absence of exogenous stress (Figs 2 and 3). Consistently, a recent report has shown that Timeless depletion leads to ssDNA accumulation, probably because of replication failure, which in this report leads to activation of Chk1 in NIH3T3 cells (Smith et al., 2009). Therefore, it is straightforward to suggest that Timeless-Tipin has both Chk1-dependent and -independent functions. Timeless might act early in the response to fork arrest and function, as a mammalian replication fork protection complex ($FPC^{Tim\text{-}Tip}$), to modulate replication fork or replisome structures in a replication-competent state (Fig. 7). It is also possible that

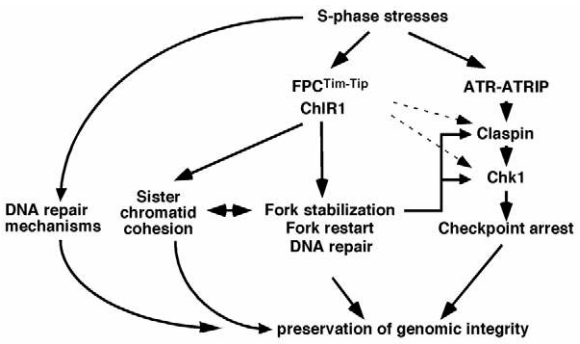


Fig. 7. S-phase stress-response mechanisms. The timeless-tipin fork protection complex ($FPC^{Tim-Tip}$) is involved in the ATR-Chk1-dependent replication checkpoint. $FPC^{Tim-Tip}$ has checkpoint-independent functions that are important for fork protection, DNA repair, and sister chromatid cohesion. In this model, $FPC^{Tim-Tip}$ stabilizes replication forks in a configuration that is recognized by replication checkpoint sensors. In addition, $FPC^{Tim-Tip}$ cooperates with ChlR1 to promote proper establishment of sister chromatid cohesion.

Timeless-Tipin is directly involved in the repair of collapsed replication forks, although further investigation is required to support this notion.

The role of Timeless-Tipin in sister chromatid cohesion

The cohesin complex loaded onto chromatin during G1 phase is thought to fully encircle the unpaired chromatid. During S phase, replication forks encounter cohesin rings, and sister chromatid cohesion is established. In one model, replication forks are proposed to pass through the ring, thereby trapping duplicated sister chromatids inside the ring to establish cohesion (Gruber et al., 2003; Lengronne et al., 2006; Skibbens, 2005). In this model, cohesin rings might transiently dissociate when forks pass through them because the replisome complex is too large to fit through the cohesin ring (Lengronne et al., 2006). In another model, called a 'handcuff model', cohesin rings are separately transferred to each replicated chromatid; therefore, the rings must temporarily dissociate from the parental chromatid and reassociate with replicated chromatids (Zhang et al., 2008). Importantly, in both models, replisome components have a chance to physically interact with cohesins, and there is a transient dissociation of cohesin from chromatin at replication forks. Therefore, specific fork components such as the Timeless-Tipin complex might aid in maintaining stable association of cohesin subunits on DNA when replication forks encounter cohesin rings. Consistently with this idea, we showed that downregulation of Timeless-Tipin leads to defective chromosomal cohesion and that Timeless-Tipin interacts with cohesin subunits in cell extracts (Fig. 4). We have also demonstrated that Smc1, Smc3 and SA1 are partially dissociated from chromatin in Timeless-depleted cells (Fig. 4C). Interestingly, association of Timeless with Smc1 and SA1 was independent of DNA, but Timeless-Smc3 interaction was DNA dependent. In addition, Tipin appeared to interact with cohesin subunits in a DNA-dependent manner. Therefore, it is possible that Timeless directly interacts with Smc1 and SA1 to promote stable association of the cohesin complex with chromatin, probably at replication forks. In yeast and frog, inactivation of Timeless homologues causes cohesion defects although physical interaction between Timeless and cohesin was not determined in these organisms (Ansbach et al., 2008; Mayer et al., 2004; Tanaka et al., 2009; Warren et al., 2004). In *C. elegans*, Tim-1 (Timeless homolog) has been shown to interact with cohesins and involved in the loading of non-SMC cohesin subunits on chromatin (Chan et al., 2003). In the present study, using human cells, we found that Timeless is more closely associated with Smc1 (an SMC subunit) and SA1 (a non-SMC subunit) independently of DNA. We have also found that Timeless is required for efficient loading of both SMC and non-SMC subunits on chromatin, indicating that the association of Timeless-Tipin and cohesin complex differ from that of *C. elegans*. Nevertheless, our current data provides mechanistic insight into understanding of replication-dependent sister chromatin cohesion.

We also found that the human ChlR1 DNA helicase, which is shown to be involved in cohesion establishment (Parish et al., 2006), interacts with Timeless and that ChlR1 overexpression partially suppresses the cohesion defect of Timeless-Tipin-depleted cells (Fig. 5). Furthermore, we found that Timeless is required for the stability of ChlR1 (Fig. 4C). Therefore, our studies suggest that Timeless-Tipin cooperates with ChlR1 to maintain stable association of cohesin complex with chromatin, which helps to establish sister chromatid cohesion during S phase (Fig. 7). Timeless-Tipin might operate upstream of ChlR1 to stabilize the replisome and allow for proper localization of ChlR1 on chromatin. It is possible that ChlR1 is degraded when it is dissociated from chromatin in the absence of Timeless. Interestingly, ChlR1 has been reported to be involved in processing the lagging strand (Farina et al., 2008), suggesting that ChlR1 has a role in preventing the formation of abnormal DNA structures at replication forks, which in turn allows replication forks to pass through the cohesin ring. Therefore, it is possible that ChlR1 and Timeless cooperate to maintain fork structure, contributing to the proper establishment of sister chromatid cohesion.

It is also possible that Timeless-Tipin is required to recruit cohesins at damaged DNA sites, because it has been shown that double-strand breaks also trigger cohesin loading onto chromatin (Strom et al., 2007; Unal et al., 2007). As Timeless-Tipin is required for recovery of collapsed forks (Fig. 2C), the complex might facilitate efficient DNA repair through maintenance of cohesins at damaged fork sites. Therefore, it would be interesting to examine in the future whether Timeless-Tipin is involved in DNA-damage-induced cohesin loading at collapsed forks and subsequent DNA repair.

Materials and Methods

Cell culture

HeLa cells were cultured in RPMI 1640 medium supplemented with 10% cosmic calf serum (Thermo Scientific HyClone, Logan, UT), 10 mM HEPES-KOH pH 7.5, and 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293 cells were cultured in DMEM supplemented with 10% cosmic calf serum, penicillin and streptomycin as above. HeLa cells were synchronized at the onset of S phase twice in the presence of 2 mM thymidine for 15 hours, with a 9 hour interval of growth without the drug, and returned to growth for the indicated times. HEK293 cells were synchronized at the G1-S boundary with 350 μM L-mimosine for 20-22 hours.

Plasmids

Full-length cDNAs against human Timeless, Tipin and ChlR1/DDX11 were amplified by PCR and ligated to 3× FLAG in the pcDNA3 vector, resulting in pcDNA3-Timeless-3FLAG, pcDNA3-Tipin-3FLAG, and pcDNA3-ChlR1-3FLAG, respectively.

Antibodies

Antibodies to Timeless and Tipin were generated by immunizing rabbits with the purified glutathione S-transferase (GST)-fused Timeless C-terminal region (914-1208 amino acids, Timeless-CT) and GST-fused Tipin, respectively. Sera were affinity purified over GST-Timeless-CT or GST-Tipin crosslinked to glutathione-Sepharose using dimethylpimelimidate, as described (Harlow and Lane, 1988). Antibodies to proliferating cell nuclear antigen (PCNA) (PC10), centromere protein B (CENP-B) (2D-7), Cdc45 (M-300) and actin (C-11) were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA); SA-1 (ab4455) from Abcam (Cambridge, MA); Smc1 (PC737) from Calbiochem (San Diego, CA); Smc3 (BL313) from Bethyl Laboratory (Montgomery, TX); ChlR1/DDX11 from Novus Biologicals (Littleton, CO); FLAG (M2) from Sigma (St Louis, MO); and BubR1 from BD Biosciences (San Jose, CA).

siRNA

Transfection of small interfering RNA (siRNA) duplexes was performed by using Oligofectamine (Invitrogen, Carlsbad, CA) as recommended by the supplier. siRNA oligonucleotides were purchased from Invitrogen. The sense strands of siRNA oligonucleotides for Timeless (Tim-#1, Tim-#2), Tipin (Tip-#1) and control (luciferase) were reported previously (Unsal-Kacmaz et al., 2007; Yoshizawa-Sugata and Masai, 2007). The sequences of ChlR1 siRNA are as follows: ChlR1-#1, 5′-UCC UGC AUG GCU GAG AGC CAG GCU U-3′; ChlR1-#2, 5′-CCA ACU GGC ACU GGG AAG UCC UUA A-3′; ChlR1-#3, 5′-CCU GUG UCU GUC UUC UUC CUG CGA A-3′.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described elsewhere (Nelson et al., 2006) with modification. Briefly, cells were fixed in culture medium with 1.42% formaldehyde for 15 minutes. The cells were then quenched with 125 mM glycine for 5 minutes, and washed twice in PBS and collected by centrifugation at 2000 ***g*** at 4°C. Cells were resuspended in ChIP lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1.0% Triton-X-100, 0.2 mM *p*-APMSF (4-amidinobenzylsulfonyl fluoride hydrochloride), and Roche Complete EDTA-free protease inhibitor cocktail), incubated on ice for 10 minutes, and sonicated using a Misonix Sonicator 3000 (Misonix, Farmingdale, NY) until chromatin DNA was sheared into 500-700 bp fragments. Cell lysates were clarified by maximum-speed centrifugation in an Eppendorf 5415D centrifuge at 4°C. Immunoprecipitations were performed in the cell extracts using either affinity-purified anti-Timeless, Cdc45 antibodies or Rabbit IgG in combination with Protein-A-Sepharose. Immunoprecipitated DNA and input DNA were recovered using Chelex-100 resin (Bio-Rad) as described (Nelson et al., 2006). Recovered DNA was analyzed by triplicate SYBR Green-based real-time PCR (Bio-Rad) using primers that are designed to amplify the *MYC* origin, *MYC* 11-kb proximal, *HBB* origin, *HBB* 31 kb proximal, *MCM4* origin, and *ATCG1* gene regions. Primer sequences were reported previously (Schaarschmidt et al., 2002; Sibani et al., 2005b; Tan et al., 2006). Raw percentage precipitated DNA values (percentage raw precipitation) were calculated based on ΔCT between input and immunoprecipitated samples and corrected for PCR efficiency. To obtain percentage precipitation values, raw percentage precipitated DNA values of IgG control samples were subtracted from percentage raw precipitation values of Timeless or Cdc45 ChIP samples.

Immunoprecipitation

HEK293 cells were transfected with pcDNA3-3FLAG, pcDNA3-Timeless-3FLAG, pcDNA3-Tipin-3FLAG, or pcDNA3-ChlR1-3FLAG with *Trans*IT LT-1 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's protocol and harvested 48 hours after transfection. Cells were washed twice in ice-cold PBS and lysed in immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 5 mM *N*-methylmaleimide, 1 μM okadaic acid, 0.2 mM *p*-APMSF, and Roche Complete EDTA-free protease inhibitor cocktail) by Branson Digital Sonifier (Danbury, CT) for eight cycles of 2 seconds at output 9%, with a 5 second interval on ice between each cycle. Protein extracts were clarified by maximum-speed centrifugation in an Eppendorf 5415D centrifuge for 10 minutes at 4°C. When needed, protein extracts were incubated with 5 U of DNase I at room temperature for 1 hour, followed by 3 hours at 4°C. Protein extracts were then mixed with anti-FLAG M2 agarose (Sigma), Protein-A/G-Sepharose (Sigma), or Protein-G dynabeads (Invitrogen) and incubated for 2 hours at 4°C. The beads were collected and washed three times in IP lysis buffer. Proteins associated with the beads were analyzed by western blotting.

Cell fractionation

Cell fractionation was performed as described elsewhere (Yoshizawa-Sugata et al., 2005). Cells were synchronized in S phase by L-mimosine as described above, rinsed twice and released into growth medium. Harvested cell were incubated in cell fractionation (CF) buffer (20 mM PIPES, pH 6.8, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 300 mM sucrose, 1 mM dithiothreitol, 1 mM Na_3VO_4, and Roche Complete EDTA-free protease inhibitor cocktail) containing 0.1% Triton X-100 for 20 minutes on ice. Triton-soluble and -insoluble fractions were separated by centrifugation in an Eppendorf 5415D centrifuge at 800 ***g*** for 4 minutes at 4°C. The insoluble fraction was washed twice in CF buffer and resuspended in the same buffer. The soluble fraction was clarified by maximum speed centrifugation. Fractions were analyzed by western blotting.

Pulsed-field gel electrophoresis

Preparation and pulsed-field gel electrophoresis (PFGE) of chromosomal DNA were performed as described (Blocher et al., 1989; Joshi and Grant, 2005), using CHEF-DR II system (Bio-Rad) at the following settings. Block 1: field strength, 1.9 V/cm; initial and final switch times, 30 and 120 seconds, respectively; running temperature, 14°C; pump speed, 70; running time, 30 hours. Block 2: field strength, 1.9 V/cm; initial and final switch times, 120 seconds and 42 minutes, respectively; running temperature, 14°C; pump speed, 70; running time, 51 hours. Gels were stained with 0.5 μg/ml ethidium bromide in water for 30 minutes and destained in water for 1 to 2 hours. DNA amount in each lane was quantified using EZ Quant-Gel software (EZQuant LTD, Tel-Aviv, Israel).

In situ cell fractionation, immunofluorescence and chromosome spreads

In situ fractionation of HeLa cells and immunofluorescence were performed as described (Mirzoeva and Petrini, 2001). Chromosome spreads were conducted as described elsewhere (Henegariu et al., 2001).

Cell cycle analysis

Cells were fixed via incubation in 80% ethanol while vortexing at maximum speed, then incubated overnight at –20°C. Fixed cells were pelleted, rehydrated in 1× PBS for 15 minutes, pelleted once again, and resuspended in Tris-HCl, pH 8.0, containing 0.2% Triton X-100, 100 U/ml RNaseA and 20 μg/ml propidium iodide (Sigma). Cells were incubated at 25°C for 30 minutes and processed for cell cycle analysis using a Guava EasyCyte Plus (Millipore, Billerica, MA).

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Supplementary material available online at
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