TIMELESS Suppresses the Accumulation of Aberrant CDC45-MCM2-7-GINS Replicative Helicase Complexes on Human Chromatin*

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The replication licensing factor CDC6 recruits the MCM2-7 replicative helicase to the replication origin, where MCM2-7 is activated to initiate DNA replication. MCM2-7 is activated by both the CDC7-Dbf4 kinase and cyclin-dependent kinase and via interactions with CDC45 and go-ichi-ni-san complex (GINS) to form the CDC45-MCM2-7-GINS (CMG) helicase complex. TIMELESS (TIM) is important for the subsequent coupling of CMG activity to DNA polymerases for efficient DNA synthesis. However, the mechanism by which TIM regulates CMG activity for proper replication fork progression remains unclear. Here we show that TIM interacts with MCM2-7 prior to the initiation of DNA replication. TIM depletion in various human cell lines results in the accumulation of aberrant CMG helicase complexes on chromatin. Importantly, the presence of these abnormal CMG helicase complexes is not restricted to cells undergoing DNA synthesis. Furthermore, even though these aberrant CMG complexes interact with the DNA polymerases on human chromatin, these complexes are not phosphorylated properly by cyclin-dependent kinase/CDC7-Dbf4 kinase and exhibit reduced DNA unwinding activity. This phenomenon coincides with a significant accumulation of the p27 and p21 replication inhibitors, reduced chromatin association of CDC6 and cyclin E, and a delay in S phase entry. Our results provide the first evidence that TIM is required for the correct chromatin association of the CMG complex to allow efficient DNA replication.

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The abbreviations used are: ssDNA, single-stranded DNA; GINS, go-ichi-ni-san complex; DDK, CDC7-Dbf4 kinase; CDK, cyclin-dependent kinase; TIM, TIMELESS; TIPIN, TIM-interacting protein; CMG, CDC45-MCM2-7-GINS complex; PG, phosphorylation-generated; ATR, ataxia telangiectasia- and Rad3-related protein; APC/C, anaphase-promoting complex/cyclosome; Rb, retinoblastoma protein; ORC, origin recognition complex; APH, aphidicolin; WCE, whole-cell extract(s); RNAPII, RNA polymerase II; IP, immunopurification.

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TIM-TIPIN in cohesion establishment is consistent with the discovery of Csm3 and Tof1 mutations in genetic screens for chromosome segregation defects (14, 25).

Here we report a novel function of human TIM for the correct association of the CMG complex on chromatin. We found that TIM-TIPIN interacts with MCM2-7 not only during S phase but also throughout the whole cell cycle. Human cell lines treated with TIM siRNAs contain elevated amounts of the p21 and p27 replication inhibitors, and this phenotype coincides with a delay in S phase entry and decreased association of CDC6 and cyclin E with chromatin. As a consequence, there is reduced recruitment of MCM2-7 to the active replication origin. Unexpectedly, despite the inefficient recruitment of MCM2-7 to the active replication origin during G1 phase in TIM-deficient cells, the levels of chromatin-bound CMG complexes remain unchanged, and the presence of these CMG complexes on the chromatin is no longer restricted to S phase. Although these CMG complexes interact with DNA polymerases, the MCM4 subunit has an altered phosphorylation pattern at the DDK- and CDK-dependent PG sites, which are important for efficient DNA replication (26, 27). Our data unveil a novel role for TIM in preventing the accumulation of aberrant CMG complexes on the chromatin outside of S phase. We propose that the presence of these non-S phase CMG complexes with altered post-translational modifications acts as a false negative feedback signal to prevent CDC6 and cyclin E from binding to DNA, thereby hindering DNA replication in TIM-deficient cells.

Results

TIM Deficiency Leads to Inefficient S Phase Entry—Mammalian TIM is a component of the replication fork progression complex and is required for the efficient progression of replication forks during S phase (21, 22, 28). In addition, TIM promotes the sister chromatid cohesion necessary for proper chromosomal segregation during mitosis (23, 24). Reduced levels of cohesin complexes during early G1 phase can also lead to slow replication progression and can lengthen S phase by limiting the number of replication origins that fire (29). It is therefore expected that TIM deficiency would lead to the accumulation of S phase cells. To test this, we depleted TIM using two separate siRNAs in HEK293 cells (Fig. 1A, left panel) and human osteosarcoma U2OS cells (Fig. 1B, left panel). Consistent with a published report (11), we observed a destabilization of TIPIN in TIM knockdown cells using either of the TIM siRNAs (Fig. 1, A and B, left panels, second blots). The percentage of the asynchronous cell population undergoing DNA synthesis was monitored by measuring DNA content (Fig. 1, A and B, right panels, x axis) and BrdU incorporation by pulse-labeling the cells with BrdU prior to analysis (Fig. 1, A and B, right panels, y axis). Unexpectedly, BrdU incorporation analysis of the TIM siRNA-treated HEK293 (Fig. 1A, right panel) and U2OS (Fig. 1B, right panel) cells showed that the S phase population was reduced compared with the control cells.

An accelerated replication fork progression rate could lead to a shorter S phase and, hence, a reduction in the S phase population. Alternatively, a decrease in the number of cells undergoing DNA synthesis may result from inefficient S phase entry. To test these possibilities, we evaluated the cell cycle progression of HEK293 cells that were transfected with either TIM siRNAs for 48 h and then synchronized to G1/M phase by nocodazole treatment for an additional 20 h. After nocodazole release, cell cycle progression was monitored by flow cytometry. Within the first 4–6 h after release, the number of G1 phase cells increased in both the control and TIM siRNA knockdown cultures (Fig. 1C, first and second columns). 12 h after nocodazole release, the majority of the control knockdown cells showed positive BrdU staining (Fig. 1C, fourth column). However, the initiation of DNA synthesis was delayed at 12 h after nocodazole release in the majority of TIM-depleted cells that had completed mitosis and entered G1 phase. Overall, these cell cycle analyses revealed an unexpected defect in S phase entry caused by TIM depletion.

High Levels of the Replication Inhibitors p21 and p27 Are Present in TIM-depleted Cells without Evidence of DNA Damage—Consistent with the observation of a reduced S phase population, elevated levels of p21 and p27 were present in TIM knockdown HEK293 and U2OS cells compared with control cells (Fig. 1, A and B, left panels, third and fourth blots). Expression of the SV40 large T antigen has been reported to stimulate the initiation of host DNA replication and reduce the expression levels of p21 and p27 (30, 31). However, HEK293T cells, which contain the SV40 large T antigen, also accumulated p21 and p27 when TIM was depleted (Fig. 2A, third and fourth panels). p21 and p27 block DNA replication by inactivating CDK2-cyclin E, which is important for G1/S phase transition (32). For cells to enter S phase, p21 and p27 must be ubiquitinated and degraded during late G1 phase to allow the activation of CDK2-cyclin E for replication initiation. Therefore, we asked whether the delay in S phase entry in TIM knockdown cells was associated with the persistent presence of p21 and p27. Indeed, when we synchronized HEK293T cells with nocodazole to various cell cycle stages (Fig. 2B), we found that, unlike in control knockdown cells, both p21 and p27 failed to be degraded in TIM-depleted cells 11 h after nocodazole release, when most of the control knockdown cells were in S phase (Fig. 2, B and C). TIM is important for replication fork stability and efficient DNA replication progression (21, 22). Therefore, we tested the possibility that the delay in S phase entry observed in TIM-depleted G1 cells was due to the presence of unreplicated or damaged DNA inherited from the previous cell cycle. Because DNA damage activates the ATR-CHK1-dependent checkpoint response during G1 phase (33, 34), we analyzed the level of p-CHK1 in TIM knockdown cells. Consistent with a previous report that TIM facilitates efficient CHK1 activation (35), we found that UV irradiation induced CHK1 phosphorylation in both control and TIM knockdown cells. However, the amount of UV-induced p-CHK1 was reduced in TIM knockdown cells (Fig. 2D). As anticipated, TIM depletion alone did not activate CHK1 in the absence of UV treatment (Fig. 2D).

To further confirm that TIM depletion does not stimulate DNA damage, we asked whether ssDNA accumulates during G1 phase. This ssDNA accumulation can occur because of either unreplicated or unresolved damaged DNA inherited from the previous cell cycle in TIM-depleted cells. To test this hypothesis, we carried out immunofluorescence microscopy...
using an anti-BrdU antibody to detect ssDNA foci formed under non-denaturing conditions in cells precultured with BrdU for 18 h. Under non-denaturing conditions, only the BrdU at ssDNA regions is recognized by the anti-BrdU antibody. ssDNA foci were normally visible only in cells synchronized to S phase (13 h) but not in those synchronized to G1 phase (6 h; Fig. 2, E–G). Even though ssDNA-positive cells were also observed during G1 phase when TIM was depleted (Fig. 2H), the number of ssDNA-positive G1 cells remained low (<7%). These results indicate that the inefficient S phase entry seen in the majority of the TIM-depleted cells (Fig. 1C) is not due to the presence of unreplicated or damaged DNA. In addition, even though at 13 h post nocodazole release ssDNA foci were detected in TIM-depleted cells, the number of ssDNA-
positive cells (Fig. 2E) and the number of ssDNA foci per cell were reduced in these cells compared with control knockdown cells (Fig. 2, compare I with G). Together, these results are consistent with reduced efficiency of DNA replication initiation rather than DNA damage.

Reduced Chromatin Association of CDC6 and Cyclin E in TIM-depleted Cells—We further determined the cause for the persistent presence of p21 and p27 in TIM-depleted cells. The degradation of p21/p27 during late G1 phase is mediated by SCF^SKP2^-dependent ubiquitination (36). We found that the SKP2 protein level was reduced in HEK293T, U2OS, and HT1080 cells (Fig. 2J). This reduction is most likely due to the fact that even though SKP2 was visible during M phase, it failed to accumulate during G1 and S phases in TIM knockdown cells (Fig. 2C, fourth panel). APC/C is known to degrade SKP2 (37). However, the level of cyclin A, which is an APC/C substrate (38), remained similar to the level in control cells (Fig. 2C, fifth panel). Therefore, the low level of SKP2 in the absence of TIM is unlikely to result from increased APC/C activity.

In addition to APC/C-dependent degradation of SKP2, the cellular level of SKP2 is also controlled by E2F-dependent transcription (39–41). Prior to replication initiation, E2F activity is...
suppressed by the binding of Rb at promoters (42–45). During late G1 phase, cyclin E-CDK2 is recruited to the chromatin to phosphorylate Rb and disrupt the Rb-E2F interaction at promoters, allowing the activation of E2F-dependent transcription (42, 46). Therefore, we analyzed the levels of chromatin-bound cyclin E and found that the amount of cyclin E on the chromatin was substantially reduced in TIM knockdown cells (Fig. 3A).

Furthermore, the level of chromatin-bound CDC6, which is required for chromatin recruitment and the activation of cyclin E-CDK2 activity (47–49), was also reduced in TIM knockdown cells (Fig. 3A).

The chromatin-bound CDC6 is necessary for the recruitment of MCM2-7 to the active origins during G1 phase and the subsequent interaction with CDC45 and GINS to form the CMG complex and initiate DNA replication (4, 6, 7). Therefore, in CDC6 siRNA knockdown cells, chromatin recruitment of the components of the CMG complex, such as SLD5 and MCM7, was reduced (Fig. 3B). Because the amount of chromatin-bound CDC6 was reduced in TIM-depleted cells, we expected that the recruitment of MCM2-7 helicase to the active origin would be compromised in TIM knockdown cells. Indeed, the amount of MCM5 bound to the active lamin B2 origin in HEK293 cells (D) and HEK293T cells (E) 6 h after nocodazole release, when cells were predominantly in G1 phase, was reduced in TIM knockdown cells compared with control knockdown cells synchronized to G1 phase (Fig. 3, C–E).
CMG Helicase Complexes Accumulate on Human Chromatin in Non-S Phase, TIM-depleted Cells—Surprisingly, even though the levels of the chromatin-bound CDC6 and the origin-bound MCM2-7 in TIM knockdown cells were reduced, we found that components of the CMG complex, including the GINS (SLD5 and PSF2) and CDC45, were still found on the chromatin (Fig. 3, A, fifth through seventh panels, and B). To further determine whether these chromatin-bound GINS and CDC45 proteins were part of CMG complexes, we purified the CMG complexes using FLAG-CDC45 from U2OS (Fig. 4A) and HEK293 (Fig. 4B) cells that were treated with either control or TIM siRNA. Unexpectedly, the protein–protein interactions among the components of the CMG complex in TIM knockdown cells were still detected (Fig. 4, A and B, compare the first and second columns). Given that CDC6 is required to establish the pre-replication complexes and subsequently assemble the CMG complexes in normal cells (5), we tested whether depleting CDC6 in TIM knockdown cells would eliminate these CMG complexes. When the cells were treated with CDC6 siRNA for 60 h, we found that all of the CMG complexes were abolished in these cells (Fig. 4, A and B, compare the first and third columns). However, depletion of CDC6 in TIM knockdown cells (e.g., TIM-CDC6 double knockdown) failed to eliminate the chromatin-bound CMG complexes (Fig. 4, A and B, compare the third and fourth columns). To exclude the possibility that the chromatin-bound CMG complexes in the CDC6-TIM double knockdown cells were due to an increase in the S phase population compared with CDC6-deficient cells, we analyzed the cell cycle distribution of HEK293 knockdown cells by flow cytometry. We found that the S phase population was decreased in TIM, CDC6, and TIM-CDC6 knockdown cells compared with control cells (Fig. 4C). In addition, even though CMG complexes were formed in TIM-CDC6 knockdown cells, these cells still showed severely disrupted cell cycle distributions similar to CDC6 knockdown cells (Fig. 4C). To further confirm that the accumulation of these CMG complexes on the chromatin were caused by TIM deficiency, we exogenously expressed an siRNA-resistant TIM construct in CDC6-TIM double knockdown cells. We found that the exogenously expressed TIM proteins stabilized endogenous TIPIN (Fig. 4B, second panel, right lane) and suppressed the CMG complexes (Fig. 4B, bottom four panels, compare the fifth and sixth lanes).

Because the accumulation of CMG complexes in TIM knockdown cells cannot be eliminated by CDC6 depletion, we...
next asked whether the chromatin association of these CMG complexes still takes place only in S phase. When we purified chromatin-bound FLAG-CDC45 from control knockdown HEK293T cells synchronized to either early G1 (4 h) or S phase (12 h) after nocodazole release (Fig. 5A), we found that, as expected, MCM2-7 and GINS were co-purified with CDC45 during S phase but not during early G1 phase (Fig. 5B). However, CMG complexes were detected in an abundant amount in both G1 phase and S phase cells, when TIM was depleted (Fig. 5, A and B), suggesting that the presence of CMG is no longer restricted to S phase when TIM is absent. Previously, we showed that human RECQ4 DNA helicase forms a chromatin-specific complex with MCM10, MCM2-7, CDC45, GINS, and TIM-TIPIN during S phase to promote efficient DNA replication initiation (51). Here we found that the interaction of RECQ4 and MCM10 with CMG also failed to be eliminated by CDC6 depletion in TIM knockdown cells (Figs. 4B and 5, C and D). Importantly, in control knockdown cells, RECQ4 only interacted with CMG in cells harvested 11 h after nocodazole release, when a substantial portion of the cells were in S phase (Fig. 5, E, bottom panel, and F, fifth lane). However, in TIM knockdown cells, we found that an increasing amount of CMG was co-purified with FLAG-RECQ4 from the human chromatin as early as 2 h after nocodazole release, when cells were undergoing mitosis (Fig. 5, E, top panel, and F, second lane). Together, these results provide evidence that, in TIM knockdown cells, the CMG complexes are present on the chromatin even when they are not undergoing DNA synthesis.

The CMG Complexes in TIM-depleted Cells Exhibit Altered Patterns of MCM4 Phosphorylation—Despite the increased amount of the RECQ4-CMG helicase complex (Fig. 5F), TIM knockdown cells showed inefficient DNA synthesis. We hypothesized that the RECQ4-CMG replicative helicase complexes that accumulate in TIM knockdown cells could be catalytically defective. Indeed, when the helicase activity of equal amounts of CMG-containing FLAG-RECQ4 helicase complex purified from the TIM knockdown and control cells was compared (Fig. 5G), the amount of single-stranded dissociation product in the presence of the TIM knockdown RECQ4-CMG complex was significantly reduced compared with that of the control cell complex (Fig. 5H).

We next wished to determine the cause of the decreased catalytic activity of the CMG-containing helicase complex in TIM knockdown cells. It has been established that both CDK and DDK are required for the assembly and activation of the CMG complex at active replication origins and that MCM4 is a direct target of DDK and CDK during replication initiation (27, 52–55). Indeed, chromatin-bound MCM4 was present in multiple modified forms that exhibited slower mobility on SDS-PAGE, and the presence of this modified MCM4 protein was abolished with treatment with inhibitors of either the CDC7 component of DDK or CDK (Fig. 6A). Given that, in the absence of TIM, the interactions of CDC45 and GINS with the MCM2-7 helicase are no longer restricted to S phase (Fig. 5, B and F), we next determined whether the CMG complexes that formed in TIM knockdown cells contained the expected post-translational modifications. First, we measured the levels of phosphorylated MCM4 by SDS-PAGE mobility shift in the purified chromatin-bound FLAG-MCM7 complex. We found a reduction in phosphorylated MCM4 when TIM was depleted (Fig. 6B, fourth panel). We used an antibody against phosphorylated Ser/Thr residues to further evaluate the presence of phosphorylated MCM4. Consistent with the observed change in MCM4 mobility, we also observed reduced levels of phosphorylated polypeptides with SDS-PAGE mobility between 100 and 150 kDa, where phosphorylated MCM4 was found in these purified complexes (Fig. 6B, fifth panel).

We next tested whether the altered phosphorylation pattern of the chromatin-bound MCM4 is due to the accumulation of stalled replication forks. It has been shown that replication fork arrest by hydroxyurea or APH leads to ATR-induced MCM4 hyperphosphorylation as part of the replication block checkpoint response (56). Indeed, we observed an increase in hyperphosphorylated MCM4 in APH-treated cells (Fig. 6C, compare the first and third lanes). However, this APH-induced hyperphosphorylation of MCM4 is distinct from the MCM4 phosphorylation pattern found in TIM knockdown cells (Fig. 5C, compare the second and third lanes). This APH-induced MCM4 hyperphosphorylation is not dependent on TIM, as this phenomenon was also observed in TIM knockdown cells treated with APH (Fig. 6C, fourth lane). This result indicates that the reduced phosphorylation of MCM4 on chromatin in the absence of TIM is not likely to be a consequence of replication fork arrest.

In yeast, multiple DDK-dependent phosphorylation sites, which are important for DNA replication, have been identified within the N terminus of MCM4 (27). In humans, the MCM4 N terminus contains three intrinsic DDK target sites (Ser or Thr followed by Asp or Glu (Ser/Thr-Asp/Glu)), and they are Ser26, Ser131, and Ser141 (Fig. 6D, underlined residues). We found that a phosphomimetic mutant containing Ser-to-Glu mutations at the three intrinsic DDK sites (Fig. 6E, 3 E-D/E) failed to rescue the alternation of MCM4 phosphorylation levels in TIM knockdown cells (Fig. 6E, compare the four left lanes), suggesting that the phosphorylation of these intrinsic DDK target sites is not affected by TIM. In addition to the intrinsic DDK target sites, DDK can also target the first Ser/Thr of the MCM4 PG sites (phosphorylated Ser/Thr-Ser/Thr-Pro) when the second Ser/Thr is already phosphorylated by CDK (26, 27, 57). Human MCM4 contains six PG sites within the N terminus: Ser2, Ser6, Ser31, Thr53, Ser70 and Ser87 (Fig. 6C, residues in bold). We found that phosphomimetic mutations at these PG sites (e.g., Fig. 6E, 6E-EP) or phosphodefective mutations (e.g., Fig. 6F, 6A-AP) eliminated the difference in MCM4 mobility on SDS-PAGE between control and TIM knockdown cells, suggesting that the chromatin-bound MCM2-7 helicase in the absence of TIM may not contain properly phosphorylated PG sites.

The PG phosphorylation events on MCM4, which are necessary for CMG assembly and DNA synthesis, are tightly regulated during the cell cycle (26, 27). Restricting CDC45 and GINS to only interacting with the PG-phosphorylated MCM2-7 helicase ensures the correct timing of CMG activation and replication initiation in normal cells. Indeed, CDC45 normally interacts only with the MCM2-7 helicase, which contains hyperphosphorylated MCM4. This hyperphosphorylated MCM4 can be converted to unphosphorylated MCM4 by a phosphatase, as demonstrated by FLAG-CDC45 immunopre-
Aberrant CMG helicase complexes accumulate on the chromatin of non-S phase cells depleted with TIM.

A, flow cytometry of control cells (left panel) and TIM knockdown HEK293T cells (right panel) stably expressing FLAG-CDC45 at different time points after release from nocodazole block.

B, Western blotting analysis of FLAG-CDC45 complexes immunopurified from the soluble chromatin fractions of control and TIM knockdown cells synchronized at different cell cycle stages, as described in A, to detect the presence of CDC45 (FLAG), TIM, MCM2-7 (MCM5 and MCM7), and GINS (SLD5 and PSF2).

Noc, nocodazole.

C, the protein levels of TIM, CDC6, and SLD5 were analyzed by Western blotting using WCE prepared from control, TIM knockdown, CDC6 knockdown, and TIM-CDC6 double knockdown HEK293T cells expressing FLAG-RECQ4.

D, Western blotting analysis of FLAG-RECQ4 complexes immunopurified from the soluble chromatin fractions of control and TIM knockdown cells described in C using antibodies against FLAG, MCM10, MCM7, CDC45, and SLD5.

E, flow cytometry of control cells (left panel) and TIM knockdown cells (right panel) stably expressing FLAG-RECQ4 at multiple time points after release from nocodazole block.

F, Western blotting analysis of FLAG-RECQ4 complexes immunopurified from the soluble chromatin fractions of control and TIM knockdown cells synchronized at various cell cycle stages, as described in E, to detect the presence of RECQ4 (FLAG), TIPIN, MCM10, MCM7, MCM2-7 (MCM7), CDC45 and GINS (SLD5 and PSF2).

G, Western blotting analysis of FLAG-RECQ4 complexes immunopurified from the chromatin fractions prepared from control cells and TIM siRNA knockdown cells using anti-FLAG antibody.

H, helicase activity of equal amounts of FLAG-RECQ4 complex purified from control cells or TIM knockdown HEK293T cells assayed using 32P-labeled splayed-arm substrates. The 32P-labeled ssDNA products were visualized by autoradiography following neutral PAGE.

All data are representative of a minimum of three independent experiments. All Western blots in each subfigure were from the same lysate or experiment. 2C and 4C represents cell containing one or two copies of each chromosome, respectively.
precipitation (Fig. 6G, compare the first and third lanes). Because there is an excessive amount of inactive MCM2-7 protein on the chromatin (1, 2), the majority of the chromatin-bound MCM2-7, which was purified using FLAG-MCM7, was not hyperphosphorylated (Fig. 6G, second lane). Hence, the CDC45-associated, hyperphosphorylated MCM2-7 was a minor species of the total MCM2-7 helicases (Fig. 6G, compare the first and second lanes). When we analyzed the phosphorylation states of the CMG complexes purified from HEK293T, U2OS, and HEK293 cells stably expressing FLAG-CDC45, we found that the complexes purified from TIM knockdown cells contained differently modified forms of MCM4 with faster
The Interaction of TIM with MCM2-7 Is Independent of the Cell Cycle—The ability of TIM to prevent the accumulation of the aberrant CMG complexes in non-S phase cells suggests that TIM may associate with the MCM2-7 helicase independent of DNA synthesis. However, TIM is only known to be a component of the replication fork progression complex (22). Therefore, we looked at the kinetics of the interaction between TIM and MCM2-7 throughout the cell cycle. First, we found that the stable interaction of TIM-TIPIN with MCM2-7 takes place on the chromatin (Fig. 7A). Interestingly, when we examined a HEK293T cell line stably expressing FLAG-MCM7, we found that, although CDC45 and GINS primarily associate with the MCM2-7 helicase during S phase (11 h), TIM-TIPIN interacts with MCM2-7 on the chromatin throughout the cell cycle (Fig. 7, B and C). On the other hand, TIM was primarily enriched in the FLAG-CDC45 complex purified from S phase cells (11 h) compared with the other phases (Fig. 7, D and E), and this result further supports the interaction of TIM with MCM2-7 prior to the interaction between MCM2-7 and CDC45 to form CMG. Consistent with this finding, we showed that CDC6 was also co-purified with TIM in the CB fraction (Fig. 7A). In addition, MCM2-7 could still be co-purified with TIM and TIPIN in the absence of CDC6, RECQ4, or MCM10 (Fig. 7, F and G), all of which are proteins that interact with MCM2-7 during G1 phase and G1/S transition to facilitate CMG assembly in human cells (51, 58). Therefore, we concluded that the interaction of TIM-TIPIN with MCM2-7 is independent of the cell cycle.

Discussion

This is the first report that demonstrates a function for human TIM in ensuring the correct chromatin association of the CMG complexes during the cell cycle (Fig. 8). During the normal cell cycle, CMG is only present on the chromatin during S phase to initiate DNA replication and allow cells to progress through S phase (5). We showed that TIM and TIPIN stably interact with the MCM2-7 helicase throughout the cell cycle (Fig. 7) and that TIM is important for preventing aberrant CMG complexes from accumulating on the chromatin when cells are not in S phase (Fig. 5, B and F). As a consequence, in the absence of TIM, aberrant CMG complexes containing MCM4, with altered phosphorylation at PG sites (Fig. 6), accumulate on the chromatin even in non-S phase cells (Fig. 8). Despite their abundance in TIM knockout cells, these abnormal CMG complexes are likely to be catalytically altered (Fig. 5H).

What is the molecular mechanism by which TIM prevents the accumulation of aberrant CMG complexes in non-S phase cells? After DNA synthesis is completed, CMG is disassembled by CDC48-dependent ubiquitination (59). It is possible that
TIM has a role in the disassembly or dissociation of the CMG complexes from the chromatin by facilitating or preserving this modification (Fig. 8). In TIM-deficient cells, the persistent chromatin association of the CMG complexes after DNA replication may continue to the next cell cycle, leading to the appearance of these CMG complexes on the chromatin prior to the next round of DNA replication. Because CDC6 is not known to be involved in the disassembly of the CMG complex, CDC6 depletion fails to eliminate these aberrant CMG complexes accumulated on the DNA in TIM knockdown cells (Fig. 4). Even though these CMG complexes remain on the DNA, they might still be targeted for dephosphorylation, leading to the altered phosphorylation pattern observed on the CMG complexes (Fig. 6). Alternatively, we cannot exclude the possibility that, in the absence of TIM, the accumulation of the CMG complex in the non-S phase cells may be a consequence of aberrant MCM2-7 chromatin loading or CMG assembly. In cells, CDC6 has been implicated in loading MCM2-7 at both active origins and the excess dormant origins, and this may be achieved by CDC6 continuously loading MCM2-7 at the active origin and releasing the loaded MCM2-7 to allow the helicase to slide away from the active origin (60, 61). However, in vitro, purified MCM2-7 helicase alone is capable of binding to DNA (53, 62). Furthermore, interactions among MCM2-7, CDC45, and GINS can form in the absence of CDC6 in vitro using recombinant proteins (53, 63). Most likely, negative regulatory mechanisms to minimize CDC6-independent DNA binding of MCM2-7 and CMG assembly may exist in cells. Indeed, multiple phosphorylation events that inhibit MCM2-7 binding to DNA have been reported (64, 65). In addition, in yeast, the SLD2 and SLD3 initiation factors interact with MCM2-7 helicase to block its interaction with CDC45 and GINS, and phosphorylation of SLD2 by CDK removes the block, thereby allowing the CMG complex to assemble (63, 66, 67). It is possible that TIM interacts with MCM2-7 prior to S phase to ensure that the MCM2-7 helicase can only be loaded onto the DNA via a CDC6-dependent pathway to form proper CMG complexes. In addition, mammalian TIM-TIPIN can interact with the MCM3, 4, 5, 6, and 7 subunits (9), whereas CDC45 and GINS interact with MCM2, 3, and 5 (68). It is plausible that TIM-TIPIN behaves similarly to yeast SLD2 and SLD3 and that the interactions of MCM2-7 with TIM-TIPIN and CDC45/GINS are normally mutually exclusive but can simultaneously interact with the MCM2-7 helicase after post-translational modification when cells enter S phase. Hence, in the absence of TIM, the interactions of MCM2-7 with CDC45 and GINS are not regulated and may form prior to DNA synthesis (Fig. 8). If so, it will be interesting to determine whether the interface of the MCM2-7 helicase that interacts with TIM-TIPIN can be modified at active replicons by CDK or DDK in a CDC6-dependent manner to allow the simultaneous interaction of CDC45 and GINS with MCM2-7 when TIM-TIPIN is present.

We further demonstrated that, in addition to preventing the accumulation of aberrant CMG complexes in non-S-phase cells, TIM contributes to the stable chromatin association of CDC6 and cyclin E (Fig. 3A). We propose the following possible mechanism by which TIM promotes the association of CDC6 with chromatin. CDC6 binding to DNA is tightly regulated to avoid re-replication during each round of the cell cycle. Possible mechanisms to prevent CDC6 from binding to DNA after rep-
lication initiation include nuclear export and cyclin A-dependent phosphorylation (4). It is possible that the assembly of CMG complexes may also provide a signal to release CDC6 from DNA for nuclear export to avoid re-replication. If so, the presence of the aberrant CMG complexes on the chromatin during $G_1$ phase could provide false signals that either prevent CDC6 from binding to chromatin or weaken its DNA binding (Fig. 8).

When cells enter $G_1$ phase of the cell cycle, CDC6 is recruited by ORC to the origin of replication (69), and this is followed by the chromatin recruitment of the MCM2-7 helicase and CDK2-cyclin E (Fig. 8) (70, 71). The chromatin binding and activation of CDK2-cyclin E provide a positive feedback loop that further enriches CDC6 on the DNA. This enrichment of CDC6 on the DNA can be achieved by protecting CDC6 from APC/C-dependent degradation (72) and activating the E2F transcription factor (73), which further amplifies the expression of CDC6 (74, 75). E2F-dependent transcription is also necessary for accumulation of SKP2, a component of the SCFSKP2 ubiquitin ligase, which promotes replication initiation by degrading p27 and p21 (36) during late $G_1$ phase (39–41, 76). The reduced chromatin loading of CDC6 in TIM knockdown cells may explain why these cells also exhibited decreased levels of DNA-bound cyclin E (Fig. 3A), reduced recruitment of MCM2-7 helicase to the active replication origin (Figs. 3D and 8), and reduced MCM4 phosphorylation (Fig. 6), low levels of SKP2 in G1 and S phases (Fig. 2C), high levels of p21 and p27 throughout the cell cycle (Figs. 1, A and B, and 2A), and a delay in S phase entry (Figs. 1C and 2B). The presence of these altered CMG helicase complexes may also explain the slow rate of replication fork progression that is observed in TIM knockdown cells when the cells start DNA synthesis (12).

**Experimental Procedures**

**Plasmids**—The pCMV-FLAG-RECQ4 plasmid was constructed as described previously (51). The MCM4, MCM7, and CDC45 cDNAs were purchased from Open Biosystems. The MCM4 and MCM7 cDNAs were subcloned into the NdeI and EcoRI sites of the pCMV-FLAG vector. The CDC45 cDNA was subcloned into the NdeI and XhoI sites of the pCMV-FLAG vector. The CDC45 cDNAs were purchased from Open Biosystems. The siRNA sequences for RECQ4, TIMELESS, and the DNA Replication Complex

**Antibodies**—Mouse anti-CDC6 (sc-9964), goat anti-actin (sc-1616), rabbit anti-p27 (sc-528), rabbit anti-H3 (sc-10809), rabbit anti-DNA pol β (sc10784), mouse anti-β-tubulin (sc-5274), and rabbit anti-CDC45 (sc-20685) were purchased from Santa Cruz Biotechnology. Mouse anti-p21 (ab16767), mouse anti-Cyclin E (ab3927), mouse anti-Cyclin A (ab16726), mouse anti-RNAPII Ser(P)5 (4H8), and rabbit anti-MCM7 (ab52489) were purchased from Abcam. Goat anti-MCM2 (A300-122A), rabbit anti-TIM (A300-961A-1), rabbit anti-TIPIN (A301-474A), rabbit anti-MCM4 (A300-193A), rabbit anti-MCM5 (A300-195A), rabbit anti-Skp2 (A302-436A), and rabbit anti-ORC2 (A302-734A) were purchased from Bethyl Laboratories. Mouse anti-Ser/Thr(P) (61548) was purchased from BD Biosciences. Rabbit anti-RECQ4 was generated as described previously against residues 71–80 of human RECQ4 (51, 77). Rabbit anti-MCM10 (12251-1-AP) and rabbit anti-Psf2 (16247-1-AP) were purchased from ProteinTech. Rabbit anti-FLAG (F7425) was purchased from Sigma. Rabbit anti-Sld5 (2-79.00.02) was purchased from Strategic Diagnostics. Rat anti-BrdU (MCA2060T) was purchased from AbD Serotec. Goat anti-rat DyLight 488-conjugated (112-485-167) and donkey anti-rat IgG conjugated with Rhodamine (712-026-150) were purchased from Jackson ImmunoResearch Laboratories. Rabbit anti-phospho-CHK1 (p-CHK1 Ser164, 133D3, 2348) and rabbit anti-CHK1 (2345) were purchased from Cell Signaling Technology. Mouse anti-ORC2 (M055-3) was purchased from MBL.

**Cell Culture, siRNA, Cell Fractionation, and Immunopurification**—The human cell lines HEK293T and HT1080 were generous gifts from Dr. Stephen West (The Francis Crick Institute). The human cell lines HEK293 and U2OS were obtained from Dr. Jeremy Stark (City of Hope). The cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator. Plasmid transfection, chromatin fractionation, and immunopurification were performed as described previously (51). The siRNA sequences for RECQ4, MCM10, and CDC6 were also described previously (51). The TIM stealth siRNAs #1 (5'-GGUUCCGAGAGAUGACUGAG-3') and #2 (5'-GGAACTGCGAGCGAGAGAG-3') were synthesized by Dharmacon (Lafayette, CO).
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GCUAU-3’ (11)) and #2 (5’-UCCAGGGUAGCUU CUUUCAAA-3’) were purchased from Invitrogen. The siRNAs were transfected into the cells using DharmaFECT 1 transfection reagent (Thermo Scientific) according to the protocol of the manufacturer. For TIM siRNA or TIM-CDC6 double siRNA knockdown, the cells were treated with 25 nM siRNA for 24 h followed by a second 25 nM siRNA treatment. The cells were harvested 36 h after the second siRNA transfection. For cell cycle synchronization, the cells were cultured with nocodazole-containing medium 24 h after the second siRNA transfection. For UV treatment, cells in log phase were irradiated with 30 J/m² UV light and harvested after 1 h of incubation. For APH treatment, the cells were cultured with 1 μg/ml of APH for 24 h prior to harvest. For CDC7 and CDK inhibitor treatments, PHA rinsed with PBS and lysed with cytoplasmic lysis buffer (New England Biolabs). The samples were separated by SDS-PAGE and detected on Western blots using an anti-MCM4 antibody. For CDC7 and CDK inhibitor treatments, PHA rinsed with PBS and lysed with cytoplasmic lysis buffer (New England Biolabs). The samples were separated by SDS-PAGE and detected on Western blots using an anti-MCM4 antibody. For CDC7 and CDK inhibitor treatments, PHA rinsed with PBS and lysed with cytoplasmic lysis buffer (New England Biolabs). The samples were separated by SDS-PAGE and detected on Western blots using an anti-MCM4 antibody.

Helicase Assay—FLAG-RECQ4 co-purified complexes were eluted with 3× FLAG peptide in Elution-A buffer (10 mM HEPES (pH 7.9), 0.2 mM NaCl, 0.2 mM EDTA, 0.05% Triton X-100, and 10% glycerol). The eluates were then dialyzed against dialysis buffer (100 mM NaCl, 0.2 mM EDTA, and 10% glycerol). An aliquot was incubated in 30 μl of the buffer supplied by the manufacturer with or without 400 U of λ protein phosphatase (New England Biolabs). The samples were separated by SDS-PAGE and detected on Western blots using an anti-MCM4 antibody.

ChIP and Real-time PCR—The cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and the reaction was stopped by adding glycerine to a final concentration of 0.125 m for 5 min at room temperature. The fixed cells were rinsed twice with PBS and lysed with cytoplasmic lysis buffer (10 mM Tris (pH 8.0), 0.34 mM sucrose, 2 mM CaCl₂, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.5% Nonidet P-40) plus protease inhibitors. Lysates were pelleted by centrifugation at 5000 rpm for 5 min at 4 °C. The pellets were resuspended in ChIP lysis buffer (1.0% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.0)) plus protease inhibitors, and chromatin was sheared by sonication to generate DNA fragments of <1 kb. Chromatin was diluted 10 times in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.0), and 167 mM NaCl) plus protease inhibitor and precleared with protein A/G beads (Thermo Scientific) for 1 h at 4 °C. Antibodies (ORC2, MCM5, and RNA polymerase II) were used for immunoprecipitation. A rabbit IgG (Santa Cruz Biotechnology) was used as a negative control. ChIP complexes were collected with protein A/G beads and washed sequentially twice with low-salt buffer (0.1% SDS, 1.0% Triton X-100, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.15 M NaCl), high-salt buffer (0.1% SDS, 1.0% Triton X-100, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5 M NaCl), LiCl buffer (0.25 M LiCl, 1.0% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris (pH 8.0)), and Tris-EDTA buffer. The ChIP complexes were eluted with 300 μl of elution buffer (0.1 M sodium bicarbonate and 1.0% SDS) at room temperature for 15 min. The chromatin was reverse cross-linked by adding 20 μl of 5 M NaCl and incubated at 65 °C overnight. The DNA was digested with RNase A and proteinase K and purified by phenol-chloroform and ethanol precipitation. Real-time PCR was performed to amplify the human Lamin B2 origin region (B48) (79) with an ABI 7500 fast system using SYBR Green fluorescence. Enrichment was calculated using the comparative Ct method.

Author Contributions—X. X. conducted most of the experiments and analyzed the results. J. T. W. contributed to the analysis of CMG complex formation. M. L. contributed to the cell cycle analysis. Y. L. conceived the idea for the project, analyzed the results with X. X., and wrote the paper.

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