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Coupling of Human Circadian and Cell Cycles by the Timeless Protein

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The Timeless protein is essential for circadian rhythm in *Drosophila*. The Timeless orthologue in mice is essential for viability and appears to be required for the maintenance of a robust circadian rhythm as well. We have found that the human Timeless protein interacts with both the circadian clock protein cryptochrome 2 and with the cell cycle checkpoint proteins Chk1 and the ATR-ATRIP complex and plays an important role in the DNA damage checkpoint response. Down-regulation of Timeless in human cells seriously compromises replication and intra-S checkpoints, indicating an intimate connection between the circadian cycle and the DNA damage checkpoints that is in part mediated by the Timeless protein.

The circadian and cell cycles are two global regulatory systems that have pervasive effects on organismal and cellular physiology. Circadian rhythm is the oscillation in the physiology and behavior of organisms with a 24-h periodicity (17, 33, 40). The rhythm consists of light and dark phases which coincide with the phases of the solar day. Cell cycle checkpoints are regulatory pathways that ensure completion of biochemical reactions unique to each phase of the cell cycle (G_1, S, G_2, and M in proliferating mammalian cells) prior to initiation of subsequent phases (26, 30, 35, 41). While these two regulatory systems involve distinct mechanisms, there is some evidence that these cycles are linked. Most mammalian diploid cells exhibit an approximately 24-h cell cycle period, and the circadian clock has been implicated in regulation of the phases of cell division (3). The emerging field of chronotherapy aims to coordinate the time of delivery of chemotherapeutic drugs with the circadian and cell cycles so as to minimize side effects while optimizing therapeutic efficacy (4).

Although a few recent studies have shown that some cell proliferation and cell cycle checkpoint genes in mammals (such as c-*myc*, *Wee1*, and *cyclin D1*) are first- and second-order clock-controlled genes (10, 23), the circadian cycle-cell cycle connection remains ill-defined. Here we present evidence that the mammalian Timeless (Tim) protein (18, 36), which appears to be required for a robust circadian rhythm (1), is also a core component of the cell cycle checkpoint system, suggesting a possibly more intimate and direct connection between the circadian cycle and cell cycle checkpoints in mammals.

Despite its initial identification as a homologue of the *Drosophila* clock protein Tim, the closest phylogenetic relatives of the mammalian Tim protein are actually cell cycle-related proteins: budding yeast Tof1 (9, 32), fission yeast Swi1 (29, 19), *Caenorhabditis elegans* TIM-1 (5), and *Drosophila* Tim-2/Timeout (dTim2/dTimeout) (2). Tof1 and Swi1 have been implicated in DNA damage checkpoint activation as mediators, and Swi1 plays an additional role in preventing replication fork collapse (29). TIM-1 is essential for chromosome cohesion in *C. elegans*, and Timeless null mutation results in embryonic lethality in both *C. elegans* (5) and mice (12). Based on these findings we reasoned that the human Tim (hTim) protein may also have a checkpoint function and set out to test this hypothesis. Our results show that Tim is a checkpoint protein and may directly couple the cell cycle and the circadian cycle in humans.

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MATERIALS AND METHODS

Flag-Tim protein. The full-length cDNA of human Tim was a gift from M. Young (40). From this full-length cDNA, Flag-tagged Tim was amplified by PCR and cloned into the pcDNA4.1 (Invitrogen) expression vector. The 5′ primer contained an ATG codon followed by a Flag epitope in frame with the coding region that was amplified. This PCR product was digested with EcoRV and NotI restriction enzymes and ligated into the pcDNA4.1 expression vector through the same enzyme sites to generate the N-terminal Flag epitope-tagged Tim.

Immunoprecipitation. For immunoprecipitations, HEK293T cells (3×10^6/15-cm tissue culture dish) were either singly transfected or cotransfected with the indicated plasmids by a calcium phosphate method as described previously (37). After 16 h of incubation at 37°C in a 5% CO_2 incubator, cells were washed twice in serum-free Dulbecco's modified Eagle's medium (DMEM), and fresh medium (DMEM, 10% fetal bovine serum) was added to the cells for a further 48 h of incubation. Cells were washed with phosphate-buffered saline (PBS) and lysed in 1.5 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM β-glycerophosphate, 10% glycerol, 1% Tween-20, 0.1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors [Roche Molecular Biochemicals]) for 30 min on ice. The cell lysates were centrifuged for 30 min at 30,000 × *g*, and the supernatants from the spun lysates were incubated at 4°C for 4 h with anti-Flag M2 affinity resin (Sigma). The beads were then washed three times with TBS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), and bound proteins were eluted with TBS buffer containing 200 μg of Flag peptide (Sigma)/ml.

siRNA. The small interfering RNA (siRNA) duplexes were 21 bp, including a 2-base deoxynucleotide overhang. The sequences of the Tim siRNA oligonucleotides were GUAGCUUAGUCCUUUCAAAdTdT and UUUGAAAGGACU AAGCUACdTdT (synthesized by Dharmacon Research Inc., Lafayette, Colo.). The sequences of control siRNA oligonucleotides were UUCUCCGAACGUG UCACGUdTdT and ACGUGACACGUUCGGAGAAdTdT (synthesized by QIAGEN).

For transfections, HeLa cells were plated in six-well plates and were transfected at 40% confluency with the siRNA duplex, using Oligofectamine (Invitrogen) transfection reagent following the manufacturer's suggested protocol. Transfections were repeated 24 h later, and cells were analyzed 48 or 72 h after the first transfections.

Cell lines and culture conditions. All cell lines were maintained in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. For normal human fibroblasts (NHF1), cell culture medium was supplemented with 2% glutamine.

For cell cycle analyses, NHF1 cells were arrested in G_0 by contact inhibition and then induced to reenter the cell cycle by replating at low densities. G_1 cells were harvested 8 h after release. For S, G_2, and M phases, cells were treated with 2 μg of aphidicolin/ml for 24 h. Arrested cells were released into the cell cycle by removal of the drug and addition of fresh cell culture medium, and cells were harvested at the following time points: S = 3 h postrelease, G_2 = 8 h postrelease, and M = 12 h postrelease. To obtain M cell cycle stage-enriched NHF1 cells, cells were additionally treated with Colcemid (100 ng/ml) in the last 4 h of a total 12-h postrelease time. For each time point, DNA content was determined by fluorescence-activated cell sorter (FACS) analysis of propidium iodide-stained cells. Cells in mitosis were determined by staining with propidium iodide and antibody to phosphohistone H3 (P-H3), followed by fluorescein isothiocyanate-conjugated secondary antibody, and the percentage of M-phase cells was determined by flow cytometry.

For cell irradiation, prior to treatment with UVC, the culture medium was removed and reserved. Cultures were washed with PBS and then placed uncovered under a General Electric germicidal lamp emitting primarily 254-nm radiation at a fluency rate of 0.5 J/m^2/s. Following irradiation, reserved medium was replaced and the cultures were incubated for the indicated periods of time. Sham-treated cultures were handled exactly the same way, except that they were not exposed to UVC.

Antibodies. Rabbit polyclonal Tim antibody was a generous gift from P. Minoo (39). Monoclonal Flag antibody was purchased from Sigma. Cyclin B and actin antibodies were purchased from Santa Cruz Biotechnology Inc. Green fluorescent protein (GFP) antibody was purchased from Clontech. P-H3 and fluorescein isothiocyanate-conjugated secondary antibodies were from Cell Signaling and Jackson Immunoresearch Laboratories, respectively. Rabbit polyclonal Claspin antibody was from Bethyl. Guinea pig Per2 antibody was a generous gift from C. Lee (20a). Phosphospecific Chk1 antibody (P-S345) was purchased from Cell Signaling.

Radioresistant DNA synthesis (RDS) assay. DNA synthesis after UV irradiation was measured as described previously (16). Briefly, cells were plated in DMEM containing 10 nCi of [^{14}C]thymidine (ICN Radiochemicals)/ml to uniformly label DNA. The next day cells were transfected with siRNA oligomers in the presence of [^{14}C]thymidine. The medium containing [^{14}C]thymidine was replaced with fresh medium, and cells were transfected for the second time with siRNA oligonucleotides. Twenty-four hours later cells were either sham treated or exposed to UV (2 J/m^2) and incubated at 37°C for 30 min and then incubated for 15 min with 25 μCi of [^{3}H]thymidine/ml. Cells were washed twice with PBS and harvested by scraping with a rubber policeman into 0.5 ml of 0.1 M NaCl containing 0.01 M EDTA (pH 8) per plate. An aliquot (200 μl) was added to a separate tube containing 200 μl of lysis buffer (1 M NaOH, 0.02 M EDTA), acid-insoluble DNA was collected on a glass microfiber (GFC) filter and air dried, and the amount of radioactivity was assayed in a liquid scintillation counter. The resulting ratios of ^{3}H counts per minute to ^{14}C counts per minute, corrected for those counts per minute that were the result of channel crossover, were a measure of DNA synthesis.

Mitotic spread. HeLa cells were transfected with siRNA oligonucleotides, and 48 h after the first transfection cells were treated with 2 mM hydroxyurea (HU) for 20 h. Colcemid (100 ng/ml) was added to the drug-containing medium in the last 4 h. Mitotic spreads were performed as described previously (27). Cells resuspended in Carnoy's fixative (3 parts methanol, 1 part glacial acetic acid) were dropped from a height of 1 m onto a glass slide and allowed to dry. Cells were stained with Giemsa solution and air dried, and a coverslip was placed above them.

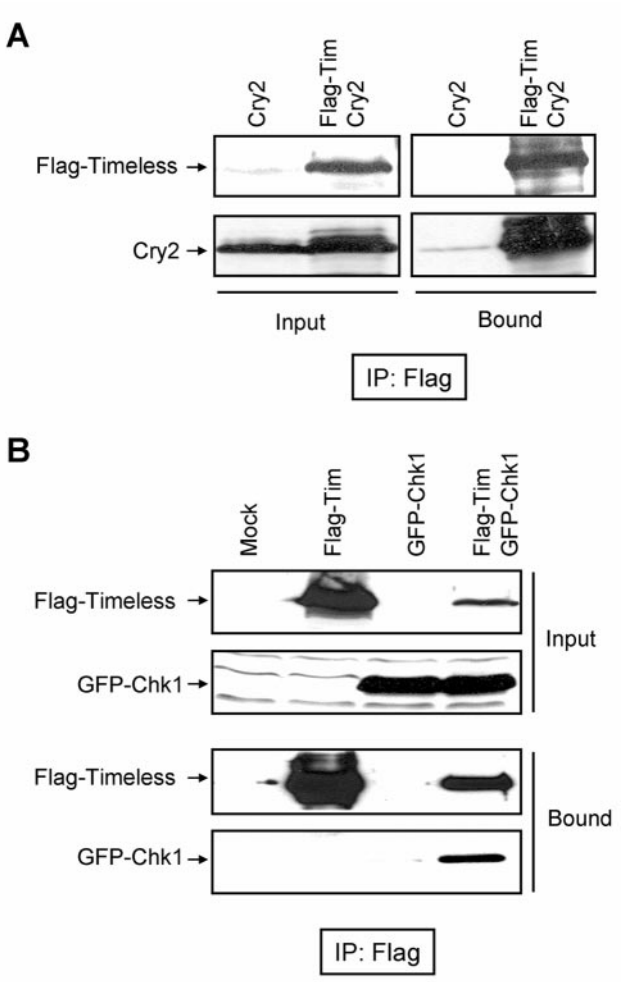

FIG. 1. Tim associates with the circadian clock protein Cry2 and the checkpoint protein Chk1 in vivo. (A) Tim-Cry2 interaction. HEK293T cells were transfected either with Cry2 alone or with Cry2 and Flag-tagged Tim. Equal amounts of cell lysate were mixed with anti-Flag antibodies linked to agarose beads, and the bound proteins were eluted by Flag peptides and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was done with anti-Flag and anti-Cry2 antibodies. Input represents 1/30 of the cell lysate used for immunoprecipitation (IP). (B) Tim-Chk1 interaction. HEK293T cells were mock transfected or transfected with either Flag-tagged Tim or GFP-tagged Chk1 or both and then immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were separated by SDS-PAGE and then immunoblotted with antibodies to Flag and GFP as indicated. Input represents 1/30 of the cell lysate used for immunoprecipitation.

RESULTS

Binding of Tim to Clock (Cry2) and Checkpoint (Chk1) proteins. First, we wished to confirm the reports (8, 13, 20) of a specific interaction between hTim and human cryptochrome 2 (hCry2), a known component of the core clock machinery in mammals (17, 33, 34, 40). We transfected HEK293T cells with Flag-tagged Tim and Cry2 and observed a significant amount of Cry2 in the anti-Flag immunoprecipitates (Fig. 1A) from these cells, in agreement with results from previous studies, including results of yeast two-hybrid assays (13), and consistent with the recently demonstrated role of Tim in the core circadian clock (1).

Next, we wished to determine whether hTim interacted with known checkpoint proteins. In fission yeast the activation of the Cds1 signal transduction kinase is strongly dependent on Swi1, a Tim orthologue, although it is unknown whether these two proteins directly interact (29). We tested for interaction between hTim and hChk1 kinase, which is the functional homolog of spCds1, by coimmunoprecipitation. As shown in Fig. 1B, hTim and hChk1 interact specifically in a manner analogous to the hTim-hCry2 interaction, raising the possibility that Tim, in addition to its reported function in the circadian cycle (1), may have a cell cycle checkpoint function as well.

It has been previously shown that mammalian Tim exhibits

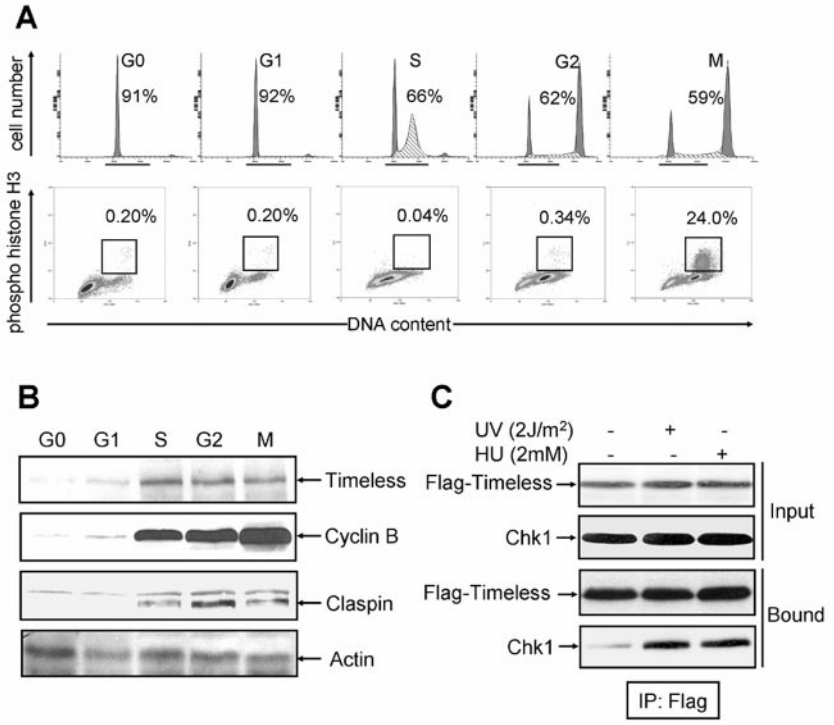

FIG. 2. Tim expression is cell cycle regulated, and its interaction with Chk1 is damage dependent. (A) Synchronization of normal human fibroblasts (NHF1). For cell cycle analyses, cells were treated with 2 mg of aphidicolin/ml for 24 h. Arrested cells were released into the cell cycle by removal of the drug and addition of fresh cell culture medium, and cells were harvested at the time points indicated (see Materials and Methods). To obtain M-phase-enriched cells, Colcemid (100 ng/ml) was added in the last 4 h of a total 12-h postrelease time. For each time point, DNA content was determined by FACS analysis of propidium iodide-stained cells (upper panel). Cells in mitosis were determined by staining with propidium iodide and antibody to P-H3, and the percentage of the M-phase cells was determined by flow cytometry (lower panel). (B) Tim expression is cell cycle regulated. Protein lysates were prepared from synchronized NHF1 cells, and 200 μg of total protein was immunoblotted with anti-Tim, anti-cyclin B1, anti-Claspin, and antiactin antibodies. (C) Interaction of Chk1 with Tim is stimulated by replication arrest. HEK293T cells transfected with Flag-Tim were either left untreated or treated with UV and lysed 1 h later or were incubated with HU for 20 h before lysis. An equal amount of cell lysate (2 mg) was immunoprecipitated with anti-Flag agarose and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Input represents 1/30 of the whole-cell lysate used for immunoprecipitation (IP). Western blot analysis was done with anti-Flag and anti-Chk1 antibodies.

circadian regulation of expression (1), with the peak level occurring at zeitgeber time 12 (ZT12 = time of lights off under a 12-h light–12-h dark cycle). Therefore, we examined whether Tim also exhibited a cell cycle-dependent oscillation because of the presumed connection between the two physiological cycles. Towards this end we determined the levels of Tim protein by Western blotting as human diploid fibroblasts synchronously entered and passed through a cell division cycle (Fig. 2A). Tim expression was low in G_0 and G_1 phases and high in S, G_2, and M, with the highest level occurring in S phase (Fig. 2B). This cell cycle-regulated Tim expression provided further evidence that Tim might be a cell cycle protein with special roles in the S, G_2, and M phases of the cell cycle.

In a subsequent experiment, we examined the effects of replication stress and DNA damage on the Tim-Chk1 interaction. HEK293T cells were transfected with Flag-tagged Tim and were treated either with HU or with UV light. Tim was immunoprecipitated, and the immunoprecipitates were analyzed for endogenous Chk1. Both HU and UV treatment significantly stimulated the Tim-Chk1 interaction (Fig. 2C). This enhanced interaction between a clock protein (Tim) and a checkpoint protein (Chk1) following treatments which activate DNA damage checkpoint pathways suggests that Tim may play a role in the cell cycle checkpoint through regulation of Chk1. We tested for such a role in the following series of experiments.

Tim transduces the replication checkpoint signal from ATR to Chk1. It has previously been shown that Chk1 Ser 345 is phosphorylated in response to replication stress by HU and that this phosphorylation in response to HU treatment is carried out by ATR (14, 22). Therefore, we reasoned that Tim might be acting as a mediator between ATR and Chk1 and interacting with ATR itself. A number of ATR interactions are mediated through association with a small ATR subunit called ATRIP (6, 37). Thus, we tested the ATR-ATRIP-Tim interaction by coimmunoprecipitation. Figure 3A shows that Tim immunoprecipitates contain both ATRIP and ATR and that HU treatment significantly stimulates the Tim-ATRIP interaction. Taken together, these data support the notion that Tim is a mediator between ATR and Chk1 in checkpoint signaling.

To determine the functional consequences of the ATR-Tim-Chk1 interaction, we used siRNA to down-regulate Tim in HeLa cells and measured the HU-induced Chk1 phosphorylation on Ser 345 with anti-phospho-Chk1 antibodies (Ser345)

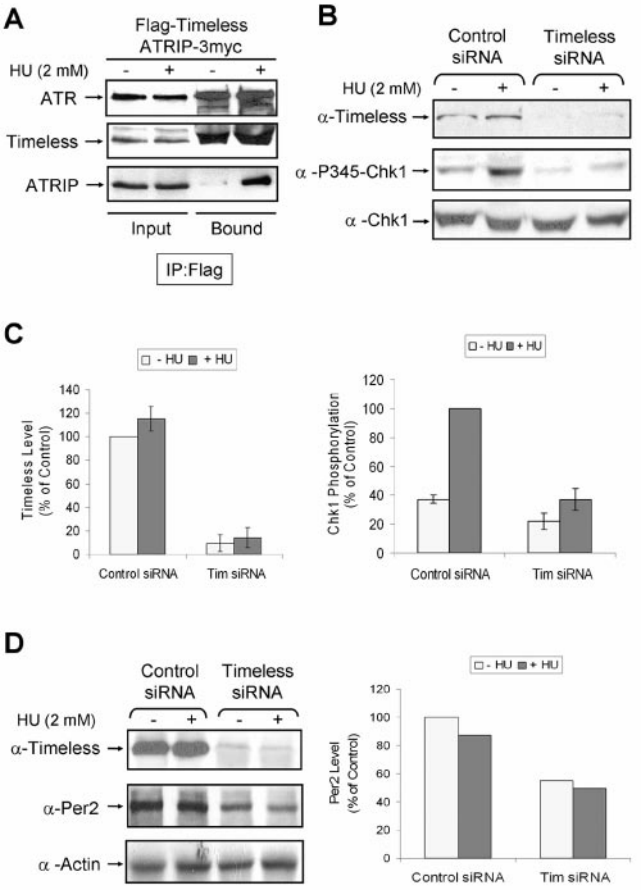

FIG. 3. Loss of Tim down-regulates Chk1 activation and the expression level of Per2 protein. (A) Interaction of Tim with ATR-ATRIP. HEK293T cells transfected with Flag-Tim and ATRIP-3myc were either left untreated or incubated for 20 h with HU before lysis. An equal amount of cell lysate (2 mg) was immunoprecipitated with anti-Flag agarose and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Input represents 1/30 of the whole-cell lysate used for immunoprecipitation (IP). Western blot analysis was done with anti-Flag, anti-Myc, and anti-ATR antibodies. We have reproducibly observed that HU increases the level of ectopically expressed ATRIP interacting with Tim without a corresponding increase in the level of Tim-associated ATR. This is most likely due to the excess of ATRIP that is not associated with ATR under this experimental setup. (B) Tim is required for Chk1 activation. HeLa cells were transfected with control or Tim siRNA two times over a 3-day period. Seventy-two hours after the initial transfection, cells were treated with 10 mM HU for 1.5 h or left untreated. Two hundred micrograms of cell lysates was immunoblotted with anti-Tim (α-Timeless), anti-P-Chk1 (Ser345), and anti-Chk1 antibodies. (C) The densities of Tim and the phosphorylated form of Chk1 bands were quantified, and data are expressed as percentages of the control sample (control siRNA, no HU treatment for Tim quantitation; control siRNA, HU treatment for Chk1 phosphorylation). Mean values and standard deviations were calculated from three independent experiments including the one for which results are shown in panel B. (D) Knockdown of Tim reduces the expression level of Per2 protein. HeLa cells were transfected with control or Tim siRNA as above. Exposure to Tim siRNA reduced the expression level of Per2 protein by at least 50% relative to the control siRNA-treated cells. Three hundred micrograms of cell lysates was immunoblotted with anti-Tim, anti-Per2, and antiactin antibodies. The density of the Per2 band was quantified, and data are expressed on the left as percentages of the control sample (control siRNA, no HU treatment).

(Fig. 3B). Down-regulation of Tim markedly reduces both basal and damage-induced Chk1 phosphorylation, suggesting that Tim interacts with Chk1 to mediate its activation by DNA damage (Fig. 3B and C).

It has been reported that conditional knockdown of Tim protein in the rat suprachiasmatic nucleus (SCN) caused a significant decrease in the level of other core clock proteins, namely Per1, Per2, and Per3, and disrupted circadian oscillation (1). HeLa cells under normal growth conditions do not exhibit an overt circadian rhythm. Nevertheless, it was of interest to ascertain if hTim plays a role in the expression of circadian proteins in our system since the role of mammalian Tim in circadian regulation has been controversial (1, 12). As seen in Fig. 3D knockdown of hTim by Tim siRNA causes a significant down-regulation of Per2, in agreement with the study by Barnes et al. (1) confirming that loss of Tim negatively impacts the level of other members of the core clock. Thus, Tim expression appears to be critical for the function of both a circadian cycle (Per2) and a cell cycle checkpoint (Chk1) protein.

Effect of Tim depletion on an HU-induced replication checkpoint. Chk1 Ser 345 phosphorylation by ATR is required for the G_2/M and replication checkpoints (14, 22). In light of our findings that Tim interacts with ATR-ATRIP and Chk1 and is required for the activation of Chk1 after DNA damage, along with previous findings for *C. elegans* that suggest that TIM-1 plays a role in the regulation of chromosome cohesion (5), we reasoned that Tim might have a role in the regulation of mitosis. To test for the role of Tim in mitotic regulation, we transfected HeLa cells with control and siRNA oligonucleotides against Tim and measured mitosis using Ser 10 phosphorylation of histone H3 as a marker. Flow cytometric analysis of control and Tim siRNA-transfected cells revealed that the total numbers of mitotic cells in the two groups were unchanged in the absence of damage (Fig. 4A). However, in the presence of HU, total levels of P-H3-positive cells were twofold greater after Tim down-regulation. Intriguingly, we observed cells with a sub-4N DNA content (presumably G_1- and S-phase cells) that noticeably exhibited P-H3 in both control and Tim down-regulated cells (Fig. 4A). A quantitative examination of these sub-4N cells revealed a reproducible 1.6- to 2-fold increase in Ser 10 phosphorylation in the absence of Tim protein. These data suggested that a reduced level of Tim may cause a defect in the replication checkpoint resulting in entry into mitosis before completion of DNA replication.

Inhibition of other proteins in the replication checkpoint pathway (ATR-Chk1) promotes premature chromatin condensation (PCC) when combined with DNA damage (27). Therefore, we tested whether Tim participates in the ATR-Chk1-dependent replication checkpoint. HeLa cells were transfected with Tim or control siRNA and treated with HU, and mitotic spreads were examined to determine whether fully functional Tim, like ATR and Chk1, was necessary for the replication checkpoint and prevention of PCC (Fig. 4B). Mitotic spreads with fragmented and disintegrated chromosomes were counted as cells undergoing PCC. We found that transfection with Tim siRNA, but not with control siRNA, increased the HU-induced PCC (Fig. 4B), indicating that the increase in sub-4N cells detected by flow cytometry in Tim siRNA-treated cells following HU treatment was due to PCC. Interestingly, there

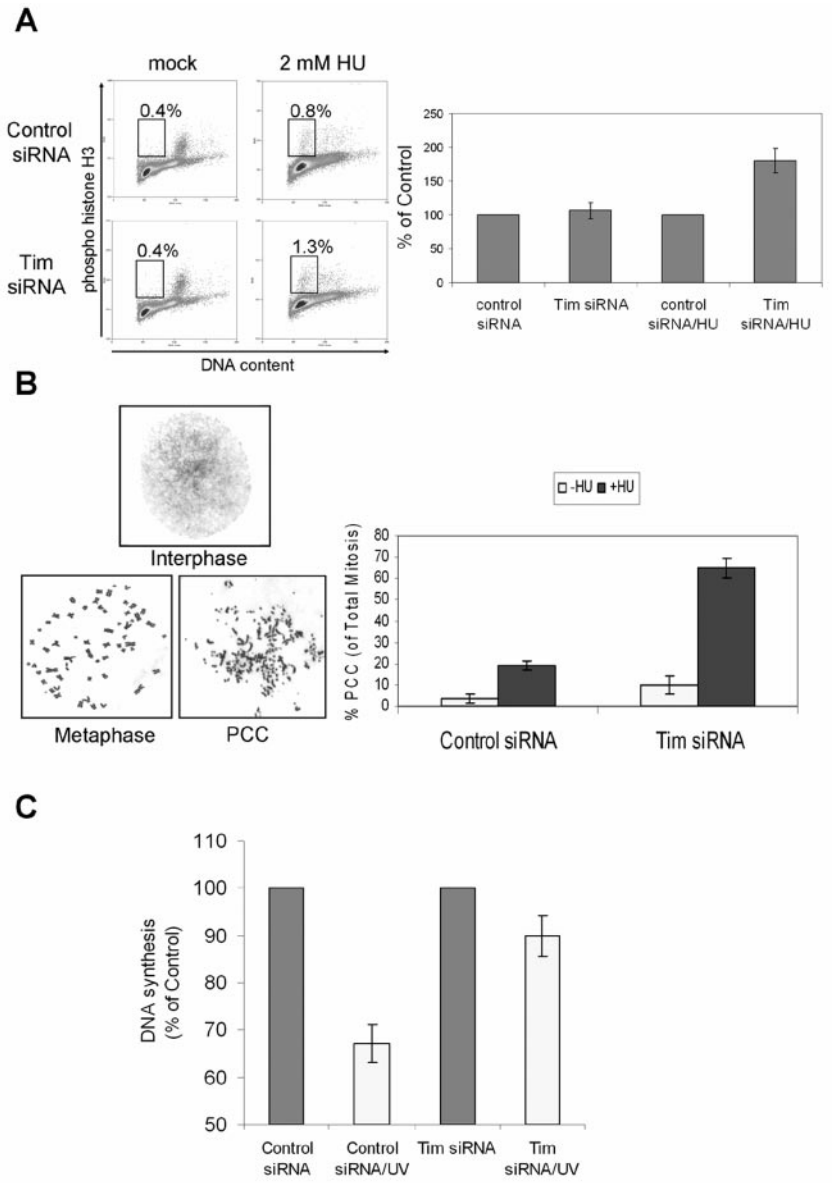

FIG. 4. (A) Tim prevents PCC. Analysis of PCC by FACS. HeLa cells were transfected with control or Tim siRNA and then either mock treated or treated with HU (2 mM) for 20 h. Cells in mitosis were determined by staining with propidium iodide and antibody to P-H3, and the percentage of the P-H3 reactivity in S-phase cells by flow cytometry was considered as PCC. One representative of three experiments is shown. Data are expressed as percentages of the control samples (control siRNA) and plotted as the means ± standard deviations. Quantitation of the data represents the averages of three independent experiments. (B) Tim prevents PCC after replication stress. HeLa cells were transfected with control or Tim siRNA and then either treated with HU (2 mM) for 20 h or left untreated. To obtain M-phase-enriched cells, cells were additionally treated with Colcemid in the last 4 h. Mitotic spreads were prepared, and cells that had characteristic features of either a normal mitosis or PCC were determined by fluorescence microscopy. Interphase cells and cells that were intermediate in morphology between normal and PCC were not counted. The three frames on the left show three characteristic DNA-staining patterns. For quantitative analysis approximately 100 mitotic cells were counted per condition. The values represent the means of three independent experiments, and the error bars indicate standard deviations. (C) Tim inhibition causes RDS. HeLa cells transfected with control or Tim siRNA were grown in the presence of [^{14}C]thymidine for 40 h to label DNA uniformly until the second transfection and then grown in nonradioactive medium for an additional 24 h. Cells were exposed to UV (2 J/m^2) or left untreated, incubated at 37°C for 30 min, and then labeled for 15 min in medium containing [^{3}H]thymidine. Relative DNA synthesis was estimated from the incorporated [^{3}H]thymidine normalized to total DNA by the ^{14}C radioactivity. Data are expressed as percentages of the control samples (no UV irradiation) and plotted as means ± standard deviations. The data represent the averages of three independent experiments.

was also a modest increase of PCC in cells transfected with Tim siRNA without HU treatment, indicating that PCC may occur in untreated control cells in the absence of Tim, consistent with an essential role of Tim in the replication checkpoint even under physiological conditions.

Tim depletion confers an RDS phenotype. Finally, we examined the role of Tim in the intra-S checkpoint. In the intra-S checkpoint, stalled replication forks after deoxynucleoside triphosphate pool depletion or DNA damage activate a signal transduction pathway that inhibits firing of new origins of rep-

lication, ultimately resulting in overall inhibition of DNA synthesis (30, 35, 41). Elimination of the intra-S checkpoint causes continuous firing of replication origins in the presence of replication blocks and results in unabated DNA synthesis, which is, for historical reasons, referred to as radioresistant DNA synthesis (RDS). In Fig. 4C, we show that down-regulation of Tim seriously compromised the intra-S checkpoint, as revealed by RDS after UV treatment. Thus, in addition to its reported role in the core clock machinery (1), Tim appears to be a direct participant in the cell cycle checkpoints, regulating the intra-S and replication checkpoints, which might explain why Tim null mutation causes embryonic lethality (12).

DISCUSSION

Tim as a cell cycle checkpoint protein. Even though the mammalian Tim protein was first discovered as the mammalian homolog of the *Drosophila* circadian clock protein dTim (18, 36, 42), the closest phylogenetic relatives of the mammalian Tim protein are actually cell cycle-related proteins: budding yeast Tof1 (9, 32), fission yeast Swi1 (29), and *C. elegans* TIM-1 (5). In addition, a distant relative of the *Drosophila* dTim clock protein called dTim2/dTimeout (2, 12) exhibits higher homology to mammalian Tim than dTim itself, although currently it is unknown whether Tim2 is a checkpoint and/or clock protein. The data we present in this paper strongly indicate that the human Tim protein, like its yeast orthologues, plays an important role in the cell cycle checkpoint response and are in agreement with the prediction made by Gotter (11) that hTim and its low-molecular-weight partner TIPIN may connect the circadian clock to the cell cycle checkpoints. Regarding the checkpoint function of hTim, our findings indicate that hTim is required for the phosphorylation and activation of Chk1 by ATR (14, 22), and therefore it may function as an adaptor/mediator between damage sensors and signal transducers for checkpoint activation. However, its functional homolog in fission yeast, Swi1, plays an important role in stabilization of stalled replication forks (29), suggesting that both Swi1 and its human homolog, Tim, may act as damage sensors as well. It is likely that these apparently disparate functions, as well as the role of TIM-1 in sister chromatid cohesion (5), reflect different facets of a common biochemical pathway. Further genetic and biochemical studies are required to understand the function of hTim and its homologs in mechanistic terms in various organisms.

Tim as a circadian clock protein. Following the discovery of mammalian Tim as a putative circadian clock protein (36, 42) that interacts with the core clock proteins mCry1 and mCry2 in the mouse (13, 20), several reports were published suggesting that the mammalian Tim is not a clock protein. First, as mentioned above, the mammalian Tim has a higher sequence homology to a *Drosophila* protein of unknown function, called dTim2/dTimeout, than to the clock protein dTim (2, 12). dTim2 appears to be highly expressed during larval stages, suggesting a developmental function (2). In support of such a role in mammals, Tim has been implicated in branching morphogenesis of embryonic kidney (21) and lung (39). Second, it was reported that mammalian Tim, despite being expressed in the master circadian clock of the mouse (SCN), in contrast to its presumptive *Drosophila* ortholog dTim, did not exhibit circadian oscillation (15). Finally, mammalian Tim knockout caused embryonic lethality (12), a phenomenon not associated with the knockout of any of the so-called "canonical clock genes" (the Clock, BMal1, Cry1, Cry2, Per1, and Per2 genes). Of equal significance, mammalian Tim mutant heterozygotes did not exhibit a circadian phenotype despite expressing significantly reduced levels of mammalian Tim protein (12).

In contrast to the reports summarized above suggesting that Tim is not a clock protein in mammals, a recent study presented compelling evidence that mammalian Tim is a circadian state variable that is essential for robust molecular and electrochemical circadian rhythm in the SCN (1). The discrepancy between this and the previous studies appears to be due, in part, to the presence of two splicing forms of mammalian Tim in the SCN and other tissues. The full-length Tim exhibits circadian oscillation in the SCN and interacts with other clock proteins, whereas the more abundant shorter form does not oscillate and apparently has no clock function. Importantly, down-regulation of full-length mammalian Tim in SCN slices drastically affects the expression levels of Cry1, Cry2, Per1, Per2, and Per3 and abolishes electrochemical oscillations of SCN output. Similarly, down-regulation of full-length Tim in HEK293 cells affects the expression of the clock protein Per2 (1). Indeed, our own experiment with HeLa cells showing that down-regulation of Tim decreases Per2 expression is consistent with similar results reported by Barnes et al. (1) and with a clock function of Tim in mammalian cells. Clearly, further work is required to reconcile the contrasting views and findings on the role of Tim in the mammalian circadian clock. It should be noted, however, that the essential role of Tim in development should not necessarily exclude it as a "canonical" clock protein because the definition of canonical in the circadian field is currently in flux.

Coupling of cell cycle and circadian cycle. Circadian and cell cycles are two global regulatory mechanisms that affect all aspects of cellular physiology. Therefore, it is to be expected that these two regulatory pathways would exhibit some overlap. It has been noted that in organisms ranging from *Chlamydomonas* (28) to zebra fish (7) to humans (3) the circadian rhythm affects the phasing of the cell cycle. Two recent reports showed that mammalian fibroblasts exhibit cell-autonomous and self-sustained circadian clocks in culture (25, 38). Moreover, it was found that the circadian clock gates cytokinesis to a defined circadian time and mitosis elicits a phase shift in circadian cycle (25), further evidence for the intimate relation between these two global regulatory systems. The mechanistic details of these coupling mechanisms are not known at present. However, conceptually there are two possible mechanisms of coupling the two cycles (24) (Fig. 5). In one mechanism, which might be called serial coupling (or a "two-process model"), the circadian clock machinery changes the threshold for propensity of occurrence of certain reactions with a periodicity of about 24 h and thus locks in the cell cycle with the circadian cycle period and phase. The regulation of cell growth (c-*myc*) (10) and cell cycle (*Wee1*) (23, 31) genes as output clock-controlled genes and the inhibition of circadian gene transcription during mitosis (25) might be considered examples of serial coupling (Fig. 5, top). In the second model, which might be called "direct coupling" (or parallel coupling), the two cycles share a key protein whose expression exhibits a circadian pattern (Fig.

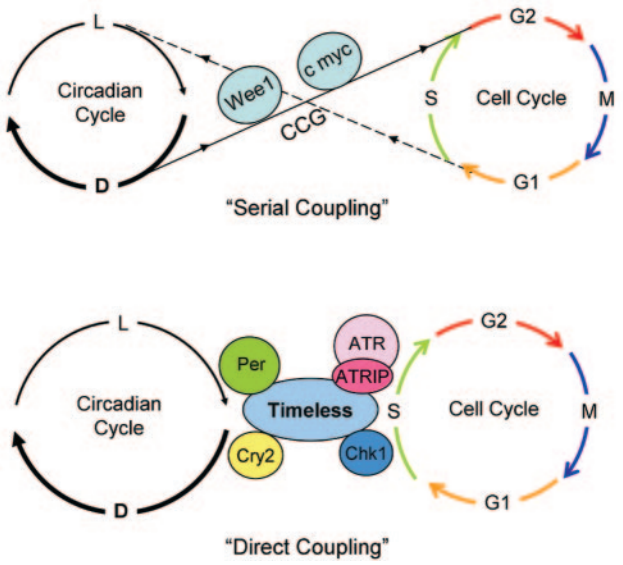

FIG. 5. Two models for coupling of circadian and cell cycles. (Top) Serial coupling. In this mode of synchronization of the two cycles, proteins or reactions belonging primarily to one cycle regulate the expression of genes controlling the other cycles. The circadian clock controls the cell cycle because, in part, it regulates the expression of *Wee1* and c-*myc* cell cycle and proliferation genes. Conversely, the cell cycle may control the circadian cycle by shutting down cellular transcription activity during mitosis (broken lines), regardless of when mitosis takes place with regard to the phase of the circadian cycle, and as a consequence may shift the phase of the circadian cycle. Disruption of one cycle may or may not significantly affect the functioning of the other cycle depending on the strength of coupling and those of homeostatic mechanisms specific for the individual cycles. (Bottom) Direct coupling. In this mode a protein such as Tim directly participates in the molecular machineries of both cycles, and as a consequence elimination of such protein may result in collapse of both cycles. Note that the model for direct coupling is drawn to show the protein-protein interactions of Tim in carrying out its checkpoint and putative clock functions and does not imply a defined biochemical pathway or the presence of a supramolecular complex of clock-checkpoint proteins participating in the two cycles simultaneously or alternately.

5, bottom). Assuming that Tim is a circadian protein, our data could be considered as evidence for the second mechanism: direct coupling of the two cyclic processes by a protein (Tim) essential to both circadian and cell cycle rhythms. Despite the compelling evidence of the coupling of the two cycles, however, it must be noted that the circadian cycle operates normally in the absence of the cell cycle, such as the circadian cycling of nondividing neural, muscle, and liver cells (33, 40). Similarly, there is currently no strong evidence that the canonical clock proteins Clock, BMal1, Cry1, Cry2, Per1, and Per2 are required for cell cycle checkpoints because mice mutated in the corresponding genes by and large exhibit normal growth and have no overt defects in cell cycle checkpoints, perhaps because homeostatic control of the cell cycle compensates for the loss of input from the circadian clock. In contrast, mutations in Tim, and perhaps yet-to-be identified essential proteins with clock and cell cycle functions, would inevitably disrupt both the circadian clock and the cell cycle checkpoint, as we have documented in this study.

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