Repression of Nascent Strand Elongation by Deregulated Cdt1 during DNA Replication in *Xenopus* Egg Extracts

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Excess Cdt1 reportedly induces rereplication of chromatin in cultured cells and *Xenopus* egg extracts, suggesting that the regulation of Cdt1 activity by cell cycle-dependent proteolysis and expression of the Cdt1 inhibitor geminin is crucial for the inhibition of chromosomal overreplication between S phase and metaphase. We analyzed the consequences of excess Cdt1 for DNA replication and found that increased Cdt1 activity inhibited the elongation of nascent strands in *Xenopus* egg extracts. In Cdt1-supplemented extracts, overreplication was remarkably induced by the further addition of the Cdt1-binding domain of geminin (Gem79-130), which lacks licensing inhibitor activity. Further analyses indicated that fully active geminin, as well as Gem79-130, restored nascent strand elongation in Cdt1-supplemented extracts even after the Cdt1-induced stalling of replication fork elongation had been established. Our results demonstrate an unforeseen, negative role for Cdt1 in elongation and suggest that its function in the control of replication should be redefined. We propose a novel surveillance mechanism in which Cdt1 blocks nascent chain elongation after detecting illegitimate activation of the licensing system.

INTRODUCTION

To maintain genome integrity, chromosomes are precisely duplicated only once per cell division cycle. In eukaryotic cells, the replication licensing system ensures accurate DNA replication. The prereplication complex associates with origins of replication before S phase through the stepwise assembly of the origin recognition complex, Cdc6, Cdt1, and Mcm2-7, after which licensing takes place (Diffley, 2004; Blow and Dutta, 2006). Mcm2-7 is thought to act as the replicative helicase, and the loading of Mcm2-7 onto chromatin is considered to be the key initiating event of the licensing reaction (Pacek and Walter, 2004). Licensed origins are presumably activated in S phase by Cdc7- and cyclin-dependent kinase (CDK)-dependent processes, leading to the formation of replication forks and the recruitment of DNA polymerases.

Repression of licensing after the onset of S phase is crucial for preventing rereplication (Fujita, 2006; Arias and Walter, 2007). In fission yeast, overexpression of Cdc18, an orthologue of Cdc6 in budding yeast and higher eukaryotes, induces rereplication (Nishitani *et al.*, 2000; Yanow *et al.*, 2001), suggesting that Cdc18/Cdc6 is a major target of mechanisms that repress licensing. In contrast, Cdt1 seems to be the crucial target in higher eukaryotes, because unreg-

ulated Cdt1 activity alone induces rereplication (Vaziri *et al.*, 2003; Nishitani *et al.*, 2004; Li and Blow, 2005; Arias and Walter, 2005; Maiorano *et al.*, 2005). Geminin represses licensing by binding and inhibiting Cdt1 (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001), and it is inactivated or degraded on mitotic exit (McGarry and Kirschner, 1998; Li and Blow, 2004). The nuclear import of geminin during S phase reactivates it to restrict licensing (Hodgson *et al.*, 2002; Yoshida *et al.*, 2005). Crystallographic analysis revealed that geminin forms homodimers that interact with Cdt1 through a central coiled-coil domain. In contrast, the C-terminal regions of geminin sterically hinder the interaction between Cdt1 and the Mcm2-7 complex (Lee *et al.*, 2004; Saxena *et al.*, 2004).

Furthermore, Cdt1 is polyubiquitinated and degraded during S phase. In *Xenopus* egg extracts, Cdt1 binds to proliferating nuclear antigen (PCNA) through a consensus PCNA-binding motif (PIP box) located in its N-terminal end and is degraded in a Cul4–Ddb1-dependent manner (Li and Blow, 2005; Arias and Walter, 2006; Yoshida *et al.*, 2005). In addition, SCF-Skp2 functions cooperatively with Cul4–Ddb1 in Cdt1 proteolysis in human cells (Sugimoto *et al.*, 2004; Lovejoy *et al.*, 2006; Nishitani *et al.*, 2006; Senga *et al.*, 2006). Although neither geminin depletion nor treatment with proteasome inhibitors significantly affects replication, a combination of these two induces substantial amounts of rereplication (McGarry, 2002; Arias and Walter, 2005; Li and Blow, 2005; Yoshida *et al.*, 2005; Kerns *et al.*, 2007), suggesting that these two regulatory pathways redundantly suppress Cdt1 activity to prevent rereplication.

Even if these two regulatory pathways are disrupted and rereplication is initiated, checkpoint mechanisms block further fork progression, thereby acting as another barrier to

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extensive overreplication (Vaziri *et al.*, 2003; Archambault *et al.*, 2005; Li and Blow, 2005). Recently, it was reported that ATM and Rad3-related (ATR)/Chk1, but not ataxia telangiectasia mutated (ATM)/Chk2, prevents rereplication (Lee *et al.*, 2007; Lin and Dutta, 2007; Liu *et al.*, 2007), presumably because ATR/Chk1 suppresses the Cdk2 activity required for the initiation of rereplication (Abraham, 2001; Luciani *et al.*, 2004). Checkpoint activation also requires multiple rounds of rereplication, suggesting that activation is induced by the aberrant DNA structures created by multiple replication forks (Davidson *et al.*, 2006).

In this study, we report that replication can be inhibited in *Xenopus* egg extracts, independently of proteolysis and checkpoint pathways, by the exogenous addition of supplementary Cdt1. Moreover, the Cdt1-binding domain of geminin counteracted this inhibition, resulting in overreplication in Cdt1-supplemented extracts. A detailed analysis of replication products revealed that the addition of exogenous Cdt1 inhibited strand elongation in a rereplication-independent manner. Our results point to a novel mechanism for preventing strand elongation after the illegitimate activation of replication licensing.

MATERIALS AND METHODS

Preparation of *Xenopus* Egg Extracts

Metaphase-arrested *Xenopus* egg extracts and demembrated *Xenopus* sperm nuclei were prepared as described previously (Chong *et al.*, 1997). Egg extracts were used after release into interphase after incubation with 0.3 mM CaCl₂ for 15 min at 23°C. Under standard conditions, extracts were supplemented with 250 µg/ml cycloheximide, 25 mM phosphocreatine, and 15 µg/ml creatine phosphokinase. Other reagents or proteins were added as indicated for each experiment.

DNA Replication Assay

Sperm nuclei (10,000 nuclei) were incubated in *Xenopus* egg extracts (10 µl) supplemented with [α -³²P]dATP for the indicated periods at 23°C. The extent of DNA synthesis was calculated from the amount of radioactivity incorporated into acid-insoluble fractions after proteinase K treatment, and values were expressed as a percentage of the values obtained under standard conditions, in which DNA synthesis was first detected at ~30 min and reached a plateau after 90 min of incubation (Figure 1C).

To examine DNA synthesis in extracts containing the CDK inhibitor p21, sperm nuclei were incubated for 60 min with egg extracts supplemented with aphidicolin and caffeine. The nuclear fraction was then isolated and transferred to fresh egg extracts supplemented with [α -³²P]dATP and p21, and further incubated in the presence of the indicated supplements.

To monitor Gem79-130 stimulation of His-Cdt1-induced overreplication, sperm nuclei (10,000 nuclei) were first incubated in egg extracts (8 µl) supplemented with [α -³²P]dATP for 90 min. Then, glutathione transferase (GST)-Cdt1 or His-Cdt1 was added to the extracts with or without Gem79-130, and the reaction volume was adjusted to 10 µl by an addition of buffer EB (50 mM KCl, 50 mM HEPES-KOH, pH 7.6, 5 mM MgCl₂, and 2 mM 2-mercaptoethanol). After a further 90-min incubation, the amount of DNA synthesized during the first and second incubations was measured.

To examine the restart of replication blocked by GST-Cdt1 addition, replication was first suppressed by incubating sperm nuclei that had almost completely replicated during a previous 90-min incubation in egg extracts supplemented with GST-Cdt1 for 60 min. Then, the reaction mixture was supplemented with caffeine, Gem79-130 or geminin in the presence or absence of p21 and incubated for 90 min. DNA synthesis in the reaction mixture was monitored after the addition of GST-Cdt1.

For nuclear transfer in replication assays, *Xenopus* egg extracts containing sperm nuclei (1000 nuclei/µl) were diluted in 1 ml of NIB (50 mM HEPES-KOH, pH 7.6, 50 mM KCl, 2 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) containing 0.01% Triton X-100 and 2.5 mM ATP. After layering 100 µl of 15% sucrose in the same buffer under the diluted extracts, the nuclei were isolated by centrifugation of the extracts at 6000 × g in a swinging bucket rotor for 5 min at 4°C. After removal of the supernatant, the precipitates were washed once with the above-mentioned buffer, and the isolated nuclei were resuspended with fresh extract for the following incubation.

Immunoblotting Analyses

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed according to previously described protocols (Tsuyama *et al.*, 2005).

To monitor the licensing activity of a Cdt1 mutant (GST-Cdt1-NGB) with a substitution of amino acids 241-248 (KAPAYQRF) by AAAAAAAAAA, which results in a marked reduction in Cdt1 affinity for geminin (Kerns *et al.*, 2007), sperm nuclei (20,000 nuclei) were added to egg extracts (20 µl) with or without geminin. The extracts were also supplemented with GST-Cdt1 or GST-Cdt1-NGB, and the mixtures were incubated for 20 min at 23°C. To address GST-Cdt1-induced loading of replication-related proteins onto over-replicating chromatin, sperm nuclei (22,000 nuclei) were incubated with egg extracts (18 µl) for 90 min, the reaction mixture was supplemented with GST-Cdt1, caffeine, and/or Gem79-130, and the reaction volume was adjusted to 22 µl. The samples were further incubated for 30 or 90 min. The resulting chromatin fractions were isolated and immunoblotted as described previously (Tsuyama *et al.*, 2005).

To examine the phosphorylation of Chk1 by immunoblotting, nuclei in egg extracts were isolated as described previously (Kumagai *et al.*, 1998), with a slight modification. After a 90-min incubation of *Xenopus* egg extracts with sperm nuclei (34,000 nuclei in 29 µl), the reaction mixture was further incubated for 90 min with 5 µl of buffer or GST-Cdt1 in the presence or absence of caffeine or Gem79-130. The mixture was then suspended in 300 µl of INIB (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 40% sucrose, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) and centrifuged at 5000 × g for 5 min in a swinging bucket rotor at 4°C. After discarding the bulk of the supernatant, the samples were resuspended with 1 ml of INIB and centrifuged again under the same conditions. The precipitated fraction was subjected to SDS-PAGE followed by immunoblotting with an anti phospho-Chk1 (Ser345) antibody.

Immunodepletion of Geminin

An anti-*Xenopus* geminin antibody coupled to protein A-Sepharose Fast Flow (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was prepared as described previously (Tsuyama *et al.*, 2005). Extracts depleted in geminin were prepared by incubating *Xenopus* egg extracts for 60 min at 4°C with a 25% volume of anti-geminin coupled to protein A-Sepharose Fast Flow beads. Mock extracts were prepared in the same manner but with a nonspecific antibody.

Flow Cytometry Analysis

After incubating sperm nuclei (35,000 nuclei) with *Xenopus* egg extracts (35 µl) for 120 min, the extracts were suspended in 900 µl of NIB supplemented with 0.25% Triton X-100, 0.25 mM phenylmethylsulfonyl fluoride and 0.3 mg/ml RNase A and left on ice for 10 min. Nuclei were fixed by the addition of 37% formaldehyde (100 µl) on ice for 10 min, followed by incubation at 20°C for 20 min. The fixed nuclei were then stained with propidium iodide (16 µg/ml) on ice for 30 min. After filtration with a Cell Strainer (40 µm; BD Biosciences, San Jose, CA), the distribution of DNA contents in the nuclei was analyzed with a FACScan (BD Biosciences). Nuclei did not aggregate after sample preparation for flow cytometry analyses, as confirmed by microscopic observation (data not shown).

Alkaline Agarose Gel Electrophoresis

After sperm nuclei (10,000 nuclei) were incubated with 10 µl of *Xenopus* egg extract, the reactions were stopped by adding 140 µl of Stop N (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA, and 0.5% SDS) containing 0.2 mg/ml proteinase K and 0.3 mg/ml RNase A, and after incubation for 60 min at 37°C, the DNA was recovered by ethanol precipitation and resuspended in 25 mM NaOH, 3 mM EDTA, 1.25% Ficoll, and 0.025% bromocresol green (20 µl). The DNA samples were then loaded onto 1% agarose gels equilibrated with 50 mM NaOH and 1 mM EDTA. Electrophoresis was done in the same buffer at 8 V/cm for 4 h on ice. After electrophoresis, the gels were fixed in 7% trichloroacetic acid and 1.4% sodium pyrophosphate for 30 min and dried between 3 MM papers (Whatman, Maidstone, United Kingdom). The radioactivity in the dried gels was visualized by exposure to x-ray film. λ -HindIII (New England Biolabs, Ipswich, MA) was used as a DNA size marker after end labeling with [α -³²P]dATP by ExTaq (Takara Bio, Madison, WI).

To address geminin-induced restoration of replication stalled by Cdt1, sperm nuclei in egg extracts were incubated with [α -³²P]dATP and 80 nM GST-Cdt1 for 60 min after a prior 90-min incubation without [α -³²P]dATP or GST-Cdt1. Geminin was then added to the extracts with 1 mM dATP, which severely attenuated the incorporation of [α -³²P]dATP into DNA (Figure 5D), and the extracts were further incubated for the indicated periods. The resulting DNA was analyzed by alkaline electrophoresis.

Coprecipitation of Gem79-130 or PCNA with GST-Cdt1

To validate the interaction of Gem79-130 with GST-Cdt1, glutathione-Sepharose (GE Healthcare) was incubated with 2.8 µg of GST-Cdt1 or GST-Cdt1-NGB for 90 min at 4°C in a buffer containing 40 mM HEPES-KOH, 20 mM potassium phosphate buffer, pH 8.0, 50 mM KCl, 1 mM EGTA, 2 mM MgCl₂, 2 mM dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 10% sucrose, 2.5 mM ATP, and 0.01% Triton X-100. The Sepharose beads were then washed with the same buffer and incubated with Gem79-130 (4 µM) for 20 min at 23°C. After thorough washing, GST-tagged proteins and their interacting

proteins were eluted with 20 mM glutathione in the same buffer and visualized by immunoblotting.

To examine the interaction of GST-Cdt1 with PCNA, recombinant human PCNA (300 ng) was mixed with 280 ng of GST-Cdt1 or GST- Δ PIP-Cdt1, in which the PCNA-interacting PIP box was mutated (Arias and Walter, 2006), and then it was incubated with glutathione-Sepharose for 60 min at 4°C in a buffer containing 40 mM HEPES-KOH, 20 mM potassium phosphate buffer, pH 8.0, 50 mM KCl, 1 mM EGTA, 2 mM MgCl₂, 2 mM dithiothreitol, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 10% sucrose, 2.5 mM ATP, and 0.05% Triton X-100. After thorough washing, GST-tagged proteins and their interacting proteins were eluted with 20 mM glutathione in the same buffer and visualized by immunoblotting.

Reagents, Antibodies, and Recombinant Proteins

Caffeine and aphidicolin were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO), respectively.

Anti-*Xenopus* Cdt1, anti-*Xenopus* Cdc6, and anti-*Xenopus* Mcm6 rabbit polyclonal antibodies were raised as described previously (Tsuyama *et al.*, 2005; Kumata *et al.*, 2007). Anti-*Xenopus* geminin rabbit polyclonal antibody was raised against recombinant His $\times$ 6-tagged geminin. The anti-Mcm4 antibody was a kind gift from Yukio Ishimi (Ibaraki University) and Hiroshi Kimura (Kyoto University). Anti-*Xenopus* Cdc45, anti-*Xenopus* Rpa30, and anti-chicken DNA polymerase α p180 monoclonal antibodies were courteously provided by Haruhiko Takisawa (Osaka University), Hiromu Murofushi (Yamaguchi University), and Fumiko Hirose (University of Hyogo), respectively. Anti-PCNA mouse monoclonal antibody (mAb), anti-human histone H3 rabbit polyclonal antibody, and anti-phospho-Chk1 (Ser345) rabbit mAb were purchased from BD Biosciences Transduction Laboratories (Lexington, KY), Abcam (Cambridge, MA), and Cell Signaling Technology (Beverly, MA), respectively.

Xenopus Cdt1 fused to glutathione-transferase (GST-Cdt1) or His6-tagged human p21 was expressed and purified as described previously (Tsuyama *et al.*, 2005, 2006). His6-tagged geminin lacking the destruction box was expressed and purified by a previously described method (McGarry and Kirschner, 1998), and used at 13 μ M as fully active geminin. GST-Cdt1-NGB (Kerns *et al.*, 2007) and GST- Δ PIP-Cdt1 (Arias and Walter, 2006) were expressed in BL21-Codon Plus RIL cells (Stratagene, La Jolla, CA) and purified with glutathione-Sepharose (GE Healthcare). His6-tagged mouse Gem79-130 was expressed in BL21 (DE3) pLysS cells (Stratagene) and purified with nickel-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA) and Mono Q HR5/5 (GE Healthcare). The purified fraction was dialyzed against 10% sucrose in EB. A plasmid expressing human PCNA was courteously provided by Toshiki Tsurimoto (Kyushu University; Fukuda *et al.*, 1995). Human PCNA was expressed in BL21-Codon Plus RIL cells and purified by serial column chromatography by using Q Sepharose Fast Flow (GE Healthcare) and Mono Q HR5/5 in a buffer containing 25 mM HEPES-NaOH, pH 7.8, 1 mM EDTA, 0.01% NP-40, and 10% glycerol.

Data Collection and Presentation

All figures in this article indicate representative results obtained from at least three independent experiments to verify their reproducibility.

RESULTS

Gem79-130 Induces Overreplication in *Xenopus* Egg Extracts Supplemented with Cdt1

To investigate the regulatory mechanisms that restrain DNA from undergoing rereplication, we first measured replication activity in *Xenopus* egg extracts supplemented with recombinant Cdt1. Sperm nuclei were incubated for 180 min with egg extracts containing various amounts of glutathione-transferase-tagged Cdt1 (GST-Cdt1) (Figure 1A). It should be noted that although Cdt1 is proteolyzed after the onset of S phase, the GST-Cdt1 used in this study was inefficiently degraded during replication in *Xenopus* egg extracts, probably because the N-terminal GST-tag significantly disturbed the polyubiquitination of Cdt1 (Senga *et al.*, 2006; Figure 3B). Despite the expected positive function of Cdt1 in replication, we found that 80 nM or more GST-Cdt1 inhibited DNA synthesis. This was not observed when M13 single-stranded DNA was used as a template (Supplemental Figure 1). The addition of recombinant Cdt1 to egg extracts was suggested previously to induce rereplication after a first round of DNA replication; however, the resulting chromatin structures stimulate the checkpoint machinery to repress further origin firing (Li and Blow, 2005; Maiorano *et al.*, 2005;

Figure 1. Overreplication is markedly enhanced by Gem79-130 in egg extracts supplemented with GST-Cdt1. (A and B) Egg extracts were supplemented with the indicated concentrations of GST-Cdt1 alone (A and B, circles) or with 5 mM caffeine (A, triangles), 13 μ M geminin (B, diamonds), or 8 μ M Gem79-130 (B, squares), and DNA synthesis was assayed after a 180-min incubation. After incubation, synthesized DNA was measured and plotted as a percentage of that synthesized in extracts without additives. (C) Sperm nuclei (5000 nuclei) were incubated for the indicated periods with 5 μ l of egg extracts supplemented with buffer (open circles), 80 nM GST-Cdt1 (closed circles), or 80 nM GST-Cdt1 and 8 μ M Gem79-130 (closed squares). After incubation, synthesized DNA was measured and plotted as a percentage of that synthesized during a 90-min incubation of extracts and sperm nuclei without additives. (D) Sperm nuclei (20,000 nuclei) were incubated in 20 μ l of egg extracts in the presence or absence of GST-Cdt1 (80 nM) for the indicated periods. After incubation, the chromatin fractions were isolated and immunoblotted to detect DNA polymerase α (Pol α), Mcm4, Cdt1, Cdc45, PCNA, and Rpa30 (RPA). Histone H3 (H3) was also visualized as a loading control. Arrowhead and arrow represent bands corresponding to GST-Cdt1 and endogenous Cdt1, respectively. (E) The distribution of nuclear DNA contents was analyzed by flow cytometry after incubation of sperm nuclei with egg extracts containing GST-Cdt1 supplemented with caffeine (5 mM), geminin (13 μ M), or Gem79-130 (8 μ M). Dashed lines indicated by arrowheads or arrows represent peak positions of unreplicated and replicated nuclei, respectively.

Davidson *et al.*, 2006). We therefore investigated whether the addition of GST-Cdt1 abrogated replication by activating checkpoint pathways in egg extracts. Replication in GST-Cdt1-supplemented egg extracts was measured in the presence of caffeine, an inhibitor of the ATM and ATR kinases that act upstream of the checkpoint pathways (Figure 1A). We observed that caffeine induced a remarkable increase of

DNA synthesis in the presence of 40–80 nM GST-Cdt1, but DNA synthesis was reduced in a Cdt1 dose-dependent manner when GST-Cdt1 was supplemented at concentrations higher than 80 nM, suggesting that rereplication induced by GST-Cdt1 is suppressed by multiple mechanisms, including checkpoint pathways. In our standard replicating conditions, DNA synthesis reached a plateau before 90 min of incubation (Figure 1C). Therefore, we refer to an increase in replication greater than that observed after a 90-min incubation under standard conditions as “overreplication.”

We next addressed whether this effect of Cdt1 was linked to its function in replication licensing. For this purpose, we used a construct containing only amino acids 79–130 of geminin (Gem79–130), whose binding affinity for Cdt1, but not its licensing inhibitory activity, is comparable with that of the wild-type protein because it lacks the domain essential for steric hindrance (Lee *et al.*, 2004; Figure 2B). To our surprise, Gem79–130 induced overreplication in a GST-Cdt1-dependant manner; in contrast, full length geminin restored replication but did not induce overreplication under these experimental conditions (Figure 1B).

We then evaluated the effect of GST-Cdt1 on the kinetics of replication in the absence or presence of Gem79–130 (Fig-

ure 1C). Replication was initially observed at 30 min and was mostly completed within 60 min in extracts without GST-Cdt1. Although the onset of replication did not seem to be noticeably changed by the addition of GST-Cdt1, DNA synthesis continued at a much lower rate until at least 120 min in GST-Cdt1-supplemented extracts. The addition of Gem79–130 to GST-Cdt1-supplemented extracts resulted in a remarkable enhancement of DNA synthesis during the first 60 min, which was comparable with or greater than that under normal replicating conditions, and DNA synthesis continually increased until at least 120 min.

To validate that replication had not terminated in extracts supplemented with GST-Cdt1, we examined the chromatin association of replication-related proteins (Figure 1D). In the absence of GST-Cdt1, the levels of chromatin-associated replication proteins peaked at 40 min and decreased afterward (Figure 1D), which confirmed that replication was mostly complete by 60–80 min under standard conditions. Although replication proteins similarly accumulated in GST-Cdt1-supplemented extracts by 40 min, the association of the proteins persisted until at least 80 min, suggesting that replication was inhibited at the stage at which nascent strands elongate under these conditions, whereas the onset of replication was hardly affected.

Next, we analyzed replication by flow cytometry, which showed that nuclei with incompletely replicated DNA accumulated in extracts supplemented with 80 nM GST-Cdt1 (Figure 1E). Caffeine induced a remarkable increase in nuclear DNA content in the presence of 80 nM GST-Cdt1. This result agrees with previous studies showing that rereplication is stimulated after a first round of DNA synthesis in egg extracts supplemented with Cdt1 (Li and Blow, 2005; Maiorano *et al.*, 2005). A similar increase in DNA content was observed after Gem79–130 addition, suggesting that rereplication was also induced under these conditions.

To determine whether Gem79–130 elicits its effect by interacting with Cdt1, we used a Cdt1 mutant (GST-Cdt1-NGB) with a markedly reduced affinity for geminin, which showed substantial licensing activity even in the presence of excess geminin (Figure 2A; Kerns *et al.*, 2007). GST-Cdt1-NGB was also reduced in its affinity for Gem79–130 (Figure 2B); therefore, we examined whether Gem79–130 suppressed the inhibition of replication induced by GST-Cdt1-NGB. We found that Gem79–130 had an almost undetectable effect in extracts supplemented with GST-Cdt1-NGB, whereas caffeine still stimulated overreplication, suggesting that Gem79–130 enhances overreplication through its ability to associate with Cdt1 (Figure 2C).

The Induction of Overreplication by Gem79–130 Is Not Due to Competition with Endogenous Geminin or Repression of Cdt1 Proteolysis

Cdt1 is supposedly down-regulated by an increase in geminin expression after the onset of S phase. Although protein synthesis was inhibited by cycloheximide in our experiments, *Xenopus* egg extracts are known to contain inactivated geminin that can be reactivated by nuclear transport (Hodgson *et al.*, 2002; Yoshida *et al.*, 2005). Therefore, it was possible that Gem79–130 could induce rereplication by competing with endogenous geminin for the Cdt1 binding site. To investigate this possibility, Gem79–130 was added to geminin-depleted egg extracts supplemented with GST-Cdt1. We found that Gem79–130 had a similar or slightly increased effect on overreplication in geminin-depleted extracts compared with its effect in mock-treated extracts (Figure 3A), indicating that the effect of Gem79–130 is not due to its competition with endogenous geminin.

Figure 2. Caffeine, but not Gem79–130, induces overreplication by a GST-Cdt1 protein with a mutation in its geminin-binding domain. (A) Sperm nuclei were incubated for 20 min with egg extracts supplemented with geminin (13 μ M), GST-Cdt1, and/or GST-Cdt1-NGB as indicated. After incubation, Mcm4 and Cdt1 in the isolated nuclei were visualized by immunoblotting. The arrowhead and arrow indicate bands corresponding to GST-Cdt1 and endogenous Cdt1, respectively. (B) After adsorption of 2.8 μ g of GST-Cdt1 or GST-Cdt1-NGB to glutathione-Sepharose, the Sepharose beads were incubated with Gem79–130 (4 μ M) for 20 min at 23°C, and bead-bound proteins were eluted with 20 mM glutathione. The elution fractions (pull down) and input proteins (input) were analyzed by immunoblotting to detect GST-Cdt1 or Gem79–130. (C) Sperm nuclei were incubated for 180 min with egg extracts containing the indicated concentrations of GST-Cdt1-NGB alone (circles) or with 5 mM caffeine (triangles) or 8 μ M Gem79–130 (squares). After incubation, synthesized DNA was measured and plotted as a percentage of that synthesized in extracts without additives.

Figure 3. The stimulatory effect of Gem79-130 on overreplication is not due to competition with endogenous geminin or to the degradation of Cdt1. (A) Sperm nuclei were incubated for 180 min with mock-treated (squares) or geminin-depleted egg extracts (circles) containing the indicated concentrations of GST-Cdt1 without (closed symbols) or with 8 μ M Gem79-130 (open symbols). After incubation, synthesized DNA was measured and plotted as a percentage of that synthesized in mock-treated extracts without GST-Cdt1 or Gem79-130. Inset, aliquots of mock-treated (mock) and geminin-depleted extracts (Δ Gem) were resolved by SDS-PAGE and immunoblotted to verify the immunodepletion of geminin. (B) Sperm nuclei (2500 nuclei) were incubated with 2.5 μ l of egg extracts alone or with Gem79-130 (8 μ M) and/or GST-Cdt1 (80 nM) for the indicated periods. After incubation, the reaction mixture was subjected to immunoblotting to detect Cdt1. Bands corresponding to GST-Cdt1 and endogenous Cdt1 (endo. Cdt1) are indicated at right. Mcm6 was also visualized on the same blot as a loading control. (C) Sperm nuclei were incubated in egg extracts for 90 min and then GST-Cdt1 (80 nM), His-Cdt1 (80 nM), and/or Gem79-130 (8 μ M) were added to the reactions as indicated. After a further 90-min incubation, the amount of DNA synthesized during the first and second incubation was measured and plotted as a percentage of that synthesized in a 180-min incubation without additives. Inset, immunoblot of Cdt1 and Mcm6 from extracts incubated for the indicated periods with sperm nuclei in the presence of 80 nM His-Cdt1 alone or with 8 μ M Gem79-130.

Cdt1 is also down-regulated through cell cycle-dependent degradation mediated by the Cul4-Ddb1 ubiquitin ligase in *Xenopus* egg extracts as well as in human cells (Arias and Walter, 2006; Lovejoy *et al.*, 2006; Nishitani *et al.*, 2006). However, Gem79-130 did not significantly alter the degradation of GST-Cdt1 or endogenous Cdt1 during S phase (Figure 3B). Although GST-Cdt1 was inefficiently degraded as mentioned above, histidine x 6-tagged Cdt1 (His-Cdt1) was degraded with kinetics similar to that of endogenous Cdt1, even in the presence of Gem79-130 (Figure 3C, inset). Although efficient overreplication was not observed unless His-Cdt1 was added after the first round of replication, Gem79-130 also enhanced overreplication induced by His-Cdt1 (Figure 3C). Together, these results show that overreplication induced by Gem79-130 cannot be ascribed to the suppression of the negative regulation of licensing activity targeting Cdt1.

Elongation of Nascent Strands Is Inhibited by Exogenous Addition of GST-Cdt1

We observed that GST-Cdt1 addition repressed replication below the level of the normal reaction and caused replica-

tion-related proteins to accumulate on chromatin (Figure 1D), suggesting that the first round of replication was also suppressed by GST-Cdt1 at the elongation stage. Thus, we examined the effect of GST-Cdt1 addition on replication in the absence of rereplication (Figure 4A). Sperm nuclei were incubated with egg extracts containing 75 μ g/ml aphidicolin (APH), which results in nearly complete inhibition of DNA polymerase activity (Luciani *et al.*, 2004; Tsuyama *et*

Figure 4. Elongation of nascent strands is inhibited by the addition of Cdt1 in *Xenopus* egg extracts. (A) Schematic representation of the experimental procedure. Sperm nuclei were incubated in egg extracts supplemented with 75 μ g/ml aphidicolin and 5 mM caffeine for 60 min. The nuclear fraction was isolated (APH-nuclei) and incubated for 90 min (B–D) or the indicated periods (E) in fresh egg extracts supplemented with [α - 32 P]dATP and p21 (5 μ g/ml). After incubation, radioactivity incorporated into DNA during the second incubation was measured and plotted as a percentage of that incorporated into sperm DNA incubated for 90 min with egg extracts under standard conditions (B and C); radioactivity was also visualized after alkaline agarose gel electrophoresis (D and E). (B) The indicated concentrations of GST-Cdt1 were added to the second incubation. (C and D) GST-Cdt1 (80 nM), caffeine (5 mM), geminin (13 μ M), and Gem79-130 (8 μ M) were added to the second incubation as indicated. (E) GST-Cdt1 (80 nM) and/or geminin (13 μ M) were added to the second incubation. r.p. indicates aliquots of replication products incubated for 90 min under standard conditions.

al., 2006; data not shown), and caffeine, which inhibits the checkpoint pathway that inactivates later-firing origins in response to APH-arrested replication forks. Thus, the resulting nuclei (APH-nuclei) are presumably arrested at early steps of nascent DNA elongation after the firing of potential origins. APH-nuclei were isolated and incubated with fresh egg extracts containing the CDK inhibitor p21 (second reaction), by which GST-Cdt1-induced overreplication was inhibited, even in the presence of caffeine or Gem79-130 (Supplemental Figure 2). Thus, p21-containing extracts should provide conditions under which elongation arrest is relieved but further origin firing and consequent rereplication are prevented. We found that the addition of GST-Cdt1 to these extracts reduced DNA synthesis in a dose-dependent manner, suggesting that Cdt1 inhibits replication at the elongation step in a manner independent of rereplication (Figure 4B).

Next, we assessed the effect of Gem79-130 or caffeine on nascent DNA elongation suppressed by the addition of GST-Cdt1 (Figure 4C). We observed that the replication of APH-nuclei repressed by GST-Cdt1 was restored by the further addition of Gem79-130 but not caffeine, suggesting that the suppression of DNA synthesis under these conditions is not due to activation of the checkpoint machinery. Furthermore, it should be noted that the addition of fully active geminin also enhanced DNA synthesis to a level comparable with that observed with Gem79-130, probably because licensing activity in the second reaction did not influence the extent of DNA synthesis under CDK-inhibited conditions. This result suggests that wild-type geminin can also inhibit the suppressive effect of Cdt1 on replication, as was demonstrated with Gem79-130.

This result raised the possibility that Cdt1 arrests the elongation of nascent strands during replication in a manner independent of the checkpoint machinery or the licensing activity of Cdt1. To test this notion, DNA synthesized in APH-nuclei was analyzed by alkaline agarose gel electrophoresis (Figure 4D), which showed that the majority of the nascent strands migrated around 4 kb when the extracts were supplemented with GST-Cdt1. This is in contrast to the longer labeled DNA seen under normal replication conditions. This result provides more direct evidence that nascent DNA elongation is arrested by the addition of Cdt1. Although the effect of GST-Cdt1 addition was not reversed by caffeine, elongation products more similar to those produced under normal conditions were observed upon the further addition of geminin or Gem79-130. A marked reduction in elongation products was also observed when GST-Cdt1 and p21 were added after a 35-min incubation of normally replicating extracts untreated with aphidicolin or not subjected to nuclear isolation, in which the initiation of replication presumably occurred at many origins, but significant DNA synthesis was not observed yet (Supplemental Figure 3). This effect of GST-Cdt1 was also cancelled by geminin or Gem79-130, but not by caffeine, suggesting that the reduction in elongation products caused by Cdt1 is not due to prior arrest of replication forks by aphidicolin.

To examine the elongation of nascent strands during the incubation, we compared the length of labeled DNA by alkaline agarose gel electrophoresis after 10- and 40-min incubations in the second reaction (Figure 4E). Without the addition of GST-Cdt1 or geminin, highly smeared bands of ~4 kb were observed at 10 min, which became more elongated by 40 min. Whereas nascent DNA synthesized in GST-Cdt1-supplemented extracts was slightly shorter than that produced in the absence of GST-Cdt1 after a 10-min incubation, the elongation observed after 40 min was severely compromised by the addition of GST-Cdt1. The fur-

ther addition of geminin to GST-Cdt1-supplemented extracts resulted in elongation products similar to those in extracts lacking supplemented Cdt1 or geminin. Together, these results confirm the idea that the addition of Cdt1 induces stalling of nascent strand elongation during replication and that this effect is cancelled by geminin.

Figure 5. Cdt1-induced arrest of replication is not due to Cdt1 activity in the loading of Mcm2-7 onto chromatin or interaction with PCNA. (A, B, D, and E) Sperm nuclei were incubated in egg extracts supplemented with 75 μ g/ml aphidicolin and 5 mM caffeine for 60 min. The nuclear fraction was isolated (APH-nuclei) and incubated with fresh egg extracts supplemented with p21 (5 μ g/ml). (A) APH nuclei were incubated with extracts containing p21 and [α - 32 P]dATP for 30 min (lanes 3–5) or 90 min (lanes 1 and 2). For lanes 4 and 5, Gem79-130 and geminin were added, respectively, and the mixtures were further incubated for 60 min. Radioactivity incorporated into the nascent DNA was detected by autoradiography after alkaline agarose gels electrophoresis. r.p. indicates aliquots of replication products incubated for 90 min under normal conditions. (B) APH-nuclei were incubated for 0 or 60 min with p21-containing extracts supplemented with GST-Cdt1 (80 nM), caffeine (5 mM), geminin (13 μ M), and/or Gem79-130 (8 μ M) as indicated. After the incubation, nuclear fractions were isolated and immunoblotted to detect Mcm4 and Cdt1. Histone H3 (H3) in the chromatin fraction was also visualized as a loading control. (C) Two-hundred eighty nanograms of GST-Cdt1 (WT) or GST- Δ PIP-Cdt1 (Δ PIP) was mixed with recombinant human PCNA (300 ng) and adsorbed to glutathione-Sepharose. The proteins bound to the Sepharose beads were eluted with 20 mM glutathione. The elution fractions (pull down) and aliquots of input proteins (input) were applied to immunoblotting to detect GST-Cdt1 or PCNA. (D) APH-nuclei were incubated with extracts supplemented with p21 and [α - 32 P]dATP for 90 min in the presence of the indicated concentrations of GST-Cdt1 (circles) or GST- Δ PIP-Cdt1 (squares). (E) APH nuclei were incubated with extracts supplemented with p21 and [α - 32 P]dATP for 90 min in the absence or presence of 80 nM GST- Δ PIP-Cdt1 without or with caffeine (5 mM), geminin (13 μ M), or Gem79-130 (8 μ M). For D and E, radioactivity incorporated into nascent DNA was measured and plotted as a percentage of that incorporated into sperm DNA incubated for 90 min with egg extracts under standard conditions.

The Inhibition of DNA Replication by GST-Cdt1 Is Not Due to the Excessive Reloading of Mcm2-7 or the Cdt1-PCNA Interaction

Because the addition of Cdt1 to *Xenopus* egg extracts after replication reportedly causes reassociation of Mcm2-7 followed by rereplication (Yoshida *et al.*, 2005; Li and Blow, 2005), it is possible that the reloading of Mcm2-7 during DNA synthesis could disturb the smooth advance of the replication machinery, which in turn could severely inhibit nascent strand elongation. To examine this possibility, APH-nuclei were incubated for 30 min in p21- and GST-Cdt1-supplemented extracts, in which short nascent DNA was produced (Figure 5A, lane 3). The short DNA synthesized during a 30-min incubation was not markedly elongated by a further incubation to 60 min (lane 2). In contrast, the addition of geminin or Gem79-130 after the first 30-min incubation resulted in markedly elongated products (lanes 4 and 5) similar to those produced without GST-Cdt1 (lane 1). Under these conditions, although chromatin-bound Mcm4 was significantly reduced during incubation in p21-treated extracts, Mcm4 further accumulated on chromatin in the presence of GST-Cdt1 (Figure 5B). These results suggest that geminin or Gem79-130 can restore the replication of chromatin on which Mcm2-7 had been already loaded by the addition of Cdt1. Furthermore, Gem79-130, in contrast to geminin, did not significantly affect the chromatin loading of Mcm4 under these conditions, as reported previously (Figure 5B; Lee *et al.*, 2004), suggesting that Gem79-130 restores elongation synthesis suppressed by Cdt1 under conditions in which Mcm2-7 reloading is induced.

Cdt1 is known to interact with PCNA, which triggers the degradation of Cdt1 (Arias and Walter, 2006; Nishitani *et al.*, 2006; Senga *et al.*, 2006). Although Cdt1 proteolysis did not seem to be directly involved with the inhibition of nascent strand elongation by GST-Cdt1 (Figure 3, B and C), it is possible that Cdt1 suppresses the polymerization of nucleotides by interacting with PCNA, because PCNA is a well-known auxiliary protein of DNA polymerase δ . To test this idea, we produced a mutant form of GST-Cdt1 (GST- Δ PIP-Cdt1) in which the PCNA-binding motif (PIP box) was mutated (Arias and Walter, 2006). Coprecipitation analysis using glutathione-Sepharose confirmed that, although

PCNA efficiently interacted with GST-Cdt1, GST- Δ PIP-Cdt1 did not detectably associate with PCNA, as reported previously (Figure 5C; Arias and Walter, 2006). We then addressed whether GST- Δ PIP-Cdt1 inhibited replication in APH-nuclei in p21-supplemented extracts as indicated above by using GST-Cdt1 (Figure 5D). As a result, GST- Δ PIP-Cdt1 inhibited replication more efficiently than did GST-Cdt1, probably because the degradation of GST-Cdt1 was severely attenuated by the mutation in the PIP box. This inhibition of replication was sensitive to Gem79-130 or geminin, but not caffeine, an effect similar to that induced by GST-Cdt1 (Figure 5E). These results suggest that the inhibition of nascent strand elongation by Cdt1 is not related to its ability to interact with PCNA.

The Addition of GST-Cdt1 Suppresses Nascent Strand Elongation under Overreplicating Conditions

We next addressed whether GST-Cdt1 also suppresses the elongation of rereplicating strands after a first round of DNA synthesis under conditions that were used previously to analyze the effect of excess Cdt1 on rereplication in *Xenopus* egg extracts. After GST-Cdt1 and Gem79-130 were added to egg extracts incubated for 90 min and further incubated for another 90 min, the nascent DNA synthesized during the second incubation was detected by alkaline agarose gel electrophoresis (Figure 6A). Short nascent strands around 3–4 kb were found to accumulate in the GST-Cdt1-supplemented extracts, an effect that was enhanced by caffeine. In contrast, the replication products were significantly elongated when Gem79-130 was included with extracts containing GST-Cdt1. The elongation products further accumulated when caffeine was simultaneously added with Gem79-130. These results suggest that Gem79-130 inhibits the Cdt1-mediated arrest of rereplicating forks, whereas caffeine increases the frequency of origin firing, resulting in the initiation of rereplication.

We then investigated the chromatin binding of replication-related proteins in egg extracts to which we had added the indicated supplements after incubation under normal conditions for 90 min (Figure 6B). In the absence of supplements, Cdc6 was the only protein that was detected in the chromatin fraction, presumably indicating that very few

Figure 6. Geminin suppresses the accumulation of short DNA fragments during overreplication induced by Cdt1. (A) After sperm nuclei were incubated in egg extracts for 90 min, [α - 32 P]dATP was added along with GST-Cdt1 (80 nM), caffeine (5 mM), and/or Gem79-130 (8 μ M) as indicated. The reaction mixtures were further incubated for 90 min, and radiolabeled DNA was visualized by autoradiography after alkaline agarose gel electrophoresis. r.p. indicates aliquots of replication products incubated for 90 min under normal conditions. (B) After sperm nuclei were incubated in egg extracts for 90 min, the extracts were supplemented with buffer or 80 nM GST-Cdt1 with or without caffeine (5 mM) or Gem79-130 (8 μ M) as indicated. After further incubation for the indicated periods, the chromatin fractions were isolated and immunoblotted to detect DNA polymerase α (Pol α), Mcm4, Cdt1, Cdc6, Cdc45, PCNA, and Rpa30 (RPA). Histone H3 (H3) in chromatin fractions was also visualized as a loading control. (C) After incubation of sperm nuclei with egg extracts, the samples were supplemented with GST-Cdt1 (80 nM), caffeine (5 mM), and/or Gem79-130 (8 μ M) as indicated. After a further incubation for 90 min, the nuclear fraction was isolated and phosphorylated Chk1 was detected by immunoblotting. Histone H3 (H3) was also detected as a loading control.

replication forks form under these conditions. The addition of GST-Cdt1 induced the reassociation of replication-related proteins with chromatin, and this effect was significantly increased by caffeine addition. This result shows that the rereplication of chromosomal DNA is induced by the addition of GST-Cdt1, which in turn causes activation of checkpoint pathways to suppress further origin refiring. Although the progression of DNA synthesis should result in the gradual dissociation of replication-related proteins from chromatin (Waga *et al.*, 2001; Hashimoto and Takisawa, 2003), these proteins seemed to be retained on replication forks stalled by GST-Cdt1. In contrast, a decrease in chromatin-associated DNA polymerase α , Cdc45, and PCNA was observed when Gem79-130 was added to the GST-Cdt1-supplemented extracts. We confirmed that Chk1 was phosphorylated in extracts supplemented with GST-Cdt1 and Gem79-130 (Figure 6C). Thus, a checkpoint pathway activated by the addition of GST-Cdt1 presumably represses multiple rounds of origin firing and the de novo establishment of replication forks, even in the presence of Gem79-130, resulting in a gradual decrease in replication forks and chromatin-associated replication proteins by the restoration of nascent strand elongation.

Geminin Restores Replication Forks Arrested by GST-Cdt1 Addition

Because the results shown in Figure 6B suggested that replication machinery is maintained on replication forks arrested by GST-Cdt1 addition, we reasoned that Gem79-130 or geminin would restore the elongation of nascent strands in rereplication forks stalled by Cdt1. To examine this possibility, GST-Cdt1 was added to egg extracts after a 90-min reaction, and the mixtures were then incubated for 60 min to induce the arrest of rereplicating forks. After incubation, the reaction mixtures were supplemented with Gem79-130 or fully active geminin and further incubated. We first assessed DNA synthesis during the incubation after the first 90-min reaction (Figure 7A). The stimulation of DNA synthesis by caffeine was abolished by the addition of p21, which implied that caffeine inhibited the checkpoint pathway that suppressed CDK activation under these conditions. In contrast, the stimulation of DNA synthesis by Gem79-130 was still observed regardless of the presence of p21. Moreover, fully active geminin also stimulated DNA synthesis under these conditions, suggesting that it can restore replication forks arrested previously by Cdt1 without further origin firing.

Because the addition of Cdt1 induced an accumulation of short nascent DNA, which presumably resulted from replication fork arrest, we next investigated whether geminin addition could induce elongation of these short DNA products by performing alkaline agarose gel electrophoresis on the reaction products (Figure 7B). As a result, even after a 60-min accumulation of short nascent DNA induced by GST-Cdt1, the addition of geminin or Gem79-130 resulted in the disappearance of short replication products and the synthesis of longer products. When unlabeled dATP was added to chase the incorporation of radiolabeled dATP after arresting elongation with GST-Cdt1, we observed a gradual increase in the lengths of the radiolabeled DNA after geminin addition (Figure 7C). Together, these results suggest that arrested forks can be restarted when repression exerted by illegitimate Cdt1 is alleviated.

Figure 7. Cdt1-induced stalling of replication is reversed by the addition of geminin. The left panel of each figure shows a schematic representation of the experiment. (A) After incubation of egg extracts with sperm nuclei for 90 min, the extracts were supplemented with GST-Cdt1 (80 nM) and [α - 32 P]dATP and then further incubated for 60 min. After the addition of caffeine (5 mM), Gem79-130 (8 μ M), or geminin (13 μ M) as indicated, the extracts were incubated again for 90 min in the presence or absence of p21 (5 μ g/ml). DNA synthesized after the second incubation was measured and plotted as a percentage of that synthesized with the same amount of sperm nuclei in normal extracts for 90 min. (B) Numbers in parentheses in the schematic figure represent lane numbers to which samples with the indicated supplement or treatment were applied. After a 90-min incubation of egg extracts with sperm nuclei, [α - 32 P]dATP was added to the reaction mixtures and incubated for 60 min (lane 4) or 90 min (lanes 1–3, 5, and 6). Numbers at the top of the right panel represent the time after the onset of the second incubation when the samples were supplemented with GST-Cdt1 (80 nM), Gem79-130 (8 μ M), or geminin (13 μ M). The minus signs mean that the indicated protein was not included during the incubation. DNA synthesized after the addition of [α - 32 P]dATP was detected by autoradiography after alkaline agarose gel electrophoresis. r.p. indicates aliquots of replication products incubated for 90 min under normal conditions. (C) GST-Cdt1 (80 nM) and [α - 32 P]dATP were added after a 90-min incubation of extracts with sperm nuclei, and incubation was continued for 60 min. Unlabeled dATP (1 mM) was then added with geminin, and incubation was continued for the indicated periods. DNA synthesized after the addition of [α - 32 P]dATP was detected by autoradiography after alkaline agarose gels electrophoresis. r.p. indicates aliquots of replication products incubated for 90 min under normal conditions or in extracts supplemented with 1 mM unlabeled dATP.

DISCUSSION

Rereplication Is Induced and Repressed by Cdt1 in S Phase

Repression of DNA rereplication is crucial for the maintenance of genome integrity. To accomplish this, Cdt1 activity is strictly regulated by multiple mechanisms in higher eukaryotic cells. Indeed, emergence of Cdt1 activity in S phase reportedly induces overreplication of DNA in cultured cells or *Xenopus* egg extracts, suggesting that the regulation of Cdt1 is important for preventing rereplication in higher eukaryotes. In this study, we obtained the surprising result that the induction of overreplication by Cdt1 is remarkably enhanced by Gem79-130.

Consistent with previous reports, we also observed that the addition of caffeine induced overreplication in the presence of recombinant Cdt1 (Li and Blow, 2005; Davidson *et al.*, 2006). Because it has been reported that activated checkpoints inhibit rereplication (Vaziri *et al.*, 2003; Archambault *et al.*, 2005; Li and Blow, 2005), the stimulation of overreplication by caffeine is presumably due to the inhibition of checkpoint pathways that repress the activation of origins for rereplication. This idea is further supported by our result that the suppression of origin firing by the CDK inhibitor p21 severely attenuated the caffeine-induced stimulation of DNA synthesis in the presence of GST-Cdt1. Our results also indicated that the addition of GST-Cdt1 induced the loading of replication-related proteins and the accumulation of short nascent DNA on overreplicating chromatin, both of which were further stimulated by caffeine. These results suggest that GST-Cdt1 induces multiple rounds of incomplete replication, resulting in checkpoint activation and the inhibition of further origin firing.

In contrast, Gem79-130 apparently provoked the elongation of nascent strands and the dissociation of Cdc45, PCNA, and DNA polymerase α from overreplicating chromatin in GST-Cdt1-supplemented extracts. Moreover, the addition of p21 had little effect on DNA synthesis in the presence of GST-Cdt1 and Gem79-130. These results indicate that Gem79-130 enhances Cdt1-induced overreplication in a manner independent of the caffeine-sensitive checkpoint pathway. It also should be noted that Gem79-130 had little effect on Chk1 phosphorylation induced by GST-Cdt1, suggesting that the checkpoint pathway effectively repressed repetitive origin firing under these conditions. Thus, these observations suggest that there are at least two mechanisms that inhibit the extensive rereplication induced by licensing in S phase, one of which depends on ATR and the Chk1-directed checkpoint machinery (Lee *et al.*, 2007; Lin and Dutta, 2007; Liu *et al.*, 2007) and the other, which is a Cdt1-induced pathway independent of the checkpoint machinery, as indicated by Davidson *et al.* (2006), which is sensitive to Gem79-130.

Cdt1 Suppresses the Elongation of Nascent Strands during Replication

Measurements of DNA synthesis and flow cytometry analysis indicated that concentrations of at least 80 nM GST-Cdt1 inhibited replication and that this inhibition could be overcome by geminin. We investigated the effect of GST-Cdt1 on aphidicolin-arrested replication under conditions in which the activation of replication origins was inhibited by p21. We found that the addition of GST-Cdt1 inhibited replication in a manner independent of origin firing and that geminin alleviated this inhibitory effect, suggesting that Cdt1 inhibits the elongation of nascent strands in a geminin-sensitive manner. This conjecture was further endorsed by perform-

ing alkaline agarose gel electrophoresis to analyze replication products in GST-Cdt1-containing extracts. A previous report indicated that human cells expressing N-terminally truncated Cdt1 accumulate in S phase (Takeda *et al.*, 2005; Teer and Dutta, 2008), suggesting that replication arrest in the presence of illegitimate Cdt1 activity is physiologically relevant for the maintenance of genome integrity.

We also observed an accumulation of short nascent DNA on alkaline agarose gels when GST-Cdt1 was added after a first round of replication. The further addition of Gem79-130 resulted in longer replication products instead of the short DNA fragments seen under overreplicating conditions, probably because Gem79-130 abrogated the Cdt1-induced constraint on nascent strand elongation but not the Cdt1-induced relicensing of replicated chromatin. It is plausible that Gem79-130 prevents Cdt1 from binding to other factor(s) that are required for the Cdt1-induced suppression of nascent strand elongation but is dispensable for replication licensing. The present results suggest that replication and rereplication forks are stalled by the illegitimate activation of Cdt1 in S phase to prevent extensive rereplication of chromatin. It is rational that this putative feedback mechanism does not recognize Cdt1 associated with geminin, because it is not active for licensing and unable to induce abnormal rereplication.

DUP, the *Drosophila* orthologue of Cdt1 (Whittaker *et al.*, 2000), has been reported to localize to chorion loci throughout the physiological amplification of the chorion gene in ovarian follicle cells, and it colocalizes with incorporated 5-bromo-2'-deoxyuridine in the later stages of gene amplification, when elongation predominantly takes place without significant initiation (Claycomb *et al.*, 2002). This previous report proposed that DUP/Cdt1 travels with replication forks to perform an undetermined function, supporting our idea that Cdt1 plays a negative role at replication forks, although this negative function may be somehow suppressed during the amplification process.

Excess Cdt1 has been reported to induce the accumulation of short double-stranded DNA fragments, as detected by native agarose electrophoresis (Davidson *et al.*, 2006). We also observed short fragments containing nascent DNA by neutral agarose electrophoresis under our rereplicating conditions, consisting of DNA that could be elongated by the further addition of Gem79-130 (Tsuyama and Tada, unpublished observation). As indicated in previous reports, the fragmented DNA caused by Cdt1 addition remains associated with bulk chromatin and is only released when the DNA is deproteinized (Davidson *et al.*, 2006). Thus, we speculate that replication forks are stalled or slowly progress in a reversible manner, as opposed to the collapse of the replication machinery, until Cdt1 is inactivated. The DNA fragments observed by Davidson *et al.* (2006) are probably released from chromatin only after the removal of proteins from replication forks stalled by Cdt1.

It is plausible that this activity is beneficial for the efficient induction of the checkpoint pathway or apoptosis. In this regard, it is noteworthy that recent papers have argued for an antitumor function of cellular senescence induced by checkpoint responses to replication stress (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). The expression of Cdt1 increases in cells early in tumorigenesis, which presumably contributes to the polyploidy and/or chromosomal rearrangements often observed in cancer cells (Karakaidos *et al.*, 2004; Seo *et al.*, 2005; Lontos *et al.*, 2007). Therefore, the negative regulation of replication exerted by increased levels of Cdt1 may induce extensive replication stress, providing a barrier to

cancer development; thus, the function of Cdt1 is likely to be overcome or severely attenuated in cancer cells.

In summary, the present work shows that the illegitimate activation of Cdt1 in S phase can stall nascent strand elongation in addition to promoting rereplication through Mcm2-7 loading. In addition, Gem79-130, which does not inhibit the licensing activity of Cdt1, suppressed the negative effect of Cdt1 on replication, suggesting that Cdt1 impairs the elongation of nascent strands through a function that is independent of its licensing activity. We speculate that a putative negative feedback mechanism detects irregular Cdt1 activity and stalls replication forks until the licensing activity is successfully repressed. Such a mechanism would avoid the risk of genome destabilization caused by rereplication.

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