Geminin Prevents Rereplication during *Xenopus* Development*

Sarah L. Kerns, Susanna J. Torke, Jacqueline M. Benjamin, and Thomas J. McGarry

From the Division of Cardiology, Department of Cell and Molecular Biology, and Feinberg Cardiovascular Research Institute, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

To maintain a stable genome, each origin of replication must initiate DNA synthesis only once in every cell cycle. If even a small number of replication origins were to fire more than once, cells would slowly accumulate more and more DNA every time they divided. Moreover, if the reduplicated region encompassed the centromere, the chromosome would either missegregate or break during mitosis. Rereplication would have such disastrous consequences that cells have evolved multiple redundant mechanisms to ensure that it virtually never occurs. Polyploid cells, such as placental trophoblasts or megakaryocytes, are produced by repeated successive S phases rather than by rereplication within a single S phase (1). Rereplication is prevented by controlling the assembly of prereplication complex (pre-RC), a collection of essential replication factors that forms on origins of replication during late G1 phase (2). In metazoans, replication is restricted to one round per cell cycle through regulation of a single pre-RC component, Cdt1. Cdt1 is required for incorporation of the Mcm2–7 helicase into pre-RC (3).

The inhibitory protein Geminin is thought to prevent rereplication by regulating Cdt1. Geminin binds tightly to Cdt1 and seems to inhibit it in a stoichiometric fashion (4, 5). Geminin accumulates during S and G2 phase and is quickly destroyed at the metaphase/anaphase transition by ubiquitin-dependent proteolysis (6). This pattern suggests that Geminin prevents rereplication during S and G2 phase and then is destroyed during mitosis to allow replication in the next cell cycle. Nevertheless, depletion of Geminin from *Xenopus* egg replication extracts does not consistently result in a second round of DNA synthesis (6–8). Cdt1 is also destroyed by ubiquitin-dependent proteolysis during S phase in a manner that is dependent upon origin firing (7, 9–11). Several groups have recently reported that reinitiation of DNA synthesis occurs in *Xenopus* egg replication extracts when Geminin is depleted and protein degradation is inhibited with proteasome inhibitors, whereas neither treatment by itself has any effect (7, 8, 12). It was not determined whether stabilization of Cdt1 itself was responsible for rereplication in these experiments.

The phenotypes of Geminin-deficient organisms call into question whether Geminin has a physiological role in preventing rereplication in vivo. Neither budding nor fission yeast contain a clear Geminin homolog, yet rereplication is adequately prevented by other mechanisms (13). *Caenorhabditis elegans* embryos treated with Geminin small interfering RNA develop normally to adulthood and do not exhibit a general cell cycle defect (14). A few cells show anaphase chromosome bridges that may result from rereplication at the centromere. *Drosophila* embryos carrying P-element insertions in the Geminin gene die at the third instar larval stage (15). As in *C. elegans*, there is no generalized cell cycle defect, but there are some indications of aberrant DNA replication, including occasional cells with anaphase chromosome bridges and prolonged DNA replication in cells that normally endoreplicate their DNA and become polyploid. In contrast to these invertebrates, *Xenopus* embryos that are depleted of Geminin abruptly stop dividing after the 13th cell division (16). The arrest occurs immediately after the midblastula transition, the point in development when the cell cycle slows and zygotic gene expression first begins. The cell cycle arrest is caused by activation of the checkpoint kinase Chk1; inhibition of Chk1 by expressing a dominant negative mutant suppresses the arrest. Although overreplication could cause this phenotype, the amount of DNA in the arrested cells is...
normal. Mouse embryos homozygous for a targeted deletion of the Geminin gene arrest at the eight-cell stage (17). Interestingly, all of the cells in Geminin-deficient embryos are of the trophoblast lineage. These normally polyploid cells had a greater DNA content when Geminin was absent, reminiscent of the Drosophila phenotype.

In two-hybrid assays, Geminin has recently been found to bind to several different transcription factors and chromatin-remodeling proteins (18–21). Modest increases or decreases in Geminin expression can affect the development of specific tissues, organs, or embryonic segments in ways that suggest that Geminin inhibits these proteins. In light of these studies, Geminin is proposed to inhibit cell differentiation until cell proliferation ceases.

In this study, we sought to determine whether Geminin is required to prevent rereplication in Xenopus embryos and whether the phenotype of Geminin depletion is due to abnormal DNA replication or to an effect on cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Cdt1 deletion mutants were generated by PCR amplification using plBluescript-Cdt1 as a template (3). Fragments were inserted between the XhoI and XbaI sites of either pCS2 for untagged constructs or pCS2-MT for Myc-tagged constructs. Site-directed mutagenesis was carried out using the QuikChange protocol (Stratagene).

**Antibodies**—To make anti-Cdt1 antibody, full-length *Xenopus* Cdt1 was amplified by PCR and inserted between the Ndel and Xhol sites of pET28(a). The product plasmid was transformed into *Escherichia coli* strain BL21 and Cdt1 production was induced with isopropyl-β-D-galactopyranoside. Cdt1 inclusion bodies were purified by centrifugation and used to immunize rabbits (Covance). Anti-Cdt1 antibodies were affinity-purified from crude immune serum using a column of renatured Cdt1 protein covalently bound to cyanogen bromide-Sepharose. To make the affinity resin, Cdt1 was purified from inclusion bodies on nickel-agarose columns under denaturing conditions according to standard techniques (Qiagen). The protein was renatured by diluting it 20-fold into renaturation buffer (55 mM Tris, pH 8.2, 10.56 mM NaCl, 0.44 mM KCl, 550 mM guanidium HCl, 2.2 mM MgCl₂, 2.2 mM CaCl₂, 550 mM l-arginine, and 1 mM dithiothreitol) and incubating at 4°C overnight. Renatured Cdt1 was covalently attached to cyanogen bromide-Sepharose using standard techniques (22). Anti-Geminin antibody was described previously (6). Polyclonal anti-Myc antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-345 Chk1 antibody was purchased from Cell Signaling Technology (Beverly, MA). Immunoblots were performed using standard methods. For quantitative immunoblots, the two samples that were to be compared were serially diluted 1:2, and the relative intensity of the bands was compared visually.

**DNA Replication Assays**—S-phase replication extracts were prepared from ionophore-activated *Xenopus* eggs as described (23). Metaphase extracts were prepared from unactivated eggs. The eggs were incubated in MMR containing 100 μg/ml cycloheximide for 15 min prior to activation. All solutions used thereafter and the final extract were supplemented with 100 μg/ml cycloheximide. Extract was prepared fresh for each experiment. Extract was immunodepleted of Cdt1 using one-tenth volume Affiprep beads (Bio-Rad) coated with affinity-purified Cdt1 antibody (1 μg of antibody/1 μl of beads). Extract was immunodepleted of Geminin using one-twentieth volume Affiprep beads coated with anti-Geminin antibody (2 μg of antibody/1 μl of beads). Two sequential depletions were performed at 4°C for 1 h each with tumbling. Cdt1-depleted extracts were supplemented with one-tenth volume of Cdt1 protein that had been translated in reticulocyte lysate.

Replication reactions containing demembranated sperm template and [α-32P]dATP were carried out at room temperature using standard procedures (23). Total DNA synthesis was measured by trichloroacetic acid precipitation and scintillation counting of incorporated 32P. Density substitution reactions were carried out in the same way, except that the reaction also contained 400 μM BrdUTP (Sigma). Cesium chloride gradients were performed using published procedures (24), except that before loading the gradient the samples were digested with RNase A and EcoRI. The percentage of rereplication was calculated as the number of counts in the heavy-heavy peak divided by the number of counts in the heavy-light peak multiplied by 100. We found that the amount of rereplication induced by a given mutant was variable from extract to extract. When two mutants were to be compared, they were always analyzed in the same extract. The experiment was repeated multiple times using different extracts, and the paired Student’s t test was used to calculate p values. Individual experiments were excluded from analysis if the amount of replication was less than 25% of an untreated control reaction. To measure Cdt1 ubiquitylation, replication extracts were supplemented with 4 μM methylated ubiquitin (Boston Biochem), and chromatin was isolated as previously described (7).

**Protein Binding Assays**—RNAs encoding Myc-tagged Cdt1 proteins were synthesized in vitro using SP6 polymerase and injected into stage VI oocytes. Oocyte maturation was induced with 2 μM water-soluble progesterone (Sigma). Oocytes that underwent germinal vesicle break down were homogenized in Embryo Extract Buffer (80 mM β-glycerolphosphate, pH 7.4, 15 mM MgCl₂, 20 mM EGTA), and particulates were removed by centrifugation. Myc-Cdt1 was precipitated from the cleared lysate with Myc antibody-coated protein A beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the precipitates were washed sequentially with Embryo Extract Buffer and then with Immunoprecipitation Wash Buffer (50 mM β-glycerolphosphate, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 100 mM NaCl, 10 μg/ml each leupeptin, pepstatin, and chymostatin), all at room temperature. The precipitates were separated on polyacrylamide gels and blotted for Geminin or Myc using standard procedures.

**Embryo and Oocyte Injection**—Stage VI *Xenopus* oocytes and two-cell embryos were injected with RNA or anti-Geminin oligonucleotide using published procedures (25). When the injected embryos reached stage 10.5, they were blindly scored for sectors showing a cell cycle arrest and then homogenized in Embryo Extract Buffer for immunoblot analysis.
RESULTS

The Phenotype of Geminin-depleted Embryos Is Reproduced by Expressing a Non-Geminin-binding Cdt1 Mutant—If the phenotype of Geminin depletion is caused by increased Cdt1 activity, then it should be reproduced by expressing a Cdt1 mutant that is not regulated by Geminin. To map the site on Xenopus Cdt1 that binds Geminin, we constructed a panel of Myc-tagged Cdt1 deletion mutants (Fig. 1A). RNA encoding each mutant was injected into stage VI Xenopus oocytes, and Cdt1 synthesis was induced with progesterone. Myc-Cdt1 was immunoprecipitated using anti-Myc antibody, and the amount of Geminin in the precipitate was determined by immunoblotting. Geminin was precipitated from lysates of oocytes expressing full-length Myc-Cdt1 but not from lysates of uninjected oocytes or oocytes co-injected with both Myc-Cdt1 RNA and antisense Geminin oligonucleotide (Fig. 1B, lanes 1–4). Geminin also co-precipitated with all Cdt1 deletion mutants that included amino acids 236–253 but not with mutants that lacked this region. These results indicate that the Geminin binding site on Cdt1 lies between amino acids 236 and 253. This region includes the sequence KAPAYQRF (amino acids 241–248), which is highly conserved among Cdt1 orthologs from different species and has been shown to be part of the Geminin binding site in mouse and human Cdt1 (26, 27). Mouse Cdt1 has a secondary Geminin binding site at sequences corresponding to Xenopus amino acids 386–399, but we could not detect binding to this region in our assay (Fig. 1B, lanes 7 and 8).

To see if Cdt1NGB is active, we immunodepleted endogenous Cdt1 from replication extracts made from Xenopus eggs and added back either Cdt1WT or Cdt1NGB that had been translated in reticulocyte lysate (Fig. 1C). Cdt1-depleted extracts do not show any replication activity, whereas mock-depleted extracts show the same amount of DNA synthesis as untreated extracts. Adding back either Cdt1WT or Cdt1NGB restores replication to normal levels (Fig. 1D). Adding recombinant Geminin to the extract completely inhibits the replication activity of Cdt1WT but does not affect the activity of
Cdt1\textsuperscript{NGB} (Fig. 1D). These results indicate that Cdt1\textsuperscript{NGB} is fully active and is not inhibited by Geminin.

We next tested to see if expressing Cdt1\textsuperscript{NGB} in early embryos would reproduce the phenotype of Geminin depletion. We previously showed that depleting Geminin with antisense oligonucleotides causes a cell cycle arrest at the last blastula stage (16). When one blastomere of a 2–4-cell embryo is injected, the arrest is manifest as a sector of abnormally large cells at the injection site (Fig. 2A). The arrest is caused by activation of the checkpoint kinase Chk1, which can be detected on immunoblots by increased phosphorylation at serine 345 using a phosphospecific antibody (Fig. 2D) (16, 28). We injected two-cell Xenopus embryos with 300 pg of RNA encoding either Cdt1\textsuperscript{WT} or the non-Geminin-binding mutant Cdt1\textsuperscript{NGB}. As a positive control, embryos were injected with Geminin antisense oligonucleotides. Immunoblots showing the amount of Cdt1 remaining after various times after the start of replication in untreated extract or Cdt1-depleted extract supplemented with different translated Cdt1 mutants. The asterisk indicates a nonspecific cross-reacting protein. B, replication reactions were untreated or supplemented with methyl-ubiquitin. Chromatin was isolated at the indicated times, and the amount of chromatin-bound Cdt1 was visualized by immunoblotting. The arrowheads indicate unmodified Cdt1 (WT or mutant), and the asterisk indicates a nonspecific cross-reacting protein.

A Nondegradable Cdt1 Mutant Also Reproduces the Phenotype of Geminin Depletion—During S phase, Cdt1 activity is removed both by Geminin binding and by ubiquitin-dependent proteolysis (7, 10, 30–32). We next tested to see if expressing a nondegradable Cdt1 mutant would also reproduce the phenotype of Geminin depletion. Degradation of human Cdt1 requires a QRVTDF sequence near the amino terminus and an RRL sequence located further downstream (9, 11). Both these sequences are found in the first 150 amino acids of Xenopus Cdt1 (Fig. 1A). We deleted the first 150 amino acids of Xenopus Cdt1 to create a nondegradable mutant, Cdt1\textsuperscript{N150}. Cdt1\textsuperscript{N150} restores replication to Cdt1-depleted extract and is inhibited by the same concentration of Geminin as Cdt1\textsuperscript{WT} (Fig. 1, C and D). To check its stability, Cdt1\textsuperscript{N150} was translated \textit{in vitro} and added back to Cdt1-depleted replication extract containing sperm DNA template. At various times, the amount of Cdt1 remaining was determined by immunoblotting (Fig. 3A). Endogenous Cdt1 is degraded with a half-life of about 40 min, as described previously (7). In vitro-translated Cdt1\textsuperscript{WT} is degraded with about the same half-life. Cdt1\textsuperscript{N150} appears to be completely stable, with a half-life longer than 240 min. To test if...
Cdt1\(^{N150}\) is ubiquitylated, we added methylated ubiquitin to untreated replication extract or to Cdt1-depleted extract supplemented with translated Cdt1\(^{WT}\) or Cdt1\(^{N150}\). Methylubiquitin-Cdt1 conjugates cannot be detected in crude replication extracts but are enriched in the fraction of Cdt1 that binds to chromatin (7). At different times during incubation at room temperature, we isolated the chromatin by centrifugation and determined the amount of ubiquitylated Cdt1 by immunoblotting with anti-Cdt1 antibody (Fig. 3B). Both endogenous Cdt1 and translated wild-type Cdt1 were ubiquitylated, as indicated by a ladder of bands representing differently sized Cdt1-methylubiquitin conjugates. This ladder did not appear when methylubiquitin was left out of the reaction or when the reaction contained Cdt1\(^{N150}\). These results indicate that Cdt1\(^{N150}\) is neither ubiquitylated nor degraded.

To determine if expressing a nondegradable Cdt1 mutant would reproduce the Geminin-deficient phenotype, we injected Xenopus embryos with 300 pg of RNA encoding either Cdt1\(^{WT}\) or Cdt1\(^{N150}\). As expected, the nondegradable Cdt1\(^{N150}\) accumulated to about 8 times the level of wild-type Cdt1 (Fig. 2D, data not shown). Cdt1\(^{N150}\) induced arrested sectors in 66% of the injected embryos (Table 1 and Fig. 4). This indicates that expressing a nondegradable Cdt1 mutant also reproduces the phenotype of Geminin depletion.

When Cdt1\(^{WT}\) RNA was increased to about 20 times the normal level (not shown). This indicates that Cdt1 concentration to (Fig. 2C). Quantitative Western blotting indicated that injecting this amount of RNA increases the Cdt1 concentration to about 20 times the normal level (not shown). This indicates that vast overexpression of Cdt1 is sufficient to overwhelm all inhibitory mechanisms.

Cdt1 Mutants Induce a Second Round of Replication in Xenopus Egg Extracts—To see if the phenotype induced by expressing Cdt1\(^{NGB}\) or Cdt1\(^{N150}\) is due to overreplication of the DNA, we tested if these mutants would cause a second round of DNA replication in Xenopus egg extracts. Wild-type or mutant Cdt1 was translated \(\text{in vitro}\) and added back to Cdt1-depleted replication extract containing \([\alpha-\text{\(32\)P}]\text{dATP}\) and the density label BrdU. After replication was complete, the radioactive product DNA was fragmented with EcoRI, and its density was determined by equilibrium centrifugation on a cesium chloride gradient. If each origin fires only once, the product DNA will be substituted with bromodeoxyuridine on only one strand and will have a heavy-light density. If, however, some origins fire more than once, then some of the product DNA will be substituted with bromodeoxyuridine on both strands and will have a heavy-heavy density. We found in these experiments that the extent of rereplication varied from extract to extract, probably reflecting differences in the quality of the eggs from which they were prepared. To compare two mutants, we tested both in the same extract and repeated the experiment multiple times using different extracts (Tables 1 and 2).

When Cdt1\(^{WT}\) was added back to Cdt1-depleted extract, a trivial amount of heavy-heavy DNA is produced, similar to the amount produced in untreated extracts (2.6 versus 0.4%, \(p = 0.053\)) (Table 1 and Fig. 4). This indicates that \(\text{in vitro}\)-translated Cdt1\(^{WT}\) is regulated normally. When Cdt1\(^{NGB}\) was added back, however, a significant amount of heavy-heavy DNA was produced. In direct pairwise comparisons, this amount was significantly greater than the amount produced in untreated extracts (11.9 versus 0.4%, \(p = <0.001\)) or extracts containing translated Cdt1\(^{WT}\) (10.2 versus 1.2%, \(p = 0.004\)). This indicates that Geminin suppresses rereplication by inhibiting Cdt1. To confirm this result, we depleted both Geminin and Cdt1 from extract and added back translated Cdt1\(^{WT}\). This gave the same amount of rereplication as adding back Cdt1\(^{NGB}\) (14.3 versus 11.5%, \(p = 0.24\)).

We also measured the amount of rereplication in extracts that had been depleted of Geminin, without depleting or adding back Cdt1. We found an average of 4.5% heavy-heavy DNA in 22 independent measurements (Fig. 5B). In direct comparisons, this amount is significantly higher than the amount produced in untreated extracts (4.4 versus 0.3%, \(p = 0.01\)) (Table 1). In contrast, depleting Geminin from metaphase-arrested extracts does not induce rereplication above background (0.7% versus 1.1%, \(p = 0.785\)), as we previously reported (6). This difference seems to be due to the amount of Cdt1 protein remaining in S phase or metaphase extracts after Geminin depletion. Depletion of Geminin from S phase extracts does not affect the Cdt1

### TABLE 1

| Replication conditions | Percentage of heavy-heavy DNA | \(n\) | \(p\) value\(^a\) |
|------------------------|-------------------------------|------|-----------------|
| Untreated              | 0.4                           | 26   |                 |
| CD + Cdt1\(^{WT}\)     | 2.6                           | 21   | 0.053           |
| CD + GD\(^a\) + Cdt1    | 10.9                          | 13   | \(<0.001\)      |
| CD + Cdt1\(^{NGB}\)    | 11.9                          | 9    | \(<0.001\)      |
| CD + Cdt1\(^{N150}\)   | 7.1                           | 9    | 0.002           |
| Untreated M phase extract | 1.1                         | 10   |                 |
| Geminin-depleted M phase | 0.7                         | 7    | 0.785           |
| Geminin-depleted S phase | 4.5                         | 22   | 0.010           |

\(^a\) Compared with untreated extract. Statistically significant differences are shown in boldface type.

\(^b\) CD, Cdt1-depleted.

\(^c\) GD, Geminin-depleted.

### TABLE 2

| GD\(^a\) | CD\(^a\) + GD + Cdt1\(^{WT}\) | CD + Cdt1\(^{NGB}\) | CD + Cdt1\(^{N150}\) | CD + GD + Cdt1\(^{N150}\) |
|---------|------------------------------|---------------------|---------------------|-----------------------------|
| CD + Cdt1\(^{WT}\) | 0.101 (\(n = 18\)) | 0.004 (\(n = 12\)) | 0.004 (\(n = 7\)) | 0.004 (\(n = 8\)) |
| GD      | 0.016 (\(n = 11\))         | 0.007 (\(n = 7\))  | 0.139 (\(n = 10\)) | 0.027 (\(n = 9\))          |
| CD + GD + Cdt1\(^{WT}\) | 0.235 (\(n = 3\))         | 0.206 (\(n = 4\))  | 0.015 (\(n = 4\))  | 0.123 (\(n = 3\))          |
| CD + Cdt1\(^{NGB}\)   | 0.235 (\(n = 3\))         | 0.206 (\(n = 4\))  | 0.015 (\(n = 4\))  | 0.003 (\(n = 9\))          |

\(^a\) GD, Geminin-depleted.

\(^b\) CD, Cdt1-depleted.

*Geminin Prevents Rereplication*
Cdt1 degradation is not sufficient to cause rereplication within a single S phase. Although expressing nondegradable Cdt1 in extracts had no effect by itself, it did enhance the amount of rereplication in geminin-depleted extracts. When Cdt1\textsuperscript{N150} was added back to extract depleted of both Geminin and Cdt1, the amount of rereplication was significantly greater than the amount in Geminin-depleted extracts (7.1 versus 2.6%, \( p = 0.03 \)) or in extracts containing Cdt1\textsuperscript{N150} but not depleted of Geminin (7.1 versus 1.2%, \( p = 0.003 \)) (Fig. 6). These results are consistent with the model that Cdt1 is inhibited both by degradation and by Geminin binding. It also indicates that proteasome inhibitors induce rereplication by stabilizing Cdt1 and not some other protein.

**DISCUSSION**

In this paper, we report that expressing a mutant of Cdt1 that is not regulated by Geminin exactly reproduces the Geminin-deficient phenotype. This finding indicates that, at least in *Xenopus*, the phenotype of Geminin depletion is the consequence of excessive Cdt1 activity. We could also reproduce the Geminin-deficient phenotype in embryos by expressing a non-degradable mutant of Cdt1 or by expressing wild-type Cdt1 at 20–30 times the endogenous level. Overexpressing Cdt1 in embryos probably overwhelms the Geminin-dependent regulatory mechanism by titrating all of the available Geminin.

Cdt1 is an essential replication factor in organisms as diverse as fission yeast and vertebrates, and the protein has no other known biological function. This strongly implies that the Geminin-deficient phenotype is due to an undetectable amount of overreplicated DNA. In support of this hypothesis, we found that expressing a non-Geminin-binding Cdt1 mutant in replication extracts induced sperm DNA templates to undergo a partial second round of DNA replication. We know that this replication occurs within a single S phase, because the cycloheximide in the extract inhibits cyclin synthesis and prevents the extract from entering mitosis. We also observed a smaller but still significant amount of rereplication in extracts that are simply depleted of Geminin. It has previously been reported, both by ourselves and by others, that depleting Geminin from *Xenopus* egg extracts does not cause rereplication (6, 7). We can offer two explanations for the discrepancy between the previous results and those reported here. First, the amount of rereplication in Geminin-depleted extracts is very small, only a few percent. Because of extract-to-extract variability, in many experiments there is no measurable rereplication above background, and statistical analysis is required to show a significant difference. Second, in our previous experiments, we depleted Geminin from metaphase-arrested extracts instead of S phase extracts. Depletion of Geminin from metaphase-arrested extracts removes most of the Cdt1 as well, whereas depleting Geminin from S phase extracts does not affect the Cdt1 level. We hypothesize that when the Cdt1 concentration is very low, Geminin-independent mechanisms (including Cdt1 degradation) are sufficient to prevent rereplication by themselves. The cause of the increased association between Geminin and Cdt1 in metaphase extract is unknown (33).

![Diagram](Image)

**FIGURE 4.** Cdt1\textsuperscript{NGB} expression induces rereplication. Replication extracts were depleted of Cdt1 (CD) and/or Geminin (GD) and supplemented with translated Cdt1\textsuperscript{WT} or Cdt1\textsuperscript{NGB} and BrdUTP. The density of the product DNA was determined on a cesium chloride gradient. **Top**, typical gradient data. **Bottom**, results averaged over several independent measurements made in different extracts. \( p \) values were calculated using the paired Student’s t test. HH, heavy-heavy; HL, heavy-light; LL, light-light.

concentration appreciably, but depletion of Geminin from metaphase extracts removes almost all of the Cdt1 (Fig. 5A, compare lanes 2 and 4). This suggests that Geminin is not required to prevent rereplication when the Cdt1 concentration is very low. It also indicates that the amount of Cdt1 in extracts is in excess of the amount required for DNA replication. Geminin-depleted S phase extracts exhibit significantly less rereplication than Cdt1\textsuperscript{NGB}-containing extracts (5.1 versus 12.3%, \( p = 0.01 \)) (Table 2). We attribute this difference to the greater concentration of in vitro-translated Cdt1\textsuperscript{NGB} protein compared with endogenous protein (about 2-fold; data not shown).

When Cdt1\textsuperscript{N150} is added back to Cdt1-depleted replication extract containing BrdUTP, it supports normal amounts of replication and does not result in the production of heavy-heavy DNA above background (Fig. 6). In direct comparisons, the amount of rereplication with Cdt1\textsuperscript{N150} was not significantly different from untreated extracts (1.3% versus 0.4%, \( p = 0.13 \)) or extracts containing in vitro-translated wild-type Cdt1 (0.8% versus 0.5%, \( p = 0.064 \)). These results indicate that preventing
We could also reproduce the Geminin-deficient phenotype in embryos by expressing a nondegradable mutant of Cdt1. Expressing this mutant in replication extracts causes no significant rereplication by itself but strongly enhances the amount of rereplication seen when Geminin is absent. These results are consistent with the hypothesis that after the first round of DNA synthesis begins, Cdt1 activity is reduced both by Geminin binding and by degradation. Our results agree with previous reports that the nonspecific proteasome inhibitor MG132 has no effect in untreated extracts but induces a second round of initiation in Geminin-depleted extracts (7, 8, 12). Here we extend these results by showing that Cdt1 itself is the critical stabilized protein. The model that Cdt1 is inhibited both by Geminin binding and by degradation explains why expressing Cdt1NGB causes more rereplication than depleting Geminin. We added about twice as much Cdt1NGB as is present endogenously, which has the same effect as inhibiting Cdt1 degradation. Cdt1NGB is ubiquitylated and degraded as efficiently as Cdt1WT (Fig. 3), indicating that Cdt1 degradation and Geminin binding are independent events, as shown previously (7).

To explain why Geminin-depleted Xenopus embryos accumulate little or no excess DNA during the first 12 embryonic cell cycles, we hypothesize that the mechanisms that regulate Cdt1 prevent rereplication during G2 phase but that a Cdt1-independent mechanism prevents rereplication during S phase. During the first 12 cell cycles, S and M phase alternate in rapid succession, and there is no G2 phase. We speculate that under these conditions, rereplication is adequately suppressed by a Cdt1-independent mechanism. Significantly, Geminin-depleted embryos arrest just after the 13th cell cycle, the point in development when G2 phase first appears (34). The theory that Cdt1 regulation operates mostly during G2 phase has some direct experimental support. It has been reported that postreplicative (G2-like) nuclei will replicate a second time when transferred to Geminin-depleted extract but not when transferred to control extract (8). Similarly, we observe significant rereplication when postreplicative nuclei are transferred to fresh extract containing Cdt1NGB but not when transferred to extract containing Cdt1WT (data not shown). These observations indicate that the Geminin is required to inhibit reinitiation after the first round of replication is complete. Moreover, adding excess recombinant Cdt1 to an extract containing postreplicative (G2-like) nuclei induces a second round of DNA synthesis, whereas adding the same amount to an extract with unreplicated (G1-like) nuclei does not (7). This suggests that a Cdt1-independent mechanism prevents reloading of pre-RC during S phase itself.

The extent of rereplication observed in our experiments was rather small, amounting to ~15% of the genome at most. We also observed marked variability in the amount of rereplication from extract to extract. For example, with Cdt1NGB, the amount of heavy-heavy DNA ranged from 5 to 20% of the heavy-light peak. Assuming that the second round of replication starts after the first round is complete, we can attribute this variability to differences in the quality of the extracts made from different
batches of eggs. Lower quality extracts might lose activity before extensive rereplication can occur, and all extracts may lose activity before the second round can be completed. We found much less variability when the same measurement was repeated several times using the same extract.

We propose the following model to explain the phenotype of Geminin depletion in *Xenopus*. Geminin prevents a second round of replication during G2 phase by binding and inhibiting Cdt1. During the first 12 embryonic cell cycles, which lack G2 phase, rereplication is suppressed by mechanisms that do not involve Geminin or Cdt1. At the midblastula transition, when G2 phase is introduced, a small amount of rereplication occurs in the absence of Geminin. This second round of initiation is responsible for the abnormalities in cell differentiation that have been ascribed to Geminin. Such studies would require eliminating Geminin or expressing Cdt1 at later stages of development.

Acknowledgments—We thank Kathryn Shinnick for technical assistance. We thank Sarah Rice and Volodya Gelfand for helpful discussions and critical reading of early versions of the manuscript.

REFERENCES

1. Edgar, B. A., and Orr-Weaver, T. L. (2001) *Cell* **105**, 297–306
2. Blow, J. I., and Dutta, A. (2005) *Nat. Rev. Mol. Cell. Biol.* **6**, 476–486
3. Maiorano, D., Moreau, J., and Mechali, M. (2000) *Nature* **404**, 622–625
4. Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J. J. (2001) *Nat. Cell Biol.* **3**, 107–113
5. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) *Science* **290**, 2309–2312
6. McGarry, T. J., and Kirschner, M. W. (1998) *Cell* **93**, 1043–1053
7. Arias, E. E., and Walter, J. C. (2005) *Genes Dev.* **19**, 114–126
8. Li, A., and Blow, J. J. (2005) *EMBO J.* **24**, 395–404
9. Arias, E. E., and Walter, J. C. (2006) *Nat. Cell Biol.* **8**, 84–90
10. Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001) *J. Biol. Chem.* **276**, 44905–44911
11. Sena, T., Sivaprasad, U., Zhu, W., Park, J. H., Arias, E. E., Walter, J. C., and Dutta, A. (2006) *J. Biol. Chem.* **281**, 6246–6252
12. Maiorano, D., Krasinska, L., Lutzmann, M., and Mechali, M. (2005) *Curr. Biol.* **15**, 146–153
13. Nguyen, V. Q., Co, C., and Li, J. I. (2001) *Nature* **411**, 1068–1073
14. Yanagi, K., Mizuno, T., Tsuyama, T., Tada, S., lida, Y., Sugimoto, A., Eki, T., Enomoto, T., and Hanaoka, F. (2005) *J. Biol. Chem.* **280**, 19689–19694
15. Quinn, L. M., Herr, A., McGarry, T. J., and Richardson, H. (2001) *Genes Dev.* **15**, 2741–2754
16. McGarry, T. J. (2002) *Mol. Biol. Cell* **13**, 3662–3671
17. Gonzalez, M. A., Tachibana, K. E., Adams, D. J., van der Weyden, L., Hemberger, M., Coleman, N., Bradley, A., and Laskey, R. A. (2006) *Genes Dev.* **20**, 1880–1884
18. Del Bene, F., Tessmar-Raible, K., and Wittbrodt, J. (2004) *Nature* **427**, 745–749
19. Kroll, K. L., Salic, A. N., Evans, L. M., and Kirschner, M. W. (1998) *Development* **125**, 3247–3258
20. Luo, L., Yang, X., Takihara, Y., Knoetgen, H., and Kessel, M. (2004) *Nature* **427**, 749–753
21. Seo, S., Herr, A., Lim, J. W., Richardson, G. A., Richardson, H., and Kroll, K. L. (2005) *Genes Dev.* **19**, 1723–1734
22. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. McGarry, T. J. (2005) *Methods Mol. Biol.* **296**, 263–278
24. Hutchison, C. I. (1993) in *The Cell Cycle: A Practical Approach* (Fantes, P., and Brooks, R., eds) pp. 188–189, Oxford Press, Oxford, UK
25. Heasman, J., Holwill, S., and Wylie, C. C. (1991) *Methods Cell Biol.* **36**, 213–230
26. Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., and Cho, Y. (2004) *Nature* **430**, 913–917
27. Saxena, S., Yuan, P., Dhar, S. K., Senga, T., Takeda, D., Robinson, H., Kornbluth, S., Swaminathan, K., and Dutta, A. (2004) *Mol. Cell* **15**, 245–258
28. Benjamin, J. M., Torke, S. J., Demeler, B., and McGarry, T. J. (2004) *J. Biol. Chem.* **279**, 45957–45968
29. Ferenbach, A., Li, A., Brito-Martins, M., and Blow, J. J. (2005) *Nucleic Acids Res.* **33**, 316–324
30. Zhong, W., Feng, H., Santiago, F. E., and Kipreos, E. T. (2003) *Nature* **423**, 885–889
31. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) *J. Biol. Chem.* **278**, 30854–30858
32. Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004) *J. Biol. Chem.* **279**, 30807–30816
33. Hodgson, B., Li, A., Tada, S., and Blow, J. J. (2002) *Curr. Biol.* **12**, 678–683
34. Iwao, Y., Uchida, Y., Ueno, S., Yoshizaki, N., and Masui, Y. (2005) *Dev. Growth Differ.* **47**, 283–294