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# Control Of DNA Replication By The Cdt1/Geminin Complex

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# ABSTRACT

## Control Of DNA Replication By The Cdt1/Geminin Complex

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It is critical to genomic integrity that DNA is replicated completely and faithfully during each cell cycle. The essential replication factor Cdt1 is a critical protein in preventing re-initiation of replication. Overexpression of Cdt1 causes re-replication in p53 null cells and transforms NIH3T3 cells, causing them to form tumors in nude mice. We sought to determine the mechanisms that shut off Cdt1 activity and prevent re-replication.

We developed an *in vitro* system using replication extract from *Xenopus laevis* eggs to analyze the mechanisms that regulate Cdt1. We found that the C-terminus of Cdt1 is required for replication whereas the N-terminus is dispensable, suggesting this region plays a regulatory role. We mapped the degradation signal and binding site for the replication inhibitor Geminin to the N-terminus. We found that both ubiquitin-dependent degradation and binding to Geminin shut off Cdt1 and prevent re-replication. Our data suggests that Geminin is required to prevent re-replication specifically during G2 phase. We found that Cdt1 is phosphorylated on 15 sites during metaphase. Cdt1 with mutations at ten putative CDK phosphorylation sites shows variable activity, suggesting that phosphorylation may consist of both activating and inhibitory mechanisms.

We also tested the activity of Cdt1 mutants *in vivo* using Xenopus as a model organism. Geminin deficient Xenopus embryos arrest in G2 phase of the cell cycle because of activation of the replication checkpoint. We show here that expression of a Cdt1 mutant that does not bind

Geminin or a mutant that is not degraded reproduces the phenotype of Geminin deficiency. This suggests that these mechanisms shut off Cdt1 *in vivo* and that the cell cycle arrest seen in Geminin deficient Xenopus embryos is caused by re-replication.

Geminin interacts with a number of transcription factors and chromatin remodeling proteins in ways that suggest it may inhibit differentiation when cells are proliferating during development. We show that Geminin deficient Xenopus embryos fail to express the early embryonic genes Brachyury and Goosecoid. This gene expression defect is reproduced by expressing mis-regulated Cdt1, suggesting it is a secondary effect of the cell cycle arrest caused by re-replication.

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## List of Abbreviations

| | |
|---|---|
| S phase | synthesis phase |
| G2 phase | gap 2 phase |
| MPF | maturation promoting factor |
| 6-DMAP | 6-dimethylaminopurine |
| BrdUTP | bromodeoxyuridine |
| Pre-RC | pre-replication complex |
| ORC | origin recognition complex |
| MCM | minichromosome maintenance |
| CDK | cyclin dependent kinase |
| RPA | replication protein A |
| UV | ultra violet |
| APC | anaphase promoting complex |
| C. elegans | Caenorhabditis elegans |
| ATR | ataxia-telangiectasia related |
| MBT | mid-blastula transition |
| bHLH | basic helix-loop-helix |
| Hox | homeobox |
| NPI | Nottingham prognostic index |
| ER | estrogen receptor |
| IPTG | isopropyl-beta-D-thiogalactopyranoside |
| HSS | high-speed supernatant |
| NPE | nucleoplasmic extract |

| | |
|---|---|
| DAPI | 4',6-diamidino-2-phenylindole |
| TCA | trichloroacetic acid |
| TE | Tris-EDTA |
| EDTA | ethylenediamine tetraacetic acid |
| RTP | replication termination protein |
| CD | Cdt1 depleted |
| GD | Geminin depleted |
| NGB | non-Geminin binding |
| PCR | polymerase chain reaction |
| PCNA | proliferating cell nuclear antigen |
| FRAP | fluorescence recovery after photobleaching |
| Xbra | Xenopus brachyury |
| Gsc | Goosecoid |
| Xnr5 | Xenopus nodal related 5 |

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# CHAPTER 1—INTRODUCTION

## *Biological Importance Of Replication Control Mechanisms*

It is critical to genomic integrity that each origin of replication initiate DNA synthesis exactly once per cell cycle. If even a small number of origins were to fire more than once before cell division, over time, greater than normal amounts of DNA would accumulate causing the cells to become aneuploid. Moreover, if the re-replicated region encompassed the centromere the chromosome would either mis-segregate or break during mitosis. This genomic instability may lead to tumor progression through amplification of oncogenes or loss of proper expression of tumor suppressor genes. Re-replication would have such disastrous consequences that cells have evolved multiple mechanisms to ensure that it never occurs. Understanding the molecular mechanisms that regulate replication and prevent re-replication will further our understanding of how cells maintain a stable genome and could help identify cancer treatment targets.

## *Early Evidence For A Mechanism To Prevent Re-Replication*

Initial evidence for a regulatory mechanism to prevent re-replication comes from cell fusion experiments by Rao and Johnson (Figure 1.1) (Rao and Johnson, 1970). They showed that if an S phase cell was fused with a G1 phase cell, the nucleus from the S phase cell would finish replication and the G1 nucleus would prematurely enter S phase and also undergo replication. This suggests that S phase cells contain a replication inducing factor that acts dominantly in G1 phase. Conversely, if an S phase cell was fused with a G2 phase cell that had already completed replication, the S phase nucleus would finish replication but the G2 phase

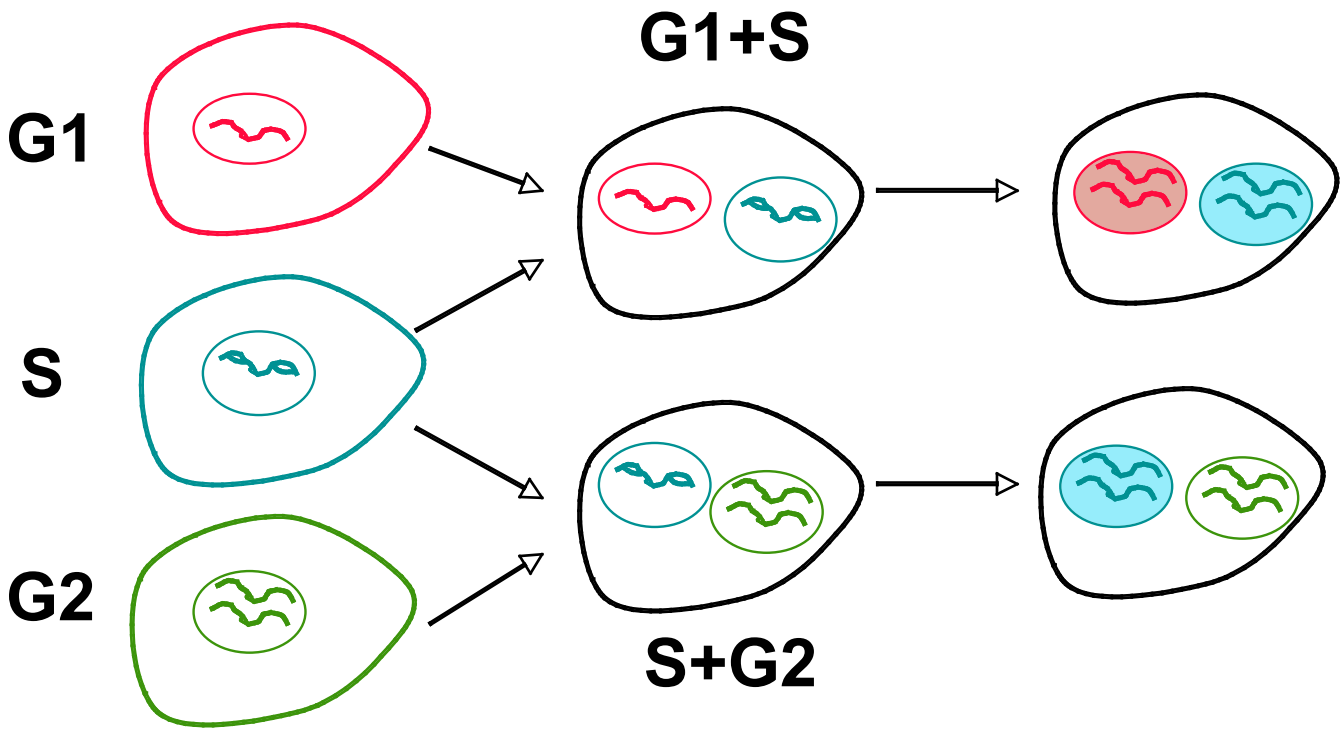



**Figure 1.1 Cell fusion experiments provide evidence for a mechanism to prevent re-replication during G2 phase (Rao and Johnson, 1970).**

nucleus would not to undergo another round of replication. This suggests that G2 phase cells contain an inhibitor of replication that acts dominantly in G2 nuclei.

Studies using a cell extract DNA replication system from Xenopus laevis eggs showed that the nuclear membrane plays an important role in preventing re-replication (Figure 1.2). If post-replicative (G2-like) nuclei are treated with mitotic extract that contains Maturation Promoting Factor (MPF), or Cyclin B/Cdc2, the nuclei re-replicate when transferred to fresh interphase extract (Blow and Laskey, 1988). As treatment with mitotic extract causes nuclear envelope breakdown, this result suggests that the nuclear envelope plays an important role in preventing re-replication. Consistent with this hypothesis, G2-like nuclei re-replicate when treated with lipid membrane destabilizers lysolecithin, Melittin, and phospholipase. These results were later extended to human cells when similar experiments were carried out using nuclei from synchronized HeLa cells in Xenopus replication extract (Leno et al., 1992).

*The Licensing Model For Replication Control*

These results lead to the development of two models to prevent re-replication (Figure 1.3). A positive licensing model states that an essential replication licensing factor is consumed in the nucleus during replication and can not be replenished until the nuclear envelope breaks down during mitosis. This model requires that the licensing factor interacts with chromatin to bring about initiation of replication. This licensing factor must be unable to freely enter the nucleus and must be inactivated following replication initiation (Blow, 1993; Blow and Laskey, 1988). A negative licensing model states that an inhibitor of replication accumulates in the G2 nucleus and prevents re-initiation until after mitosis occurs. The negative licensing factor must act dominantly in G2 nuclei.

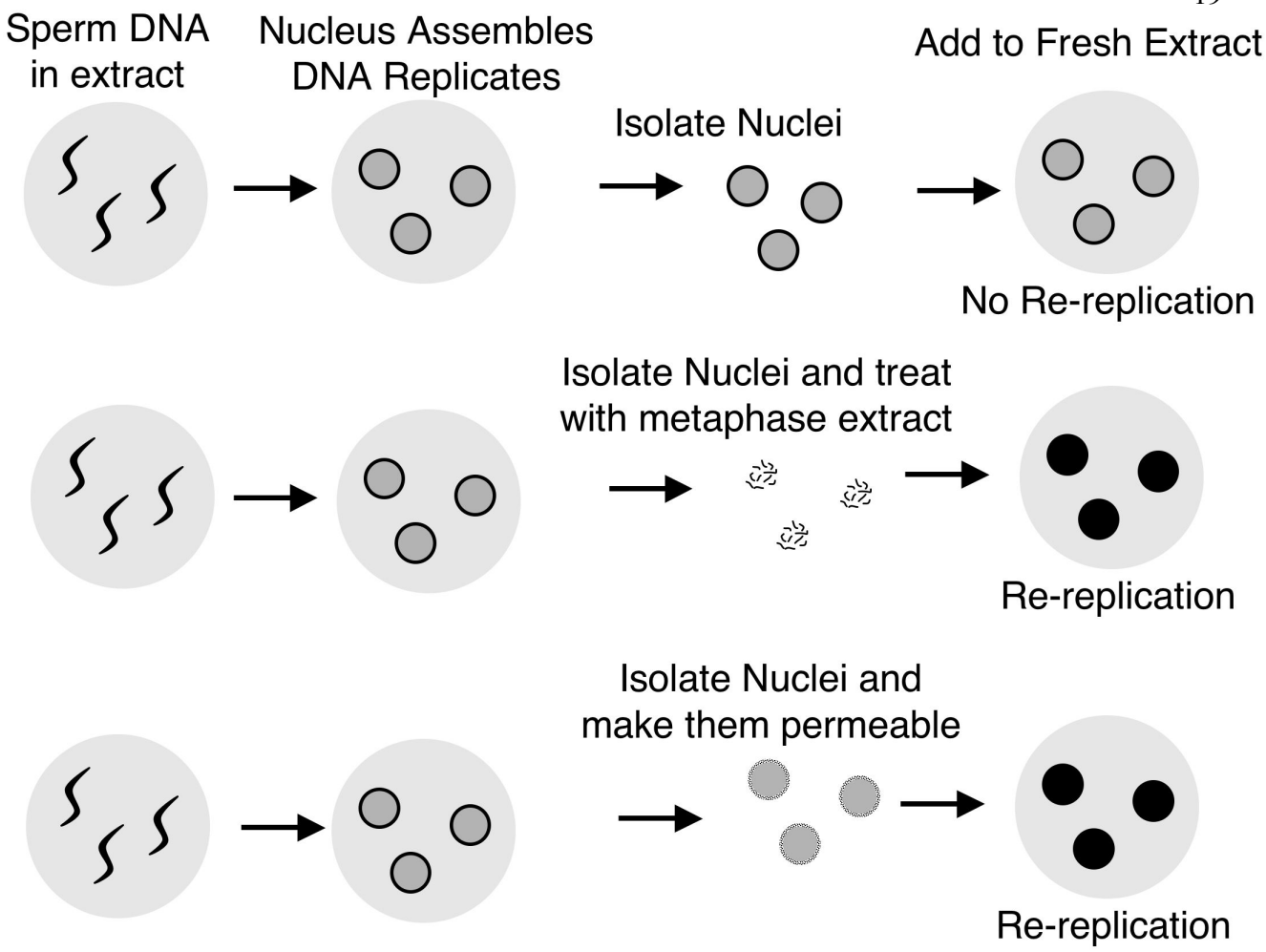



**Figure 1.2 The nucelar envelope prevents re-replication in G2 nuclei.** In tact, post-replicative (G2) nuclei will not re-initiate a second round of replication when transferred to S phase egg extract (top). Treatment with metaphase extract (middle) or lipid membrane destabilizers (bottom) causes the nuclear membrane to dissolve and allows re-replication (modified from Murry and Hunt, 1993).

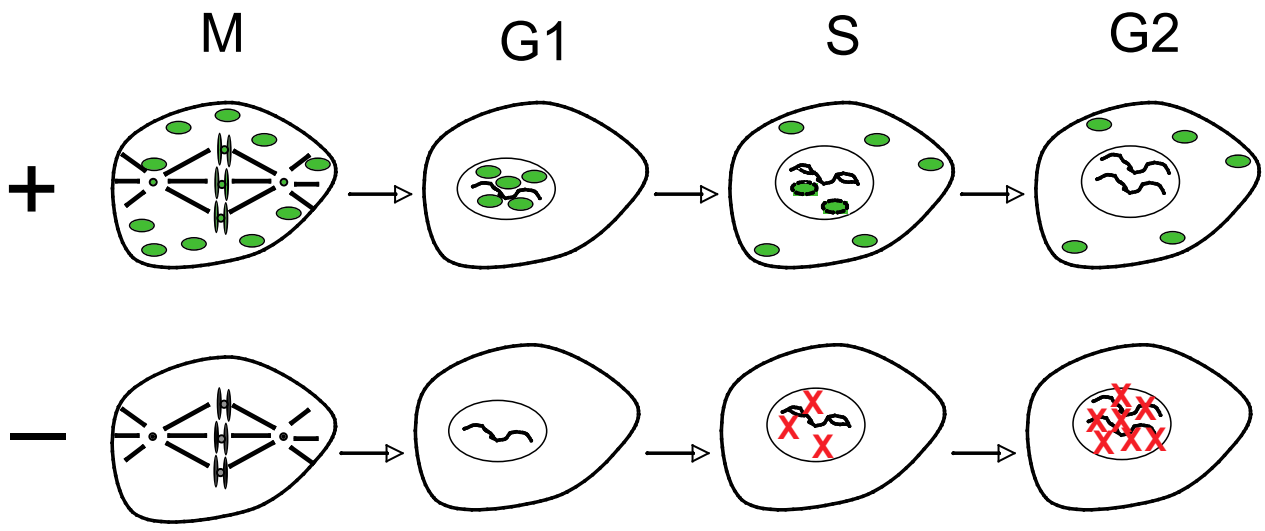



**Figure 1.3 Two models for preventing re-replication within a single cell cycle.** The positive licensing model states that an essential replication factor (green oval) is consumed during replication. The negative licensing model states that an inhibitor of replication (red X) accumulates in the nucleus during G2 phase.

Experiments in Xenopus egg replication extracts provide evidence for the existence of a positive replication licensing factor (Blow, 1993). If Xenopus extract prepared from metaphase arrested eggs is treated with the kinase inhibitor 6-DMAP, replication is inhibited. Normally, if replication occurs in untreated extract in the presence of the density label BrdUTP and then the nuclei are transferred to fresh extract, no further replication can occur. Re-replication does occur if nuclei are permeablized upon transfer to fresh extract. However, when permeablized nuclei are transferred to 6-DMAP containing extract, re-replication does not occur, suggesting that the 6-DMAP inhibits some essential replication factor that normally enters the nucleus of the permeablized nuclei. These results provide evidence that a replication licensing factor exists whose cell-cycle regulated access to DNA plays a role in preventing re-replication.

*Temporal Regulation Of DNA Replication*

Since these early experiments, many of the components involved in replication initiation have been identified, and molecular details provide insight into which components play key regulatory roles in licensing. Formation of the pre-Replication Complex (pre-RC) during G1 phase is an initiating step in the replication process (Figure 1.4). The pre-RC forms through sequential loading of replication factors. The six-subunit Origin Recognition Complex (ORC) is the template upon which the other components assemble; it remains bound to the origin throughout the cell cycle. Once ORC is assembled on origins, Cdc6 binds. Studies in yeast have shown that ORC subunits and Cdc6 possess ATPase activity although it is not entirely clear how this activity is involved in pre-RC assembly or function (Chong et al., 2000).

After Cdc6 associates with ORC, the Cdt1 protein binds and recruits the MCM complex. Cdt1 was first identified in fission yeast as a gene target of the Cdc10/Sct1 transcription factor.

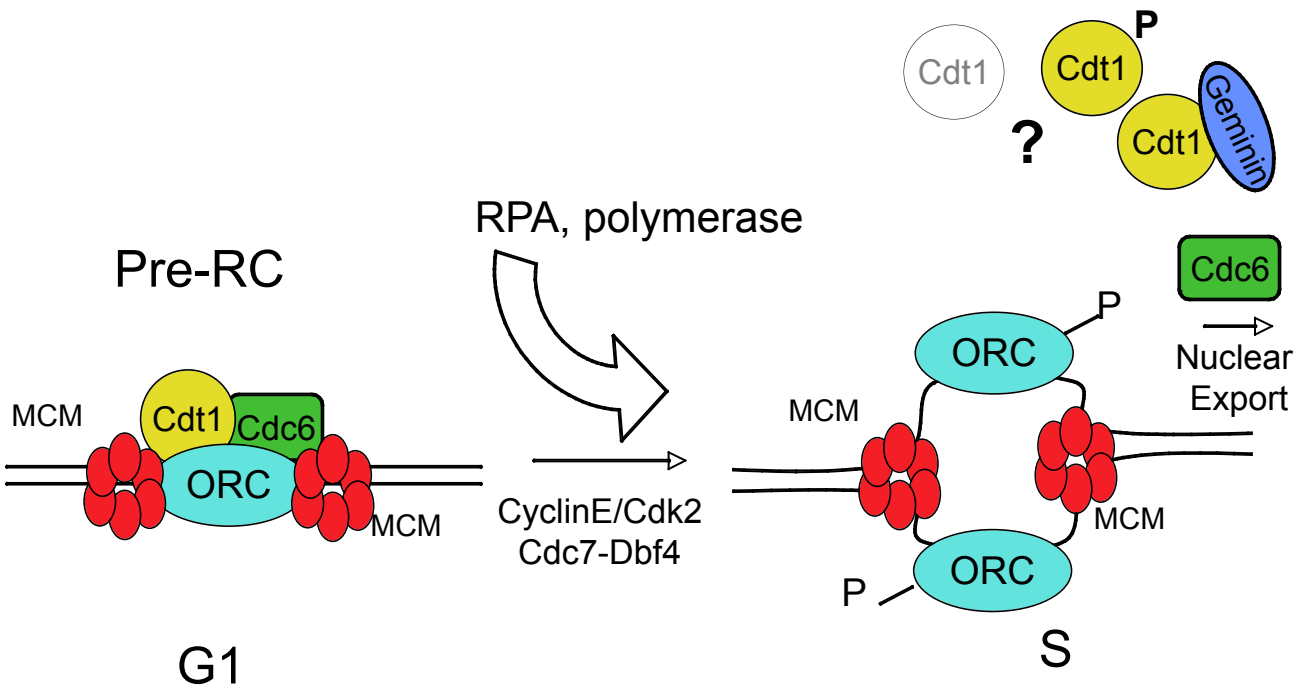



**Figure 1.4 Temporal regulation of licensing.** During G1 phase, replication origins are 'licensed' by regulated, sequential assembly of the pre-replication complex (pre-RC). Once origins fire and replication begins, the pre-RC components must be inactivated so that origins are not re-licensed until the cell divides.

A yeast strain deficient in the Cdt1 gene is defective in DNA replication (Hofmann and Beach, 1994). The requirement of Cdt1 for recruitment of MCM was first shown in Xenopus egg extract (Maiorano et al., 2000). If chromatin is incubated in Cdt1-depleted Xenopus extract, ORC proteins and Cdc6 can associate with the DNA but MCM proteins can not. Similarly , Cdt1 is required for the MCM4 homolog Cdc21 to associate with chromatin in a fission yeast strain expressing Cdt1 under an inducible promoter (Nishitani et al., 2000). Thus, Cdt1 is incorporated into pre-RC sequentially following ORC but is required for MCM incorporation. Consequently, Cdt1 depleted Xenopus egg extract is not able to undergo replication. Cdt1-repressed yeast cells that are arrested in early S phase with hydroxyurea are able to complete replication upon removal of hydroxyurea but are unable to enter another round of S phase. This finding shows that Cdt1 is not required for elongation but rather is required for initiation of replication origins.

Recruitment of the MCM complex completes pre-RC formation and renders origins licensed for replication. Unlike the other pre-RC components, the MCM complex remains associated with replication forks throughout elongation. The MCM complex has DNA helicase activity and is thought to unwind DNA as replication forks proceed (Ishimi et al., 1997 and You et al., 1999). Once pre-RC formation is complete, activity of S phase cyclin/CDK complexes triggers origins to fire and replication begins.

The components of the pre-RC are inhibited by several different mechanisms after replication starts. These mechanisms vary somewhat between yeast and metazoans. Genetic studies in yeast have shown that cyclin-dependent kinases (CDKs) phosphorylate and inhibit the activity of ORC, Cdc6, and MCMs (Nguyen et al. 2001). In mammalian cells, Orc1 is degraded after replication starts (Kreitz et al., 2001). In both yeast and mammalian cells Cdc6 is

phosphorylated by CDKs. In yeast it is subsequently removed by ubiquitin-dependent proteolysis (Drury et al., 1997). In mammalian cells, phosphorylated Cdc6 is exported from the nucleus in a Crm1 dependent manner (Saha et al., 1998). In yeast, MCM proteins are exported from the nucleus during G2 and M phase. MCM2 and MCM4 are phosphorylated by CDKs although the functional consequences are incompletely understood. In yeast, interference with any one mechanism is not sufficient to cause re-replication, but rather several mechanisms must be defeated at once (Nguyen et al., 2001).

*Cdt1: Replication Licensing Factor?*

Recent work implicates Cdt1 as the one critical replication factor that must be shut off in order to prevent re-replication in metazoan cells. This suggests that Cdt1 may be the essential replication factor described in the positive licensing model (Figure 1.3). The mechanisms that shut off Cdt1 activity are incompletely understood.

Cdt1 causes re-replication when overexpressed in cultured cells. Over-expression of Cdt1 in the H1299 human lung cancer cell line results in accumulation of greater than 2n DNA content as measured by propidium idodide staining (Vaziri et al., 2003). The over-replication likely occurs within one cell cycle as the cells were initially synchronized in S phase with aphidicolin and collected before entry into mitosis, which was monitored by phospho-histone H3 staining. Interestingly, this cell line has a mutation in the p53 gene. Overexpression of Cdt1 does not cause re-replication in four other cell lines that have wild type p53. The replication checkpoint was activated in the wild type p53 cell lines, suggesting that this pathway plays a role in protecting cells from Cdt1-induced re-replication. The mechanism through which it does so is not known.

Cdt1 overexpression also causes re-replication in other organisms. Overexpression of Cdt1 in a fission yeast strain that slightly overexpresses Cdc6 results in accumulation of greater than normal amounts of DNA (Nishitani et al., 2000). Over-expression of the Drosophila Cdt1 homolog, double-parked, results in enlarged nuclei, apoptosis, and polyploidy in cells of the ovary and imaginal disc (Thomer et al., 2004).

Because of its central role in preventing re-replication, we are particularly interested in understanding the mechanisms that inactivate Cdt1. Several potential regulatory mechanisms have been suggested to shut off Cdt1 activity. Cdt1 may be inhibited by ubiquitin-dependent proteolysis, phosphorylation by cyclin dependent kinases (CDK), and binding to the inhibitory protein Geminin (Figure 1.4). The importance of these mechanisms in preventing re-replication is not understood and the goal of this study is to determine the relative importance of each.

*Ubiquitin-dependent proteolysis of Cdt1*

The cell-cycle dependent degradation of Cdt1 was first reported in synchronized HeLa cells (Nishitani et al., 2001). Cells were arrested in early S phase by double thymidine block and Cdt1 level was assessed every two hours by immunoblot. Cdt1 was only faintly detectable during late S phase and it accumulated during late M and G1 phase. Similar results were observed when cells were synchronized by treating with nocodazole following thymidine to arrest them in mitosis. To determine whether Cdt1 is regulated at the transcriptional level or the protein stability level, both RNA and protein levels were analyzed in synchronized HeLa cells. While RNA level remained constant throughout the cell cycle, protein level fluctuated as described above. Moreover, treatment of cells with the proteasome inhibitor MG132 caused Cdt1 level to remain constant throughout the cell cycle. It also resulted in detection of a ladder

of higher molecular weight protein by Cdt1 immunoblot, suggesting that Cdt1 proteolysis is carried out by ubiquitin-dependent degradation.

The ubiquitin-dependent destruction of Cdt1 is closely linked to the onset of DNA replication. In Xenopus replication extract, chromatin-bound Cdt1 specifically is targeted for degradation (Arias and Walter, 2005). To measure this, replication extract made from Xenopus eggs is supplemented with methylated ubiquitin, a from of ubiquitin that can be conjugated to a target protein but inhibits further polymerization. In the absence of methylated ubiquitin, Cdt1 was lost from chromatin 30 minutes after the onset of replication. In the presence of methyl ubiquitin, a ladder of high molecular weight Cdt1 was detected upon isolation of chromatin, confirming that ubiquitination of Cdt1 takes place on chromatin during S phase. Furthermore, initiation of DNA replication is required for degradation of Cdt1. Specifically, depletion of either MCM, replication protein A (RPA), or DNA polymerase $\alpha$ from Xenopus extract stabilized total Cdt1 levels and prevented accumulation of ubiquitylated Cdt1 on chromatin (Arias and Walter, 2005).

The importance of Cdt1 degradation as a regulatory mechanism to prevent re-replication has not been firmly established. In C elegans, depletion of the CUL-4 ubiquitin ligase by RNA interference stabilizes Cdt1 during S phase and induces re-initiation of DNA synthesis in some epidermal cells, though a direct link to Cdt1 stabilization was not shown (Zhong et al., 2003). Cdt1 is targeted by the DDB1-CUL4A-ROC1 ubiquitin ligase in response to UV-induced DNA damage, though a role for this mechanism under non-DNA damaging conditions was not reported (Hu et al., 2004). A direct interaction between Cdt1 and Skp2, the substrate recognition subunit of the $SCF^{Skp2}$ ubiquitin ligase, was investigated based on the importance of Skp2 for normal G1/S transition (Willems et al., 1996). Cdt1 and Skp2 co-immunoprecipitate from

cultured cells, and *in vitro* immunoprecipitation assays confirmed that this interaction was direct. When Skp2 was depleted from cultured cells by siRNA techniques, the half life of Cdt1 was extended (Li et al., 2003). However, the importance of Cdt1 stability on re-replication was not investigated.

*Cyclin-dependent kinase-mediated phosphorylation of Cdt1*

Studies in *S. pombe* provide evidence of a role for CDKs in preventing re-replication. Deletion of the gene encoding the mitotic cyclin Cdc13 causes cells to accumulate greater than normal amounts of DNA (Hayles et al., 1994). Similar results are seen when CDK inhibitors are expressed in either S. pombe or S. cerevisiae (Correa-Bordes and Nurse, 1995; Dahmann et al., 1995; Jallepalli and Kelly, 1996; Moreno and Nurse, 1994). In these studies, it is not known whether the cells re-initiate replication within one S-phase or if they do not undergo mitosis properly before re-entering the next S-phase. A specific role of CDKs in preventing reformation of pre-RC is shown in chromatin crosslinking studies showing that high CDK activity prevents association of MCM proteins with chromatin (Tanaka et al., 1997).

Some evidence suggests that CDKs might prevent re-initiation by phosphorylating and inhibiting Cdt1. In Drosophila embryos, myc-Cdk2 co-immunoprecipitates with Cdt1 (Thomer et al., 2004). An in vitro kinase assay using the Cdt1 immunoprecipitate from these embryos shows high H1 kinase activity, suggesting Cdt1 associates with an active kinase, presumably Cdk2. This group also showed that Cdt1 from Drosophila larval brain is phosphorylated at one or more CDK consensus sites. When detected by western blot, Cdt1 is present as two different apparent molecular weights, the higher of which disappears with lambda phosphatase treatment. Overexpression of cyclin E caused enhanced accumulation of the higher molecular weight form.

A Cdt1 mutant with alanine replacing the serine or threonine at ten potential CDK consensus sites ({S/T}PX{K/R}) does not show a molecular weight shift upon overexpression of cyclin E. Expression of this phosphorylation mutant in Drosophila embryos induced polyploidy in wing disc cells of drosophila embryos, though it is not clear whether this was an effect of phosphorylation defects or over-expression (Thomer et al., 2004).

Cyclin/cdk2 and cyclin/cdk4 interact with Cdt1 and promote its phosphorylation in human cultured cells (Liu et al., 2004). Inhibition of Cdk activity by overexpression of p21 or p27 prevents degradation of Cdt1, and a mutant of Cdt1 that does not bind cyclin/cdk complexes and is not phosphorylated is also not degraded. While this group showed that Cdt1 phosphorylation can affect degradation, they did not show a direct functional role for phosphorylation in regulating Cdt1 licensing activity. Another group mapped a cyclin-binding motif to the N-terminus of Cdt1 and showed that this region is required for phosphorylation of Cdt1 *in vitro* (Sugimoto et al., 2004). They showed that CDK-mediated phosphorylation is required for recognition of Cdt1 by the Skp2 subunit of the E3 ubiquitin ligase. Again, a role for phosphorylation in shutting off Cdt1 activity and preventing re-replication was not reported.

*Cdt1 Interacts With Geminin*

As mentioned, the negative licensing model proposes that the nuclear envelope acts to prevent re-initiation by concentrating an inhibitor of replication in G2 nuclei (Figure 1.3). The unstable cell cycle protein Geminin fits the requirements of such an inhibitor. Geminin was first discovered by a screen to identify proteins ubiquitylated by the Anaphase Promoting Complex (APC) during mitosis (McGarry and Kirschner, 1998). In this screen, $^{35}$S-methionine labeled Geminin protein is degraded with a half life of 15 minutes when added to mitotic Xenopus

extract. It is completely stable in interphase extract. The degradation is efficiently inhibited by addition of a competitor peptide containing the destruction box of cyclin B. This peptide is comprised of the sequence RRTLKVIQP and is recognized and ubiquitylated by the APC so that it competes with normal APC substrates. Geminin itself has a similar destruction box which, when mutated, renders the protein stable in mitotic extract (McGarry and Kirschner, 1998). In cultured HeLa cells, Geminin protein accumulates during S phase and disappears at the G2/M transition. This expression pattern is consistent with an inhibitor of DNA replication. In agreement with this model, the non-degradable mutant Geminin$^{DEL}$ inhibits replication in S phase Xenopus extract. Specifically, Geminin$^{DEL}$ prevents pre-RC formation at the MCM binding step, the step at which Cdt1 is required.

Geminin interacts directly with Cdt1 (Tada et al., 2001; Wohlschlegel et al., 2000). Specifically, Cdt1 co-immunoprecipites with Geminin in asynchronously growing cultured cells and in Xenopus replication extract. As mentioned, addition of extra Geminin to Xenopus replication extract inhibits pre-RC assembly at the point of MCM recruitment (McGarry and Kirschner, 1998). Addition of extra Cdt1 restores pre-RC assembly in Geminin treated extract, suggesting that Geminin inhibits replication by binding Cdt1 (Tada et al., 2001). Expression of non-degradable Geminin$^{DEL}$ in Xenopus embryos results in anucleate cells, suggesting that Geminin inhibits replication when overexpressed (McGarry and Kirschner, 1998). This data suggests Geminin may be the replication licensing inhibitor described in the negative licensing model, and that it may inhibit licensing by binding Cdt1 (Figure 1.3).

*Does Geminin Prevent Re-Replication By Inhibiting Cdt1 In Vivo?*

Some observations call into question whether Geminin has a physiological role in inhibiting Cdt1 and preventing re-replication in vivo. Direct attempts to show that Geminin is required to prevent re-replication have proven difficult.

*C. elegans* embryos injected with Geminin siRNA reach adulthood, but a small fraction (~20%) of the individuals are sterile and have morphological defects in germ cell nuclei. Some chromosomal abnormalities were reported such as chromosome bridges in intestinal cells, but no generalized somatic cell cycle defect was reported (Yanagi et al., 2005). Drosophila embryos carrying P-element insertions into the Geminin gene die at larval stages. As in C. elegans, there is no obvious cell cycle defect but there are some indications of aberrant DNA replication, including anaphase chromosome bridges and an increased number of cells in the central nervous system that incorporate BrdUTP (Quinn et al., 2001).

Silencing of Geminin by siRNA in several human cancer cell lines causes accumulation of greater than 2n DNA content (Zhu, W. et al., 2004). Similarly, another group found that silencing of Geminin causes over-replication in both normal human fibroblast cells and tumor cell lines (Melixetian et al., 2004). In these studies, over-replication was not affected by the presence or absence of the p53 gene. Interestingly, overexpression of Cdt1 requires loss of functional p53 in order to induce re-replication (Vaziri et al., 2003). This raises the possibility that loss of Geminin may be affecting something in addition to Cdt1 inhibition.

*Geminin deficiency in Xenopus embryos*

In contrast to other organisms, Geminin deficiency produces a striking phenotype in Xenopus embryos. Geminin-deficient Xenopus embryos undergo twelve apparently normal cell divisions then abruptly arrest in G2 phase after the thirteenth cell division, the time when G2 phase first appears (Figure 1.5) (McGarry, 2002). At the arrest point, the checkpoint kinase Chk1 is phosphorylated on serine 345, indicating that the DNA replication checkpoint has been activated. This phenotype suggests that Geminin depletion causes a small amount of re-replication that is interpreted by the cell as incompletely replicated DNA. However, removal of Geminin from metaphase-arrested Xenopus egg extracts with specific antibodies, does not result in a second round of replication (McGarry, 2002).

The DNA replication checkpoint that is activated in Geminin deficient Xenopus is active during normal ongoing replication so that the cell can not enter mitosis before replication is complete (Figure 1.6). During replication, the ATR kinase localizes to DNA and becomes activated. Active ATR then activates Chk1 by phosphorylating it on serine 345. Phosphorylated Chk1 phosphorylates the Cdc25 phosphatase on an inhibitory serine. This provides a binding site for a 14-3-3 protein which sequesters Cdc25 in the cytoplasm. In the absence of active Cdc25, the Cdc2/Cyclin B complex remains phosphorylated and thus inactive, preventing the cell from entering mitosis.

Activation of this checkpoint in Geminin deficient embryos suggests two models for Geminin activity (Figure 1.6). One model is that Geminin is required for normal progression from G2 to M phase by shutting off the checkpoint to signal that replication is complete at the end of S phase. Another model is that Geminin is required to prevent re-replication, and in Geminin depleted embryos, a small amount of re-replication occurs due to activity of Cdt1. This

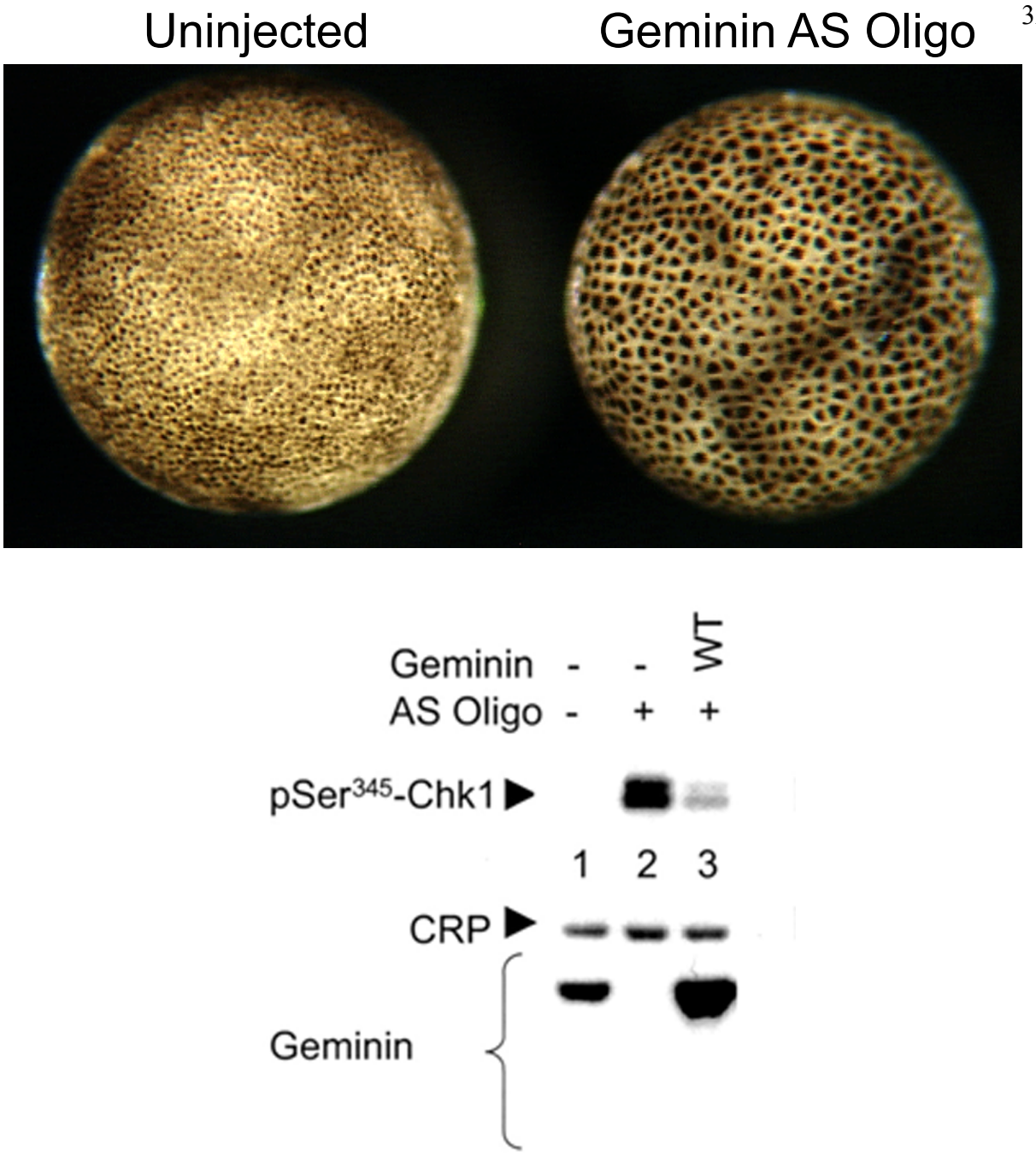



**Figure 1.5 Geminin deficient Xenopus embryos arrest after the mid-blastula transition in G2 phase of the cell cycle.** (Top) Xenopus embryos at the blastula stage. The embryo on the left was untreated and the embryo on the right was injected at the two-cell stage in each cell with Geminin antisense oligonucleotide. (Bottom) Immunoblots of homogenized embryos that were untreaed (lane 1), injected with Geminin AS oligo (lane 2), or injected with Geminin AS oligo + RNA encoding wild-type Geminin (lane 3). Top half was immunoblotted with phospho-serine345 Chk1 antibody; bottom half was immunoblotted with Geminin antibody. CRP is cross reacting protein. (Top; adapted from McGarry, 2002; Bottom adapted from Benjamin et al., 2004).

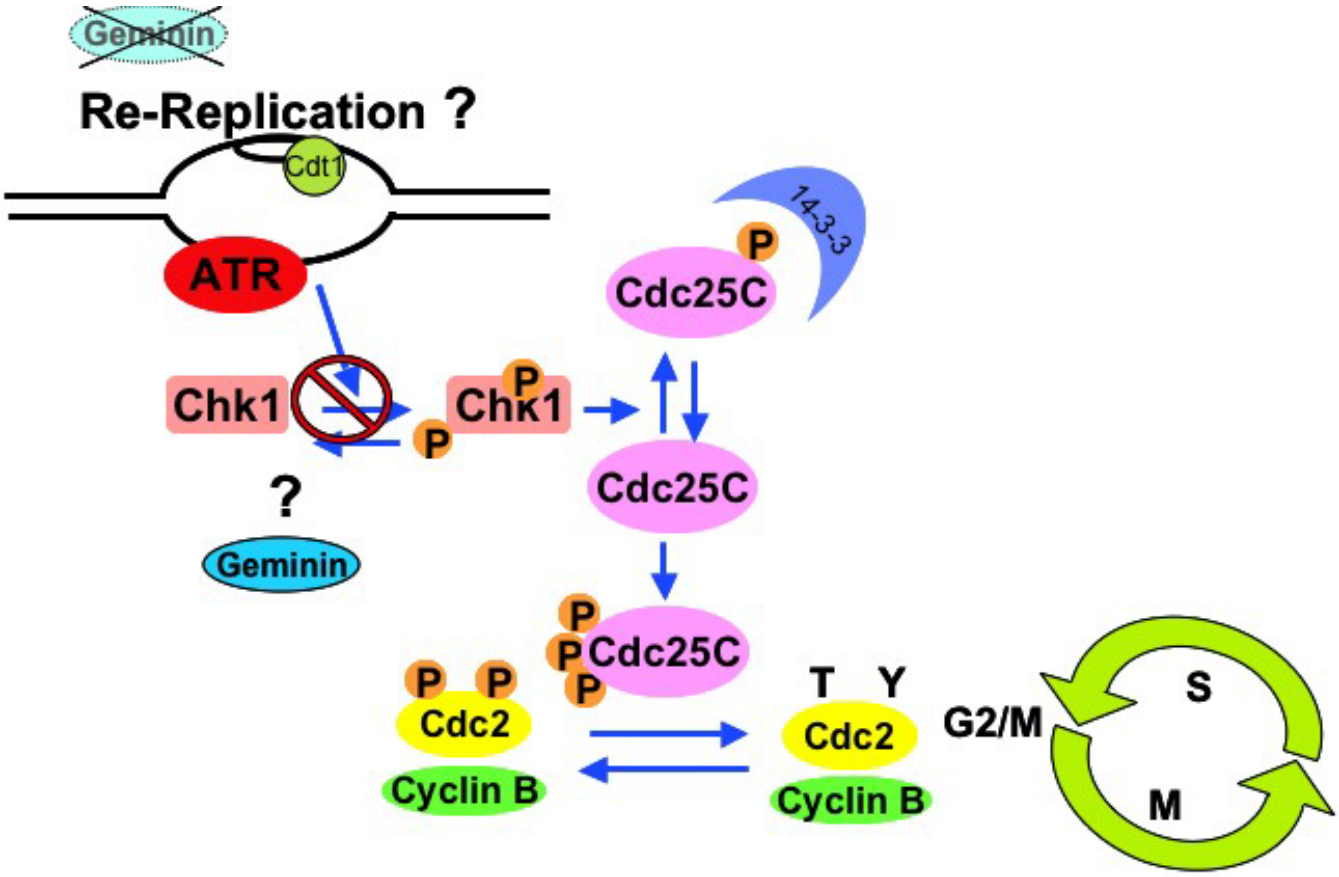



**Figure 1.6 Two models for Geminin activity in Xenopus embryos.** Geminin deficient Xenopus embryos are characterized by a cell cycle arrest accompanied by activated of the replication checkpoint. This phenotype can be explained by two models: 1) Geminin is required for normal mitotic entry and signals to shut off the checkpoint when replicatio is complete, or 2) Geminin is required to shut off Cdt1 activity and prevent re-replication which is sensed by the checkpoint as improper DNA replication.

re-replication is interpreted by the cell as incorrectly replicated DNA, leading to a G2 cell cycle arrest.

*The Role Of Geminin In Cell Differentiation And Development*

In addition to binding Cdt1, Geminin interacts with several different transcription factors and chromatin remodeling proteins that can affect embryonic gene expression. 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. Geminin appears to regulate embryonic development through several different mechanisms. In early Xenopus embryos Geminin inhibits the differentiation of neurons by antagonizing interactions between the chromatin remodeling protein Brg1 and the neuron-specific basic helix-loop-helix (bHLH) transcription factors neurogenin and NeuroD (Seo et al., 2005b). A yeast two-hybrid screen identified the bHLH transcription factor Six3 as a Geminin interacting protein. Mechanistically, Geminin does not inhibit Six3 from interacting with the Six3-binding consensus on DNA, but rather inhibits some downstream step in Six3-dependent transcription. Geminin overexpression in medaka fish embryos inhibits eye and forebrain formation, though direct involvement of the interaction with Six3 was not shown (Del Bene et al., 2004). A yeast two-hybrid screen also identified members of the homeobox (Hox) family of transcription factors as Geminin interacting proteins. In chick embryos Gemimin alters the expression of Hox transcription factors in different body segments by binding both to the Polycomb group protein Scmh1 and to the Hox proteins themselves (Luo et al., 2004). Mechanistically, Geminin interferes with the binding of Hox proteins to DNA. In each of these cases, Geminin is proposed to inhibit cell differentiation until proliferation ceases. In each case, embryos over-expressing Geminin are deficient in

specific types of cells or tissues while embryos depleted of Geminin have an overabundance. It is possible that some of the effects on gene expression and development are due to effects of altered Geminin expression on DNA replication or cell cycle progression.

*Role of Geminin and Cdt1 In Tumorigenesis and Cancer Prognosis*

Geminin has been proposed to act as an indicator of proliferative state in cancer cells because of its expression pattern with respect to the cell cycle. It has also been proposed to act as a tumor suppressor because of its ability to inhibit replication when overexpressed its potential role in preventing genomic instability. Because of these potential diagnostic and therapeutic roles, it is important to understand the complete biological function of Geminin.

Recent clinical studies have identified Geminin as a prognostic marker in several different cancer types. In a study using tissue samples from patients with primary operable breast carcinomas, Geminin expression was positively associated with tumor size, histological grade, Nottingham prognostic index (NPI) score, and tumor type, and negatively associated with estrogen receptor (ER) status (Gonzalez et al., 2004). Geminin expression was also found to be positively associated with overall survival and development of metastases, and the authors concluded that Geminin labeling index improves on current histological methods for predicting patient outcome following breast cancer surgery. This study also highlighted the fact that while other proliferation markers, such as Mcm-2 and Ki-67, are already used as prognostic indicators, they only indicate that cells have exited a quiescent state but do not indicate cells that are actively dividing. Geminin expression would distinguish cells that are actively dividing from cells that are held in G1 phase and more accurately reflect the rate at which cells are progressing through the complete cell cycle.

The activity of Cdt1 and consequences of Cdt1 overexpression suggest that Cdt1 acts as an oncogene. Cdt1 was found to be overexpressed in several tumor cell lines, and NIH3T3 cells overexpressing Cdt1 can form tumors in nude mice (Arentson et al., 2002). Over-expression of Cdt1 in p53 null mice causes lymphoblastic lymphoma and decreased lifespan (5.5 months) compared to p53 null mice expressing normal levels of Cdt1 (10 months) (Seo et al., 2005a). The tumors in $p53^{-/-}$ mice overexpressing Cdt1 are larger and greater in number than those found in mice with p53 loss alone. Chromosome analysis of NIH3T3 cells overexpressing Cdt1 showed translocations, inversions, chromosome end fusions, double minute chromosomes, and robertsonian translocations, suggesting that the increased tumorigenesis in the Cdt1 overexpressing p53 null mice is due to increased genomic instability. In another study, mantle cell lymphomas showed an unbalanced increase in Cdt1 expression relative to Geminin expression (Pinyol et al., 2006). The lymphomas that showed increased Cdt1 expression also showed significantly more chromosomal imbalances as assessed by comparative genomic hybridization, suggesting increased genomic instability.

*Focus Of This Study*

Several pieces of evidence suggest that the interaction between Geminin and Cdt1 plays a key role in the control of DNA replication. We propose that Cdt1 is the positive licensing protein and that Geminin is the negative licensing protein described in the model for preventing re-replication. Cdt1 may also be regulated by Geminin-independent mechanisms, including degradation and phosphorylation. In this study, we investigated the mechanisms that inhibit Cdt1 activity and prevent re-replication. We also sought to determine whether Geminin is required to prevent re-replication *in vivo*. Finally, we sought to determine whether the phenotype

of Geminin deficiency is due to activity of misregulated Cdt1 or some other effect of Geminin on cell cycle progression or gene expression.

# Chapter 2—Materials and Methods

## *I. Plasmid Construction*

Cdt1 deletion mutants were generated by PCR amplification using pBluescript-Cdt1 as a template (Maiorano et al., 2000). Fragments were inserted between the Xho I and Xba I sites of either pCS2 for untagged constructs or pCS2-MT for myc-tagged constructs. Geminin point mutants were generated by PCR amplification using degenerate oligonucleotides. Site-directed mutagenesis was carried out using the Quikchange protocol (Stratagene).

## *II. Protein Expression*

Full-length Xenopus Cdt1 was amplified by PCR and inserted between the Nde I and Xho I sites of pET28(a). The product plasmid was transformed into E. coli strain BL21 and Cdt1 production was induced with IPTG. Cdt1 inclusion bodies were purified by centrifugation and Cdt1 was purified on nickel-agarose columns under denaturing conditions according to standard techniques (Qiagen). The protein was renatured by diluting it twenty-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 DTT) and incubating at 4˚C overnight. Geminin protein was expressed and purified as previously described (McGarry and Kirschner, 1998).

Cdt1-Geminin complex was expressed using the pET-DUET vector. Geminin was inserted between BamHI and EcoRI sites 3’ of the poly-histidine tag. Cdt1 was inserted between NdeI and KpnI sites. The product plasmid was transformed into E. coli strain BL21 and protein

expression was induced with IPTG. Cdt1-Geminin complex and free Geminin was purified on a nickel-agarose column (Qiagen).

*III. Antibody Production*

Cdt1 inclusion bodies were 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 using standard techniques (Harlow and Lane, 1988) . Geminin antibody was described previously (McGarry and Kirschner, 1998). Polyclonal myc antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phospho-serine 345 Chk1 antibody was purchased from Cell Signaling Technology (Beverly, MA). Immunoblots were performed using standard methods. Cdt1, Geminin, and Myc antibodies were used at 1ug/ml and phospho-serine 345 Chk1 antibody was used at 1:1000 dilution. For immunoblot of replication reactions, the equivalent of 1ul of extract for each sample was run on 10% polyacrylamide gels. 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.

*IV Preparation of Xenopus Replication Extract*

S-phase replication extracts were prepared from ionophore-activated Xenopus eggs as described (McGarry, 2005) . The eggs were pre-incubated in MMR containing 100 μg/ml cycloheximide for 15 minutes prior to activation. All solutions used thereafter and the final extract were supplemented with 100 μg/ml cycloheximide. Metaphase extracts were prepared from unactivated eggs using Extract Buffer containing 5mM EGTA. For both types, final extract was prepared by centrifugation for 10 minutes at 9,500g. S-phase extract was supplemented with

cycloheximide (100μg/ml), protease inhibitors (10μg/ml each leupeptin, pepstatin, and chymostatin), cytochalasin B (10ug/ml), and energy mix (150mM phosphocreatine, 20mM ATP, 20mM $MgCl_2$). CSF extract was supplemented with the same additions but CSF energy mix was used (150mM phosphocreatine, 20mM ATP, 20mM $MgCl_2$, 2mM EGTA). Extract was prepared fresh for each experiment.

Extract was immunodepleted of Cdt1 using 1/10 volume Affiprep beads (BioRad) coated with affinity-purified Cdt1 antibody (1 μg antibody/1μl beads) or Geminin using 1/20 volume Affiprep beads coated with affinity-purified Geminin antibody (2ug antibody/1ul beads). Two sequential depletions were performed at 4˚C for one hour each with tumbling.

High-speed supernatant extracts (HSS) were made by centrifugation of S-phase extract for 2 hours at 50,000rpm using an SW-55 rotor. The cytoplasmic layer was removed from the lipid and membrane layers and supplemented with an ATP regeneration system (2mM ATP, 20mM phosphocreatine, 100ug/ml creatine kinase).

To make nucleoplasmic extract (NPE), S-phase extract was supplemented with the ATP regeneration system and 4000 sperm/ul. This nuclear assembly reaction was incubated at room temperature and inverted every 15 minutes. It was monitored for nuclei formation every 15 minutes by fixing 1ul samples with DAPI stain and visualizing nuclei by fluorescence microscopy. Once nuclei form, the reaction incubates for an addition 30 minutes. Nuclei were then collected by centrifugation for 2 minutes at 16,000 rcf at 4˚C in the HB-4 rotor. Nuclei form a clear top layer which is removed, taking care not to remove any of the dark brown layer below. The purified nuclei were then crushed by centrifugation for 30 minutes at 260,000 rcf at 4˚C in the SW-55 rotor. The resulting NPE is removed from the pellet if insoluble material.

*V Replication Assays*

Replication - Replication reactions containing demembranated sperm template and α-[$^{32}$P]-dATP were carried out at room temperature for 90 minutes using standard procedures (McGarry, 2005). The reaction was stopped by adding ten volumes of Replication Stop Buffer (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS). Samples were prepared by proteinase K digestion followed by phenol:chloroform extraction. Total DNA synthesis was measured by TCA precipitation of each sample and scintillation counting of incorporated [$^{32}$P]. Replication was calculated by dividing incorporated counts by total counts (non TCA precipitated sample) and multiplying by 13.1 to account for the amount of sperm DNA template. Cdt1and/or Geminin-depleted extracts were supplemented with either 1/10 volume of Cdt1 protein that had been translated in reticulocyte lysate, Xenopus extract, or recombinant protein as indicated.

Re-Replication - Density substitution reactions were carried out in the same way as standard replication reactions except that the reaction also contained 400 μM BrdUTP (Sigma-Aldrich). Samples were prepared for separation by cesium chloride gradient by proteinase K digestion followed by phenol:chloroform extraction. They were then digested with RNase A (in TE buffer) and EcoRI (in EcoRI buffer; Promega) for 1 hour at 37°C each. DNA was recovered by ethanol precipitation between each digestion. Final samples were diluted into 6ml of 100% CsCl in TE buffer, overlayed with mineral oil, and centrifuged for at least 40 hours at 170,000rcf. Gradients were collected in 30 fractions of 200μl each. Incorporated [$^{32}$P] was measured by TCA precipitation of 50ul from each fraction. The percentage re-replication 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 re-replication 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.

Nuclear Transfer - Standard replication reactions (85μl) were carried out using S-phase extract as described above. After incubation at room temp. for 90 minutes, each reaction was diluted in 400ul of Buffer A (60mM KCl, 1 mM Tris-HCl pH 7.4, 15mM NaCl, 1mM β-mercaptoethanol, 0.5mM spermidine HCl, and 0.15mM spermidine 4 HCl). Each sample was transferred to a 500ul Beckman tube (5 x 41mm) and underlayered with 10ul of Buffer A plus 15% sucrose, then underlayered with 10ul of Buffer A plus 70% sucrose. The samples were centrifuged at 2,500rcf for 2 minutes. The 10ul 70% sucrose layer containing the nuclei was removed and added to 75ul of fresh S-phase extract (either untreated or depleted of Cdt1 or Geminin as indicated). Fresh BrdUTP and [$^{32}$P]-dATP was added and the reactions were incubated at room temp. for an additional 120 minutes. Reactions were prepared and counted as described above.

*VI 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-Aldrich). Oocytes that underwent germinal vesicle breakdown were homogenized in Embryo Extract Buffer (EEB; 80 mM ß-glycerol phosphate 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, Santa Cruz, CA) and the precipitates were washed sequentially with EEB then with IP Wash buffer (50 mM ß-Glycerol Phosphate pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 100 mM NaCl, 10 μg/ml each leupeptin, pepstatin, and chymostatin), all at room temperature. The precipitates were separated on polyacrylamide gels and immunoblotted for Geminin using standard procedures.

*VII Ubiquitylation Assay*

To detect Cdt1 ubiquitylation, replication reactions were supplemented with 4μM methylated ubiquitin (Boston Biochem) and chromatin was isolated as previously described (Arias and Walter, 2005). Specifically, 15ul of replication reaction was dilute into 300ul of cold Extract Buffer containing 0.25% Triton X-100 and underlayered with 300ul of Extract Buffer containing 30% sucrose. The samples were centrifuged for 15 minutes at 15,000rpm in an HB-4 swinging bucket rotor. Liquid was aspirated off leaving approximately 10ul of buffer containing chromatin to which SDS sample buffer was added.

*VIII Embryo Injection*

Xenopus embryos were injected at the two-cell stage with RNA or anti-Geminin oligonucleotide using published procedures (Heasman et al., 1991). Anti-Geminin oligonucleotide was used as previously described (McGarry, 2002). Cdt1 RNA was transcribed from pCS2-Cdt1 plasmid described above (Plasmid Construction). Geminin RNA was transcribed from plasmids previously generated in the lab (Benjamin et al., 2004). The RNA synthesis reactions consisted of 2.5ug linearized plasmid, 1X Sp6 RNA polymerase buffer, 10mM each rNTP, 5mM GppG

cap, 100mM DTT, 50U RNasin, and 40U Sp6 RNA polymerase. When the injected embryos reached stage 10.5 they were blindly scored for sectors showing a cell cycle arrest. Embryos were prepared for immunoblot analysis by homoginization in Gerhart's Extract Buffer (80mM β-glycerol phosphate pH 7.4, 15mM $MgCl_2$, 20mM EGTA, 25nM calyculin A, 0.5mM sodium pervanadate, and protease inhibitors). The extract was centrifuged at 13,000rcf for 5 minutes to separate cytoplasm from lipids and yolk components. The equivalent of 1 embryo per sample was run on 10% polyacrylamide gels for immunoblots.

*IX In Situ Hybridization of Xenopus Embryos*

Embryo Injections - Xenopus embryos were injected with indicated RNA or anti-Geminin oligonucleotide plus β-galactosidase RNA and fixed at the mid-blastula stage (stage 10.5) by tumbling in 5ml of MEMFA (0.1M MOPS pH 7.4, 2mM EGTA, 1mM $MgSO_4$, 3.7% formaldehyde) for 1 hour. For detection of β-galactosidase activity, fixed embryos were incubated in 1X PBS containing 5mM $K_3Fe(Cn)_6$, 5mM $K_4Fe(Cn)_6$, 1mg/ml X-gal, and 2mM $MgCl_2$ at room temp. for approximately 1hour. Embryos were then re-fixed in MEMFA for an additional hour.

Probe Synthesis – Xbra and Gsc probes were made using pGEM-7Z-Xbra and pGEM-7Z-Gsc respectively. Xnr5 probe was made using pBluescriptSK(-)-Xnr5. The probe synthesis reaction consisted of 2.5ug linearized plasmid, 1X Sp6 RNA polymerase buffer (Promega, Madison WI), 2.5mM digoxigenin-NTPs, 100mM DTT, 40U RNasin, and 2U RNA polymerase. Sp6 polymerase was used for pGEM-7Z based plasmids and T7 polymerase was used for pBluescriptSK(-) based plasmid. Reactions were carried out at 37°C for 2 hours. They were

supplemented with DNase and incubated at 37°C for an additional 30 minutes. RNA was recovered by phenol:chloroform extraction and ethanol precipitation.

In Situ Hybridization - In Situ RNA hybridization was carried out essentially as described (Sive et al., 2000) Specifically, embryos were washed once with 1X PBS and three times with 1X PBS, 0.1% Tween-20 (PBST). They were then washed twice with 100mM triethanolamine pH7.6 then twice with 100mM triethanolamine pH7.6 + 12.5ul acetic anhydride per vial, followed by two washes in PBST. All washes were 5-10 minutes at room temp. Embryos were pre-hybridized for 1 hour at 60°C in hybridization buffer (50% formamide, 5X SSC, 1mg/ml Torula RNA, 100ug/ml heparin, 1X Denhart's, 0.1% Tween 20, 0.1% CHAPS, 10mM EDTA), and incubated with the appropriate digoxigenin-labelled RNA probe (1ug/ml) overnight at 60°C. After hybridization, embryos were washed for 30 minutes with hybidization solution followed by two 60 minute washes in 2X SSC, three 60 minute washes in 0.2X SSC, all at 60°C.

Embryos were washed twice in Maleic Acid Buffer, MAB (100mM maleic acid, 150mM NaCl, pH7.5) then incubated in MAB containing 2% blocking reagent (Roche Diagnostics Corporation) for 1 hour at room temp. MAB/2% blocking was replaced with MAB/2% blocking plus alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics Corporation) and embryos were incubated overnight at 4°C. Embryos were washed with MAB several times with at least one wash overnight. Embryos were washed twice with alkaline phosphatase buffer (100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20, 2mM levamisol-added fresh) then incubated in BM Purple substrate reagent (Roche Diagnostics Corporation) until staining became visible (typically 2 hours). The embryos were scored blindly by two people and these scores were averaged for each experiment.

# CHAPTER 3—RESULTS

## Part I: Geminin Binding and Ubiquitin-Dependent Degradation Inactivate Cdt1 and Prevent Re-Replication

### Introduction

Recent work suggests that the essential replication factor Cdt1 plays a key role in limiting replication to exactly once per cell cycle. Cdt1 is part of the pre-RC which licenses DNA for replication during G1 phase of the cell cycle. Over expression of Cdt1 in $p53^{-/-}$ cultured cells or in yeast cells overexpressing Cdc6 causes accumulation of greater than 2n DNA content. Overexpression of Cdt1 in NIH3T3 cells results in chromosomal instability and causes these cells to form tumors in nude mice. The mechanisms that control Cdt1 activity are incompletely understood. Cdt1 is degraded via the ubiquitin-proteasome system and is phosphorylated by several mitotic CDKs. Cdt1 also binds the unstable cell cycle protein Geminin. We investigated the relative importance of each of these potential regulatory mechanisms in preventing re-replication.

### Results

*Development of an in vitro replication system to analyze Cdt1 mutants*

We have developed a Xenopus extract-based system to test the replication activity of Cdt1 mutants (Figure 3.1). Cell cycle extract made from the eggs of the African clawed frog *Xenopus laevis* faithfully reproduces the events of DNA replication that occur *in vivo*. We deplete the endogenous Cdt1 from extract using specific antibodies then add back either wild-

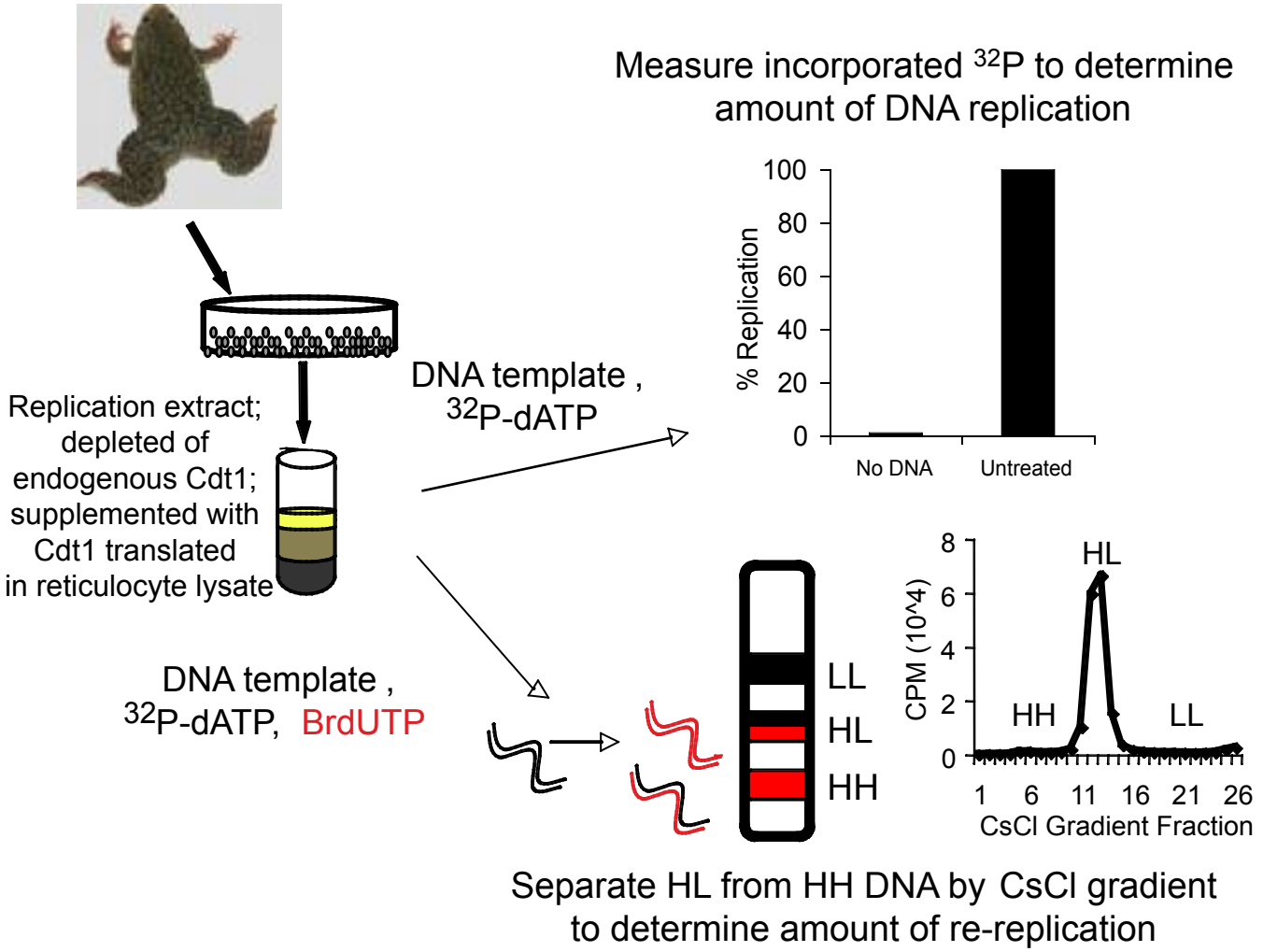



**Figure 3.1 Extract-based system to measure replication and re-replication.** A cytoplasmic extract is made by centrifugation of eggs from Xenopus laevis. The cytoplasm is supplemented with sperm DNA template and [32]-P-dATP, causing nuclei to form and the DNA template to be replicated. Product DNA is TCA precipitated and incorporated [32]-P-dATP is quantified to measure the amount of replication. Re-replicated DNA is quantified by supplementing the extract with BrdUTP and sparating product DNA by CsCl density gradient.

type or mutant Cdt1 that has been produced by in vitro translation using reticulocyte lysate. Replication begins when sperm DNA template and $^{32}$P dATP are added to the extract. The amount of replication is determined by measuring incorporation of $^{32}$P dATP into TCA precipitable material. If we add the density label BrdUTP and separate the replicated DNA by CsCl density gradient, we can measure the amount of re-replication that different mutants support.

To deplete the endogenous Cdt1 from replication extracts, we first raised an antibody against Cdt1. Rabbits were immunized with inclusion bodies from bacteria expressing the protein. The antibody was affinity purified from serum using a column of renatured Cdt1 protein bound to cyanogen bromide sepharose. The immune serum and purified antibody recognize Cdt1 translated in reticulocyte lysate (Figure 3.2, Top). The purified antibody recognizes a band of 75kDa, the calculated molecular weight of Cdt1, in Xenopus oocytes that disappears when the oocytes are injected with anti-Cdt1 oligonucleotide (Figure 3.2, Bottom).

To show that this antibody can be used to deplete Cdt1, replication extracts were prepared from activated Xenopus eggs and treated with affinity-purified Cdt1 antibody bound to protein A beads. This treatment removes >90% of the endogenous Cdt1 as judged by immunoblotting, while nonspecific rabbit IgG has no effect (Figure 3.3, Left). To demonstrate that Cdt1 had been completely depleted, we added sperm DNA as a replication template and $^{32}$P dATP to the extract and measured the incorporation of the label into TCA-precipitable material. Extracts depleted of Cdt1 do not show any replication above background while extracts treated with nonspecific antibody show about the same amount of DNA synthesis as untreated extracts (Figure 3.3, Right). This confirms that our antibody can sufficiently deplete endogenous Cdt1. We could restore replication to Cdt1-depleted extract by adding back Cdt1 that had been

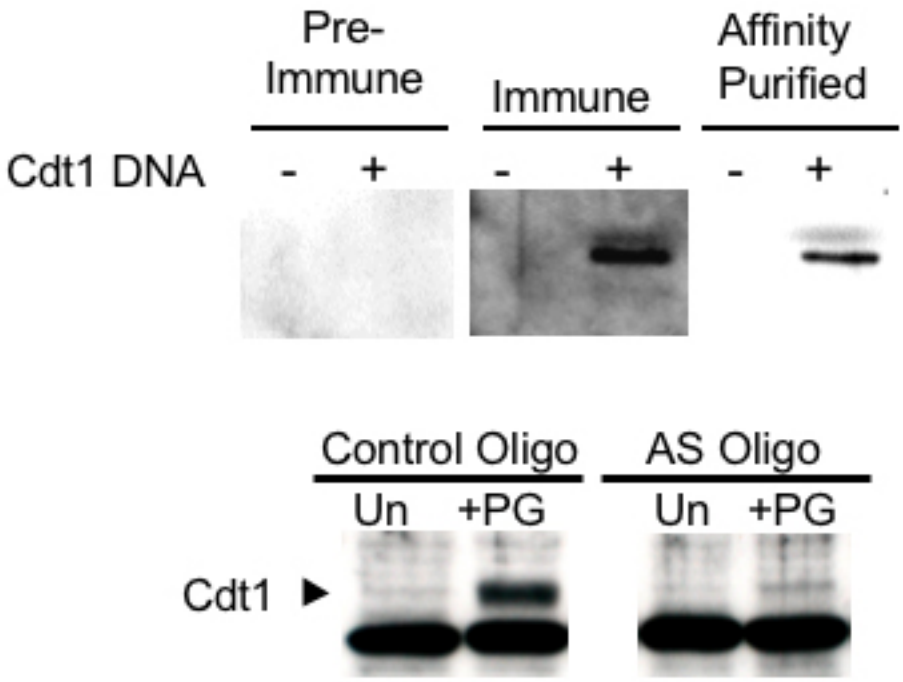



**Figure 3.2 Specificity of Cdt1 antibody.** (Top) Cdt1 DNA was transcribed and translated in reticulocyte lysate and immunoblots were performend using pre-immune serum, immune serum, or affinity purified antibody. (Bottom) Stage VI Xenopus oocytes were injected with anti-Cdt1 (AS Oligo) or control oligonucleotide (Control Oligo), then either induced to enter meiosis with progesterone (+PG) or left untreated (Un). Cdt1 was detected by immunoblot using the affinity purified antibody.

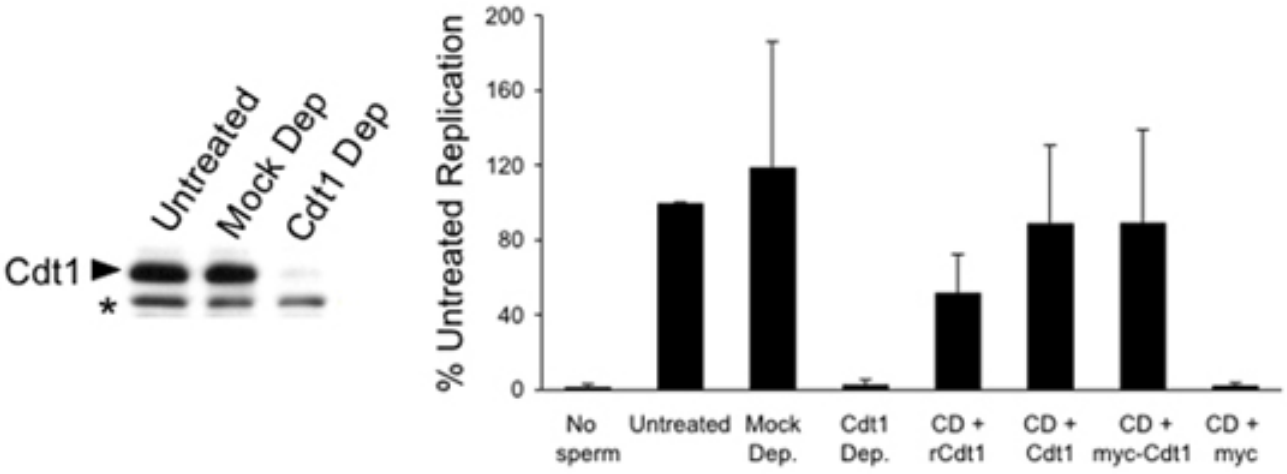



**Figure 3.3 Rescue of replication in Cdt1 depelted extract.** (Left) Extract was untreated, mock depleted with non-specific IgG, or Cdt1 depleted with Cdt1 antibodies, and the amount of Cdt1 was analyzed by immunoblot. (Right) Replication was measured in extract that had been untreated, mock depleted, or Cdt1 depleted and supplemented with Cdt1 that had been translated in reticulocyte lysate or recombinant Cdt1 (rCdt1) purified from bacteria as indicated.

translated *in vitro* using reticulocyte lysate or recombinant Cdt1 renatured from bacterial inclusions. Both untagged and myc-tagged Cdt1 are fully active, while translation of the empty myc-tagging vector has no effect, confirming that replication does not occur in the depleted extract specifically because of the loss of Cdt1. These results demonstrate that this system can be used to study the specific effects of various Cdt1 mutants without interference by endogenous protein.

*Mapping Of Cdt1 Catalytic And Regulatory Domains*

The structure of the Xenopus Cdt1 protein is illustrated in Figure 3.4. The carboxy terminal half of the protein includes a domain that structurally resembles the contrahelicase domain of the replication termination protein (RTP) of Bacillus subtilis and a domain that binds MCM6, a component of the MCM2-7 helicase (Lee et al., 2004; Yanagi et al., 2002). Three regions are predicted to form coiled-coil structures, one located within the MCM6 binding site and the other two in the N-terminus. The protein contains a number of potential CDK phosphorylation sites (SP or TP sites) that are clustered near the amino terminus.

To determine which parts of Cdt1 are required for DNA replication, we constructed a panel of myc-tagged Cdt1 deletion mutants, translated them in reticulocyte lysate, and added each one separately back to Cdt1-depleted replication extract to see how much replication it would support (Figure 3.4). Equal translation of the mutants was demonstrated by an immunoblot using an antibody against the N-terminal myc tag (Figure 3.5, Top). We found that deletion mutants that remove up to 253 amino acids from the N-terminus restore replication to nearly the same level as full-length protein, while mutants that remove residues past amino acid 253 do not restore any replication activity at all (Figure 3.5, Bottom). This indicates that the first

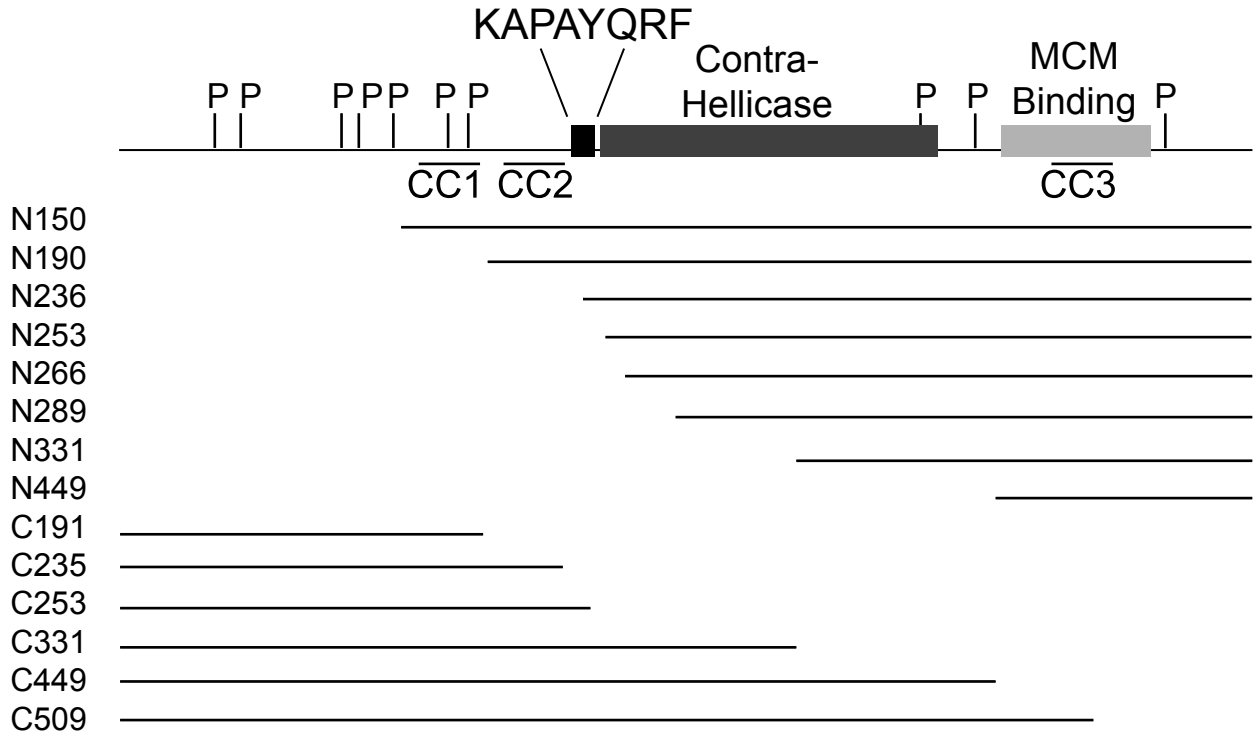



**Figure 3.4 Domain map of Xenopus Cdt1.** (Top) Structural and functional domains are indicated by grey boxes. Putitive CDK phosphorylation sites are labelled P and coiled-coil domains are indicated by CC1-3. (Bottom) Map of Cdt1 deletion mutants used in replication experiments.

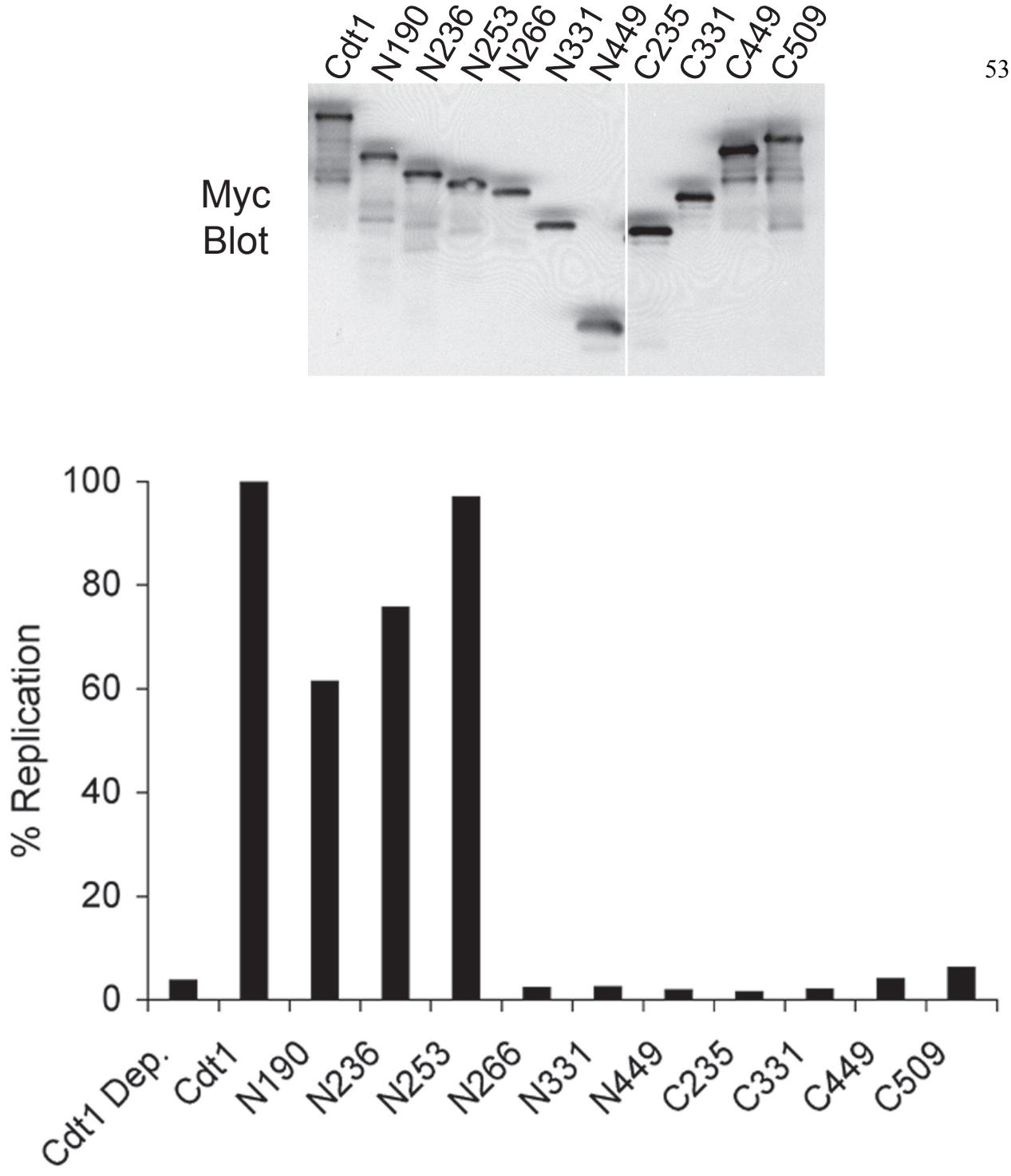


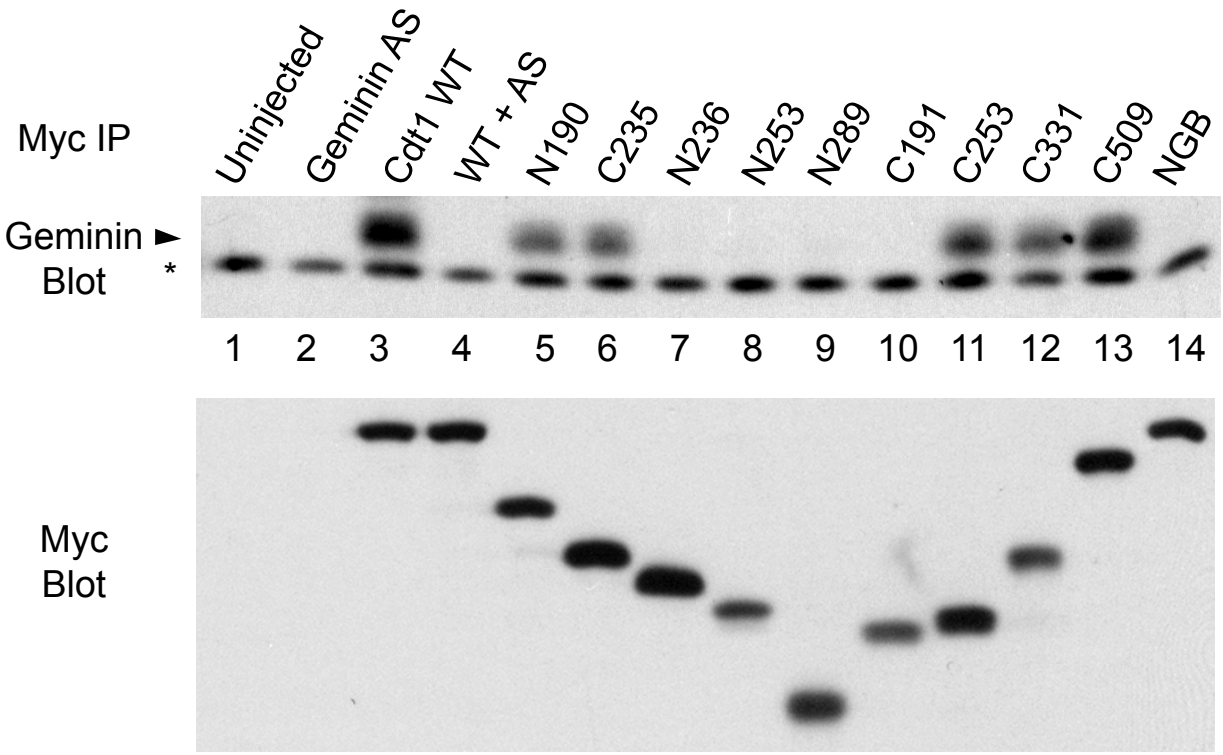

**Figure 3.5 Replication activity of Cdt1 deletion mutants.** (Top) Immunoblot of myc-tagged Cdt1 deletion mutants translated in reticulocyte lysate. (Bottom) Replication was measured in extract depleted of endogenous Cdt1 and supplemented with the indicated myc-tagged Cdt1 deletion mutants. % Replication was normalized to full-length Cdt1.

253 amino acids are not required for DNA replication. In contrast, deletion of as little as 112 amino acids from the carboxy terminus (myc-Cdt1$^{C509}$) completely destroys the protein's replication activity (Figure 3.5, Bottom). We conclude that the C-terminal portion of Cdt1 (amino acids 253-620) is absolutely required for DNA replication while the N terminal portion (amino acids 1-253) is dispensable. We hypothesized that this region serves as a regulatory domain.

*Geminin Binds Cdt1 Between Amino Acids 236 and 253*

To map the site on Xenopus Cdt1 that binds Geminin, we used our panel of myc-tagged Cdt1 deletion mutants in a Geminin binding assay. RNA encoding each mutant was injected into stage VI Xenopus oocytes and the synthesis of Geminin and Cdt1 was induced with progesterone. Myc-Cdt1 was immunoprecipitated using a myc antibody and the amount of Geminin in the precipitate was determined by immunoblotting with Geminin antibody (Figure 3.6, Top). 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 (Figure 3.6, lanes 1-4). Geminin also co-precipitated with all Cdt1 deletion mutants that included amino acids 236 through 253 (Figure 3.6, lanes 5, 6, and 11-13) but not with mutants that lacked this region (Figure 3.6, lanes 7-10). These results indicate that the Geminin binding site on Cdt1 lies between amino acids 236 and 253. This region lies within the domain we found to be dispensable for replication activity, consistent with a role as a regulatory domain.

The Geminin binding region includes the sequence KAPAYQRF (amino acids 241-248), which is highly conserved among Cdt1 orthologs from different species (Figure 3.6, Bottom). It

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | - | E | P | C | G | E | K | A | P | A | Y | Q | R | F | H | A | L | A | Q |
| Mouse | - | Q | P | C | V | E | K | A | P | A | Y | Q | R | F | H | A | L | A | Q |
| Drosophila | M | N | P | H | V | M | T | A | - | R | M | Q | R | F | Q | S | L | L | L |
| Xenopus | - | A | Q | E | S | E | K | A | P | A | Y | Q | R | F | H | N | L | A | Q |
| Cdt1$^{NGB}$ mutant | - | A | Q | E | S | E | A | A | A | A | A | A | A | A | H | N | L | A | Q |

**Figure 3.6 Mapping the Geminin binding site on Cdt1.** (Top) RNA encoding myc-tagged Cdt1 mutants was injected into Xenopus oocytes. The Cdt1 was immunoprecipitated using a myc antibody and Geminin was detected by immunoblot using Geminin antibody. Equal expression of myc-Cdt1 was measured by immunoblot using a myc antibody. (Bottom) Sequence alignment of the Geminin-binding region of Cdt1. The amino acids mutated to alanine are highlighted in the black box.

has also recently been shown to be part of the Geminin binding site in mouse and human Cdt1 (Lee et al., 2004; Saxena et al., 2004). 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 (Figure 3.6, lanes 7 and 8). We constructed a non-Geminin binding mutant, $Cdt1^{NGB}$, by mutating the KAPAYQRF sequence to AAAAAAAA. Geminin does not bind to $Cdt1^{NGB}$ when both are expressed in oocytes (Figure 3.6, lane 14).

To see if $Cdt1^{NGB}$ is active, we immunodepleted endogenous Cdt1 from replication extracts made from Xenopus eggs and added back either $Cdt1^{NGB}$ or $Cdt1^{WT}$ that had been translated in reticulocyte lysate. Cdt1-depleted extracts do not show any replication above background. Adding back either $Cdt1^{WT}$ or $Cdt1^{NGB}$ restores replication to normal levels (Figure 3.7). Adding recombinant Geminin to the extract completely inhibits the replication activity of $Cdt1^{WT}$ but does not affect the activity of $Cdt1^{NGB}$ (Figure 3.7). These results indicate that $Cdt1^{NGB}$ is fully active and is not inhibited by Geminin.

*Geminin is Required to Prevent Re-replication in Extract*

To see if loss of Geminin binding is required to shut off Cdt1 activity, we tested if $Cdt1^{NGB}$ would cause initiation of a second round of DNA replication in Xenopus egg extracts. Wild-type or mutant Cdt1 was translated in vitro and added back to Cdt1-depleted replication extracts containing $\alpha$-[$^{32}$P]dATP and the density label BrdUTP. 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 BrdU on only one strand and will have a heavy-light (HL) density. If, however, some origins fire more than once, then some of the product DNA will be

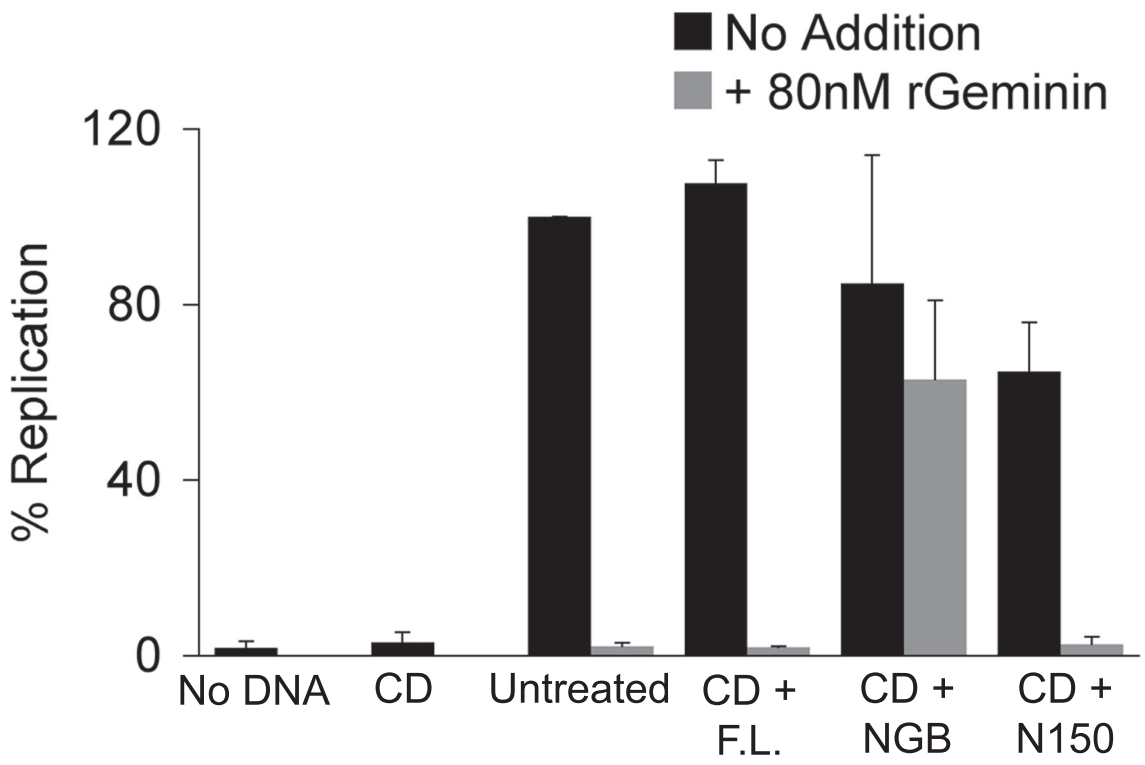


**Figure 3.7 Replication activity of Cdt1 NGB and Cdt1 N150.** Replication was measured in untreated extract, Cdt1 depleted extract (CD), and Cdt1 depleted extract supplemented with Cdt1 translated in reticulocyte lysate as indicated (Black bars). Reactions were repeated in te presence of 80nM recombinant Geminin protein (grey bars). % Replication was normalized to untreated extract.

substituted with BrdU on both strands and will have a heavy-heavy (HH) density. We found in these experiments that the extent of re-replication 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 $Cdt1^{WT}$ is 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% vs 0.4%, $p = 0.053$, Table 1 and Figure 3.8). This indicates that in vitro-translated $Cdt1^{WT}$ is regulated normally. When $Cdt1^{NGB}$ was added back, however, a significant amount of heavy-heavy DNA was produced. We know that this re-replication occurs within a single cell cycle because the extracts contain cycloheximide which inhibits cyclin synthesis prevents the extract from entering mitosis. In direct pairwise comparisons, this amount was significantly greater than the amount produced in untreated extracts (11.9% vs 0.4%, $p = < 0.001$; Table 1) or extracts containing translated $Cdt1^{WT}$ (10.2% vs 1.2%, $p = 0.004$; Table 2). This indicates that Geminin suppresses re-replication 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 re-replication as adding back $Cdt1^{NGB}$ (14.3% vs 11.5%, $p = 0.24$; Table 2).

We also measured the amount of re-replication 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 twenty-two independent measurements (Figure 3.9). In direct comparisons, this amount is significantly higher than the amount in untreated extracts (4.4% vs 0.3%, $p = 0.01$, Table 1). In contrast, depleting Geminin from metaphase-arrested extracts does not induce re-replication above background (0.7% vs. 1.1%, $p=0.785$, Table 1), as previously reported (McGarry and

| | % HH DNA | n | p-value |
|---|---|---|---|
| **Untreated** | 0.4 | 26 | |
| **CD + Cdt1** | 2.6 | 21 | 0.530 |
| **Geminin depleted S phase** | 4.5 | 22 | **0.010** |
| **CD + GD + Cdt1** | 10.9 | 13 | **<0.001** |
| **CD + NGB** | 11.9 | 9 | **<0.001** |
| **CD + N150** | 1.2 | 12 | 0.132 |
| **CD + GD + N150** | 7.1 | 9 | **0.002** |
| **CD + NP** | 1.0 | 4 | 0.408 |
| **CD + GD + NP** | 9.7 | 2 | |
| **CD + myc-Cdt1** | 15.9 | 12 | **<0.001** |
| **Untreated M phase** | 1.1 | 10 | |
| **Geminin depleted M phase** | 0.7 | 7 | 0.785 |

**Table 1. Average extent of re-replication under different conditions.** The percent re-replication was averaged for each condition in different extracts. The number of repetitions in different extracts is shown (n). Statistically significant differences are shown in boldface type. P-values were calculated compared to untreated extract.

| | CD + WT | Gemin in Dep | CD + GD + Cdt1 | CD + NGB | CD + N150 | CD + GD + N150 | CD + NP | CD + myc-Cdt1 |
|---|---|---|---|---|---|---|---|---|
| **Untreated** | 0.053 (n=20) | 0.01 (n=21) | 0.000 (n=13) | 0.000 (n=8) | 0.132 (n=10) | 0.002 (n=9) | 0.408 (n=4) | 0.000 (n=10) |
| **CD + WT** | | 0.101 (n=18) | 0.004 (n=12) | 0.004 (n=7) | 0.064 (n=9) | 0.004 (n=8) | 0.423 (n=3) | 0.000 (n=10) |
| **Geminin Dep** | | | 0.016 (n=11) | 0.007 (n=7) | 0.139 (n=10) | 0.027 (n=9) | 0.460 (n=3) | 0.002 (n=8) |
| **CD + GD + Cdt1** | | | | 0.180 (n=3) | 0.206 (n=4) | 0.147 (n=4) | / (n=2) | 0.713 (n=4) |
| **CD + NGB** | | | | | 0.015 (n=4) | 0.123 (n=3) | 0.041 (n=3) | 0.218 (n=8) |
| **CD + N150** | | | | | | 0.003 (n=9) | / (n=1) | 0.010 (n=5) |
| **CD + GD + N150** | | | | | | | / (n=1) | 0.681 (n=3) |

**Table 2. P values comparing the extent of re-replication under different conditions.** Pairwise comparisons were made between two conditions in the same extract. Statistically significant differences are in boldface type. CD, Cdt1 depleted; GD, Geminin depleted.

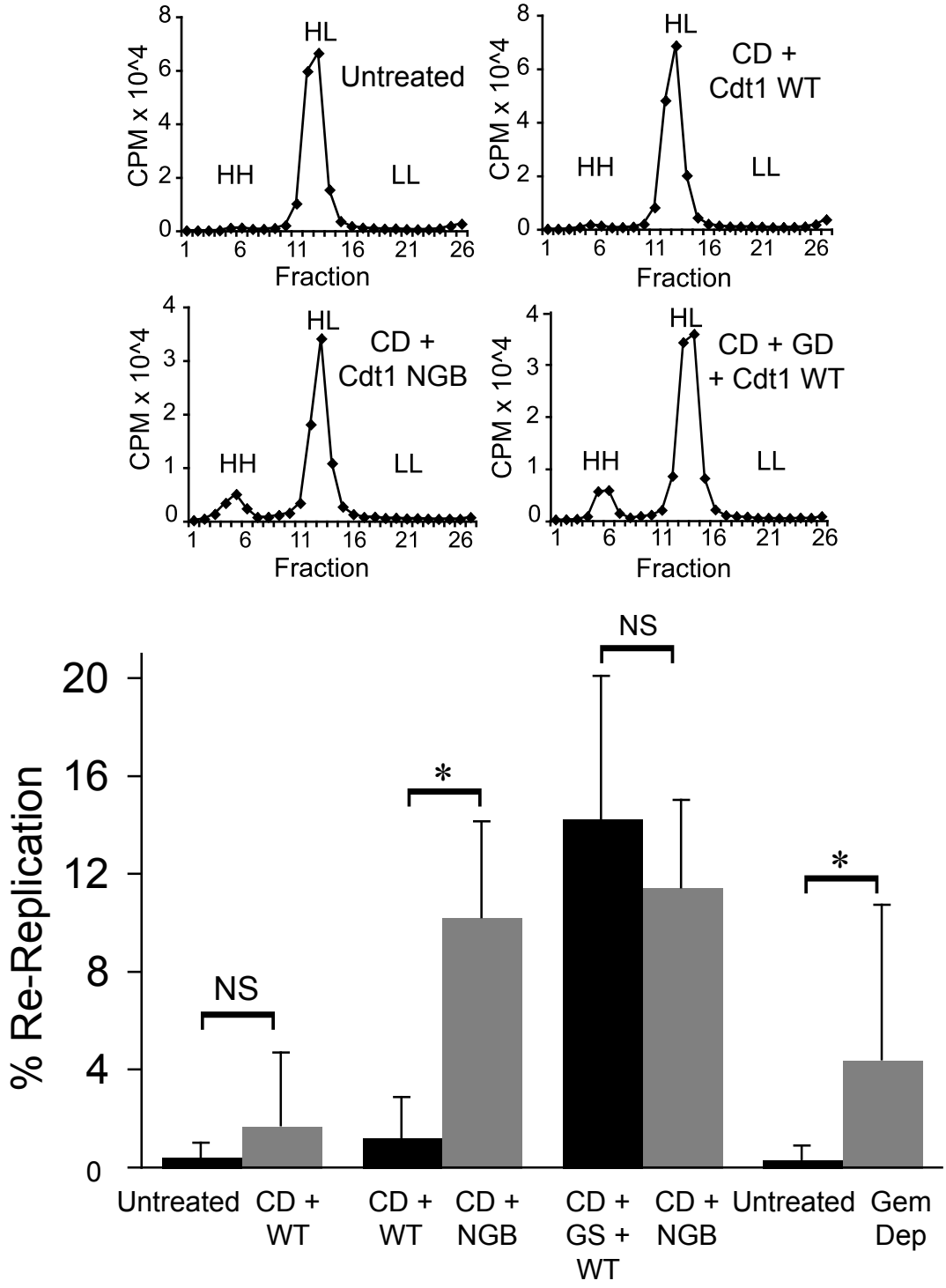


**Figure 3.8 Geminin is required to prevent re-replication within one cell cycle.** Replication extracts containing BrdUTP were depleted of Cdt1 (CD) and/or Geminin (GD) and supplemented with Cdt1 translated in reticulocyte lysate. The density of the product DNA was determined on a CsCl gradient. (Top) representative gradient data. (Bottom) Results averaged over several independent measurements made in different extracts. p values were calculated using the paired Student's t test and statistically significant differences ($p<0.05$) are indicated by the *. NS, not significant; HH, heavy-heavy; HL, heavy-light; LL, light-light.

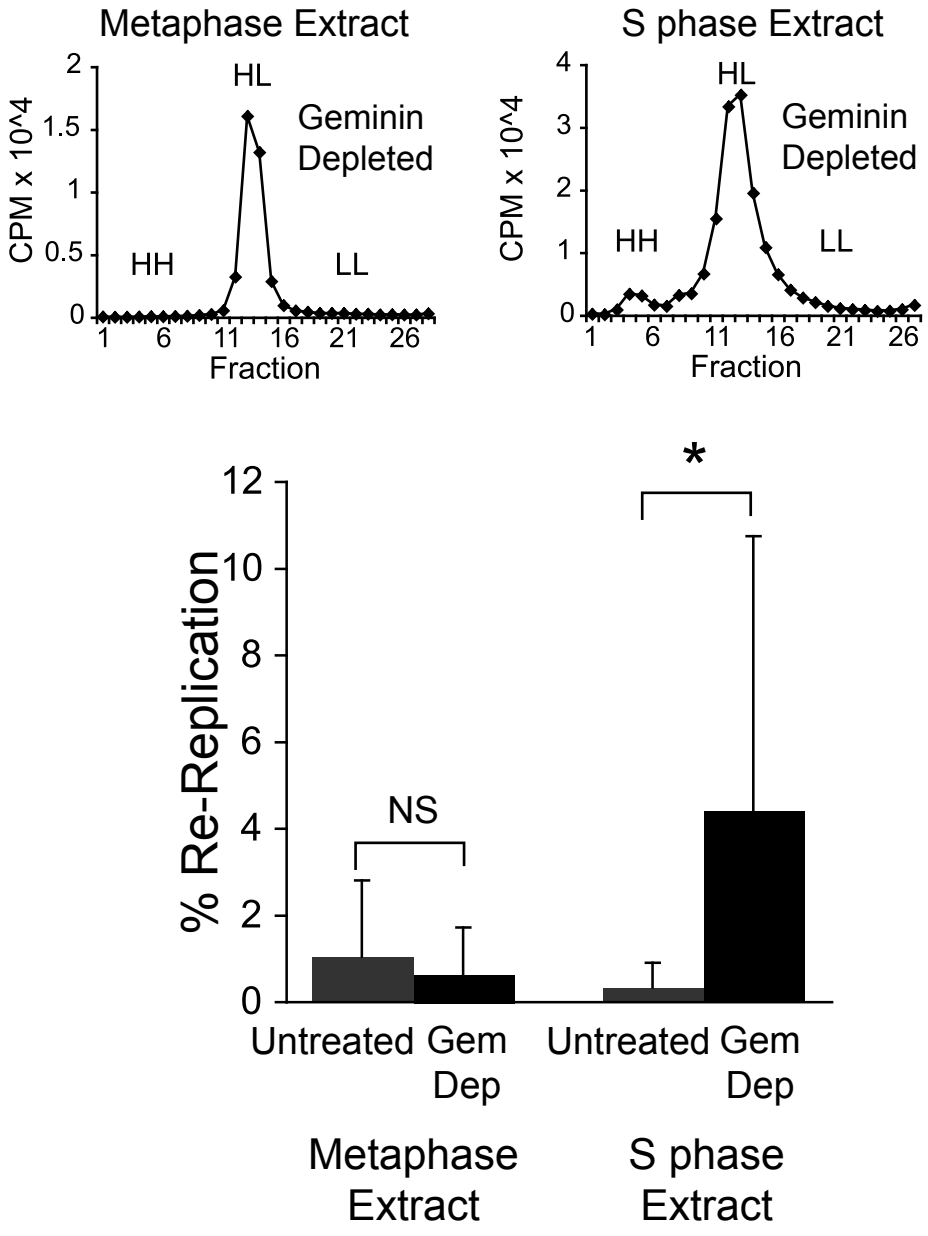


**Figure 3.9 Geminin depletion causes re-replication in S phase extract but not M phase extract.** Replication extracts containing BrdUTP were untreated or depleted of endogenous Geminin. The densityof the product DNA was determined on a CsCl gradient. (Top) Representative gradient data. (Bottom) Results averaged over several independent measurements made in defferent extracts. Statistically significant differences ($p<0.05$) are indicated by the *; NS, not significant; HH, heavy-heavy; HL, heavy-light; LL, light-light.

Kirschner, 1998). 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 removes some of the Cdt1, but depletion of Geminin from metaphase extracts removes almost all of the Cdt1 (Figure 3.10, compare lanes 2 and 4). This suggests that Geminin is not required to prevent re-replication 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 re-replication than $Cdt1^{NGB}$-containing extracts (5.1% vs 12.3%, P=0.01, Table 2). We attribute this difference to the greater concentration of *in vitro*-translated $Cdt1^{NGB}$ protein compared to endogenous protein (about two-fold; Figure 3.11).

*Cdt1 Proteolysis Provides a Secondary Mechanism to Prevent Re-Replication*

During S phase Cdt1 activity is also removed by ubiquitin-dependent proteolysis (Arias and Walter, 2005; Li et al., 2003; Nishitani et al., 2004; Nishitani et al., 2001; Zhong et al., 2003) We added our panel of Cdt1 deletion mutants to replication extract that had been depleted of endogenous Cdt1. At various times after the start of replication the amount of Cdt1 remaining was determined by immunoblotting. Endogenous Cdt1 is degraded with a half-life of about 40 minutes, as described previously (Figure 3.11) (Arias and Walter, 2005) . *In vitro*-translated $Cdt1^{WT}$ is degraded with about the same half-life. $Cdt1^{NGB}$ appears to have a similar half life and we conclude that Geminin binding does not affect Cdt1 degradation. We find that deletion of the first 150 amino acids ($Cdt1^{N150}$) results in a completely stable protein. $Cdt1^{N150}$ appears to have a half-life longer than 240 minutes (Figure 3.11). This suggests that the degradation signal for

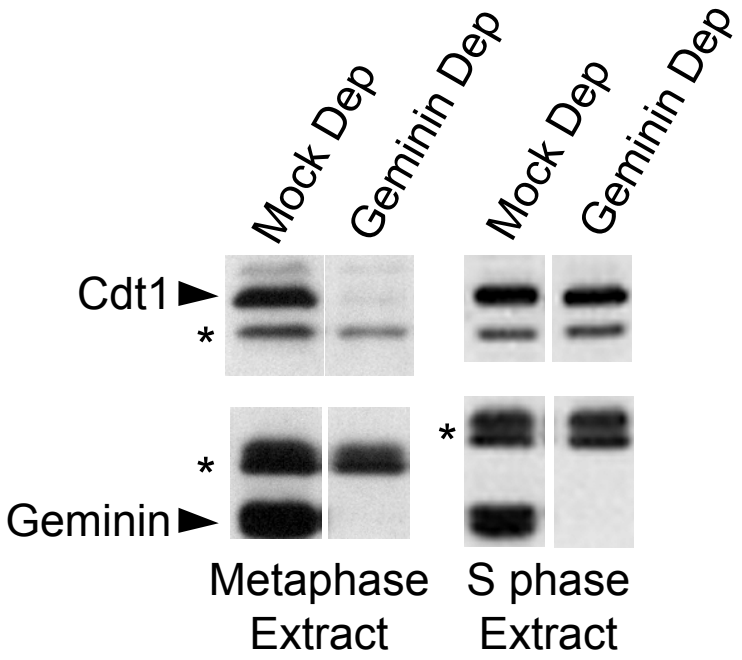


**Figure 3.10 Cdt1 is mostly Geminin-bound in metaphase extract and unbound in S phase extract.** Metaphase or S phase extract was treated with either Geminin antibody (Geminin Dep) or nonspecific rabbit IgG (Mock Dep). The amount of Cdt1 and Geminin remaining after depletion was determined by immunoblot using the indicated antibody. The asterisks indicate cross-reacting proteins.

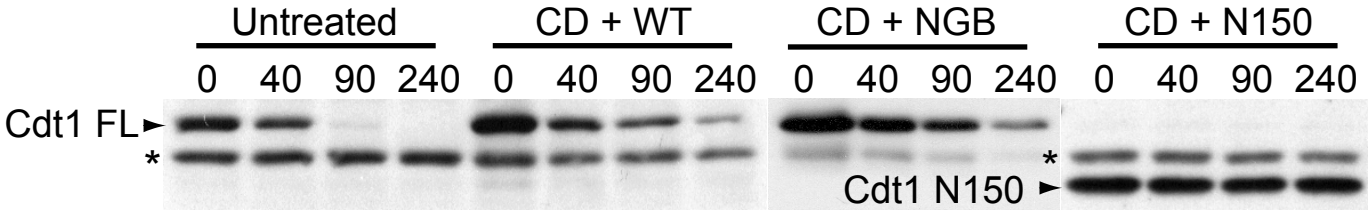


**Figure 3.11 The degradation signal of Cdt1 lies in the N-terminus.** Extracts were untreated or depleted of endogenous Cdt1 (CD) and supplemented with Cdt1 translated in reticulocyte lysate. Immunoblots show the amount of Cdt1 remaining at various times after the start of replication. The asterisk indicates a cross-reacting protein. FL, full length.

Xenopus Cdt1 lies in the amino terminal 150 amino acids and that deletion of this region renders the protein completely stable during S phase.

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 (Arias and Walter, 2005). At different times during incubation at room temperature, we isolated the chromatin by centrifugation and determined the amount of ubiquitylated Cdt1 by immunoblotting with Cdt1 antibody (Figure 3.12). 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 methylated ubiquitin was omitted from the reaction or when the reaction contained $Cdt1^{N150}$. These results indicate that $Cdt1^{N150}$ is neither ubiquitylated nor degraded. When $Cdt1^{N150}$ is added back to Cdt1-depleted replication extracts it restores replication similar to wild-type Cdt1 and is inhibited by Geminin like wild type Cdt1 (Figure 3.7).

When $Cdt1^{N150}$ 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 (Figure 3.13). In direct comparisons, the amount of re-replication with $Cdt1^{N150}$ was not significantly different from untreated extracts (1.3% vs 0.4%, $p=0.13$, Table 1) or extracts containing in vitro translated wild-type Cdt1 (0.8% vs 0.5%, $p=0.064$, Table 2). These results indicate that preventing Cdt1 degradation alone is not sufficient to cause re-replication within a single S phase.

Although expressing non-degradable Cdt1 in extracts had no effect by itself, it did enhanced the amount of re-replication in Geminin-depleted extracts. When $Cdt1^{N150}$ was added

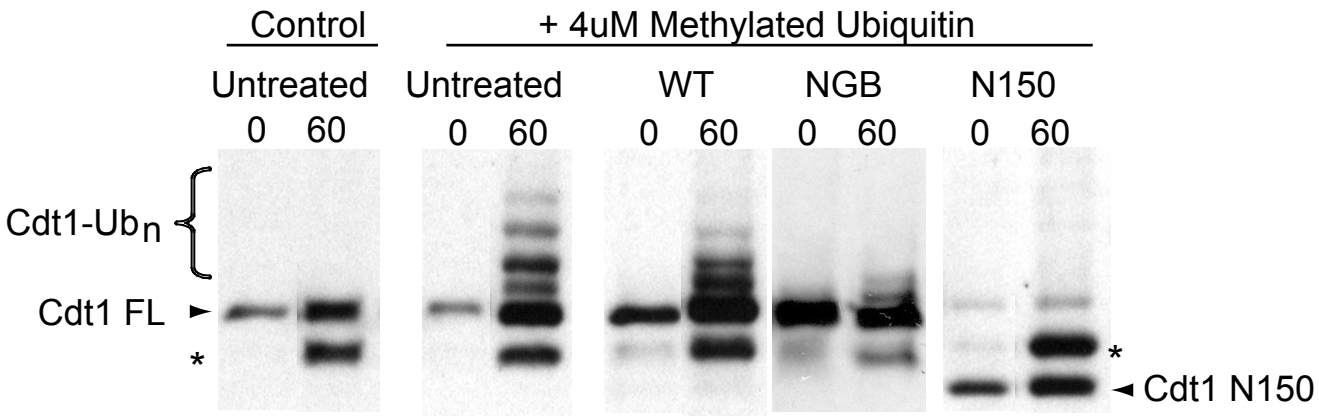


**Figure 3.12 Cdt1 N150 is not ubiquitylated.** Replication reactions were supplemented with 4uM methylated ubiquitin where indicated. Chromatin was isolated immediately and 60 minutes after the start of replication and ubiquitylated Cdt1 was detected by immunoblot using a Cdt1 antibody. The asterisk indicates a cross-reacting protein.

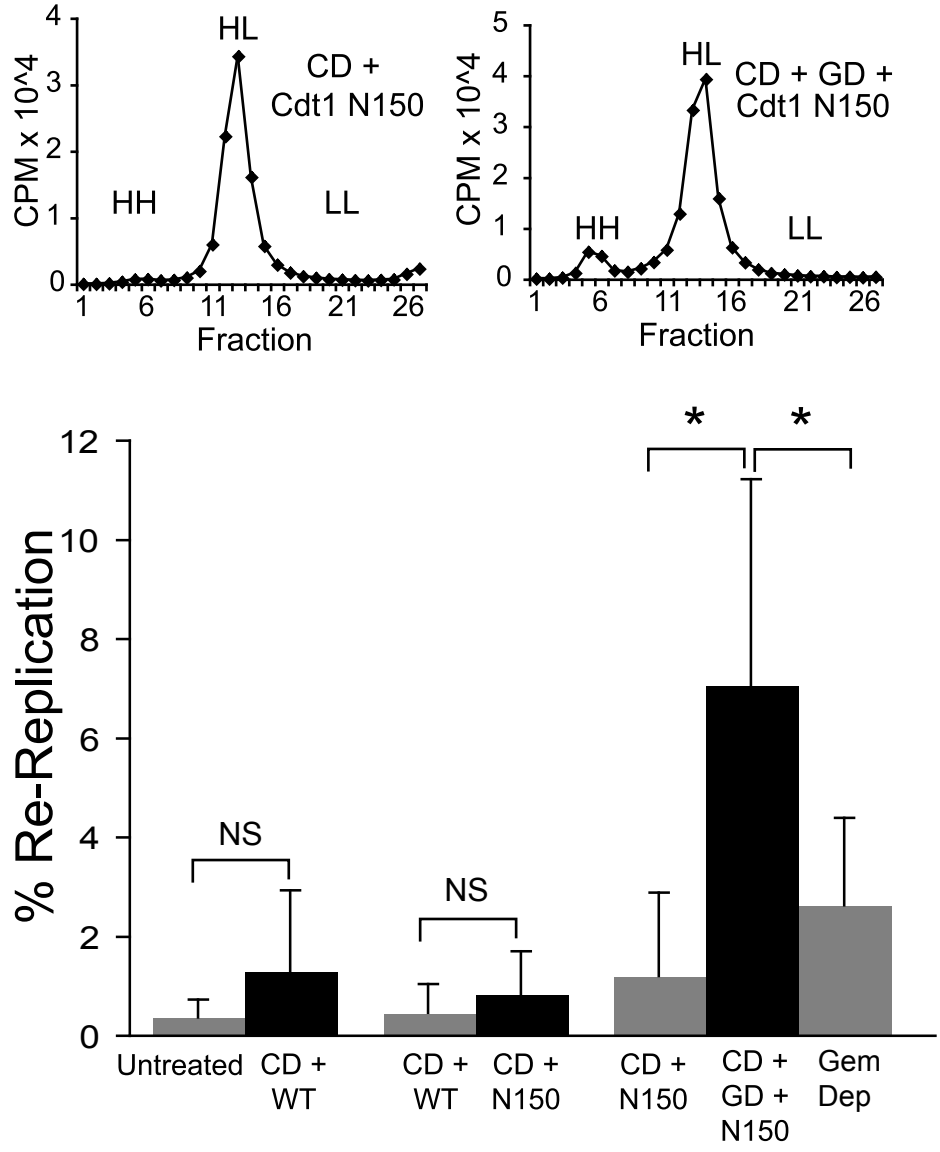


**Figure 3.13 A non-degradable Cdt1 mutant enhances the amount of re-replication caused by Geminin depletion.** Replication extracts containing BrdUTP were depleted of Cdt1 (CD) and/or Geminin (GD) and supplemented with Cdt1 that had been translated in reticulocyte lysate. The density of the product DNA was determined by CsCl gradient. (Top) Representative gradient data. (Bottom) Results averaged over several independent measurements made in different extracts. Statistically significant differences ($p<0.05$) are indicated by the *. NS, not significant; HH, heavy-heavy; HL, heavy-light; LL, light-light.

back to extract depleted of both Geminin and Cdt1, the amount of re-replication was significantly greater than the amount in Geminin-depleted extracts (7.1% vs 2.6%, p=0.03, Table 2 and Figure 3.13) or in extracts containing $Cdt1^{N150}$ but not depleted of Geminin (7.1% vs 1.2%, p=0.003, Table 2 and Figure 3.13). These results are consistent with the model that Cdt1 is inhibited both by degradation and by Geminin binding.

*Cdt1 Is Heavily Phosphorylated When Bound To Geminin During Metaphase*

In yeast, cyclin-dependent kinases prevent DNA re-replication by inhibiting multiple replication factors including ORC subunits, Cdc6, and MCM subunits (Nguyen et al., 2001). Human Cdt1 is phosphorylated by cyclin/cdk2 and cyclin/cdk4 complexes, though the residues phosphorylated have not been identified and the role of phosphorylation in preventing re-replication is not known (Liu et al., 2004; Sugimoto et al., 2004). Cdt1 runs at a higher apparent molecular weight in mitotic Xenopus egg extracts suggesting that it is phosphorylated (Maiorano et al., 2000). The shift disappears when the extracts are induced to enter S phase. It is not known which sites are phosphorylated, which kinase is involved, or how phosphorylation affects the protein's activity. We took two approaches to investigating these areas. First, we identified potential CDK sites in Xenopus Cdt1 by consensus sequence matching and made a non-phosphorylatable mutant that we tested for replication activity. Second, we isolated phosphorylated Cdt1 from mitotic Xenopus extract and identified phosphorylation sites by mass spectrometry.

We identified 10 potential CDK sites based on TP and SP consensus sequences (Figure 3.4). We mutated each threonine or serine residue to alanine using mutagenesis PCR. When this mutant, $Cdt1^{NP}$, is added to metaphase Xenopus extract, it does not show a shift in

electrophoretic mobility that is seen with endogenous Cdt1 (Figure 3.14). This suggests that this shift is due to phosphorylation at one or more of these ten putative CDK sites.

Interestingly, we found that $Cdt1^{NP}$ shows variable activity in supporting replication. When $Cdt1^{NP}$ is translated in vitro and added back to replication extract depleted of endogenous Cdt1, it only rescues replication 3 out of 6 (50%) of the times tested (Figure 3.15). These results suggest that Cdt1 phosphorylation may be complex and affect both replication activity and inhibition after replication is initiated. Since $Cdt1^{NP}$ has all ten putative CDK sites mutated, it is possible that multiple activities are affected.

To determine if phosphorylation at one or more of these sites is required to shut off Cdt1 activity, we measured re-replication in Xenopus extract depleted of endogenous Cdt1 and supplemented with $Cdt1^{NP}$. In the experiments where $Cdt1^{NP}$ rescued replication, we found that $Cdt1^{NP}$ does not cause significant re-replication above background compared to either endogenous Cdt1 ($p=0.408$, Table 2) or wild-type Cdt1 translated in vitro ($p=0.423$, Table 2).

It is possible that a redundant mechanism, such as Geminin binding is sufficient to prevent re-replication when phosphorylation in impaired. To check whether Geminin inhibition acts as a redundant mechanism with phosphorylation at one or more of these 10 putative CDK sites, we measured re-replication in extract depleted of endogenous Cdt1 and Geminin and supplemented with $Cdt1^{NP}$. We found that in the absence of Geminin, $Cdt1^{NP}$ did not, on average, cause more re-replication than wild type Cdt1 (9.7% vs 10.9%; Table 1).

In a more direct approach to investigate the nature of Cdt1 phosphorylation, we analyzed its phosphorylation status by mass spectrometry. Since the shift in electrophoretic mobility is seen during metaphase, we immunoprecipitated Geminin-bound Cdt1 from metaphase arrested Xenopus extract using Geminin antibody bound to protein A beads. We know that almost all of

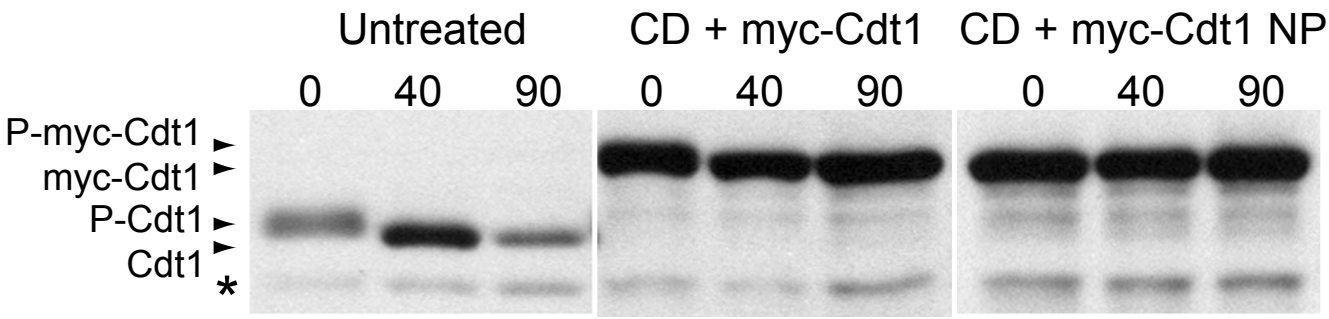


**Figure 3.14 Cdt1 is phosphorylated at one or more CDK sites in metaphase extract.** Replication extracts were made from metaphase-arrested Xenopus eggs. The extract was untreated or depleted of endogenous Cdt1 (CD) and supplemented with myc-Cdt1 that had been translated in reticulocyte lysate. The asterisk indicates a cross-reacting protein.

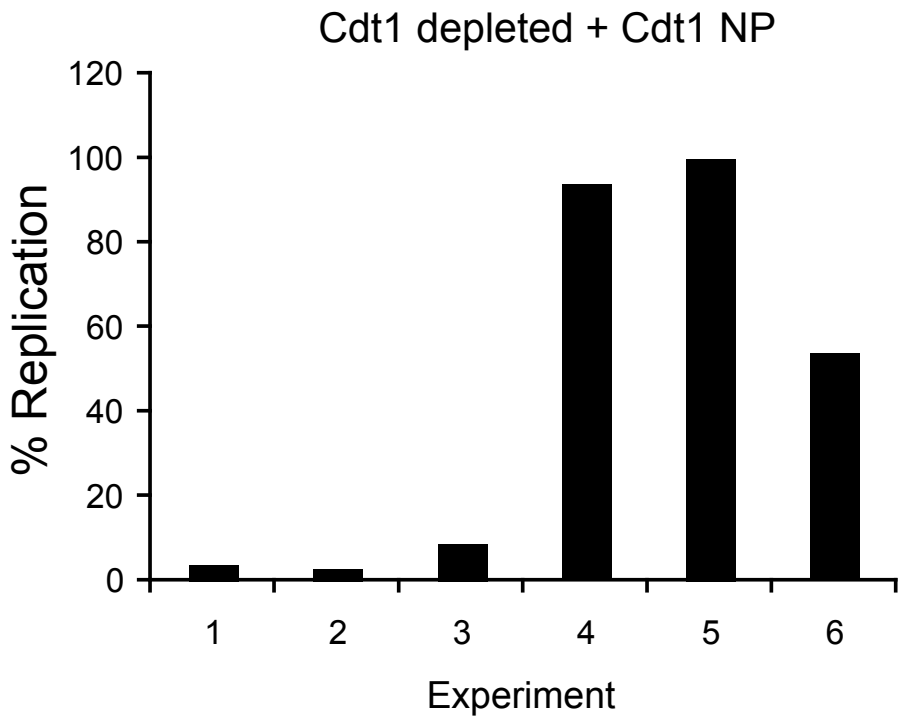


**Figure 3.15 Cdt1 NP shows variable replication activity.** Replication was measured in extracts depleted of endogenous Cdt1 and supplemented with Cdt1 NP that had been translated in reticulocyte lysate. Each bar represent an individual experiment and each experiment was done in a different extract. % Replication is normalized to untreated extract from the same extract.

the Cdt1 in metaphase extract is bound to Geminin, so immunoprecipitating Geminin is an efficient way to recover Cdt1 from metaphase extract (Figure 3.10). We ran the immunoprecipitate on a polyacrylamide gel and isolated the band corresponding to phosphorylated Cdt1. We had the protein analyzed by mass spectrometry by Dr. John Asara at the Beth Israel Deaconess Medical Center. The analysis covered 444/620 amino acids or 72% of the protein. Mass spectrometry analysis showed 11 serine and 4 threonine residues that were phosphorylated in moderate-high confidence (Figure 3.16). Three of the sites, S147, T159, and S164 were detected with a very high frequency and are highly conserved (Figure 3.16, residues marked with asterisks).

We used mutagenesis PCR to constructed $Cdt1^{S147A}$, $Cdt1^{T159A,S164A}$, and $Cdt1^{S147A,T159A,S164A}$ point mutants. We found that all three mutants support normal levels of replication in Xenopus extract depleted of endogenous Cdt1 (Figure 3.17). We added each of the mutants to Cdt1 depleted replication extract containing BrdUTP and measured the product DNA after separation by CsCl gradient. In this experiment, wild type Cdt1 caused some re-replication compared to untreated extract. However, neither of the phosphorylation mutants caused more re-replication relative to wild type Cdt1 (Figure 3.18). This suggests that either these phosphorylation events do not play an important role in replication activity or that a redundant mechanism, such as degradation or Geminin binding are sufficient to prevent re-replication when phosphorylation is impaired.

*An N-Terminal Myc Tag Impairs Inhibition of Cdt1*

When we initially tested wild type Cdt1 to see if it was regulated properly in replication extracts, we used both myc-tagged and untagged Cdt1. Interestingly, we found that myc-Cdt1

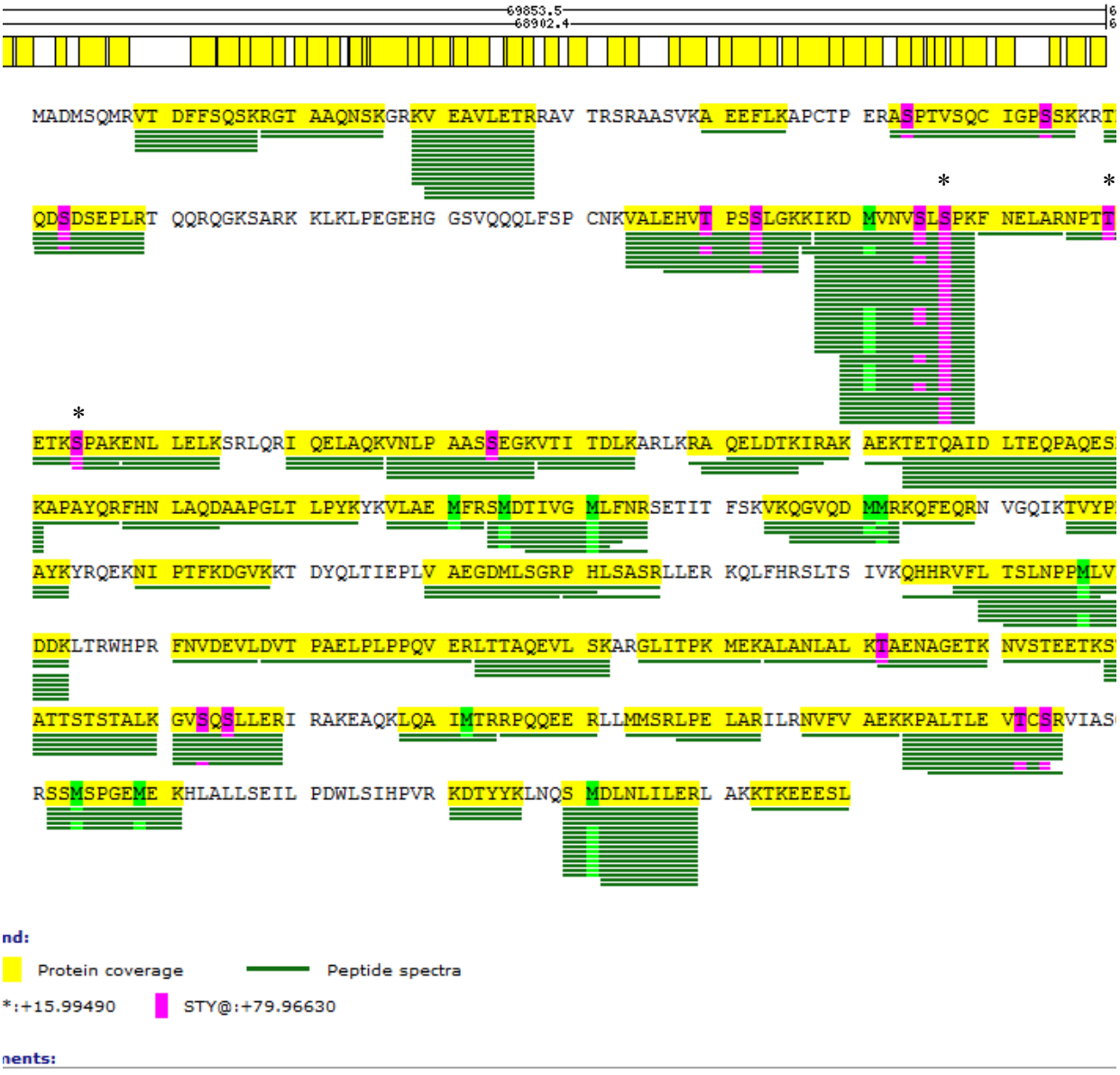


**Figure 3.16 Cdt1 from metaphase extract is phosphorylated on 15 serine and threonine residues.** Mass spec analysis of Cdt1 protein that was co-immunoprecipitated from metaphase arrested extract with Geminin antibody. Residues highlighted in pink are serine, threonine, or tyrosine sites that were detected as phosphorylated. Asterisks indicate residues that were detected in the phosphorylated form at high frequency.

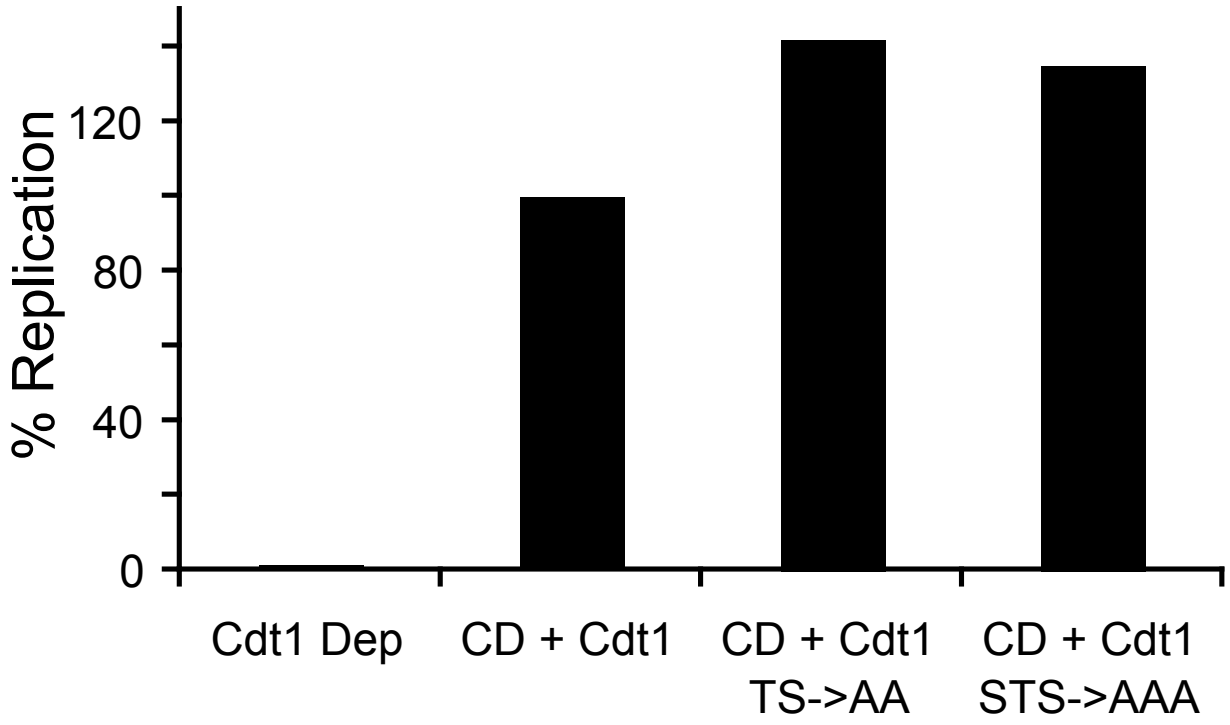


**Figure 3.17 Cdt1 Phosphorylation point mutants are active.** Replication was measured in extract depleted of endogenous Cdt1 (CD) and supplemented with Cdt1 or the indicated Cdt1 phosphorylation mutants that had been translated in reticulocyte lysate. Replication was normalized to wild type Cdt1.

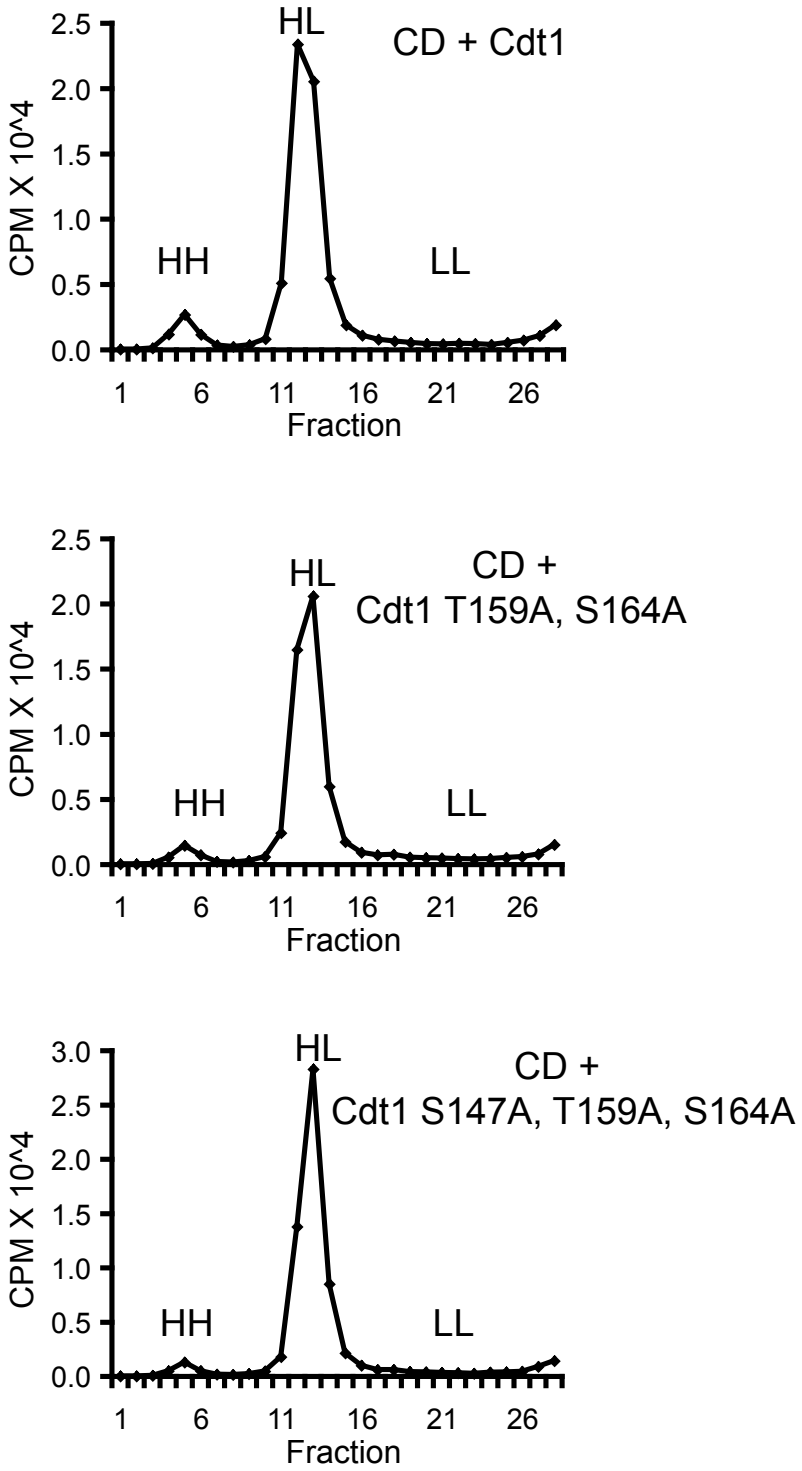


**Figure 3.18 Re-replication with Cdt1 phosphorylation point mutants.** Replication extracts containing BrdUTP were depleted of Cdt1 (CD) and supplemented with Cdt1 wild type or point mutants (as indicated) that had been translated in reticulocyte lysate. The density of the product DNA was determined by CsCl gradient. HH, heavy-heavy; HL, heavy-light; LL, light-light.

caused on average 16% re-replication (Table 1). This is significantly more re-replication than we see in untreated extracts or extracts containing wild type Cdt1 (Table 2).

It is possible that the myc tag interferes with degradation and/or Geminin binding. We measured the half-life of myc-Cdt1 by immunoblot of samples collected during the timecourse of replication. Myc-Cdt1 is degraded slightly slower than endogenous Cdt1 but appears to have the same half life as untagged Cdt1 (Figure 3.19, Top). However, we find that myc-Cdt1 is not ubiquitylated in replication extract containing methylated ubiquitn (Figure 3.19, Bottom). This suggests that the myc tag may interfere with the normal ubiquitin-dependent proteolysis that regulates Cdt1 activity.

We have shown that non-degradable Cdt1 does not cause re-replication unless the Geminin binding mechanism is also defeated. Also, in direct pairwise comparisons, myc-Cdt1 caused significantly more re-replication than $Cdt1^{N150}$ (10.2% vs 1.2%, p=0.010, Table 2). This suggests that interference with degradation by the myc tag does not fully explain why myc-Cdt1 causes re-replication. We have previously shown that the myc tag does not interfere with the ability of Cdt1 to bind Geminin because we used myc-tagged proteins in co-immunoprecipitations to map the Geminin binding site on Cdt1 (Figure 3.6). It is possible that Geminin can bind to myc-Cdt1 but does not inhibit its activity. To see if the myc tag interferes with Geminin-mediated inhibition, we added either untagged Cdt1 or myc-Cdt1 to replication extracts supplemented with increasing amounts of Geminin protein (Figure 3.20). We found that Geminin inhibits both untagged Cdt1 and myc-Cdt1 at the same concentration.

Our results suggest that myc-Cdt1 is not degraded normally but is inhibited by Geminin. As we have found that inhibition of degradation alone is not sufficient to cause re-replication, we

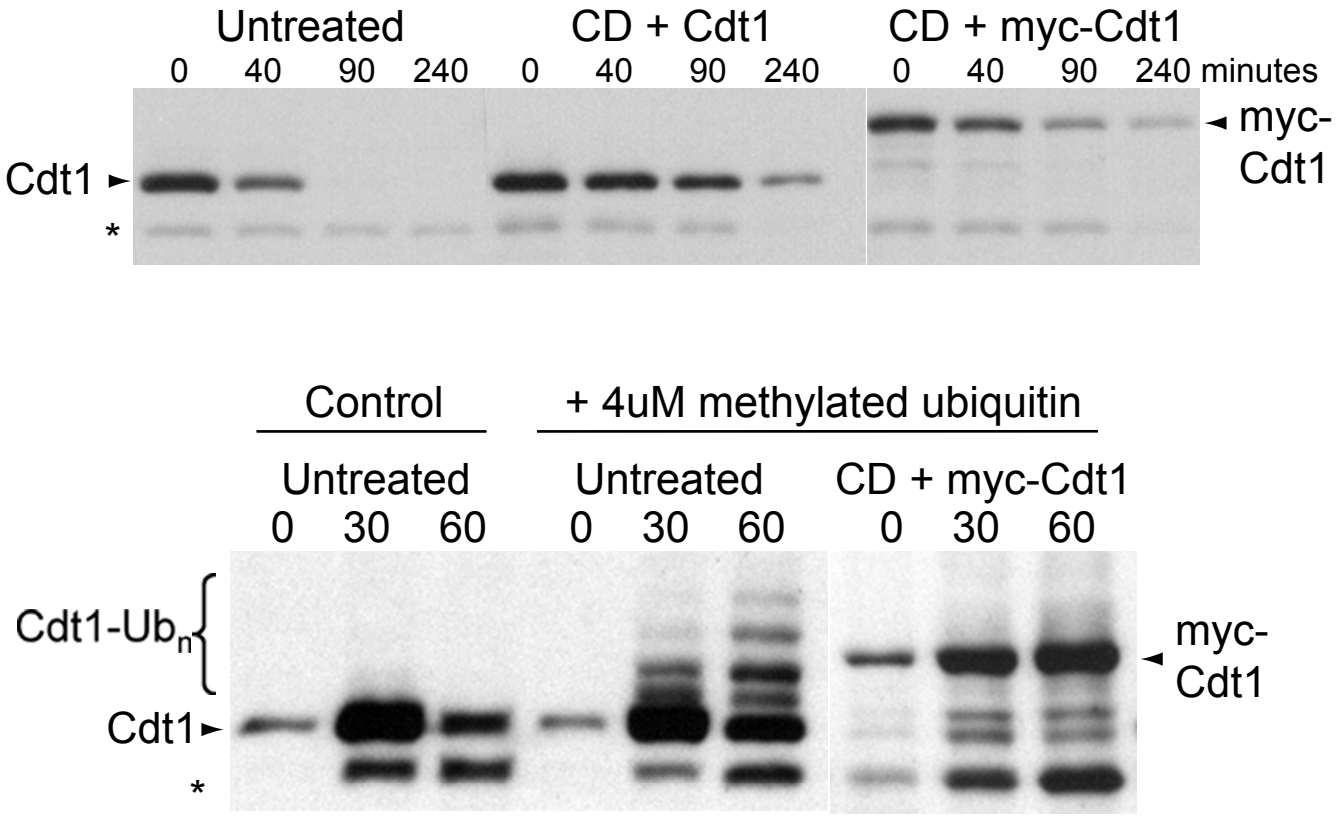


**Figure 3.19 Myc-Cdt1 is not ubiquitylated or degraded.** (Top) Extracts were untreated or depleted of endogenous Cdt1 (CD) and supplemented with Cdt1 translated in reticulocyte lysate. Immunoblots using Cdt1 antibodies show the amount of Cdt1 remaining at various times after the start of replication. (Bottom) Replication reactions were supplemented with 4uM methylated ubiquitin where indicated. Chromatin was isolated immediately and 60 minutes after the start of replication and ubiquitylated Cdt1 was detected by immunoblot using a Cdt1 antibody. The asterisk indicates a cross-reacting protein.

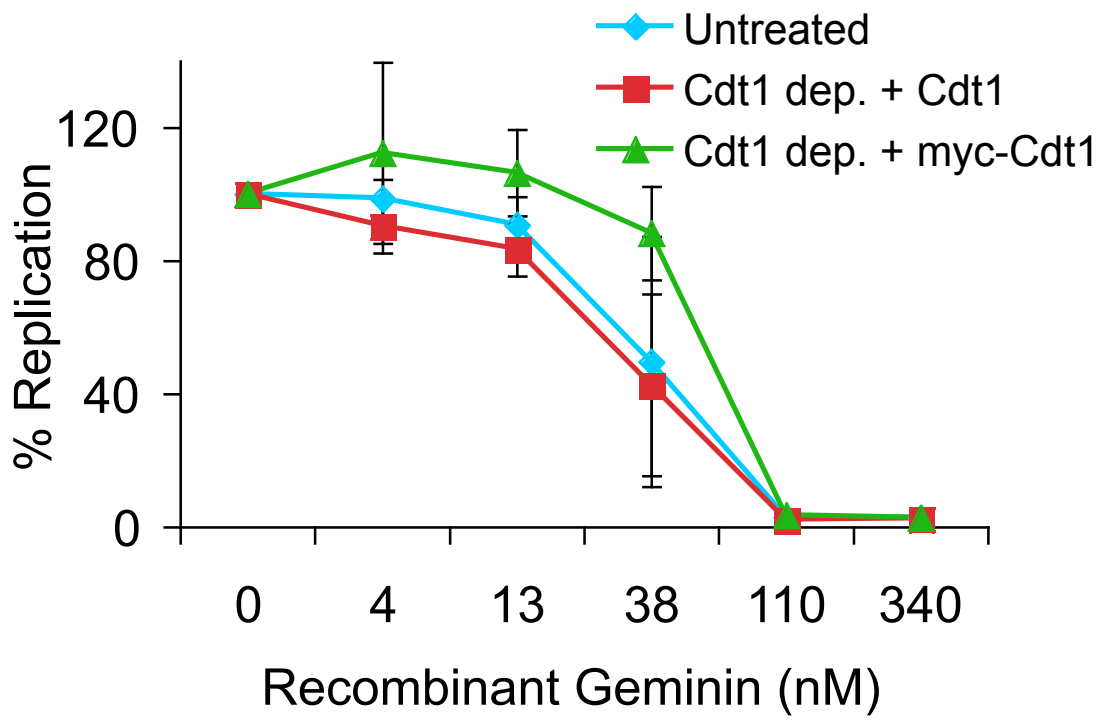


**Figure 3.20 Myc-Cdt1 is inhibited by Geminin.** Replication reactions were either untreated or depleted of endogenous Cdt1 and supplemented with Cdt1 or myc-Cdt1 translated in reticylocyte lysate. Recombinant Gemini protein was added at the indicated concentrations. Graph represents % replication normalized to '0nM Geminin' averaged from three separate experiments.

think that the myc tag may interfere with an additional uncharacterized regulatory mechanism. This will be addressed in the Discussion in Chapter 4.

*Geminin Prevents Re-Replication During G2 Phase*

We have shown that depletion of Geminin from Xenopus replication extract results in re-replication. However, if Geminin is depleted from Xenopus embryos using antisense oligonucleotides, there is no detectable re-replication (McGarry, 2002). To explain why Geminin-depleted Xenopus embryos accumulate little or no excess DNA during the first twelve embryonic cell cycles, we hypothesize that Geminin regulates Cdt1 and prevents re-replication during G2 phase but that a Cdt1-independent mechanism prevents re-replication during S phase. During the first twelve cell cycles in Xenopus embryos, S and M phase alternate in rapid succession and there is no G2 phase. Significantly, Geminin-depleted embryos arrest just after the 13th cell cycle, the point in development when G2 phase first appears. We speculate that during the early embryonic cell cycles re-replication is adequately suppressed during S phase by a Cdt1-independent mechanism. Then, at the $13^{th}$ cell division a small amount of re-replication occurs that is detected by the replication checkpoint.

To test if Geminin acts specifically during G2 phase, we measured re-replication in Cdt1 depleted extract supplemented with $Cdt1^{NGB}$ or Geminin depleted extract supplemented with wild type Cdt1at different timepoints after DNA was added. If Geminin was required to prevent re-replication only during G2 phase, we would expect that heavy-heavy DNA would be absent from early timepoints, when the first round of replication is ongoing, and become detectable at later timepoints, after the first round of replication is complete. We repeated the experiment four times. In one experiment, we detected only heavy-light DNA at the early, 45 and 60 minute

timepoint. By 90 minutes we detected some heavy-heavy DNA and the amount of heavy-heavy DNA was increased at 240 minutes (Figure 3.21). However, in the other three experiments, a small amount of heavy-heavy DNA was detectable at the earlier timepoints as well. There was more heavy-heavy DNA present at 240 minutes, but we can not determine whether this is because most origins did not re-fire until late in replication or because origins fired early but we are unable to detect re-replicated DNA until it had elongated later on in the time course.

To more directly test whether Geminin acts to prevent re-replication in G2 phase, we employed a nuclear transfer experiment. Here, we measured re-replication in nuclei that underwent one round of replication in untreated extra t, and were thus in a G2-like state, which were then transferred to a fresh replication extract that is either untreated or Geminin depleted. If Geminin is required to prevent re-replication specifically during G2 phase, we would expect to see re-replication in G2 nuclei that are transferred to fresh extract that does not contain endogenous Geminin. We repeated the experiment twice. As expected, if G2-like nuclei are transferred from untreated extract to a fresh aliquot of untreated extract, the DNA does not undergo a second round of replication. If G2-like nuclei are transferred from untreated extract to Geminin-depleted extract or extract supplemented with $Cdt1^{NGB}$ , we see re-replicated DNA (Figure 3.22). We see much more re-replication in these nuclear transfer experiments than we see by depletion of Geminin alone. We think that this is because the extract loses replication activity over time as the reaction sits at room temperature, and addition of nuclei to fresh extract replenishes the reaction (see Discussion). These results support our hypothesis that Geminin is required to prevent re-licensing of DNA by Cdt1 in G2 phase, after one round of replication has already occurred.

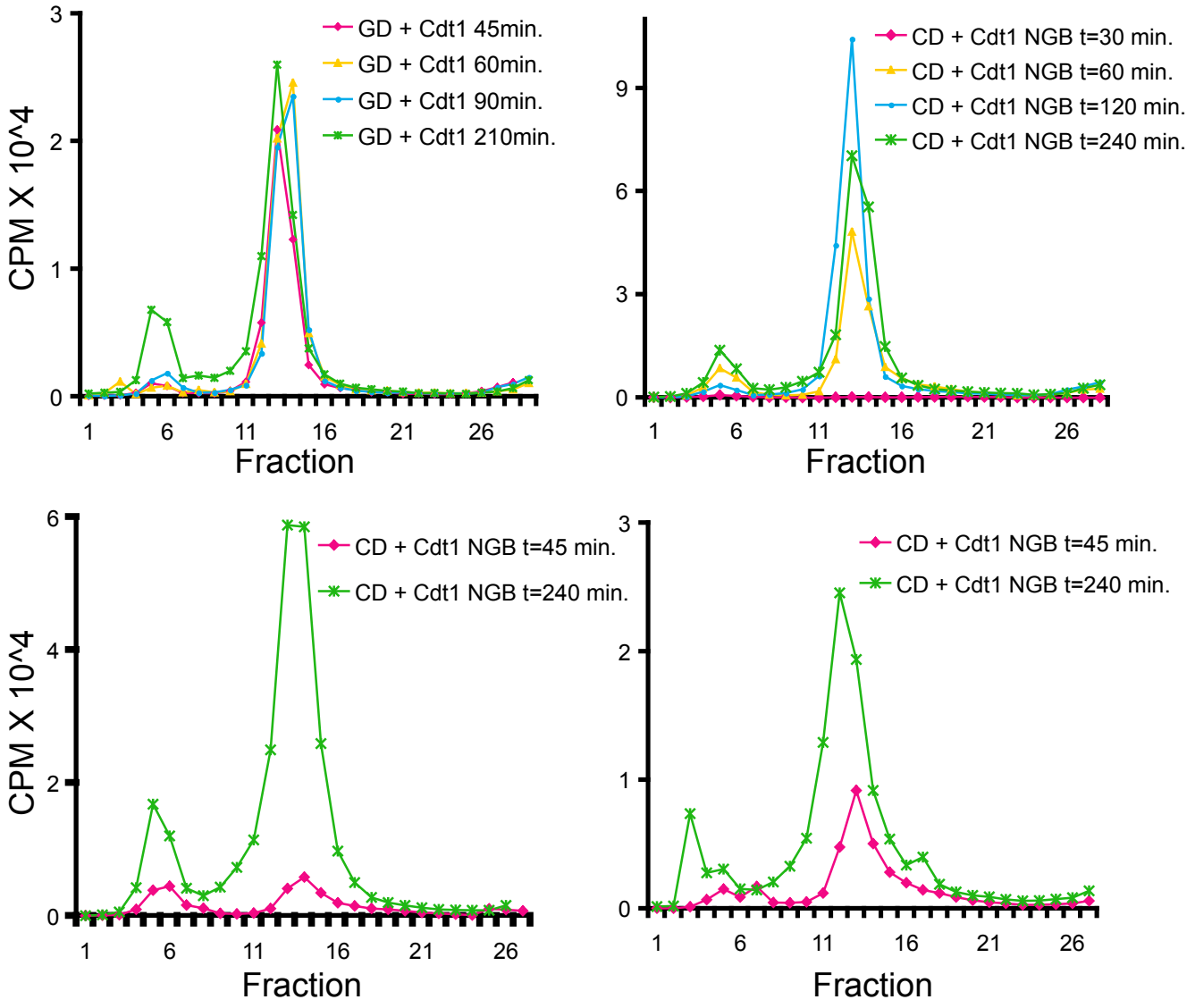


**Figure 3.21 Re-replication timecourse.** Replication extracts containing BrdUTP were either depleted of endogenous Cdt1 and supplemented with Cdt1 NGB or depleted of endogenous Geminin and supplemented with Cdt1 translated in reticylocyte lysate. Samples were collected at the indicated timepoints and the density of the product DNA at each timepoint was determined by CsCl gradient. CD is Cdt1 depleted; GD is Geminin depleted.

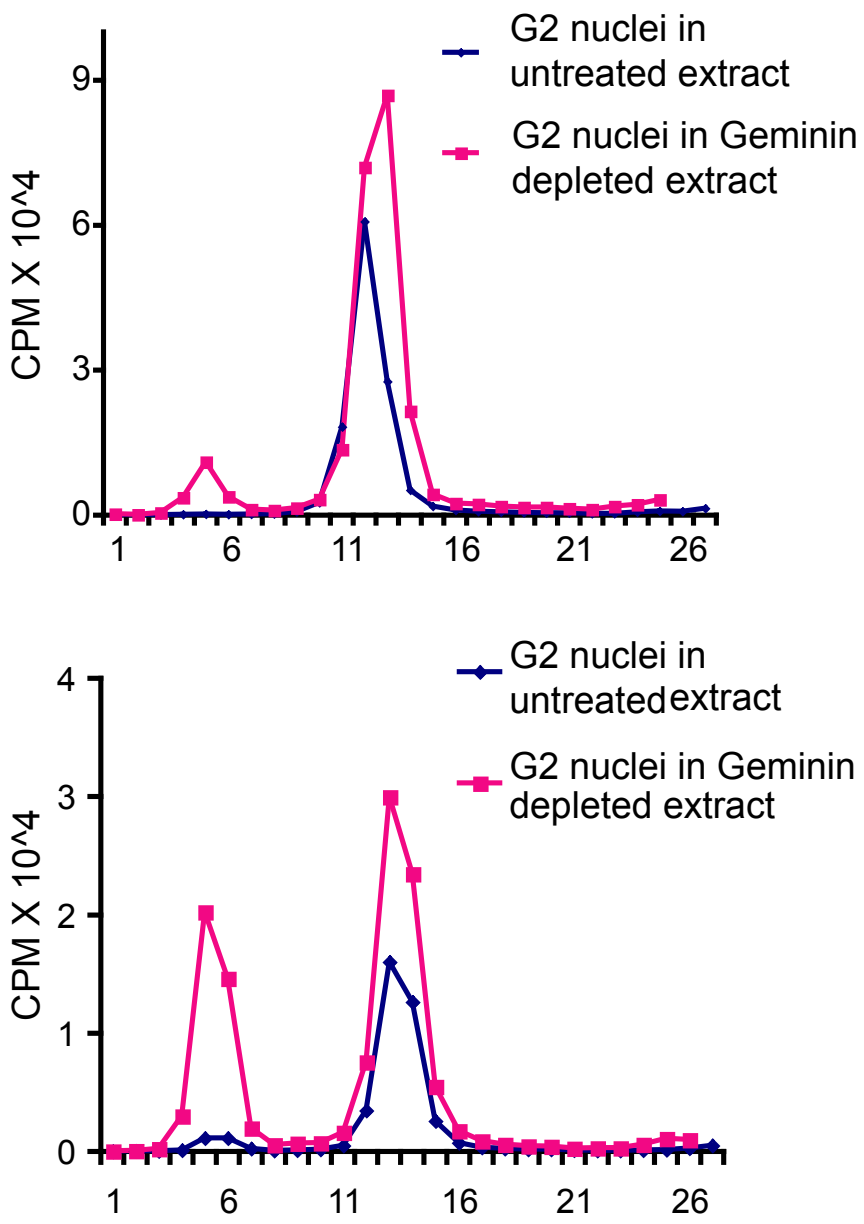


**Figure 3.22 Geminin prevents re-replication during G2 phase.** S phase extracts were supplemented with DNA template and BrdUTP and allowed to undergo replication. After 60 minutes, the G2 nulcei were collected by centrifugation and transferred to either fresh untreated S phase extract or fresh Geminin depleted S phase extract containing BrdUTP. Two separate experiments are represented.

*A Cdt1/Geminin Complex Is Active*

We wanted to investigate the nature of the inhibitory activity of Geminin to determine why Geminin only inhibits Cdt1 after replication starts and does not inhibit Cdt1 from licensing replication origins at the onset of S phase. Geminin can associate with Cdt1 on chromatin during G1 phase in an *in vitro* replication licensing system (Gillespie et al., 2001). One possible explanation is that binding of Geminin to Cdt1 is not sufficient for inhibition but rather the Cdt1-Geminin complex exists in an active state before origins fire and an inactive state after replication starts. To determine if Cdt1 bound to Geminin is active, we measured the replication activity of a purified complex of Cdt1 bound to Geminin.

We used the pET-DUET expression system to express both Cdt1 and Geminin together in bacteria. The Geminin sequence was cloned in following the poly-histidine tag and Cdt1 sequence was cloned into the same plasmid without an affinity tag. We induced expression of the proteins in bacteria and passed the lysate over a nickel-agarose column. His-Geminin and Geminin-bound Cdt1 was eluted with imidazole and the imidazole was subsequently removed by dialysis. This resulted in a highly purified Geminin-Cdt1 complex (Figure 3.23, Bottom left). The complex may contain some free Geminin in addition to Geminin-Cdt1 complex, but as the gel shows equal intensity staining of each protein and since Geminin is roughly half the size of Cdt1 we think that the prep contains roughly 2:1 Geminin:Cdt1. Geminin forms a dimer when it is active, so a 2:1 ratio would likely mean that most of the Geminin is bound to Cdt1 (Benjamin et al., 2004).

We added the complex at a concentration of 40nM to Xenopus extract that has been depleted of endogenous Cdt1 and Geminin and measured replication. Surprisingly, we found that the complex is fully active in restoring replication to the level of untreated extract (Figure

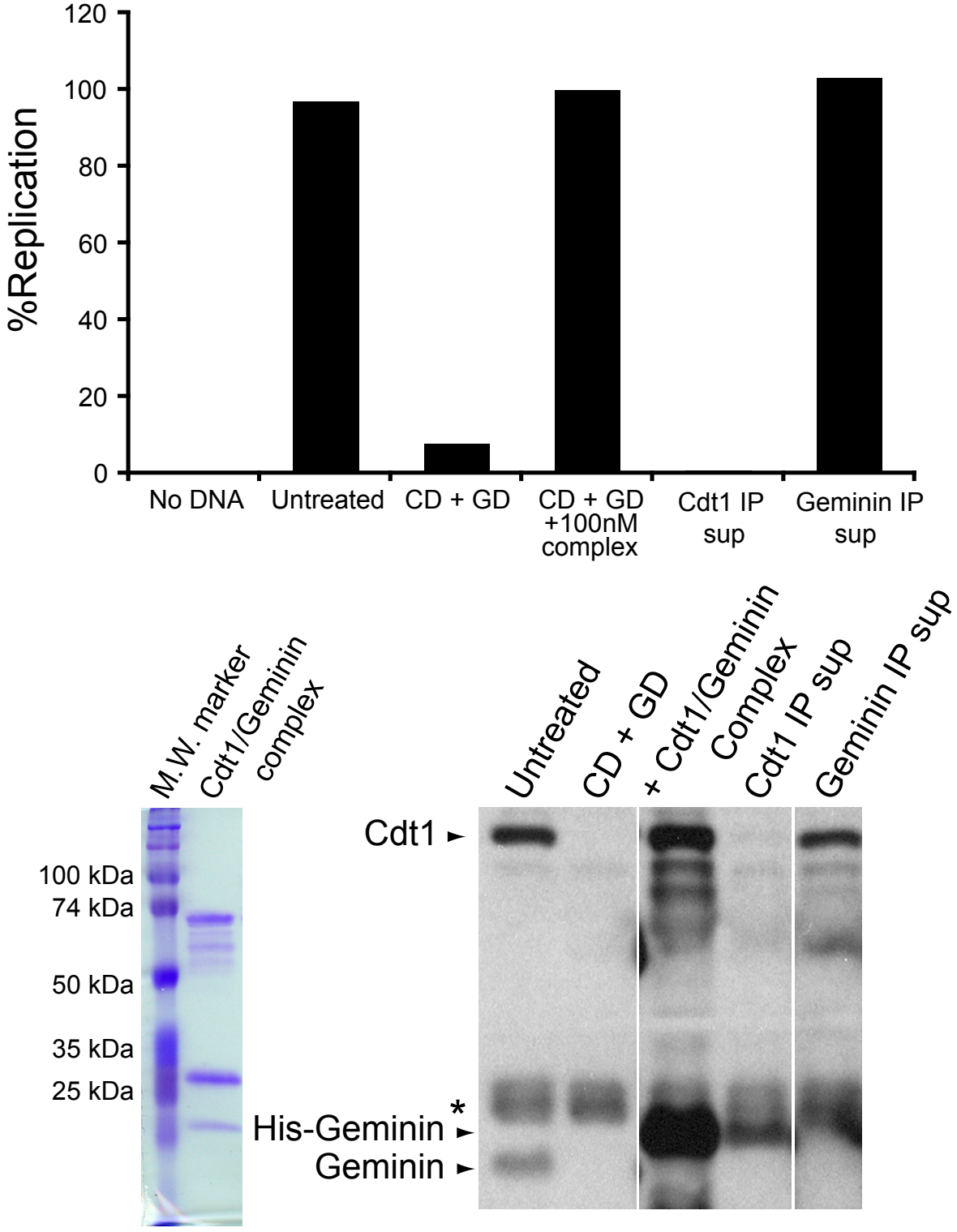


**Figure 3.23 Cdt1-Geminin complex is active in replication licensing.** (Top) Replication was measured in untreaed extract and extract depleted of endogenous Cdt1 and Geminin (CD + GD) then supplemented with Cdt/Geminin complex purified from bacteria (CD + GD + 100nM complex). Replication was also measured in samples from this reaction that were again depleted of either Cdt1 (Cdt1 IP sup) or Geminin (Geminin IP sup). (Bottom) Cdt1 and Geminin immunoblots of samples taken from replication reactions.

3.23, Top). This suggests that binding of Geminin to Cdt1 may not be sufficient for inhibition of Cdt1 licensing activity.

It is possible that the complex rescues replication because it dissociates in S phase extract. To check this, we added complex to Xenopus extract that had been depleted of endogenous Cdt1 and Geminin, let it sit for 30 minutes at room temperature, and immunodepleted either Cdt1 or Geminin (Figure 3.23, Bottom right). The Cdt1 immunodepleted extract was immunoblotted for Geminin and the Geminin immunodepleted extract was immunoblotted for Cdt1. We found that depletion of Cdt1 left free Geminin in the extract and depletion of Geminin left free Cdt1 in the extract (Figure 3.23, Bottom right). This suggests that replication is due to the free Cdt1 rather than the Cdt1 bound to Geminin.

Our initial results suggest that the Cdt1-Geminin complex can exist in an active and inactive state. But, based on our dissociation measurement, we can not exclude the possibility that the replication we see in extract containing Cdt1/Geminin complex is due to the presence of free Cdt1 that has dissociated from Geminin. This experiment also suggest that pre-replicative S phase extract is in a state that drives the Cdt1/Geminin binding equilibrium towards more free protein. This idea will be addressed further in the Discussion below.

**Discussion**

We have developed an in vitro replication system to test the replication activity of Cdt1 mutants. We found that Cdt1 has an N-terminal regulatory domain and a C-terminal replication domain that is required for activity. The N-terminus contains both the Geminin binding domain and degradation signal as well as several putative CDK phosphorylation sites. We constructed

Cdt1 mutants that interfere with each of these potential sites of regulation and analyzed their replication activity.

*Cdt1 Is Regulated By Geminin And By Ubiquitin-Dependent Degradation*

We have found that a Cdt1 mutant that does not bind Geminin causes re-replication within one cell cycle, suggesting that Geminin is absolutely required to prevent re-licensing of replication origins. While a non-degradable Cdt1 mutant does not cause re-replication on its own, it enhances the amount of re-replication seen when Geminin is absent. These results agree with recent reports that the nonspecific proteasome inhibitor MG132 has no effect in untreated extracts but induces a second round of initiation in Geminin-depleted extracts (Arias and Walter, 2005; Li and Blow, 2005; Maiorano et al., 2005). Here we extend these results by showing that Cdt1 itself is the critical stabilized protein.

The finding that Cdt1 is inhibited both by Geminin binding and by degradation could explain why expressing $Cdt1^{NGB}$ causes more re-replication than depleting Geminin. We added about twice as much $Cdt1^{NGB}$ as is present endogenously. This level likely partially overwhelms the Cdt1 degradation mechanism. Also, we find that when we deplete Geminin from extract, we also remove a small amount of the endogenous Cdt1, presumably because the proteins exist in an equilibrium between free and complex protein. So, when we measure re-replication in Geminin depleted extract, we are starting with slightly less Cdt1 and so the degradation mechanism is essentially enhanced.

This finding may also explain why previous reports, both by ourselves and by others, show that depleting Geminin from Xenopus egg extracts does not cause re-replication (Arias and Walter, 2005; McGarry and Kirschner, 1998). We can offer two explanations for the

discrepancy between the previous results and those reported here. First, the amount of re-replication in Geminin-depleted extracts is very small, only a few percent. Because of extract-to-extract variability, in many experiments there is no measurable re-replication 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 almost all of the Cdt1, while depleting Geminin from S-phase extracts only removes a small amount of Cdt1. The cause of the increased association between Geminin and Cdt1 in metaphase extract is unknown. We hypothesize that when the Cdt1 concentration is very low, Geminin-independent mechanisms (including Cdt1 degradation) are sufficient to prevent re-replication by themselves.

Recent studies have worked out some of the details of the ubiquitin-degradation mechanism that removes Cdt1 activity. Cdt1 interacts with PCNA and the Cul4-Ddb1-Cdt2 E3 ubiquitin ligase (Hu, McCall et al. 2004; Arias and Walter 2006). In Xenopus extract, the Cdt2 ortholog DCAF2 is required for Cdt1 degradation (Jin, Arias et al. 2006). The Cul4-Ddb1-DCAF2 E3 ligase associates with Cdt1 when Cdt1 is bound to chromatin, suggesting a way in which degradation is closely linked to the onset of replication. The finding that this Cul-4 containing ligase is responsible for Cdt1 degradation supports the idea that the over-replication seen in CUL-4 deficient C. elegans is specifically due to Cdt1 stabilization (Zhong et al., 2003). Two recent reports show that degradation of human Cdt1 requires a QXRVTDF sequence near the amino terminus and an RRL sequence located further downstream (Arias and Walter, 2006; Senga et al., 2006). Both these sequences are found in the first 150 amino acids of Xenopus Cdt1, consistent with our finding that $Cdt1^{N150}$ is a completely stable protein.

Another group showed that Cdt1 interacts with the F-box protein Skp2 and that an SCF-Skp2 E3 ubiquitin liagase complex could also target Cdt1 for degradation. Cdt1 co-immunoprecipitates from cultured cells with Skp2 and Cul-1 and this interaction is disrupted when cells are treated with λ phosphatase (Li, Zhao et al. 2003). It is not clear though whether phosphorylation of Cdt1 itself plays a role in this degradation process. We would like to further investigate the phosphorylation state of post-replicative Cdt1 (see below) and in doing so we would be able to more clearly determine if and how degradation is dependent on phosphorylation.

*Cdt1 Is Heavily Phosphorylated During Metaphase*

We found that mutation of all ten putative CDK phosphorylation sites in Cdt1 has a variable effect on replication activity. The times that this mutants supports replication, it does not cause re-replication. We think that phosphorylation of Cdt1 may be complex, consisting of both activating and inhibitory sites. $Cdt1^{NP}$ can bind to Geminin, and it does not cause significantly more re-replication in Geminin depleted extract compared to wild type Cdt1, suggesting it is regulated normally by Geminin.

Using mass spectrometry, we have identified fifteen sites on Geminin-bound Cdt1 that are phosphorylated during metaphase. We found that mutation of three of these sites that were detected with high frequency does not interfere with Cdt1 licensing activity and, in one experiment, does not interfere with mechanisms that shut off Cdt1. When we measured re-replication, wild type Cdt1 caused some re-replication compared to untreated extract. We found that on average, wild type Cdt1 does not cause significantly more re-replication than untreated extract. We are not sure why it did cause re-replication in this experiment. The

phosphorylation mutants caused less re-replication than wild type Cdt1, but as we only did one experiment, we could not do statistical analysis and therefore can not definitively conclude that these sites do not affect inhibition of Cdt1.

We used Cdt1 isolated from metaphase arrested extract for mass spectrometry analysis because Cdt1 is phosphorylated in metaphase. However, we think that the sites on Cdt1 that are phosphorylated during metaphase may serve a function different from sites that may be phosphorylated during S phase. It is possible that the Cdt1 that is phosphorylated during metaphase is a separate pool of protein that has not yet become active or participated in origin licensing. It would be interesting to check the replication activity of a Cdt1 mutant that has a glutamate residue substituted for S147, T159, and/or S164 to mimic a constitutively phosphorylated mutant. If these sites are phosphorylated to prevent newly synthesized Cdt1 from acting prior to the onset of G1 phase, we would expect a phosphorylation mimic mutant would not be active. To better identify phosphorylation sites that are important for shutting off post-replicative Cdt1, we plan to isolate Cdt1 from post-replicative nuclei and analyze it by mass spectrometry. We can then compare any sites identified on that pool of Cdt1 with sites we have identified on metaphase Cdt1.

*An N-terminal Myc Tag On Cdt1 Causes Re-Replication Through A Geminin-Independent Mechanism*

We have found that addition of a myc tag to the N-terminus of Cdt1 prevents the protein from being shut off properly. Myc-Cdt1 appears to be regulated by Geminin normally. The tag interferes with ubiquitylation, but we have found that interference with ubiquitylation is not sufficient to cause re-replication within one cell cycle.

There are several ways in which we think the N-terminal myc tag might interfere with Cdt1 inhibitory mechanisms. One possibility is that the myc tag prevents Cdt1 from being released from chromatin when origins fire. The mechanism that causes release of pre-RC components is not understood. One possibility is that release is connected to origin firing so that the pre-RC breaks apart as replication begins. This could depend of post-translation modification or conformational changes of pre-RC components. The N-terminal myc tag could prevent Cdt1 from undergoing a conformational change that reduces its affinity for DNA. It could also prevent Cdt1 from interacting with other pre-RC components properly so that the coordinated release from chromatin is not carried out. Myc-Cdt1 shows a shift to a higher molecular weight in metaphase extract suggesting that it is phosphorylated correctly, at least in that part of the cell cycle. It is possible though that the myc-tag interferes with some post-translational modification that takes place during S phase and is not apparent on gels.

Another possibility is that the myc tag folds back on Cdt1 in a way that blocks some other site of protein interaction, preventing Cdt1 from interacting with another inhibitory protein. Cdt1 has three predicted coiled-coils two of which lie upstream of the Geminin binding domain and one in the MCM binding domain. Coiled-coils are often sites of protein-protein interaction and it is possible that an additional inhibitory protein may interact with Cdt1 through one of these coiled-coil domains. The myc tag consisted of a six subunit repeat of the coiled-coils of the myc protein. It is possible that the tag forms a similar structural motif as a binding site on Cdt1 and competes for binding to an inhibitory protein. As mentioned above, Cdt1 interacts with PCNA at replication origins and this interaction is required for Cdt1 degradation. The myc tag may interfere with degradation by blocking the site on Cdt1 that interacts with PCNA. The myc

tag may also block the site on Cdt1 that is recognized by the substrate recognition subunit of an E3 ubiquitin ligase.

*Geminin Inhibits Re-Replication During G2 Phase*

Since we started this project, other groups have reported that post-replicative (G2-like) nuclei will replicate a second time when transferred to Geminin-depleted extract but not when transferred to control extract (Li and Blow, 2005). Moreover, adding excess recombinant Cdt1 to an extract containing post-replicative (G2-like) nuclei induces a second round of DNA synthesis, while adding the same amount to an extract with unreplicated (G1-like) nuclei does not (Arias and Walter, 2005). These results are consistent with the model that Geminin inhibits Cdt1 to prevent re-replication mainly during G2 phase. It is likely that Cdt1-independent mechanisms help to prevent re-replication in early S phase, immediately after origins fire. CDK-mediated phosphorylation and nuclear export regulate the availability of other pre-RC components including Cdc6 and MCM subunits, and the availability of these components is necessary for origins to re-fire.

This finding is also consistent with recently published data showing that addition of excess recombinant Cdt1 to replication extract does not cause re-replication when added at the start of S-phase but does cause re-replication when added 60 minutes after replication starts (Arias and Walter, 2005). The re-replication that occurs when Cdt1 is added late is enhanced if Geminin is depleted from the extract. Presumably, Geminin-independent mechanisms, including degradation, inactivate Cdt1 that is added early, but Geminin is required to inactivate Cdt1 that is added late.

The finding that Geminin acts in G2 phase can explain why the extent of re-replication observed in our experiments was rather small, amounting to ~20% of the genome at most. We also observed marked variability in the amount of re-replication from extract to extract. For example, with $Cdt1^{NGB}$ the amount of heavy-heavy DNA ranged from 5% to 20% of the heavy-light peak. Assuming that the second round of replication starts in G2 phase 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 re-replication 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.

*What Mediates Inhibition Of Cdt1 By Geminin?*

It is not clear if binding of Geminin to Cdt1 is sufficient to inhibit Cdt1 licensing activity. Geminin can be detected in association with Cdt1 on chromatin in G1 phase, before replication starts (Gillespie et al., 2001). We tried to determine if Geminin-bound Cdt1 was active by adding a complex of Cdt1-Geminin to replication extracts depleted of endogenous Cdt1 and Geminin. We found that the complex was active. Co-immunodepletion experiments showed that the complex dissociated upon incubation in the extract so we can not exclude the possibility that the replication licensing was due to the presence of free Cdt1. Another group recently published the same result that a Cdt1-Geminin complex was active, but they did not check whether or not their complex dissociated in the extract (Lutzmann et al., 2006).

A previous report showed that Geminin can inhibit Cdt1 from interacting with chromatin *in vitro* (Yanagi et al., 2002). If recombinant mouse Cdt1 is incubated with $^{32}P$ labeled-

oligonucleotides, the DNA runs at a higher mobility on polyacrylamide gels than it runs in the absence of Cdt1 protein. This high molecular weight complex disappears if Geminin protein is added to the reaction. The physiological relevance of this result is called into question by another recent publication in which live cell imaging was used to show that Geminin can interact with chromatin-associated Cdt1 in living cells (Xouri et al., 2007). They identified Cdt1 mutants that separately interfere with chromatin binding and Geminin binding, suggesting that these interactions are separable. They also used fluorescence recovery after photobleaching (FRAP) to show that Geminin does not affect Cdt1 association with chromatin in vivo. These studies suggest that Geminin does not inhibit Cdt1 by removing it from chromatin.

We have come up with three possible models to explain the inhibitory nature of the Geminin/Cdt1 complex. One model is that Geminin-bound Cdt1 is inactive and the binding reaction is driven by a change in stoichiometry of the two proteins during the cell cycle. By this model, before replication starts some Cdt1 is associated with Geminin, but the relative concentrations of the two proteins drives the equilibrium in the direction of more free Cdt1. Then, after replication starts when Cdt1 begins to be degraded and Geminin level increases, the equilibrium is driven towards Cdt1-Geminin complex.

However, in our experiments, the replication extract contains the protein synthesis inhibitor cycloheximide so the level of Geminin protein remains constant before and after replication (Figure). Cdt1 is degraded in extract, but extract containing non-degradable $Cdt1^{N150}$, in which the Geminin and Cdt1 level remains constant over the timecourse of replication, do not re-replicate. This is inconsistent with this model that changes in protein concentration alone mediate binding and inhibition. To explain this apparent discrepancy, we hypothesize that the nuclear envelope acts to regulate the local concentration of Cdt1 and Geminin even when the

total concentration does not change. According to this hypothesis, Geminin would be excluded from the nucleus until replication origins have fired, at which time Geminin would become concentrated in the nucleus and drive the Cdt1 + Geminin equilibrium towards Cdt1/Geminin complex.

A second model is that the Cdt1-Geminin complex exists in an active and inactive state that is determined by a conformational change induced upon origin firing. In the active state, Geminin binds to Cdt1 in a way that does not block its ability to recruit MCM proteins. Then, when origins fire, the complex undergoes a conformational change so that Geminin binds Cdt1 in a way that blocks further activity. This conformational change could be driven by a number of things such as phosphorylation of Cdt1 or a change in conformation of Cdc6 or ORC proteins that associate with Cdt1 in the pre-RC.

The recently published crystal structure of a fragment of mouse Cdt1 bound to a fragment of mouse Geminin indicates a second site on Cdt1 that interacts with Geminin that we did not identify in our binding assay (Lee et al., 2004). Specifically, Geminin interacts with a C-terminal LTRWHP sequence of Cdt1 that corresponds to amino acids 404-409 in the Xenopus protein. In our binding assay, the Cdt1 mutant C331 that is missing this site is able to immunoprecipitate Geminin as well as full length Cdt1 (Figure 3.6). In the context of this second model, it is possible that the interaction at this secondary site occurs when the Cdt1-Geminin complex is in the 'inhibited' conformation but not when the complex is in the 'active' conformation. We have constructed a Cdt1 mutant that has alanine substitutions at each of the amino acids in this secondary site (LTRWHP) and we plan to see if this mutant causes re-replication in extracts.

A third model is that Geminin is modified in a way that increases its affinity for Cdt1 even when the protein concentration does not change. When we immunoprecipitated Geminin and Cdt1 from metaphase extract to isolate Cdt1 for phosphorylation analysis, we also analyzed the Geminin protein by mass spectrometry. Interestingly, we found four residues that were phosphorylated on Geminin. We have not yet investigated the significance of these sites. It is possible that they may have a role in the Geminin-Cdt1 interaction. A previous publication indicates that Geminin exists in an active and inactive state and that its activity depends on its import into the nucleus during S phase (Hodgson et al., 2002a). These phosphorylation sites may play a role in nuclear import or nuclear retention of Geminin, or they may put Geminin in an active conformation once it is concentrated in the nucleus.

## Part II: The Phenotype of Geminin Depletion is Due to Mis-Regulated Cdt1

### Introduction

Our lab previously showed that depleting Geminin from Xenopus embryos with antisense oligonucleotides causes a G2 cell cycle arrest at the last blastula stage (McGarry, 2002). 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 (Figure 1.5). 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 phospho-specific antibody (Figure 1.5) (Benjamin et al., 2004; McGarry, 2002). Activation of this checkpoint suggests two models for Geminin activity. One model is that Geminin is required for normal mitotic entry. Another model is that Geminin is required to prevent re-replication by inhibiting Cdt1. Our *in vitro* replication data favors this model. We next sought to determine whether the phenotype of Geminin deficient Xenopus embryos is a consequence of mis-regulated Cdt1 activity.

### Results

*Non-Geminin Binding Cdt1 Causes a Cell Cycle Arrest in Xenopus Embryos*

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. We injected two-cell Xenopus embryos with 300 pg of RNA encoding either $Cdt1^{WT}$ or the non-Geminin binding mutant $Cdt1^{NGB}$. As a positive control, embryos were injected with anti-Geminin morpholino oligonucleotides. Immunoblots confirmed that $Cdt1^{WT}$ and $Cdt1^{NGB}$ were expressed equally (Figure 3.24, Middle blot, lanes 5 and 13). When the embryos reached the late blastula stage we

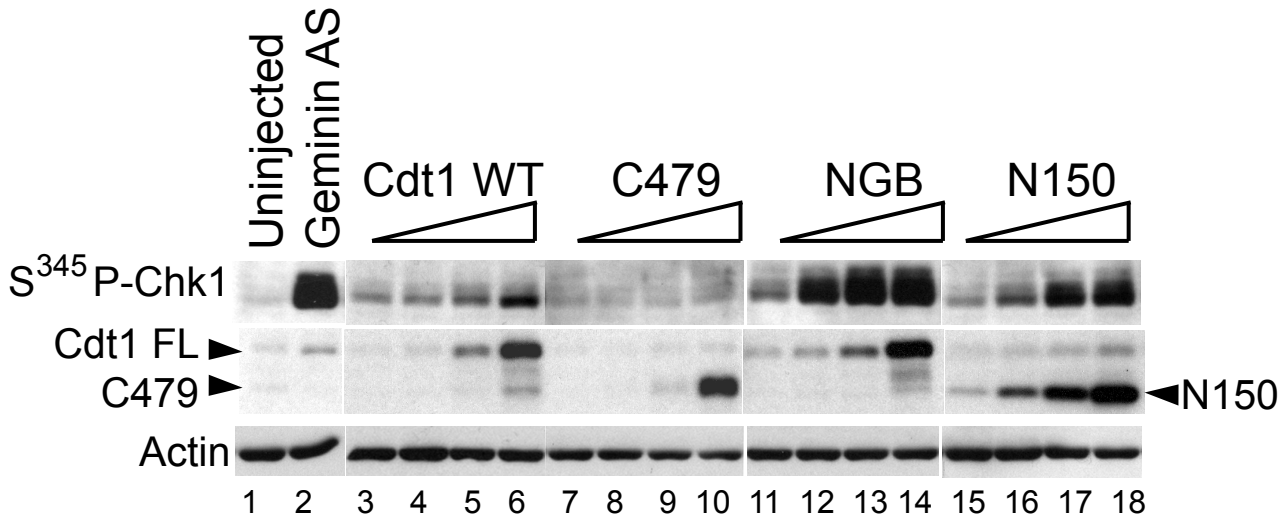


**Figure 3.24 Expression of Cdt1 mutants in Xenopus embryos.** Immunoblots of homogenized embyros that were uninjected or injected at the two-cell stage with Geminin anti-sense oligo or increasing amounts (30pg, 100pg, 300pg, 1000pg) of RNA encoding Cdt1 WT or mutants. Top, phospho-S345 Chk1 antibody; middle, Cdt1 antibody; bottom, actin antibody.

calculated the percentage of injections that produced a sector of abnormally large cells. Expressing $Cdt1^{NGB}$ induced arrested sectors in 59% of injections, whereas expressing $Cdt1^{WT}$ induced arrested sectors in just 3% (Figure 3.25). Embryos injected with $Cdt1^{NGB}$ RNA also showed a high level of Ser345-phosphorylated Chk1, while embryos injected with $Cdt1^{WT}$ did not show any increase above background (Figure 3.24, Top blot; compare lanes 1, 5, and 13). Injection of RNA encoding the inactive mutant $Cdt1^{C479}$ (Ferenbach et al., 2005) neither caused a cell cycle arrest nor induced Chk1 phosphorylation (Figure 3.24 and 3.25). This indicates that active Cdt1 is required for the phenotype and also ensures that titration of Geminin is not causing the phenotype. Because expression of a Cdt1 mutant that is not regulated by Geminin reproduces the phenotype caused by Geminin depletion, we conclude that the phenotype of Geminin depleted embryos is due to improperly regulated Cdt1 activity. Injecting anti-Geminin oligonucleotides induced a cell cycle arrest in 100% of the injected embryos and induced a greater degree of Chk1 phosphorylation than $Cdt1^{NGB}$. We attribute this difference to the more extensive diffusion of the antisense oligo, compared to RNA, throughout all parts of the injected embryo.

*Non-Degradable Cdt1 also reproduces the Phenotype of Geminin Depletion*

To determine if expressing a non-degradable Cdt1 mutant would reproduce the Geminin-deficient phenotype, we injected Xenopus embryos with 300 pg RNA encoding either $Cdt1^{WT}$ or $Cdt1^{N150}$. As expected, the non-degradable $Cdt1^{N150}$ accumulated to about 10 times the level of wild-type Cdt1 (Figure 3.24, middle blot, compare lanes 5 and 17). $Cdt1^{N150}$ induced arrested sectors in 66% of the injections, and the arrest was accompanied by increased amounts of Ser345-phosphorylated Chk1 (Figure 3.24 and 3.25, Top blot). These results indicate that

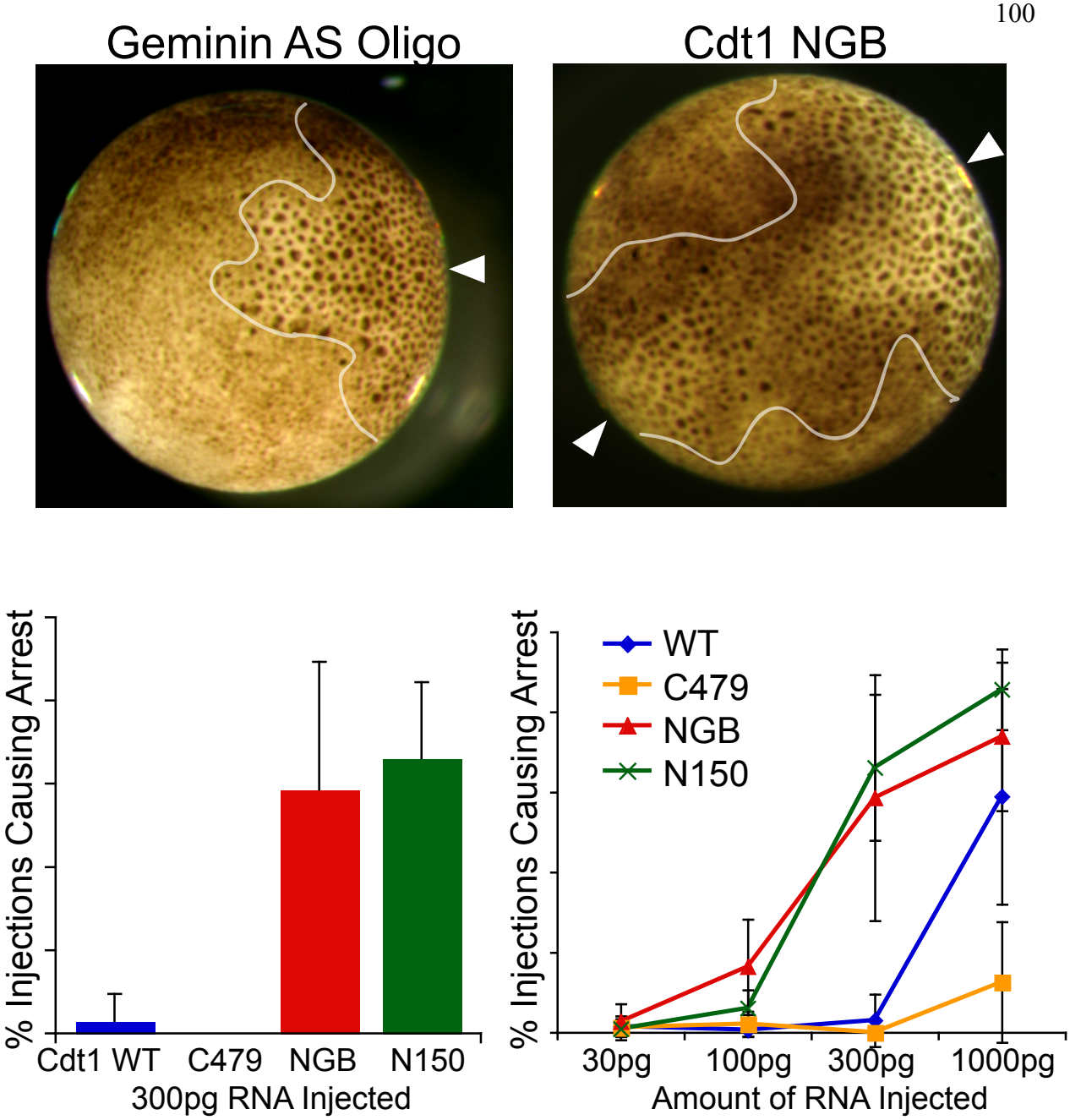


**Figure 3.25 Mis-regulated Cdt1 reproduces the cell-cycle arrest phenotype of Geminin deficient Xenopus embryos.** Two-cell Xenopus embryos were injected on both sides with increasing amounts of RNA encoding wild-type Cdt1 or Cdt1 mutants. When the embryos reached the late blastula stage, the percentage of injections producing a sector of arrested cells was calculated. (Top) Representative images of embryos injected with Geminin antisense oligo or Cdt1NGB. The arrowhead indicates the site of injection and the line indicates the boundry of the arrested sector. (Bottom) Graphs comparing the number of injections producing an arrested sector. Left, embryos were injected with 300pg of RNA; right, embryos were injected with increasing amounts of RNA.

expressing a non-degradable Cdt1 mutant also reproduces the phenotype of Geminin depletion. When the amount of Cdt1WT RNA was increased to 1000 pg, 59% of the injected embryos showed a cell cycle arrest (Figure 3.25). Immunoblot shows that injecting this amount of RNA increases the Cdt1 concentration above the endogenous level. This indicates that vast over-expression of Cdt1 is sufficient to overwhelm all inhibitory mechanisms.

*Geminin Mutants That Do Not Inhibit Replication In Vitro Do Inhibit Re-Replication*

Our lab previously reported that three Geminin mutants, YWK, RTGG and KKFEV (Figure 3.26, Top panel) bind Cdt1 and are able to rescue the cell cycle arrest in Geminin deficient embryos but do not inhibit replication in extracts (Benjamin et al., 2004). The activity of these mutants suggested that the phenotype of Geminin deficiency is caused by something other than over-activity of Cdt1. This contradicts our finding that expression of mis-regulated Cdt1 reproduces the phenotype of Geminin deficiency.

It is possible that the recombinant Geminin proteins that we use to inhibit replication in extract is mis-folded or has become inactive in some way during expression in bacteria and the purification process. As a more physiologically relevant way to test these mutants, we translated each one in Xenopus extract using RNA as the starting material. We then measured replication in fresh extract containing 25% extract in which the translation had been carried out. We find that, like bacterially expressed protein, wild type Geminin inhibits replication whereas the YWK, RTGG, and KKFEV mutants do not (Figure 3.26, Middle). Immunoblots from the replication extracts shows roughly equal translation of each Geminin protein (Figure 3.26, Bottom). These results strongly suggest that the reason these Geminin mutants rescue the cell cycle arrest in Geminin deficient embryos is because they prevent Cdt1 from causing re-replication.

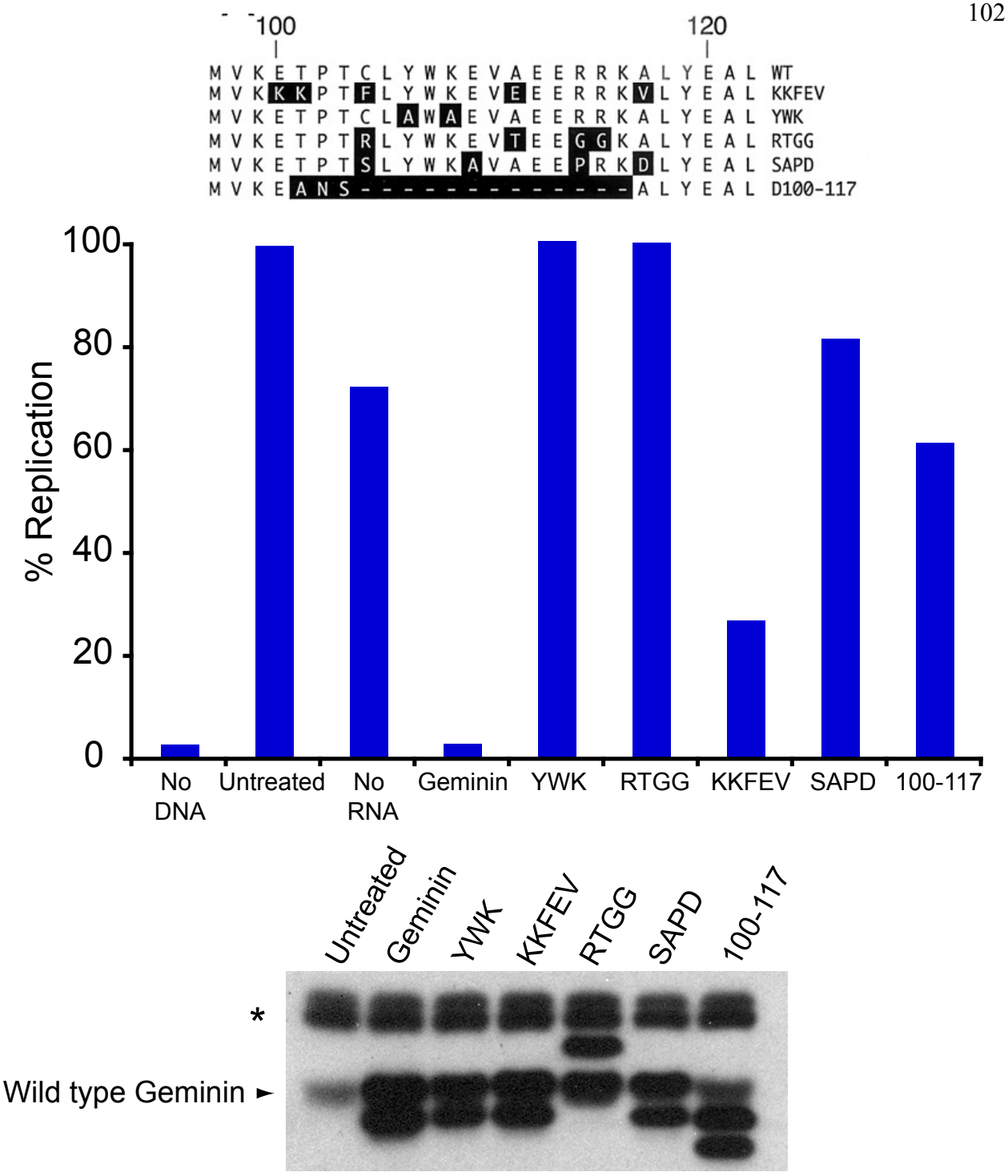


**Figure 3.26 Geminin mutants translated in Xenopus extract do not inhibit replication.** Geminin mutants were translated from RNA in Xenopus egg extract. (Top) Replication was measured in extract supplemented with translated protein. (Bottom) Equal tranlation was determined by Geminin immunoblot of samples taken from the replication reactions using.

To try to reconcile the difference in behavior of these mutants in different assays, we directly tested the ability of the YWK, RTGG, and KKFEV mutants to prevent re-replication in extract. We measured re-replication in extracts that had been depleted of endogenous Cdt1 and Geminin and supplemented with Cdt1 translated in reticulocyte lysate and either wild type recombinant Geminin or each of the Geminin mutants. Addition of equal amounts of recombinant Geminin protein was measured by immunoblot (Figure 3.27, Top panel). We found that the mutants were able to inhibit re-replication in three separate experiments in different extracts (Figure 3.27, Bottom panel). This confirms that these mutants behave like wild type Geminin and suggests that these mutants are able to rescue the cell cycle arrest in Geminin deficient embryos because they prevent Cdt1 from causing re-replication. This still does not explain why these mutants do not inhibit replication when added to S phase extracts. This discrepancy will be addressed in the Discussion below.

**Discussion**

*The Phenotype Of Geminin Deficient Xenopus Embryos Is Due To Re-Replication Caused By Mis-Regulated Cdt1*

We have found that expressing a mutant of Cdt1 that is not regulated by Geminin exactly reproduces the Geminin-deficient phenotype *in vivo*. This finding indicates that, at least in Xenopus, the consequences of Geminin depletion are the consequences of excessive Cdt1 activity. We propose that during the first twelve embryonic cell cycles, which lack G2 phase, re-replication is suppressed by mechanisms that do not involve Geminin or Cdt1. At the mid-blastula transition, when G2 phase is introduced, a small amount of re-replication occurs in the absence of Geminin. This second round of initiation introduces replication forks into the DNA

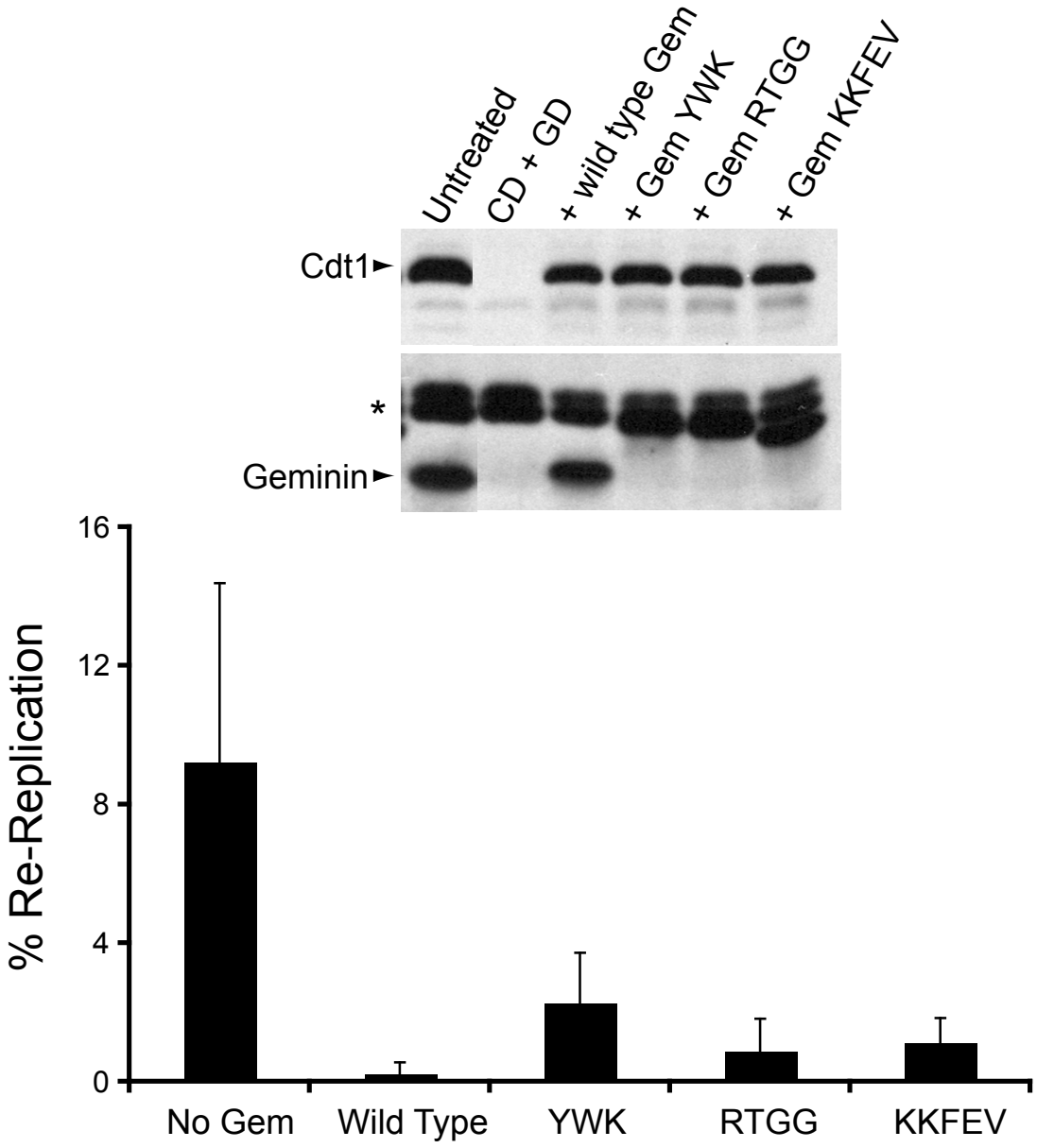


**Figure 3.27 Geminin mutants inhibit re-replication in extract.** Extract was depleted of endogenous Cdt1 and Geminin (CD + GD) and supplemented with Cdt1 translated in reticulocyte lysate plus the indicated Geminin protein purified from bacteria. (Top) Sequence alignment of Geminin WT and mutants. (Middle) The amount of each protein present in the replication reactions was determined by immunoblot using Cdt1 antibody or Geminin antibody. Geminin mutants are His-tagged and run at a slightly higher molecular weight. (Bottom) Replicatin reactions were suplemented with BrdUTP and the density of product DNA was determinedby CsCl gradient. Results were averaged over three independent experiments using different extracts.

that engage the DNA replication checkpoint machinery, activate Chk1 kinase, and arrest the cells in G2 phase.

We found that while our non-degradable Cdt1$^{N150}$ mutant does not cause re-replication in extracts, it does cause a cell cycle arrest in embryos. We can explain this discrepancy by suggesting that the degradation mechanism is not absolutely required to prevent re-replication in one cell cycle (as in extract) because Geminin efficiently inhibits Cdt1 activity. However, if Cdt1 is not degraded over several cell cycles (as in developing embryos) it accumulates to such high levels that it overwhelms the Geminin binding mechanism. This explanation is supported by immunoblots showing that in embryos, Cdt1$^{N150}$ accumulates to levels approximately 20 times greater than are present endogenously (Figure 3.24). We could also reproduce the Geminin deficient phenotype in embryos by overexpressing wild-type Cdt1. Over-expressing Cdt1$^{WT}$ in embryos probably overwhelms the Geminin-dependent regulatory mechanism by titrating all of the available Geminin.

Our finding that ubiquitin-dependent degradation is important for shutting off Cdt1 activity *in vivo* explains the previously published finding that silencing of the CUL-4 gene by siRNA in C. elegans causes accumulation of up to 100C DNA content (Zhong et al., 2003). The CUL-4 gene encodes a cullin-family ubiquitin ligase that can associate with RING finger proteins to form an E3 ubiquitin ligase complex. *C. elegans* treated with CUL-4 siRNA show higher levels of Cdt1 protein, but this study did not show that Cdt1 was the key CUL-4 substrate leading to the increased DNA content (Zhong et al., 2003). Our experiments suggest that their results were due to stabilization of Cdt1 specifically and that the increased DNA content is a result of re-initiation within a single cell cycle rather than some other cell cycle abnormality such as impaired cell division.

Our lab previously published that the YWK, RTGG, and KKFEV Geminin mutants bind to Cdt1 and rescue the cell cycle arrest in embryos that do not express endogenous Geminin but that they do not inhibit replication in extracts. This results lead us to hypothesize that the cell cycle arrest may be caused by some activity of Geminin separate from its ability to inhibit Cdt1. In this study, we have further characterized these mutants. We found that they are able to suppress re-replication in extracts that are depleted of endogenous Cdt1. This suggests that their ability to rescue the cell cycle arrest is due to their ability to inhibit Cdt1 activity.

We do not have a good model to explain why these mutants behave exactly like wild type Geminin in all assays except that they do not inhibit replication *in vitro*. The mutants may have a slightly lower affinity for Cdt1 that is not detectable in co-immunoprecipitation assays, so that they do not inhibit Cdt1 at the onset of S phase but later, in G2 phase when some Cdt1 has been degraded, they can sufficiently inhibit the remaining protein. Alternatively, replication inhibition might require a higher order multimer complex of Geminin that the mutants can not form. One way to investigate the nature of the Geminin mutants would be to co-crystallize the Geminin binding region of Cdt1 with a fragment of each mutant that contains the Cdt1 binding region. The conditions for co-crystallization of a fragment of mouse Cdt1 bound to a fragment of mouse Geminin have been published (Lee et al., 2004). The crystal structure would show that either Cdt1 interacts with the Geminin mutants at the exact same sites as wild type Geminin or that Cdt1 interacts with wild type Geminin in a way that can not occur with the mutants. The latter result could potentially reveal a novel site of interaction that takes place with wild type Geminin but not the Geminin mutants that may be important for replication inhibition.

*Effects Of Geminin In Other Organisms May Be Due To Effects On Replication*

Our results indicate that the Geminin deficient phenotype is caused by a small amount of over-replicated DNA that was undetected by techniques used in previous experiments (McGarry and Kirschner, 1998). We cannot exclude, however, the possibility that the phenotype of Geminin-depleted embryos represents a common developmental response to several different primary abnormalities. Geminin has also been depleted from Drosophila and C. elegans embryos (Quinn et al., 2001; Yanagi et al., 2005). In Drosophila, zygotic deletion of the Geminin gene does not cause an early cell cycle arrest, perhaps because of maternally supplied Geminin RNA and protein. There are, however, some indications of aberrant DNA replication, including anaphase chromosome bridges (which may result from re-duplication of the centromere) and an increased number of cells in the CNS that incorporate BrdUTP, which may reflect continued DNA replication during G2 phase. In C. elegans knockdown of Geminin expression with siRNA causes nuclear defects in the gonad that are associated with sterility. Although no cell cycle defect in somatic cells was reported, it is likely that Geminin expression was not completely suppressed in these animals. In summary, most of the phenotypes observed in Geminin deficient animals could be attributed to abnormalities in DNA replication.

Recently published experiments in cultured cells suggest a role for Geminin in controlling centrosome duplication and spindle assembly during mitosis (Tachibana et al., 2005). This group showed that silencing of Geminin expression in cultured cells with siRNA lead to increased centrosome number. Immunofluorescence staining for tubulin showed that duplication of centrosomes was accompanied by mis-shapen, multipolar spindles during mitosis. A specific role for Geminin in centrosome duplication has not yet been identified and it is not known whether these effects are due to effects on Cdt1 activity. While our results in embryos support

the model that all of the effects of Geminin are mediated through Cdt1, it is, however, possible that some of the cell cycle defects caused by Geminin depletion may be independent of replication defects. This idea will be addressed in the Discussion in Chapter 4.

## Part III: Geminin is Required for Early Embryonic Gene Expression in Xenopus

### Introduction

Geminin was initially discovered as both a cell cycle regulatory protein and a protein that promoted neuronal development (Kroll et al., 1998; McGarry and Kirschner, 1998). When Geminin is moderately overexpressed in Xenopus embryos, the neural plate is expanded (Kroll et al., 1998). Yeast two-hybrid and co-immunoprecipitation assays have identified several novel Geminin interacting proteins. These binding partners include members of the homeobox (Hox) domain family, the polycomb protein Scmh1, the SWI/SNF family member Brg1, and the transcription factor Six3 (Del Bene et al., 2004; Luo et al., 2004; Seo et al., 2005;). These proteins are involved in transcriptional regulation and chromatin remodeling, and their activity can affect embryonic gene expression and cellular differentiation. 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. Because Geminin interacts with proteins involved in replication and gene expression, Geminin has been suggested to inhibit differentiation during proliferation. We sought to determine whether Geminin is required for early embryonic gene expression in Xenopus embryos and whether the Geminin-Cdt1 interaction affects developmental gene expression.

### Results

*Geminin is required for early embryonic gene expression in Xenopus*

To see if Geminin depletion affects early embryonic gene expression in Xenopus, we examined the expression of Goosecoid (Gsc) and Brachyury (Xbra) by RNA *in situ*

hybridization. Gsc is an early embryonic marker of the dorsal side of the body axis. In Xenopus, cells expressing Gsc eventually differentiate into pharyngeal endoderm, head mesoderm, and notochord (De Roberts et al., 1992). Xbra is expressed in epithelial progenitor cells and early mesoderm (Wilkinson et al., 1990). We chose to look at expression of these two genes because they are turned on very early in development at the mid-blastula transition (MBT). Time-lapse imaging shows that Geminin deficient embryos arrest very early in development, just after the MBT (McGarry, 2002). Thus, examining expression of these early genes is a good indicator of the effects of Geminin on gene expression.

In uninjected embryos, Goosecoid is expressed on the dorsal side of the embryo close to the blastopore lip while Brachyury is expressed circumferentially in the marginal zone (Cho et al., 1991; Smith et al., 1991 and Figure 3.28, Left column). We injected anti-Geminin morpholino oligonucleotide into one cell (Xbra) or both cells (Gsc) of a two-cell embryo and saw a marked decrease in the expression of both genes when the embryos reached the early gastrula stage (Figure 3.28, Middle column). Xbra staining shows that the reduced expression is cell autonomous because it was only observed in the descendants of the cell injected with the anti-Geminin oligonucleotide. We measured Xbra expression in embryos co-injected with Geminin RNA that is mutated so that it does not interact with the antisense oligo. These embryos were co-injected with RNA encoding lacZ and treated with X-Gal so that the site of injection is marked by blue staining. Xbra expression is restored in embryos expressing Geminin RNA in addition to antisense oligo (Figure 3.28, Right column). This indicates that the loss of Geminin itself is responsible for the decreased expression.

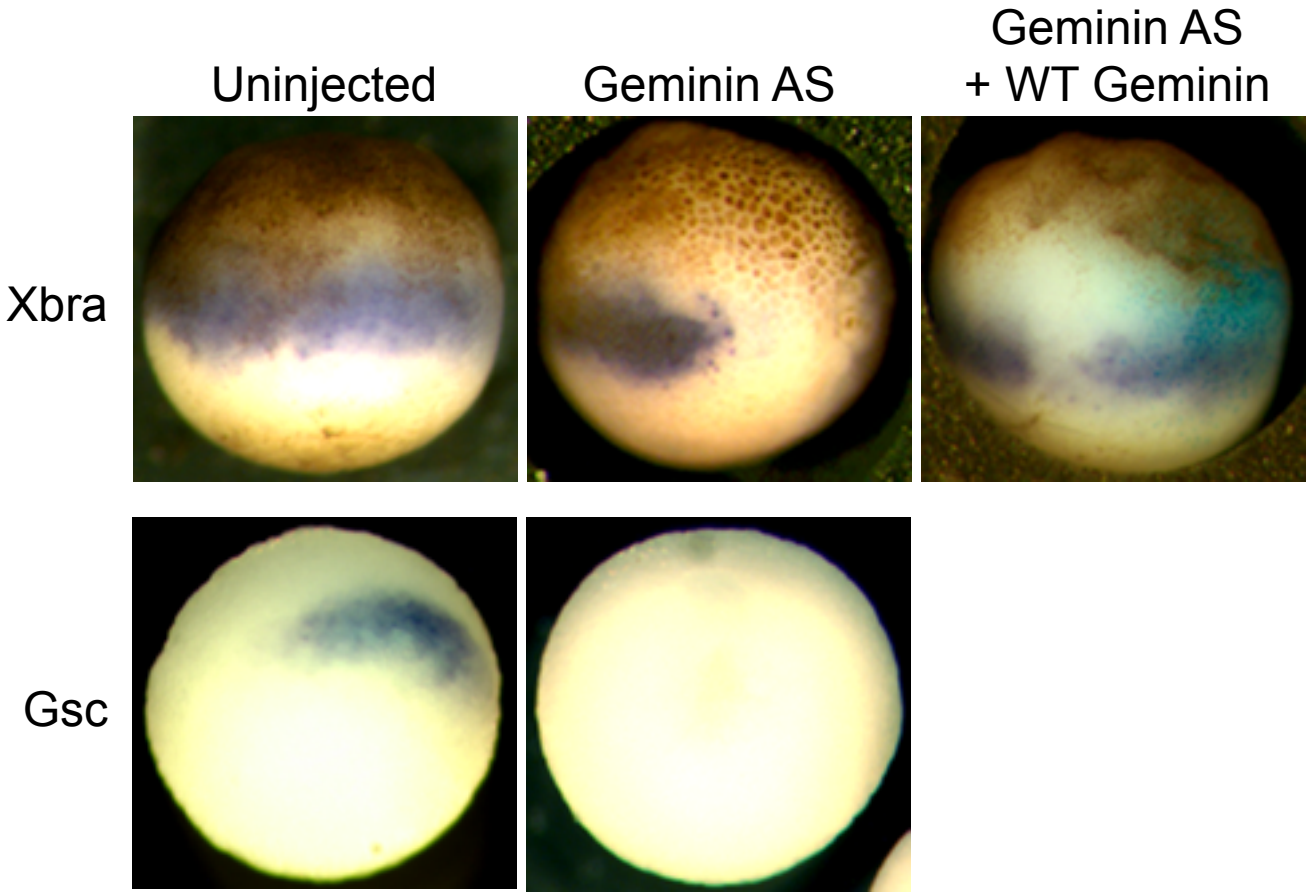


**Figure 3.28 Geminin deficient Xenopus embryos fail to express early embryonic genes.** Expression of Brachyury (Xbra) and Goosecoid (Gsc) was detected in stage 10.5 Xenopus embryos by RNA in situ hybridization. Albino embryos were used for detection of Gsc. Embryos were either uninjected (left), injected with Geminin antisense oligo (middle), or injected with Geminin antisense oligo + RNA encoding wild type Geminin along with RNA encoding lacZ (right). The injection site of embryos injected with Geminin RNA + lacZ RNA was visualized by treating the embryos with X-Gal (blue stain).

*The Cdt1 Binding Domain of Geminin is Required for Rescue of Gene Expression*

We have determined that the cell cycle arrest seen in Geminin deficient Xenopus embryos can be rescued by Geminin mutants that bind Cdt1 but not by mutants that do not bind Cdt1 (Benjamin et al., 2004). To determine whether the Cdt1 binding activity of Geminin is required for expression of these early embryonic genes, we injected a panel of Geminin deletion and point mutants that have previously been characterized by their ability to bind Cdt1 and looked at the effects on Xbra and Gsc mRNA expression (Figure 3.29). Embryos were injected at the two cell stage with anti-Geminin morpholino oligo plus 30pg of RNA encoding the Geminin mutant to be tested. We found that Geminin mutants that can not bind Cdt1 (SAPD and Δ100-117) are not able to rescue the loss in gene expression. Geminin mutants that bind Cdt1 and rescue the cell cycle arrest in Geminin deficient embryos (KKFEV, YWK, RTGG) restore gene expression as well as wild type Geminin (Figure 3.29). We scored these same embryos for the presence of a sector of arrested cells and found that the nearly all embryos that show a cell cycle arrest also show loss in Xbra expression. This suggests that the complete phenotype of Geminin deficient Xenopus embryos can be reproduced by interfering with the Geminin/Cdt1 interaction.

*Expression of Mis-Regulated Cdt1 Reproduces the Gene Expression Defects Seen in Geminin Deficient Xenopus Embryos*

We have found that expression of mis-regulated Cdt1 reproduces the G2 cell cycle arrest seen in Geminin depleted Xenopus embryos. To see if this mutants also reproduces the effects of Geminin loss on embryonic gene expression, we injected one cell of two-cell stage Xenopus embryos with a concentration series of RNA encoding $Cdt1^{NGB}$ and measured Xbra expression

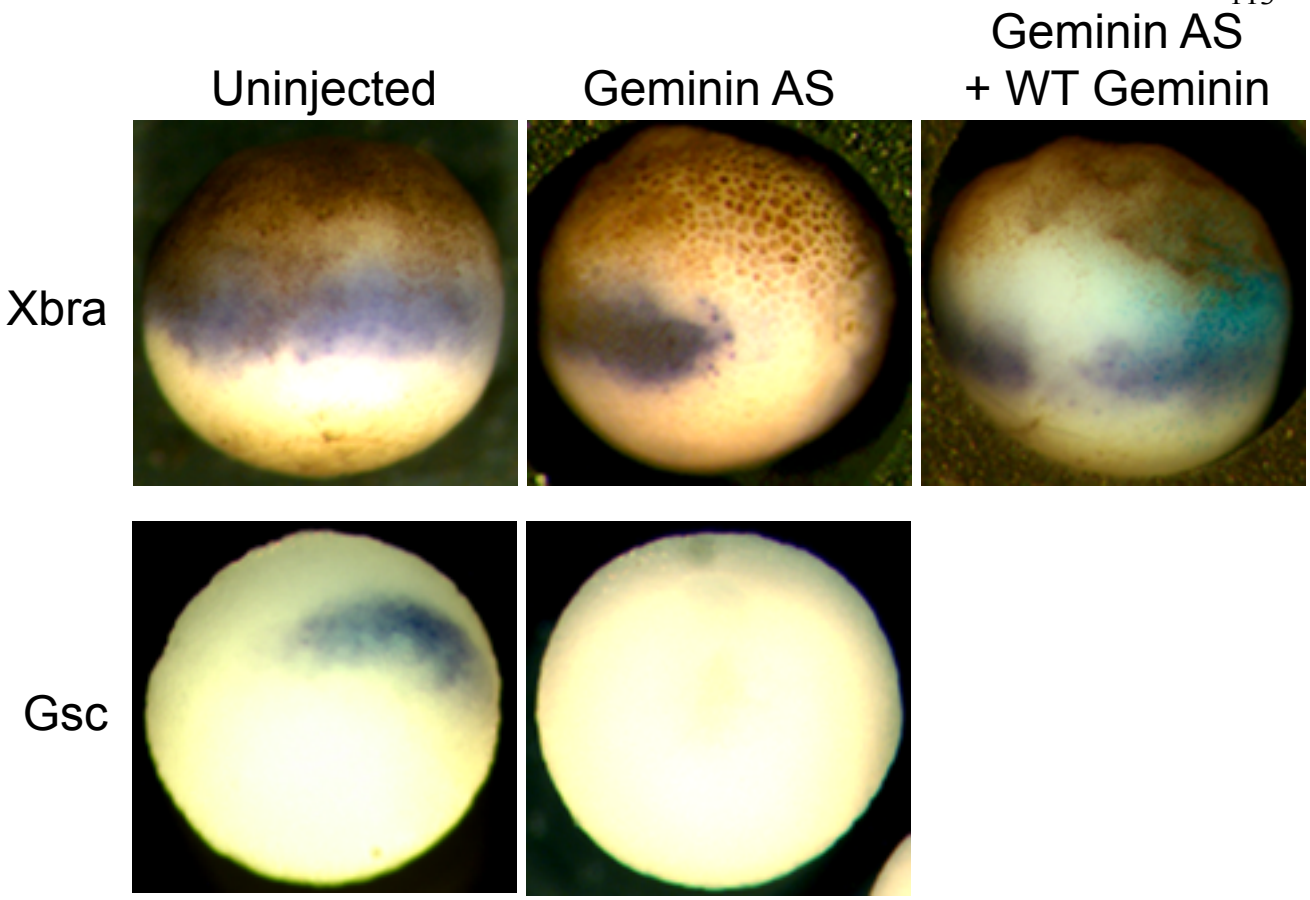


**Figure 3.28 Geminin deficient Xenopus embryos fail to express early embryonic genes.** Expression of Brachyury (Xbra) and Goosecoid (Gsc) was detected in stage 10.5 Xenopus embryos by RNA in situ hybridization. Albino embryos were used for detection of Gsc. Embryos were either uninjected (left), injected with Geminin antisense oligo (middle), or injected with Geminin antisense oligo + RNA encoding wild type Geminin along with RNA encoding lacZ (right). The injection site of embryos injected with Geminin RNA + lacZ RNA was visualized by treating the embryos with X-Gal (blue stain).

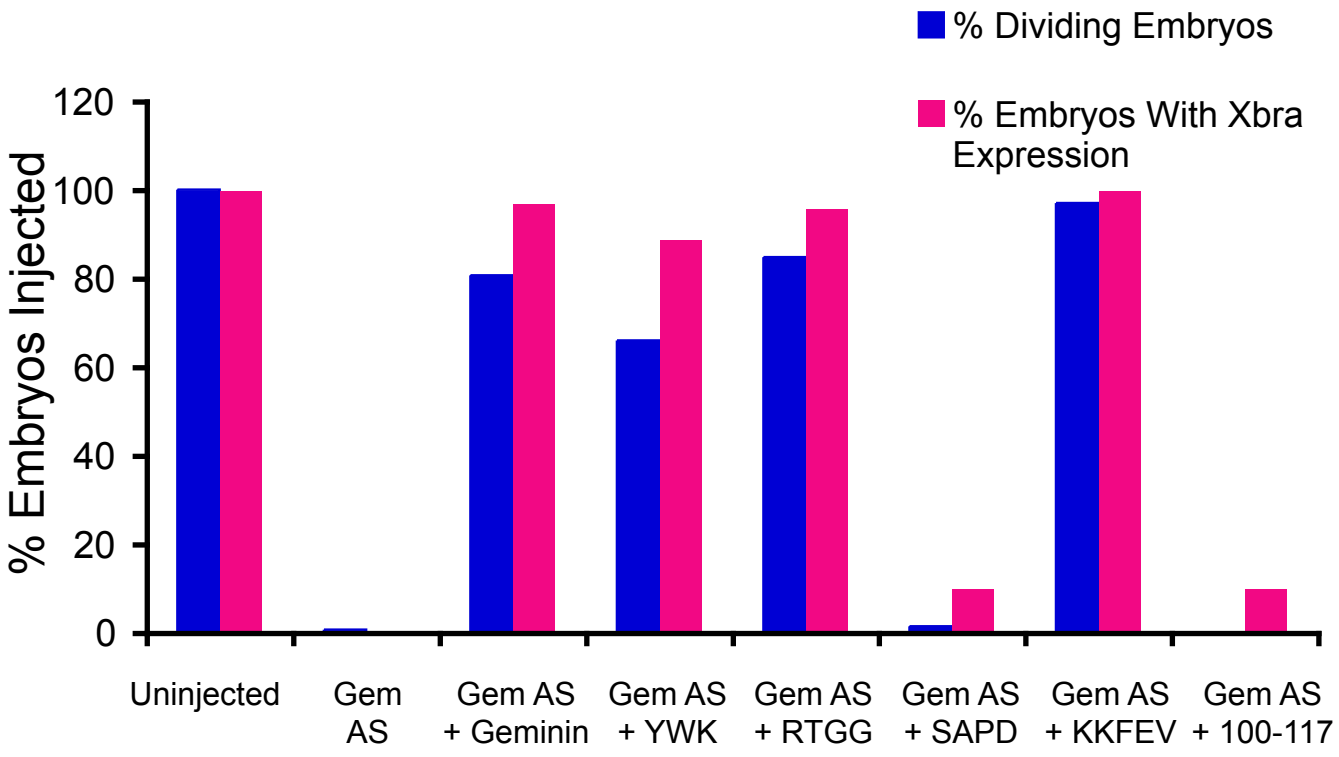


**Figure 3.29 Geminin mutants that do not bind Cdt1 do not rescue the loss in gene expression caused by Geminin deficiency.** Embryos were injected with Geminin antisense oligo + RNA encoding the indicated Geminin mutants. Embryos were blindly scored for both arrested sectors and Xbra expression by two individuals and results were averaged for each experiment.

by *in situ* hybridization. We found that at intermediate concentrations, $Cdt1^{NGB}$ causes a significant loss in Xbra expression compared to wild type Cdt1 (Figure 3.30, Left graph). At 1000pg, $Cdt1^{NGB}$ and wild type Cdt1 inhibit Xbra expression equally (Figure 3.30, Right graph). We have shown previously that the proteins are expressed at approximately 20 times endogenous levels when 1000pg of RNA is injected, suggesting that wild type Cdt1 likely overwhelms the Geminin binding mechanism (Figure 3.24). At intermediate concentrations, the inactive mutant $Cdt1^{C479}$ has no effect on gene expression, confirming that the effects are specifically due to Cdt1 activity and not due to titration of Geminin.

Expression of the non-degradable mutant $Cdt1^{N150}$ has the same effect on the cell cycle as expression of $Cdt1^{NGB}$. To test if this mutant also inhibits expression of Xbra, we injected 300pg of RNA, the concentration found to cause a cell cycle arrest, into one cell of two-cell stage Xenopus embryos. Similar to $Cdt1^{NGB}$, $Cdt1^{N150}$ prevented expression of Xbra mRNA (Figure 3.30). We conclude that the loss of Xbra and Gsc expression is due to over activity of Cdt1.

*The Loss of Gene Expression is a Secondary Effect of the G2 Cell Cycle Arrest*

The findings that Geminin interacts with transcriptional regulators and can affect embryonic development does not immediately explain how mis-regulated Cdt1 would reproduce gene expression defects seen in Geminin deficient embryos. One explanation for this common phenotype is that the loss in Xbra and Gsc gene expression is a secondary effect of the G2 cell cycle arrest cause by Geminin depletion or expression of mis-regulated Cdt1 rather than a direct effect on gene transcription. To see if Geminin deficient embryos are able to express genes that are turned on earlier in development, before the cell cycle arrest, we measured expression of Xnr5 by in situ hybridization. While most embryonic genes are not turned on until after the mid-

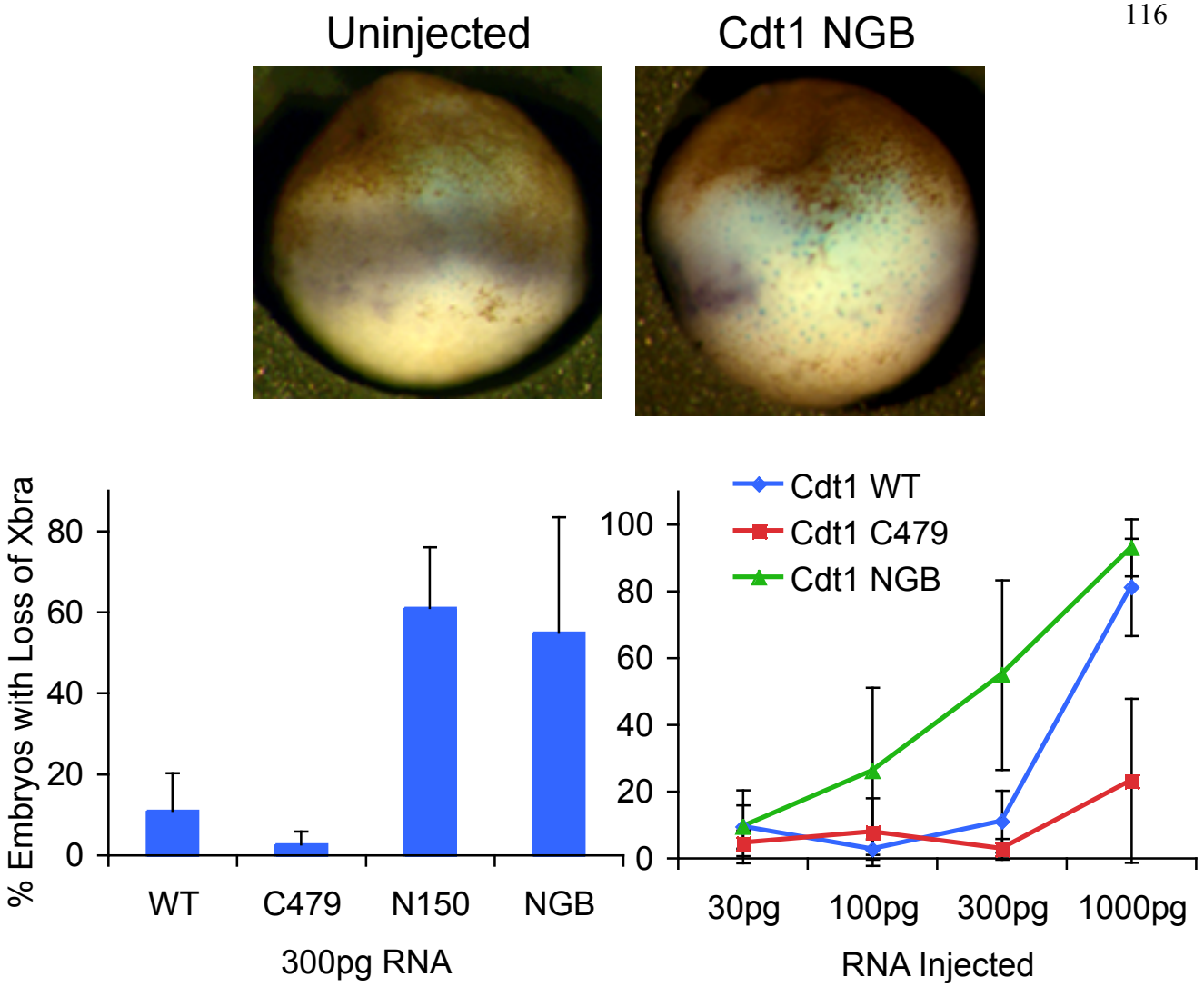


**Figure 3. 30 Expression of mis-regulated Cdt1 mutants reproduces the gene expression defects seen in Geminin deficient Xenopus embryos.** Two cell Xenpus embryos were injected with RNA encoding wild type Cdt1, Cdt1 C479, Cdt1 N150, or Cdt1 NGB and Xbra expression was determined by RNA *in situ* hybridization. (Top) Representative images of Xbra expression in embryos. (Bottom) The percent of embryos injected with 300pg Cdt1 RNA (left) or increasing amounts of Cdt1 RNA (right) that show a loss in Xbra expression. Embryos were scored blindly by two individuals and the results were averaged for each experiment.

blastula transition, a few, including Xnr5, are turned on very early (Takahashi et al., 2000). We see that Xnr5 expression is detectable in stage eight embryos, and that injection of Geminin AS oligo does not inhibit expression compared to untreated embryos (Figure 3.31). Therefore, loss of Geminin does not prevent expression of all genes but seems to affect only genes that are turned on near the point in development when Geminin deficient embryos stop dividing. We conclude that the effects on Xbra and Gsc expression are secondary effects of the cell cycle arrest rather than a specific effect of Geminin on gene expression.

**Discussion**

We have found that Geminin deficient Xenopus embryos fail to express of the early developmental genes Xbra and Gsc. This gene expression defect seems to be a secondary effect of the cell cycle arrest since expression of mis-regulated Cdt1 or Geminin mutants that do not bind Cdt1 produces the same effect. These results raise the possibility that at least some of the developmental defects seen in other organisms when the Geminin level is increased or decreased may be due to effects on the cell cycle. For example, the loss of eye and forebrain development seen when Geminin is overexpressed in the Medaka fish can be explained by reduced numbers of these cell types due to replication inhibition.

A recent report shows that deletion of the geminin gene in the mouse is lethal at the eight-cell stage (Gonzalez et al., 2006). All of the cells in these embryos express Troma-1, a marker of the trophoblast lineage, and fail to express the pluripotency marker Oct-4. One interpretation of this phenotype is that Geminin is required to maintain pluripotency, or rather inhibit differentiation, and that normally suppression of Geminin synthesis triggers trophoblast differentiation. It is also possible that loss of Geminin causes the cells to stop dividing due to

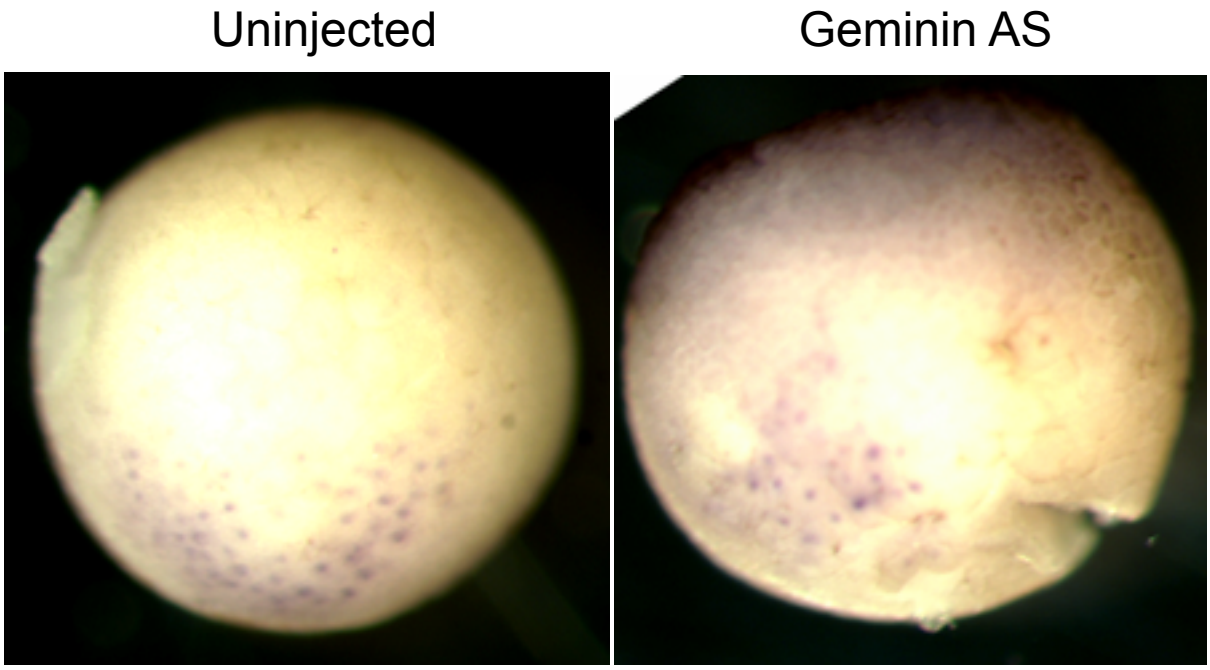


**Figure 3.31 Geminin deficient embryos express the very early embyonic gene Xnr5.** Two cell stage Xenopus embryos were uninjected or injected with Geminin antisense oligo and Xnr5 expression was detected by RNA *in situ* hybridization.

replication defects and the cells differentiate into trophoblasts as a result of the halted proliferation. Our results in Xenopus embryos support the latter model, though they do not rule out a more direct role of Geminin in maintaining pluripotency.

# CHAPTER 4—DISCUSSION

*Model for Prevention of Re-Replication*

The goal of this project was to investigate the mechanisms that prevent re-replication of DNA within each cell cycle. There have been two suggested models for preventing re-replication. The positive licensing model states that an essential replication licensing factor is consumed in the nucleus during S and G2 phase and can not be replenished until the nuclear envelope breaks down and reforms during mitosis. The negative licensing model states that a replication inhibitor accumulates in the nucleus during G2 phase and is present until the nuclear envelope breaks down during mitosis. The results of this study provide evidence that both models are correct and add to our understanding of how these two models cooperate to prevent re-replication within one cell cycle.

We propose that Cdt1 is the positive replication licensing factor described in the first model and Geminin is the negative licensing inhibitor described in the second model. We have determined that Cdt1 is shut off by binding to Geminin and by ubiquitin-dependent proteolysis. Disruption of the Cdt1-Geminin interaction causes re-replication in extracts, and this re-replication is enhanced by stabilization of Cdt1.

Our results also added more details to this model. We found that Geminin is required to prevent re-replication specifically during G2 phase and propose that additional mechanisms, including Cdt1 degradation, as well as Cdt1-independent mechanisms (such as inhibition of other pre-RC components) prevent re-replication during early S phase. We also found that Cdt1 is heavily phosphorylated in metaphase and we have identified 15 sites of phosphorylation. We think that Cdt1 phosphorylation may be complex, and we hypothesize that phosphorylation at

different sites may activate or inhibit the protein. Finally, we made the interesting observation that an N-terminal myc tag interferes with inhibition of Cdt1. We show that myc-Cdt1 is inhibited normally by Geminin, suggesting that the myc tag may interfere with a potentially novel mechanism.

We have extended our findings to an animal model by analyzing the effects of mis-regulated Cdt1 in Xenopus embryos. We have shown that the G2 cell cycle arrest and activation of the replication checkpoint seen in Geminin deficient Xenopus embryos is reproduced by expressing mis-regulated Cdt1 mutants. We have also found that Geminin deficient Xenopus embryos do not express the early embryonic genes Xbra and Gsc. Like the cell cycle arrest, this gene expression defect is reproduced by expressing Geminin mutants that do not bind Cdt1 or Cdt1 mutants that are not shut off properly. Similarly, Geminin mutants that do not bind Cdt1 are not able to rescue the cell cycle arrest or the loss in gene expression. Taken together, these results suggest that the entire phenotype of Geminin deficient Xenopus embryos is due to the cell cycle arrest that is caused by re-replication (Figure 4.1).

*Geminin As A Switch Between Proliferation And Differentiation*

Geminin has been shown to interact specifically with several transcriptional regulatory proteins. Though our data suggests that the effects of Geminin depletion on early Xenopus development are due to mis-regulated Cdt1 activity, it is possible that some of the effects of Geminin in other organisms are mediated through interactions with these proteins.

Yeast two-hybrid and *in vitro* immunoprecipitation assays show that Geminin can bind directly to seven different members of the Hox family of homeodomain proteins as well as the polycomb group protein Scmh1. ChIP assays show that Geminin associates with Hox regulatory

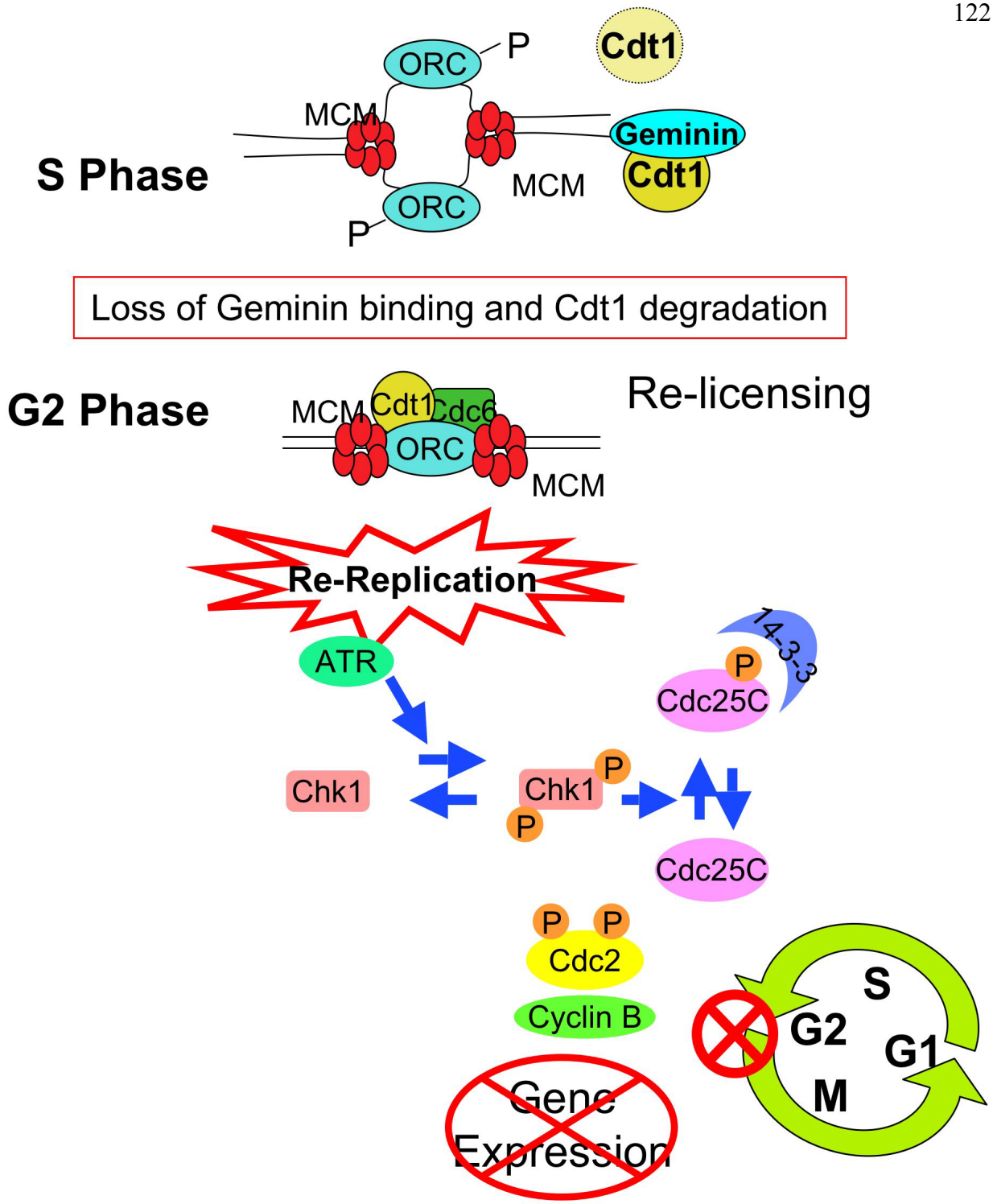


**Figure 4.1 Model for replication licensing by Cdt1 and Geminin.** Cdt1 activity is shut off by ubiquitin dependent proteolysis during S phase and by Geminin binding during G2 phase. In the absence of these mechanisms, Cdt1 can re-license replication origins and cause re-replication. Re-replicated DNA is sensed by the replication checkpoint which arrests the cell in G2 phase and prevents gene transcription.

elements (Luo et al., 2004). Geminin co-immunoprecipitates with the polycomb group protein Rae28 in vivo in mouse embryonic extracts (Luo et al., 2004). Altered levels of Geminin can affect the expression and activity of these proteins as well as embryonic development in ways that suggest Geminin has other activities in addition to Cdt1 inhibition.

Geminin also co-immunoprecipitates with the SWI/SNF chromatin-remodeling protein Brg1 in cell lysates (Seo et al., 2005b). This group showed that overexpression of Geminin or loss of Brg1 in Xenopus embryos results in failure of normal neuronal differentiation. In this study, Xenopus embryos were injected with RNA encoding a Geminin mutant that does not bind Cdt1, so that the effects of Geminin were likely due to effects on interactions with other proteins and independent from effects on DNA replication.

These results have lead to the model that Geminin may act as a switch between proliferation and differentiation. According to this model, when Geminin is present, it interacts with transcriptional regulators to keep developmentally regulated genes silenced. It also interacts with Cdt1 to prevent re-replication, thereby allowing proper entry into mitosis and progression through the cell cycle. When Geminin is absent, transcriptional regulators are free to act on genes involved in differentiation.

Since Geminin-depleted or $Cdt1^{NGB}$-expressing Xenopus embryos arrest very early in development, we were not able to investigate whether abnormal Cdt1 regulation is responsible for the abnormalities in cell differentiation that have been ascribed to Geminin. We were able to show that Geminin deficient embryos fail to express the early embryonic genes Xbra and Gsc and that this gene expression defect is reproduced in embryos expressing mis-regulated Cdt1 mutants. This suggests that, at least in Xenopus, effects on gene expression are secondary effects of mis-regulated Cdt1 activity and cell cycle arrest.

*The Role Of Cdt1 and Geminin In Tumorigenesis And Cancer Prognosis*

Our results, and the recently published results of others indicate that Geminin may act as a tumor suppressor protein by inhibiting Cdt1 from initiating a second round of replication in one cell cycle. The importance of inhibiting over-activity of Cdt1 in tumorigenesis is evidenced by the increased tumorigenicity of $p53^{-/-}$ mice that overexpress Cdt1 (Seo et al., 2005a). These mice develop a greater number of lymphomas, and the lymphomas they develop are larger than those seen in $p53^{-/-}$ mice. These mice also have a decreased lifespan compared to $p53^{-/-}$ mice. Presumably, the increased tumorigenesis seen in these mice is due to increased genomic instability resulting from re-replicated chromosomes. In support of this, NIH3T3 cells overexpressing Cdt1 form tumors in mice. Chromosomal analysis using these cells shows abnormal chromosome numbers, translocations, inversions, and end-to-end fusions. Silencing of Geminin in cultured cells results in accumulation of greater than 2n DNA content (Melixetian et al., 2004; Zhu et al., 2004). These studies provide evidence that by preventing over-activity of Cdt1, Geminin acts as a safeguard against genomic instability which can be a precursor to tumorigenic transformation.

Despite this data indicating that Geminin may act as a tumor suppressor, several studies indicate that increased Geminin expression in tumor cells is a predictor of poor clinical outcome. As mentioned, in breast tumor samples, Geminin expression was positively associated with tumor size, histological grade, Nottingham prognostic index (NPI) score, and tumor type and negatively associated with estrogen receptor (ER) status (Gonzalez et al., 2004). Geminin expression was also found to be positively associated with overall survival and development of metastases. Thus, Geminin expression can be used as a prognostic marker because of its

expression pattern with in tumor tissues. It is possible that the increased Geminin levels in tumor tissues compared to normal tissues is a consequence of increased numbers of proliferating cells. As Geminin is expressed normally during G2 phase, it is likely that, at least in some cases, increased Geminin expression is a consequence of increased proliferation and, more specifically, increased cells in S and G2 phase of the cell cycle.

As Geminin has been found to inhibit differentiation in proliferating cells, an interesting possibility is that increased Geminin expression in tumor cells is a cause rather than a consequence of tumorigenesis. By inhibiting differentiation, Geminin may confer a selective advantage on cells by preventing them from exiting the cell cycle and allowing them to remain in a proliferative state. In this way, increased Geminin expression may be indicative of a de-differentiated, or stem cell-like state. There are currently no published studies investigating mutations in the Geminin gene with respect to tumor formation. Mutations in many other genes involved in replication control, such as DNA repair genes, underlay syndromes that are characterized by a predisposition to cancer. It would be interesting to see if mutations in the Geminin gene, for example mutations in the Cdt1 binding domain, might have similar effects on tumorigenesis.

*Future Directions*

We have carried out a preliminary study to determine if Geminin binding and ubiquitin-dependent degradation are the only mechanisms that shut off Cdt1 activity. We set up a large replication reaction in extract that had been immunodepleted of Geminin and supplemented with MG132 to prevent Cdt1 degradation. Once replication was complete (as determined by 30 minutes after complete nucleus formation) we collected nuclei and centrifuged them to make a nuclear extract (nucleoplasmic extract, NPE). This removes any cytoplasmic Cdt1 that would not have participated in replication. We set up replication reactions that consisted of 75% fresh Cdt1-depleted extract and 25% post-replicative Cdt1 (from NPE). Control reactions consisted of 75% fresh Cdt1-depleted extract and 25% untreated extract or 25% high-speed supernatant (HSS). The HSS contains pre-replicative Cdt1 but can not support replication on its own because it can not form nuclei. We find that HSS (containing pre-replicative Cdt1) can support replication in Cdt1 depleted extract, but Cdt1 depleted extract supplemented with NPE (containing post-replicative Cdt1) does not replicate (Figure 4.2, Top). This suggests that Cdt1 may be inactivated following replication by mechanisms that are independent of degradation or Geminin binding. However, this experiment is not conclusive. Immunoblots show that NPE contains slightly less Cdt1 than is present in untreated extract or HSS (Figure 4.2, Bottom). So, it is possible that the reason NPE does not rescue is because there is less Cdt1, not because the Cdt1 is inactive.

We would like to repeat this experiment using a non-degradable Cdt1 mutant rather than adding MG132 to the extract. We have found that MG132 does not completely inhibit Cdt1 degradation. Also, MG132 does not prevent Cdt1 from being ubiquitylated which may have an effect of Cdt1 activity aside from directing its degradation. Use of a non-degradable protein

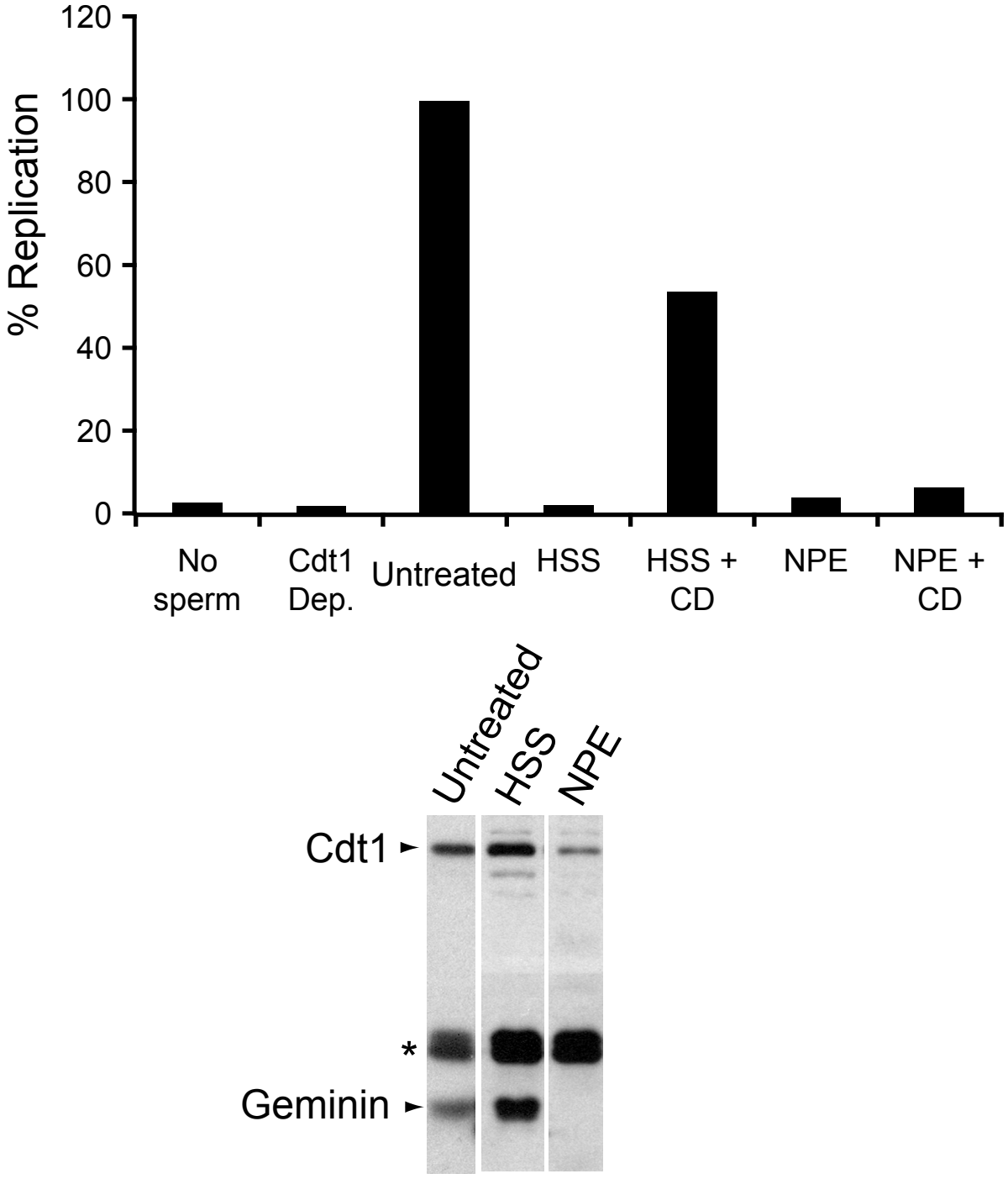


**Figure 4.2 Post-replicative Cdt1 is inactive.** (Top) Replication was measured in untreated extract, high-speed extract (HSS), high-speed extract mixed 1:1 with Cdt1 depleted extract (HSS + CD), nucleoplasmic extract made from post-replicative nuclei formed in Geminin depleted extrac (NPE), or nucleoplasmic extract mixed 1:1 with Cdt1 depleted extract (NPE + CD). % Replication is normalized to untreated extract. (Bottom) Cdt1 and Geminin immunoblots of extract used in the replication reactions.

would ensure that we are eliminating the ubiquitylation and degradation mechanism and ensure that the level of Cdt1 would not be variable.

If we find that post-replicative Cdt1 from Geminin depleted extract is not active, we would like to further investigate the additional mechanism(s) that shut Cdt1 off. One obvious mechanism is phosphorylation. The phosphorylation studies we have carried out suggest that Cdt1 phosphorylation may be complex with both activating and inhibitory components. We have mapped the phosphorylation sites on Cdt1 that is bound to Geminin during metaphase. We would like to continue this investigation by mapping the phosphorylation sites on Cdt1 during G1 phase that has not yet participated in a round of replication. We could immunoprecipitate both pre-and post-replicative Cdt1 from HSS and NPE respectively and analyze it by mass spectrometry. By comparing the phosphorylation state of pre-replicative Cdt1 with that of post-replicative Cdt1 we may be able to identify sites that are critical for both activation and inactivation of the protein. We would also like to map the phosphorylation state of post-replicative Cdt1 that is not bound to Geminin to see if phosphorylation affects Geminin binding. $Cdt1^{NP}$ can co-immunoprcipitate Geminin from Xenopus embryos but we do no know if the specific sites that we mapped by mass spectrometry are important for Geminin binding. Alternatively, phosphorylation may be a separate mechanism to shut off Cdt1 activity immediately after origins fire to prevent re-initiation before Geminin protein accumulates.

We would also like to further investigate the nature of the effect of the N-terminal myc-tag. We plan to determine if adding a different type of tag, such as GFP, to the N-terminus of Cdt1 has the same effect as the myc tag. This would help us determine if the myc tag is doing something positive, such as competing for binding to an inhibitory protein, or if it is doing something negative, such as preventing Cdt1 from being released from chromatin.

We would also like to extend our finding to a mouse model. While Geminin deletion is embryonic lethal in the mouse, a conditional knockout would allow us to analyze the effects of Geminin in development and differentiation in specific tissue types. Based on the model that Geminin acts as a tumor suppressor, we would expect to see tumor formation in the tissue type in which the Geminin gene was deleted. Based on the model that Geminin acts as a switch between proliferation and differentiation, we might expect to see reduced cell numbers or prematurely differentiated cells in the tissue type in which the Geminin gene was deleted.

A conditional transgenic $Cdt1^{NGB}$ expressing mouse would allow us to compare the effects of Geminin deletion to the effects of mis-regulated Cdt1 to more conclusively determine which are caused by a common mechanism. It might also be useful to knock out Geminin or express $Cdt1^{NGB}$ at a specific time in development using a doxycycline-inducible Cre recombinase system. This would allow us to, for example, knock out Geminin in a tissue type that is no longer actively dividing so that any effects we see would be due to effects on gene expression or interaction with chromatin remodeling proteins rather than effects on DNA replication.

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