Molecular Characterization of a Replication Licensing Checkpoint

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine.

Chapel Hill
2009

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ABSTRACT

Kathleen Rae Nevis: Molecular Characterization of a Replication Licensing
Checkpoint

(Under the directions of Dr. Marila Cordeiro-Stone and Dr. Jeanette Gowen Cook)

Each and every time a cell divides it must replicate millions of bases of DNA.
In order to carry out this large feat, it initiates replication from thousands of sites
along the DNA known as origins. Origins of DNA replication are licensed through
the assembly of a chromatin-bound pre-replication complex consisting of ORC,
Cdc6, Cdt1 and the MCM complex. Multiple regulatory mechanisms block new pre-
replication complex assembly after the G1/S transition to prevent rereplication. The
strict inhibition of licensing after the G1/S transition means that all potential origins
used in S phase must have been licensed in the preceding G1. Therefore, it would
seem crucial for the cell to sense whether enough origins have been licensed before
entering S phase. This dissertation focuses on investigation of a novel "origin
licensing checkpoint."

It is documented in this study that that depletion of either of two essential
licensing factors, Cdc6 or Cdt1, in normal human fibroblasts induces a G1 arrest
accompanied by inhibition of cyclin E/Cdk2 activity and hypophosphorylation of Rb.
The Cdk2 inhibition is attributed to a reduction in the essential activating
phosphorylation of T160 and an associated delay in Cdk2 nuclear accumulation. In
contrast, licensing inhibition in the HeLa or U2OS cancer cell lines failed to down-

regulate Cdk2 or Rb phosphorylation, and these cells died by apoptosis. Co-depletion of Cdc6 and p53 in normal cells restored Cdk2 activation and Rb phosphorylation, permitting them to enter S phase with a reduced rate of replication that was accompanied by markers of DNA damage. These results demonstrate dependence on origin licensing for multiple events required for G1 progression, and suggests a mechanism to prevent premature S phase entry that functions in normal cells.

ACKNOWLEDGEMENTS

I would like to start off by thanking my two advisors, Dr. Marila Cordeiro-Stone and Dr. Jeanette Gowen Cook.   I am grateful for having two amazing mentors who taught me so much not only about science but about life too. There enthusiasm for teaching and wanting their students to succeed was a major driving force in my success. I would like to thank my committee members: Dr. William Kaufmann, Dr. David Kaufman and Dr. Robert Duronio for their scientific discussion and expertise. They were very instrumental in my development as a scientist and as a person.

I would like to acknowledge the past and present members of the Cordeiro-Stone and Cook Lab's. They created the best lab environment that anyone could ever ask for. Thank you to Stephanie Cohen, Bruna Brylawski, and Paul Chastain who were always there for amazing scientific discussion and words of encouragement. I want to especially thank Srikripa Chandrasekaran and Karen Reidy for making the late nights in the lab so much more fun and for being great friends.

To all the friends I have made over the past 6 years. Each and every one of them has made an impact in my life and I am grateful for each of them. In particular, I want to thank Seth Maleri, Lea Beauleiu, Elizabeth Dorn, Tim Miller, Paula Miller, Cindy Swarts, and Sara Malvar.   They have been there for me during the ups and downs of graduate school, and never gave up on me. They are truly amazing and

these seven people exemplify what it means to be a friend. Without them I would have never made it through the last 6 years.

Finally, to my parent, Richard and Geraldine, I want to thank them for all of the love, support and encouragement they have provided for me throughout the last 6 years. They have always encouraged me to pursue my goals even if it meant being thousands of miles away. To my brother, Rich, for all the love and support he have provided me. He truly is my other half and I could not imagine a better brother in the world!

To All Who Have Touched My Life- Thank You!!!

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LIST OF ABBREVIATIONS

AAA+ - ATPases-Associated with various cellular Activities

AKT - v-akt murine thymoma viral oncogene homolog 1

Ankrd17 – Ankyrin repeat domain 17

APC - Anaphase promoting complex

ARS - Autonomously replicating sequences

ATCC - American Type Culture Collection

ATM - Ataxia telangiectasia mutated

ATP - Adenosine triphosphate

ATR - Ataxia telangiectasia mutated and Rad3 related

BrdU - Bromodeoxyuridine

CAK - Cdk-activating kinase

Cdc25 - Cell division cycle 25

Cdc45 - Cell division cycle 45

Cdc6 - Cell division Cycle 6

Cdk - Cyclin dependent kinase

Cdc2 - Cell division cyclin 2

Cdt1 - Cdc10 dependent transcript 1

ChIP - Chromatin immunoprecipitation

Chk1 - Checkpoint kinase 1

Chk2 - Checkpoint kinase 2

CHO - Chinese hamster ovary cells

CKI - Cyclin-dependent kinase inhibitors

CTD - C-terminal domain

Cul4 - Cullin 4

DDB1 - DNA damage binding protein 1

DDK - Dbf4-dependent kinase

DMEM - Dulbecco's Modified Eagle Medium

FBS - Fetal Bovine Serum

GSK-3β - Glycogen synthase kinase 3 beta

HDAC - Histone Deacetylase

HMGA1a - High mobility group AT-hook 1

HPV - Human Papillomavirus

KAP - Cdk-associated phosphatase

MAPK - Mitogen-activating protein kinase

Mat1 - Menage a trios homolog 1

MCM - Minichromosome maintenance complex

MEM - Modified Eagle Medium

NHF - Normal human fibroblasts

NLS - Nuclear localization signal

ORC - Origin recognition complex

PAGE - Polyacrylamide gel electrophoresis

PCNA - Proliferating cell nuclear antigen

PP2A - Protein phosphatase 2A

preRC - Pre-replication complex

PTEN - Phosphatase and tensin homolog

PVDF - Polyvinylidene Fluoride

Rb - Retinoblastoma

Raf - v-raf-1 murine leukemia viral oncogene homolog 1

RNAi - RNA interference

SCF - Skp, Cullin, F-box containing complex

SDS - Sodium dodecyl sulfate

siRNA - Small interfering RNA

Skp2 - S-phase kinase-associated protein 2

Tlk1 - Tousled-like kinase 1

UV - Ultraviolet radiation

CHAPTER 1

INTRODUCTION

A. Biological Significance

Everyday, thousands of people are diagnosed with cancer, and based on rates from 2003-2005, 40.25% of men and women born today will have cancer at some point in their lifetime (cancer.gov). It is estimated that in 2008 there will be 1.4 million new cases of cancer and a staggering 565,000 deaths (SEER Cancer Statistics Review). With the help of ongoing research, there have been major advances in the development of new therapies that are prolonging the quality of life for people diagnosed with cancer. Unfortunately, even with new therapies, cancer mortality continues to be the second leading cause of death among all Americans (http://www.cdc.gov/nchs/FASTATS/deaths.htm).

In order to foster the development of better treatments, there must be a continued effort toward a better understanding of how cancer develops. Are there cellular mechanisms that can be targeted to make cancer cells susceptible to death? If so, researchers must understand how the manipulation of these mechanisms would affect both the normal cells and the cancer cells. The discovery that disruption of origin licensing results in cell death in cancer cells but survival of normal cells will be the focus of this dissertation. Specifically, the search for the

mechanisms that protect normal cells from cell death under conditions of inefficient origin licensing will be discussed.

B. Human Cells in Culture as an Experimental Model System

A number of experimental models systems: yeast, mice, *Xenopus*, Drosophila, and mammalian cells in culture can be utilized to address a variety of experimental hypotheses. The work to be described in this dissertation will utilize mammalian cells cultured *in vitro* under a controlled environment.   There are many advantages and reasons why cell cultures are so widely utilized. First, this model allows one to examine a wide range of cell types (normal cells and various types of cancer cells) from both animals and humans. Second, because the cells are grown in culture, this allows for relatively short experimental time frames. Third, it allows for easy manipulation of proteins by down-regulation (siRNA) or over-expression (plasmid transfection or adeno/retroviral infection). And finally, all the above combined allows one to better study various molecular pathways involved in different biological responses.

With every model system there are inherent disadvantages and this is no exception for mammalian cell culture. One of the major disadvantages is that it is difficult to reproduce with cells in culture the role that the microenvironment may play in a cellular response. Tumors are surrounded by normal cells of various origins and these normal cells may secrete factors that retard or accelerate the growth of the tumor. Tumors are also able to secrete various factors, including those that promote vascularization from surrounding endothelial cells. In a cell culture system, the

microenvironment that exists *in vivo* is not replicated, so the way a cell responds to certain conditions *in vitro* may not represent how it would respond in its true environment. Another disadvantage is that many normal cells will undergo senescence and therefore can only be passaged in culture for a very short period of time. This disadvantage has become less of an issue with the ability to constitutively express telomerase, an enzyme that maintains the length of telomeres and stabilizes the ends of chromosomes. Expression of telomerase in normal cells expands their lifespan in culture (17).

C. The Cell Cycle and Maintenance of Genomic Stability

In order to ensure that genetic information is duplicated and properly segregated to each daughter, the dividing cell must traverse through four phases of the cell cycle (Figure 1.1). Gap 1 phase, or G1 phase, is the time preceding DNA replication where the cell prepares the DNA to undergo DNA replication. The cell then enters synthesis phase, or S phase, where the DNA is replicated and an exact copy of the genome is generated. Following S phase, the cell enters Gap 2 phase, or G2 phase, during which the cell verifies that the entire genome has been replicated and prepares for cell division. Finally, the cell enters mitosis, or M phase, where the cell divides to generate two genetically identical daughter cells.

As the cell traverses through the cell cycle, it can accumulate various types of DNA damage induced by exogenous insults (ultraviolet light, ionizing radiation, and alkylating agents), reactive oxygen species formed during cellular metabolism, and replication errors. This damage is detected in the cell by sensory proteins that elicit
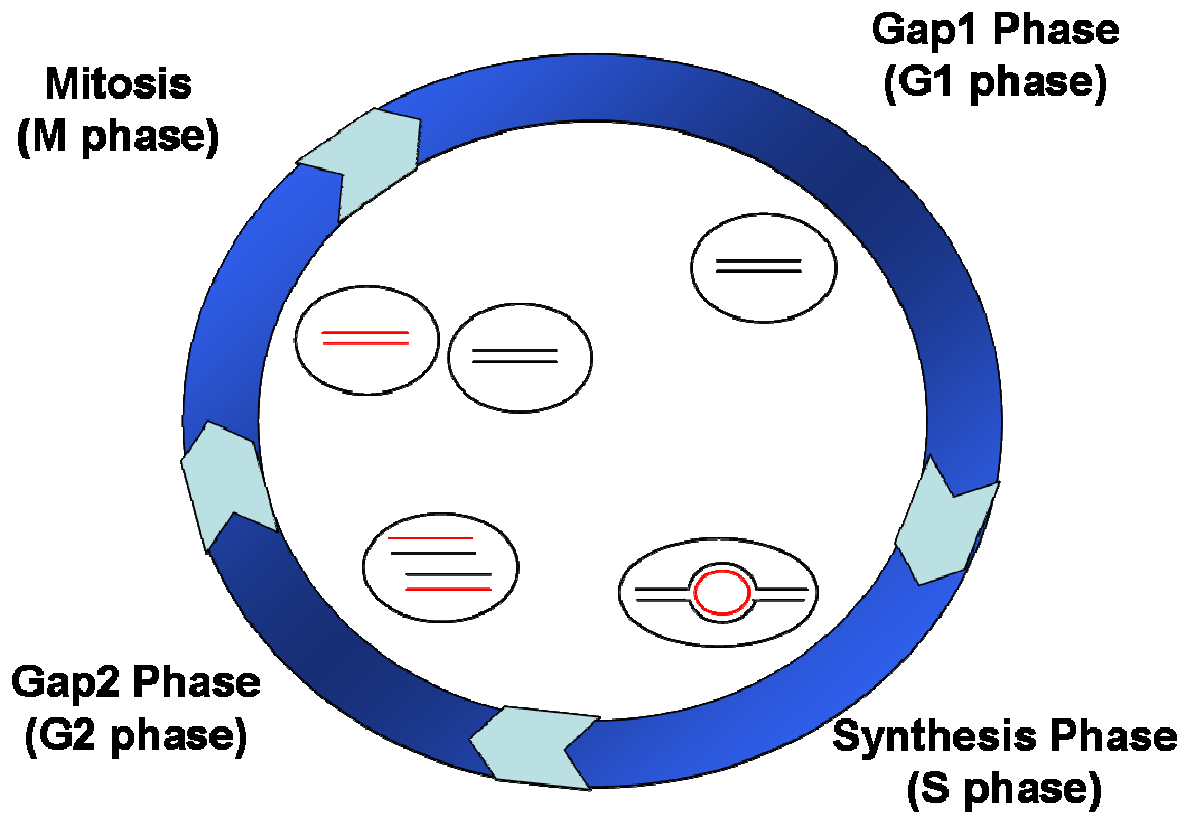
Figure 1.1: Schematic Representation of the Different Phases of the Cell Cycle. In Gap1 or G1 phase of the cell cycle, the cell prepares the DNA for replication. The cell undergoes DNA replication in S phase and completion of replication is monitored in G2 phase. Finally, the cells enter mitosis or M phase during which it divides to generate two daughter cells.

an intricate signal transduction pathway known as a checkpoint response. Cell cycle checkpoints are signaling pathways that slow or arrest progression through the cell cycle, extending the time for the repair of DNA lesions or for the completion of preceding cellular events. These checkpoints are important to the cell's ability to maintain genomic stability.

There are, in essence, two different types of checkpoints: DNA damage checkpoints and surveillance checkpoints. The DNA damage checkpoints operate by activating specific pathways that depend on the type of damage detected and the phase of the cell cycle. Proteins in the cell known as sensor proteins detect the damaged DNA and propagate a signal to activate the downstream protein kinases, ATM or ATR. Activated ATM and ATR, in turn activate downstream kinases Chk2 and Chk1, respectively (3,96,175). This cascade of events represents the initial steps required for the activation of a checkpoint that ultimately arrests the cell in G1, S, or G2 phase of the cell cycle (Figure 1.2). These arrests allow time to repair the damaged DNA before proceeding into the next phase of the cell cycle.

The most extensively studied of the surveillance checkpoints are the spindle checkpoint and the decatenation checkpoint. Surveillance checkpoints differs from the DNA damage checkpoints, in that they are present to safeguard the normal transition through the cell cycle and are turned off once specific cellular events are completed. For example, in the spindle checkpoint, the checkpoint is "on" until all chromosomes are attached to the mitotic spindle, and it is only then that the checkpoint is turned "off" and the cell is allowed to complete mitosis.
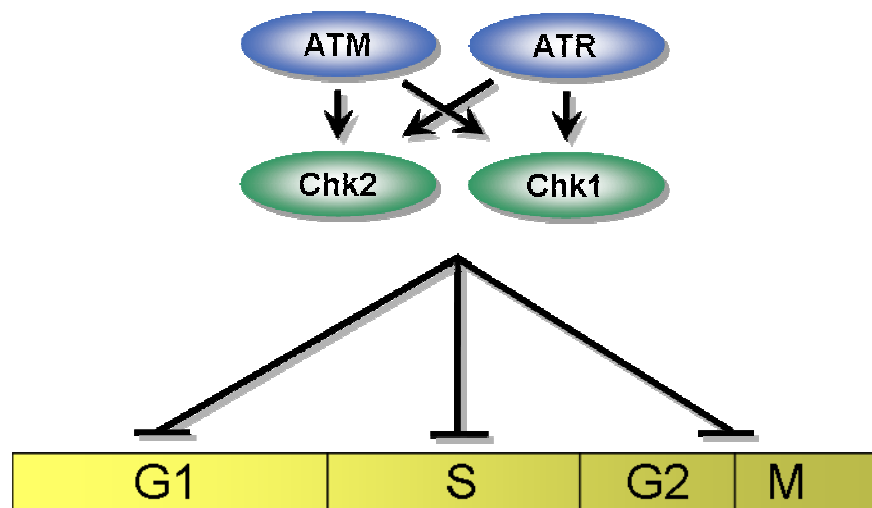
Figure 1.2: Simplified schematic of the various DNA damage checkpoints. In response to various forms of damage, ATM and ATR become activated, resulting in the downstream activation of two kinases, Chk2 and Chk1. The initiation of this cascade results in the arrest of cells in G1, S, or G2 phase of the cell cycle.

D. Mechanisms of DNA replication

One of the most important events in the G1 phase of the cell cycle is the preparation of the DNA to undergo DNA replication. In order to replicate the entire genome in S phase, the cell initiates DNA replication from thousands of sites along the DNA known as replication origins. Much of our understanding of origins comes from studies performed in yeast. *S. cerevisiae* have identifiable sequence-specific regions known as autonomously replicating sequence (ARS) elements. These ARS elements were identified as origins by insertion of the elements into plasmid DNA and demonstrating the initiation of DNA replication from these sites (146).

In mammalian cells, the identification of origins of replication has been much more difficult. Mammalian origins do not have an accepted consensus sequence like that of the ARS sequence in *S. cerevisiae*, but they do appear to contain common features among each other. They tend to be within AT-rich regions, CpG islands, and regions that contain transcriptional control elements (1,18,40,185). To date, only a handful of mammalian origins have been identified. Due to limitations in the resolution of many of the techniques utilized to identify origins of replication, there continues to be debate over whether higher eukaryotes initiate replication from specific loci or from larger regions known as initiation zones (2,24,197).

Replication is initiated only from origins where the prior assembly of a multi-protein complex, known as the pre-replication complex (preRC), has taken place during the G1 phase of the cell cycle. The preRC is comprised of the Origin Recognition Complex (ORC), Cell Division Cycle 6 (Cdc6), Cdc10-dependent Transcript 1 (Cdt1), and the Minichromosome Maintenance Complex (MCM). The

concerted actions of the preRC proteins result in the "licensing" of all potential origins that could be utilized in S phase.

E. Proposed Research: Specific Aims

It has been established by other groups that disruption of different components of the preRC results in a G1 arrest in normal cells while initiating a p53-independent apoptotic response in cancer cells (56,181). Manipulations that specifically kill cancer cells, regardless of their p53 status, but do not jeopardize the viability of normal cells would be highly desirable therapeutically. **This project tested the hypothesis that normal cells, unlike cancer cells, have the ability to sense the insufficient assembly of the pre-replication complex and activate a "pre-replicative checkpoint."**

My initial proposal was set up to address two different aims based on preliminary data that demonstrated differential phenotypes in response to reduction of Cdc6 and Cdt1. These aims were: **(1) Determine the upstream molecular mechanisms activating the G1 arrest triggered by reduction of Cdc6 expression and (2) Investigate the phenotype (G2 arrest) associated with knockdown of Cdt1 expression.** Much of the work in this dissertation focused on Aim 1, which turned out to be a more challenging goal than originally anticipated.

CHAPTER 2

REGULATION OF S PHASE ENTRY AND GENOME DUPLICAITON

A. Pre-Replication Complex

In order for DNA replication to initiate at origins, a multi-protein complex known as the pre-replication complex (preRC) must first be assembled in G1 phase of the cell cycle. The preRC is formed through the concerted actions of the origin recognition complex (ORC), Cdc6, Cdt1 and the MCM helicase complex. Binding of ORC to the origins results in the recruitment of both Cdc6 and Cdt1. Together these proteins recruit and load the minichromosome maintenance (MCM) helicase complex onto the chromatin (Figure 2.1). Once the MCM complex is loaded the origin is said to be "licensed" for replication initiation.

<u>Origin Recognition Complex</u>

The Origin Recognition Complex (ORC) is a heterohexameric ATPase and was initially identified in *Saccharomyces cerevisiae* (13). The ORC complex consists of six Orc subunits (Orc1-6), which are evolutionarily conserved. The binding of ORC to origins of replication initiates a cascade of events that ultimately result in the licensing of the origin for DNA replication.

Much of our understanding of ORC binding to DNA comes from studies performed in yeast. The reason for this is that *S. cerevisiae* origins contain origin
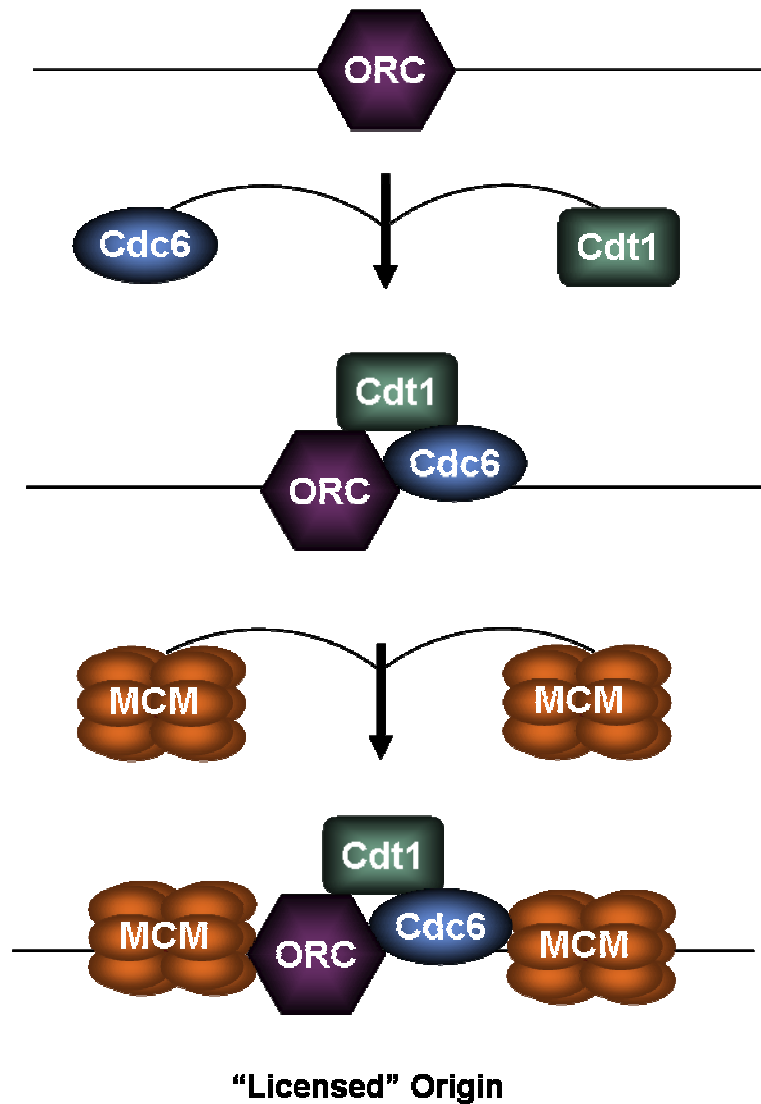
Figure 2.1: Assembly of the Pre-Replication Complex. The origin recognition complex (ORC) binds to the origins and recruits Cdc6 and Cdt1. This results in the recruitment and loading of the minichromosome maintenance (MCM) complex. Once MCMs are chromatin bound, the origin is said to be "licensed" for DNA replication.

specific sequences known as autonomously replicating sequences (ARS element) which contain an 11 base pair region that is required for ORC binding (12). The lack of any sequence specific identifier in mammalian cells [reviewed in (38)] makes it much more difficult to investigate the requirements for ORC binding and regulation. One thing that appears to be common among ORC's purified from yeast, flies, or mammalian cells is that they all exhibit a preference for asymmetrical A:T rich DNA (65,102,170,196).

Recent evidence suggests that co-factors may aid in origin identification in higher eukaryotes. It was demonstrated in mammalian cells that HMGA1a, a member of the high-motility group family of proteins, interacts with ORC both *in vitro* and *in vivo* (193). The potential significance of this interaction was demonstrated by targeting HMGA1a to a site-specific region on a plasmid which resulted in the recruitment of ORC and generating an artificial origin of replication (193). These findings lend to the possibility that in mammalian cells ORC requires the guidance of additional proteins to mark origins of replication.

In mammalian cells, ORC 2-6 protein levels as well as chromatin bound levels remains constant throughout the cell cycle (137,154). The one exception to this appears to be the Orc1 subunit (Figure 2.2, green line). There is much debate whether Orc1 remains chromatin bound or oscillates throughout the cell cycle. These differences appear to be dependent on the cells for which the studies are conducted in. In Chinese hamster ovary (CHO) cells, Orc1 levels remain constant throughout the cell cycle while in some cancer cells Orc1 appears to oscillate due to
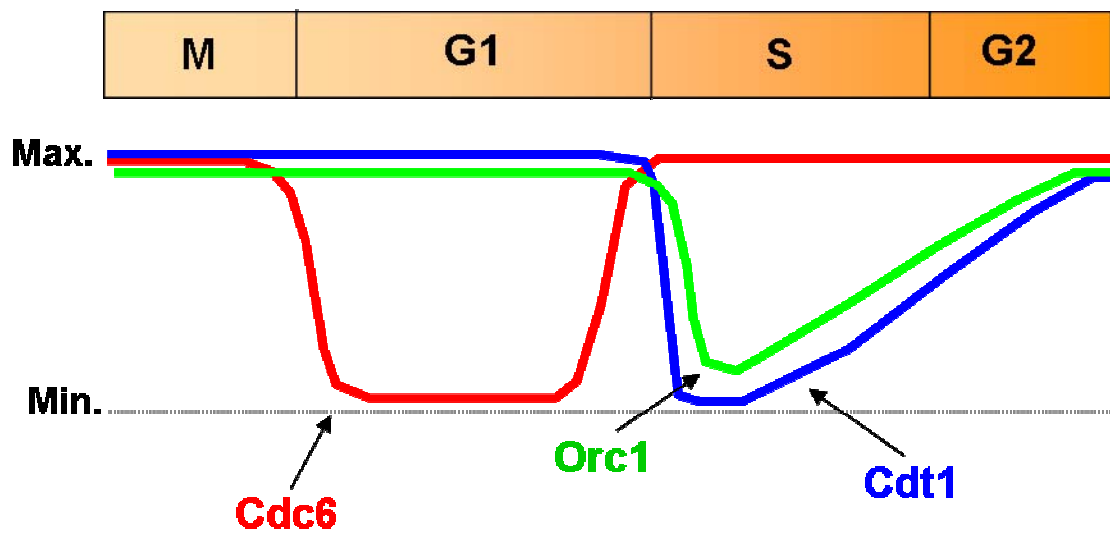
Figure 2.2: Diagram displaying cell cycle expression of Orc1, Cdc6 and Cdt1 protein. The colored lines represent the oscillations of protein expression for Orc1 (green), Cdc6 (red), and Cdt1 (blue) at various times during of the cell cycle.

the polyubiquitination by SCF^{skp2}, and degraded by the 26S proteasome in S phase (60,117,134,137). In addition to these findings, Orc1 was found to be bound to chromatin throughout the cell cycle in HeLa cells but was not detected on the chromatin outside of G1 in normal fibroblasts, WI38 (133).

The regulation of ORC is not conserved across species. In yeast ORC remains chromatin bound throughout the cell cycle but is regulated through phosphorylation by Cdk1/Clb at different stages of the cell cycle (46,59,102,118,122). In *Xenopus* ORC is released from chromatin following chromatin binding of MCM and activation of Cdk2 (187,201). And finally, in *Drosophila* there appears to be some level of cell cycle dependent regulation of ORC. Drosophila Orc1 fluctuates throughout the cell cycle, accumulating in late G1 and S phase then undergoes ubiquitinated and degraded by Fzr (APC) in mitosis (8,10).

ORC belongs to the AAA+ (ATPases-Associated with various cellular Activities) family of ATPases and *in vitro* work in *S. cerevisiae* demonstrates that Orc1 and Orc5 are able to bind ATP but only Orc1 has ATP hydrolysis activity (99). Both binding of ATP and ATPase activity of ORC are critical for origin licensing. ORC mutants that can not bind ATP, are unable to recruit Cdc6 to the chromatin (183). Mutation of the arginine ring finger residue 267 on Orc4, which is required for ATP hydrolysis, is lethal *in vivo*; in addition, interference of ORC ATP hydrolysis prevents subsequent loading of the MCM complex (19).

Cell Division Cycle 6

The cell division cycle 6 (Cdc6) protein is recruited to the chromatin upon ORC binding. Cdc6 was initially identified in a screen of budding yeast mutants that were defective in cell division cycle (75), while its role in DNA replication was discovered in an analysis of temperature sensitive yeast Cdc6 mutants that arrested at the G1-S transition (23). Cdc6 also belongs to the AAA+ family of ATPases and is responsible for a variety of the actions that take place regarding the assembly of the preRC.

In yeast, one of the main functions of Cdc6 ATPase activity is to remove ORC from non-origin DNA. Cdc6 hydrolyzes ORC bound ATP promoting the disassociation of both ORC and Cdc6 from the non-origin DNA (140,183). Cdc6 ATPase activity has also been shown to be important for stabilization of the ORC/Cdc6 complex (183). Another function of Cdc6 is facilitating the loading of the MCM complex onto chromatin. The exact role Cdc6 plays in the loading of the MCM complex is unknown but Cdc6 mutants unable to bind ATP display a defect in the ability to load the MCM complex onto chromatin (200)

Cdc6 expression and regulation is cell cycle dependent. Cdc6 is an E2F regulated gene (155,207) whose expression spikes at the end of mitosis and again in late G1. In G1 phase of the cell cycle, Cdc6 protein levels are maintained extremely low and accumulate as the cell enters S phase (Figure 2.2, red line). The low levels of Cdc6 in G1 are due to the activity of the anaphase promoting complex (APC) (Figure 2.3A). APC[Cdh1] is a ubiquitin ligase that is highly active in G1 and targets Cdc6 for ubiquitin mediated degradation in G1 phase (164). This seems counter-intuitive since G1 is where preRC assembly occurs, therefore there must be
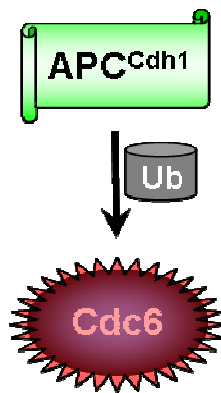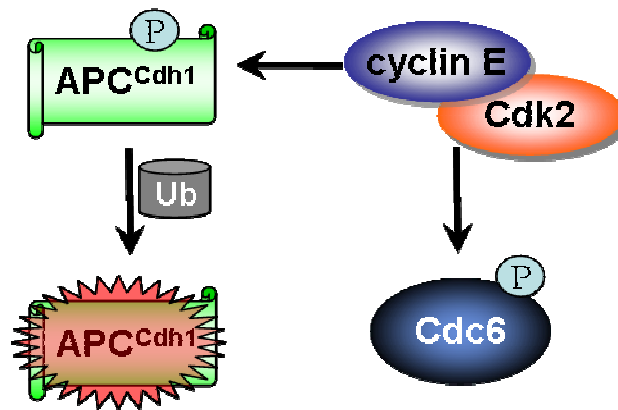
Figure 2.3: Cell cycle regulation of Cdc6. (A) In G1 phase of the cell cycle, Cdc6 protein levels are kept low through ubiquitination by APC^{Cdh1} resulting in the degradation of Cdc6. (B) At the G1/S transition, APC^{Cdh1} becomes phosphorylated by cyclin E/Cdk2 promoting its ubiquitin-mediated degradation. At the same time, Cdc6 is stabilized by the phosphorylation carried out by cyclin E/Cdk2.

a fine balance between degradation and synthesis of Cdc6 in order for origin licensing to take place.

Once the cell has passed the G1/S transition, activation of the cyclin/Cdk kinase results in the accumulation of Cdc6 protein (Figure 2.3B). Phosphorylation of Cdh1 by Cdk inactivates the APC[Cdh1] complex and therefore can not target Cdc6 for degradation (25,125,164). In addition to the inactivation of the APC complex, Cdc6 becomes phosphorylated by cyclin E/Cdk2 which stabilizes Cdc6 (50,51) .

In addition to being regulated by APC and cyclin E/Cdk2, there is some evidence that Cdc6 regulation is through nuclear/cytoplasmic localization (92,163,174). However, more recent evidence suggests that the cytoplasmic localization of Cdc6 was due to overexpression of Cdc6 and in fact endogenous Cdc6 remained in the nucleus and chromatin bound through S phase (6,36,61). Even with these more recent findings, the issue of Cdc6 localization in mammalian cells is still wildly debated.

In addition to its role in origin licensing, Cdc6 has been shown to have other functions throughout the cell cycle. It has been demonstrated that Cdc6 might have a role in checkpoint activation, in particular Chk1 activation. In the *Xenopus* cell-free system, extracts treated with aphidicolin in the absence of Cdc6, can not phosphorylate Chk1 suggesting that Cdc6 has some role in activating Chk1 in response to replication stress (153). In support of these findings, it has also been demonstrated that overexpression of Cdc6 blocks entry into mitosis and treatment of a Chk1 inhibitor, UCN-01, abrogates this arrest (30).

Additionally, recent evidence has demonstrated that Cdc6 is required both *in vitro* and *in vivo* for the reactivation of Cdk2 following S phase checkpoint activation. Under these conditions, Cdc6 competes away the Cdk inhibitor, p21, which is bound to Cdk2 allowing for the reactivation of the Cdk2 complex and S phase progression (94).

Cdc10 dependent transcript 1

The Cdc10 dependent transcript 1 (Cdt1) protein is recruited concurrently with Cdc6 upon ORC bounding to potential origins. Cdt1 was first identified in fission yeast as a cell cycle regulated gene through an immunoprecipitation assay that demonstrated that the Cdt1 promoter was immunoprecipitated with transcription factor Cdc10 (82).   Recruitment of Cdt1 is dependent on ORC, particularly Orc6. *In vitro* studies in *S. cerevisiae*, demonstrate that in the absence of Orc6, Cdt1 is not recruited to the chromatin (28). Recruitment of Cdt1 to chromatin is crucial, for its required for the loading of the MCM complex. Loss of Cdt1 does not affect the binding of ORC or Cdc6, but prevents the recruitment and loading of the MCM complex onto the chromatin (150,168,189).

The pattern of Cdt1 protein expression is the same in *S. pombe*, *Drosophila melanogaster*, and humans in that the protein levels of Cdt1 are high in G1 but low in S and G2 phase (Figure 2.2, blue line). This is not the case in budding yeast where Cdt1 is exported to the cytoplasm but protein levels remain the same throughout the cell cycle [reviewed in (58)].

Regulation of Cdt1 is cell cycle dependent with multiple mechanisms preventing its expression outside of G1 phase of the cell cycle (Figure 2.4). As cells
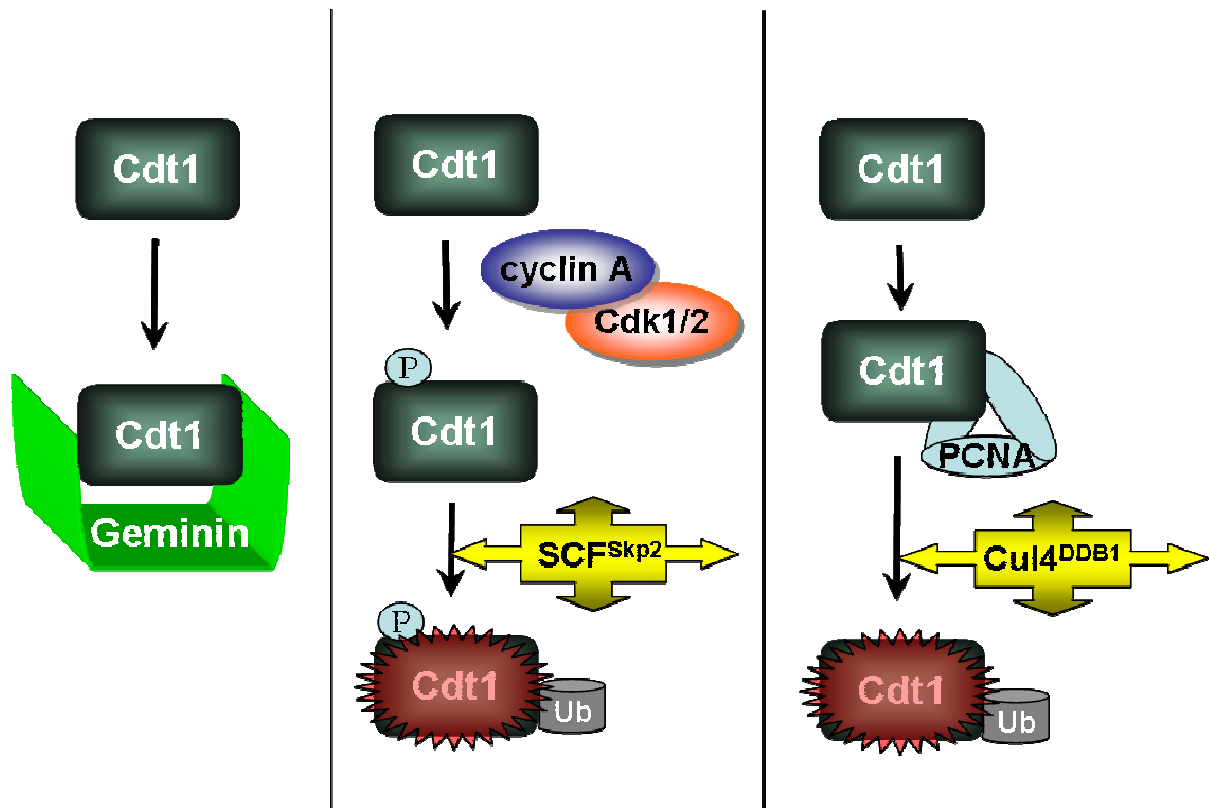
Figure 2.4: Cell cycle regulation of Cdt1. There are three different mechanisms of regulation of Cdt1 in S phase of the cell cycle. First, Cdt1 can be sequestered and inhibited by the binding of Geminin. Second, Cdt1 is phosphorylated by cyclin A-Cdk1/2 which is required for the ubiquitination by SCF^{Skp2}. Third, Cdt1 is targeted for ubiquitination by Cul4^{DDB1} upon binding of Cdt1 to PCNA. The ubiquitination by either SCF^{Skp2} or Cul4^{DDB1} results in Cdt1 degradation.

enter S phase, Cdt1 is either sequestered or degraded to prevent re-licensing of origins. One mechanism by which Cdt1 is regulated is through its interaction with an inhibitor protein, geminin (202). Geminin is highly expressed in the nucleus in S phase and is targeted for APC-mediated degradation at the end of mitosis (131,202). The binding of Geminin to Cdt1 inhibits its activity by preventing it from being able to bind to Cdc6 and MCM (32). The importance of the Geminin/Cdt1 interaction in preventing origin assembly outside G1 phase has been demonstrated through the finding that depletion of geminin results in re-replication and subsequent DNA damage (71,135,214).

Another mechanism by which Cdt1 is regulated is through the phosphorylation by cyclin A/Cdk1 or Cdk2. This phosphorylation results in the binding of Cdt1 to the F-box protein Skp2, a component of the SCF ubiquitin ligase complex, which results in the degradation of Cdt1 (138,149,186). In addition to Skp2 dependent degradation, Cdt1 is also degraded by a Cdk2-independent mechanism. During S phase, Cdt1 binds to the ubiquitin ligase Cul4-DDB1 which mediates degradation of Cdt1 through its association with PCNA (9,85). These two mechanisms of degradation ensure that Cdt1 degradation is coupled to the initiation of DNA synthesis.

Minichromosome maintenance complex

The minichromosome maintenance complex (MCM) is the last of the components of the pre-replication complex to be recruited to the chromatin. The MCM proteins (Mcm 2-7) were originally identified in genetic screens of yeast mutants defective in plasmid maintenance and cell cycle progression (127,141) and

grouped together because of their sequence similarities. The MCM complex is a six subunit complex that is comprised of Mcm2-7 which are transcriptionally regulated by E2F with the peak of transcription occurring at the end of mitosis (39,132).

The MCM complex requires the sequential binding of Cdc6 followed by Cdt1 in order to be recruited to the chromatin (195), and the combined actions of the ORC-Cdc6 ATPase to be loaded (184). Once bound to ORC, Cdc6, and Cdt1 are no longer required for the MCM complex to remain chromatin bound (19,48,53,173). In fact, in *Xenopus,* the affinity of ORC for Cdc6 is significantly reduced, going from $t_{1/2}$ 170 min to 15 min, following MCM chromatin loading (76).

In vitro work with purified MCM from yeast, mouse and *Xenopus* demonstrate that the MCM complex as a whole has very minimal helicase activity but assembly of individual subunits (Mcm4-Mcm6-Mcm7) exhibit very strong helicase activity (88,110,210). Once loaded, the helicase activity of the MCM complex is presumably responsible for the unwinding of the DNA ahead of the replication fork (105,159).

One would assume that since DNA replication in eukaryotes is bi-directional, loading of two MCM complexes per origin would be sufficient enough to successfully complete DNA replication. Interestingly, it has been demonstrated in *Xenopus,* yeast, and humans that the MCM complex gets loaded in excess (as much as 20-fold) at replication origins (48,53,112).   It is unknown if all the MCM complexes are activated or what purpose the large number of chromatin bound MCM complexes serve since reduction to approximately two MCM per origin presents no problem with replication (53,205).

Some insight into why so many MCM complexes are loaded onto the chromatin has been demonstrated in *Xenopus*. Here, the excess MCM complexes are required for the activation of "cryptic/dormant" origins under replication stress conditions (64,205) and recently, similar findings were demonstrated in mammalian cells (86). These "dormant" origins are origins that appear to only be activated when replication forks encounter pauses and are used to ensure timely completion of DNA replication [reviewed in (16)].

In addition to Mcm2-7, there is emerging evidence for the importance of other MCM, such as Mcm8 and Mcm9, in DNA replication. There is some debate to where in the progression of preRC assembly, Mcm8 functions. It was originally established that Mcm8 functioned after preRC assembly because Mcm8 did not associate with Mcm2-7 and it was found to bind to chromatin following Mcm2-7 binding (68). It has also been shown that it interacts with Orc2 and Cdc6 and absence of Mcm8 resulted in reduced loading of Cdc6 onto chromatin (198). These later findings could be explained by the fact that Cdc6 becomes chromatin bound in S phase and therefore Mcm8 is required for Cdc6 chromatin binding here and not for chromatin binding in preRC formation.

In regards to Mcm9, only very recent evidence suggests that it has a role in preRC assembly and this was through the finding that Mcm9 is required for Cdt1 recruitment and preRC assembly (121). More work must be done in regards to both Mcm8 and Mcm9 to better understand their exact role in DNA replication.

B. Cell Cycle Events Required for G1 Progression

Origin licensing is only one of the many events that must take place in G1 phase of the cell cycle. A number of other events are required throughout G1 phase in order for the cell to proceed successfully through the cell cycle. These include (but not limited to) inactivation of the retinoblastoma protein, activation of the cyclin/Cdk complexes, transcriptional up-regulation of essential S phase genes, and assembly of the pre-initiation complex.

Retinoblastoma (Rb) Protein

The retinoblastoma (Rb) protein is a tumor suppressor protein that is important in controlling cell cycle progression through its interaction with the E2F family of transcription factors. Rb is able to repress gene transcription by E2F through recruitment and binding of histone deacetylases (HDACs) and other chromatin remodeling factors to E2F bound promoters (Figure 2.5) [reviewed in (211)]. In its repressive state, Rb is hypophosphorylated and binds to E2F, preventing transcription of key regulators of the G1/S transition (Figure 2.5A). As the cell traverses through G1 phase, Rb becomes phosphorylated by cyclin D-Cdk4/6 resulting in a conformational change that releases the HDAC from the complex (Figure 2.5B). These initial phosphorylations allow for transcriptional upregulation of a minor set of genes, one being cyclin E. Cyclin E-Cdk2, further phosphorylates Rb, releasing it completely from E2F and allowing for the transcriptional up regulation of essential cell cycle progression factors (Figure 2.5C) [reviewed in (77)].
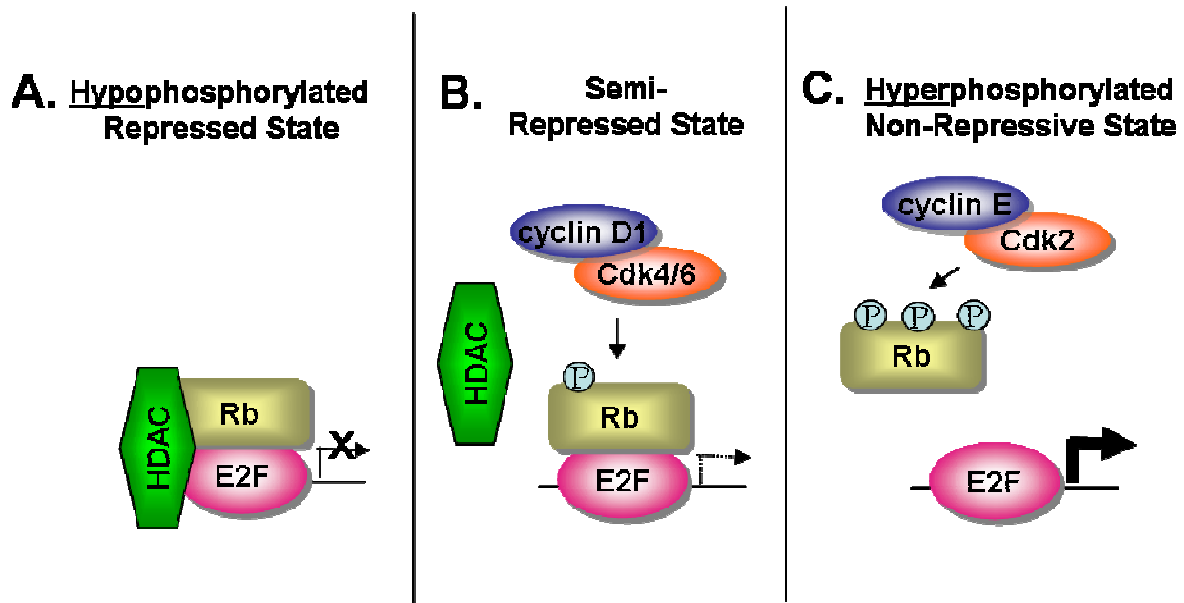
Figure 2.5: Inactivation of the Retinoblastoma (Rb) protein. (A) In the hypophosphorylated state, the retinoblastoma protein remains bound to E2F and histone deacetylase (HDAC). (B) The initial phosphorylation of Rb, carried out by cyclin D-Cdk4/6 results in a conformational change and the release of the HDAC. This release allows for transcription of a select few genes, one of which is cyclin E. (C) Cyclin E/Cdk2 phosphorylates Rb, generating a hyperphosphorylated state, which allows for full transcriptional activation of E2F regulated genes.
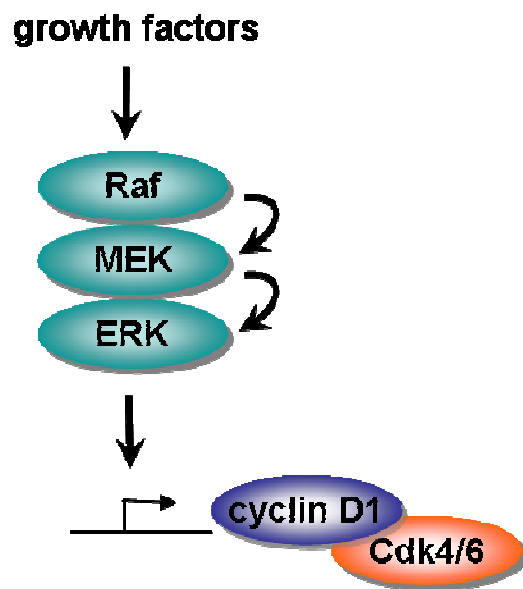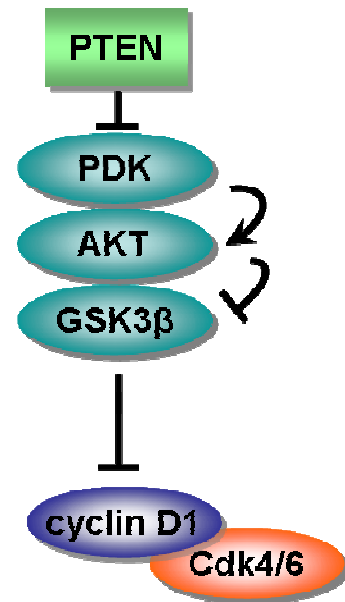
Figure 2.6: Regulation of cyclin D1. Transcriptional and translational regulation of cyclin D.

Cyclin D-Cdk4/6

        Cyclin dependent kinase (Cdk) complexes are an integral component of the cell's ability to complete cell division. As cells exit mitosis and proceed into G1 phase, cyclin D-Cdk4/6 is the first cyclin-dependent kinase to become activated. Mitogenic stimulation in G1 results in cyclin D accumulation (11). In continuously dividing cells, mitogenic stimulation activates Ras, which signals through AKT to inhibit GSK-3β, allowing for the accumulation of cyclin D (43). In addition, growth factors regulate cyclin D gene transcription upon re-entry into the cell cycle from quiescence, by activation of the Ras-Raf-MAPK pathway (Figure 2.6) (4,109,204). Even though there is this mitogenic regulation of cyclin D, unlike what its name suggests, cyclin D expression is not cyclic. Instead, once the cell enters S phase, cyclin D is exported from the nucleus until the subsequent G1 phase (66).

        Cyclin D, once abundant enough in G1, associates with its catalytic subunits, Cdk4 and Cdk6. One of the major functions of the activated complex is to phosphorylate the retinoblastoma (Rb) protein. Rb becomes partially phosphorylated by cyclin D/Cdk4, which allows for amongst other things, transactivation of *cyclin E*, an E2F target (52,73,145).   The initial phosphorylation of Rb by cyclin D-Cdk4/6 does not completely relieve the inhibition on E2F, instead that phosphorylation allows for leakiness of the *cyclin E* promoter allowing for accumulation of cyclin E. Cyclin E then binds its catalytic partner, Cdk2, and relieves the remaining inhibitory effects of Rb on E2F.

Cyclin E/Cdk2

In addition to phosphorylating Rb, cyclin E/Cdk2 carries out a number of other important events required for G1 progression. As stated above, upon accumulation of cyclin E in mid/late G1 phase, it binds to its catalytic partner, Cdk2. Cyclin E/Cdk2 kinase activity requires the activating phosphorylation on threonine 160 (T160) of Cdk2. This activating phosphorylation is carried out by the CDK-activating kinase (CAK), which is comprised of Cdk7/ Cyclin H/ Mat1 (182). Cyclin E/Cdk2 kinase activity is very important for progression through G1 and into S, for over-expression of cyclin E/Cdk2 in quiescent cells can drive cells into S phase in the absence of any detectable E2F (156). In addition, inhibition of cyclin E/Cdk2 prevents S phase entry (157). Active cyclin E/Cdk2 has also been shown to be involved in activation of histone transcription (123,208,213), regulation of centrosome duplication (158,194) and promoting and inhibiting licensing and replication. Cyclin E/Cdk2 phosphorylation of Cdc6 on S54 promotes preRC assembly and protects it from APC/C-dependent proteolysis (125). In addition, Ankrd17, whose inhibition results in loss of chromatin bound Cdc6 and cell cycle progression, is also phosphorylated by cyclin E/Cdk2 (41). This suggests that cyclin E/Cdk2 is an essential component that drives cells through the cell cycle.

Cyclin E binds preferentially to Cdk2 but in the absence of Cdk2, Cdk1 (although in the normal cycling cell it is not active until late S phase) can substitute for Cdk2 to maintain cell cycle progression in mouse fibroblasts (176) and DNA replication (5,81).
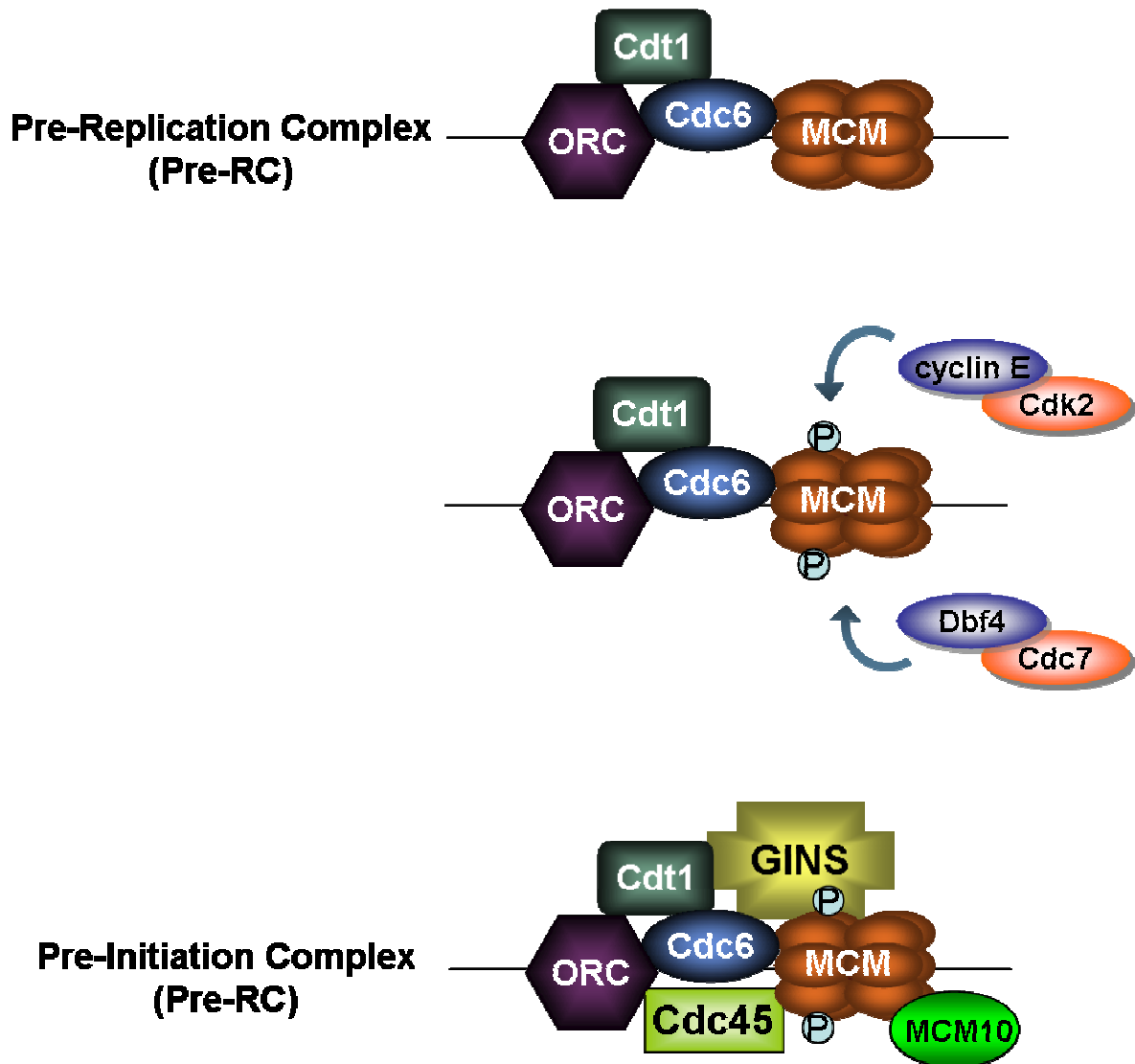
Figure 2.7: Conversion of the pre-replication complex to the replication competent pre-initiation complex. Upon entry into S phase, the pre-replication complex (particularly MCM) is phosphorylated by cyclin E/Cdk2 and Cdk7/Dbf4. This results in the recruitment of MCM10, Cdc45 and GINS forming the pre-initiation complex.

C. Activation of Replication Origins

Once origins have been licensed, a series of events must ensue that activates the helicase activity of the MCM complex allowing for the initiation of DNA replication. Two kinases, cyclin E/Cdk2 and Cdc7/Dbf4, are responsible for converting the pre-replication complex in to a pre-initiation complex which initiates DNA replication (Figure 2.7). Cyclin E/Cdk2 is responsible for the initial phosphorylations of the MCM complex which in turn help to facilitate the phosphorylation of MCM by Cdc7/Dbf4 (128).

The Cdc7/Dbf4 kinase, also known as Dbf4-dependent kinase (DDK), has been shown to have effects on multiple of the MCM subunits. In *S. cerevisiae*, phosphorylation of Mcm5 by Cdc7/Dbf4 is thought to promote conformation changes in Mcm5 that results in a more highly active MCM helicase complex (80). Phosphorylation of Mcm2 and Mcm4 *in vitro* and *in vivo* has been shown to play a critical role in the recruitment of Cdc45 (26,128,142). In addition to the recruitment of Cdc45, the phosphorylation of Mcm4 has been shown to promote a more stable MCM-Cdc45 complex. In the absence of Cdc7/Dbf4, Cdc45 (another essential initiation factor) can not bind to origins and replication is inhibited (91,215).

Association of Cdc45 with origins requires both Cdk and DDK and is recruited to early origins in early S phase and late origins in late S phase (7,91,139). Cdc45 is required for the activation of the helicase activity of the Mcm2-7 complex (129) because degradation of Cdc45 results in fork stalling which can be overcome with the re-expression of Cdc45 (192). In line with this, it has also been shown *in vitro* that Cdc45 binds with the Mcm7 subunit (103).

The Mcm2-7 complex and Cdc45 are maintained on the chromatin through their interaction with the GINS complex (63,144). The GINS complex is a four subunit complex (Sld5, Psf1, Psf2, and Psf3) that is required for the initiation of DNA replication and replisome progression. Loss of Cdc45 or any of the four subunits of the GINS complex in *Drosophila* results in an accumulation of cells in G1 and S phase (144).

In addition to the above mentioned proteins, there are other factors that have been demonstrated to be important for pre-initiation complex assembly. Mcm10 is thought to be one of the earliest initiation factors to be recruited to the chromatin. Mcm10 is not part of the preRC and does not appear to have any role in the origin licensing step. Instead Mcm10 is required for the activation of the licensed origin. It is required for facilitating phosphorylation of Mcm2-7 by Cdc7-Dbf4 (111) and recruitment of Cdc45 onto chromatin (89,177,201).

CHAPTER 3

CONSEQUENCES OF INSUFFICIENT ORIGIN LICENSING

A. Background

It is important that origins of replication are licensed in the G1 phase of the cell cycle because multiple levels of regulation prevent origin licensing from occurring once the cell has entered S phase (15,45,58,116,131,152,203). If cells enter S phase with an insufficient number of licensed origins, it is possible that sparsely distributed replication forks could result in incomplete replication. Replication forks encounter natural pause sites [reviewed in (106)] and, under normal conditions, these sites may pose few problems to the cell since replication forks from a nearby origin could rescue the fork stalled at the pause site. Prolonged stalling at natural pause sites, however, can result in genomic instability (113,169). Therefore, under conditions where origins are not sufficiently licensed, forks that stall at these pause sites for an extended period of time could become unstable and result in double strand breaks ultimately leading to genomic instability.

Since a cell with partially replicated DNA cannot revert back to G1 to license more origins, it would seem essential for the cell to determine if an adequate number of origins are licensed before entering S phase. Some evidence, although indirect, indicates that an origin licensing checkpoint exists [reviewed in (108)].

Under what circumstances could insufficient licensing occur? It is well established that environmental stressors and DNA damage result in the degradation of key factors involved in origin licensing (14,50,79,84). Degradation of these licensing factors in S phase is important to prevent origins from being re-licensed and minimizes the potential for re-replication to occur. Since the degradation of Cdc6 and Cdt1 also occurs in G1 phase, there is a potential for cells upon deactivation of a DNA damage signal to enter S phase with an insufficient number of licensed origins.   Therefore, an origin licensing checkpoint that delays S phase entry until all origins are licensed would ensure complete replication.

B. Insufficient Origin Licensing in Yeast

The consequences of insufficient origin licensing have been demonstrated in both budding and fission yeast by generation of null mutants of preRC components. Orp2/Orc2 (98), Tah11/Cdt1 (42,82), and Cdc6 (166) deletion mutants exhibit loss of origin licensing and they enter mitosis in the absence of DNA replication. These mutant cells undergo a reductional anaphase, in which unreplicated chromosomes segregate to only one spindle pole, generating non-viable daughter cells. Inability to arrest in G1 upon inadequate origin licensing suggests that a licensing checkpoint does not exist in yeast.

The null mutants mentioned above do not provide information about the effects of insufficient origin licensing, because in the absence of Cdc6 for example, no origins can be licensed. However, in strains with compromised Cdc6, Tah11/Cdt1, or Mcm2-7 expression or function, plasmids containing a single origin

are lost from the population at a higher frequency than in the wild-type controls (42,127). The loss rates of these "minichromosomes" are used as an indicator of replication origin activity, which depends on origin licensing. This elevated minichromosome loss can be suppressed by the addition of multiple ARS sequences (83), suggesting a competition for licensing factors between the origins in the plasmid and the yeast chromosomes. Together, these observations suggest that failure to fire sufficient origins on the endogenous chromosomes may also lead to loss of genetic material and this argument supports the hypothesis that origin licensing defects through their dysregulation of DNA replication are a potential source of genomic instability.

C. Effects of Insufficient Origin Licensing in Higher Eukaryotes

Budding and fission yeast are useful models to investigate the mechanisms of replication initiation, but these experimental systems do not completely recapitulate DNA replication in higher eukaryotes. The advent of RNAi technology allowed investigators to probe the consequences of origin licensing inhibition in human cells. Cultured human cells transfected with siRNA to deplete essential licensing factors are not the equivalent of a null allele, but more closely resemble hypomorphic mutants. In this regard, siRNA-treated cultures are predicted to represent more closely those physiological circumstances where origin licensing is still incomplete, either due to early G1 status or to DNA damage-dependent degradation of Cdc6 and Cdt1.

The outcome of depleting essential origin licensing components, such as Ccd6, Orc2 or Mcm5, depends on whether the cells are normal or transformed. All cancer cell lines tested thus far die by apoptosis, whereas normal cells survive and arrest in an apparent G1 state with 2N DNA content (56,124). Furthermore, overproduction of Geminin to block Cdt1 function has a similar differential effect on cancer cells compared to normal cells (181). These findings raised two possibilities: 1) robust intra-S checkpoint pathways in the normal cells trigger an arrest so early in S phase that they only appear to be in G1 based on flow cytometric profiles, or 2) normal cells have an active origin licensing checkpoint that arrests them *before* the G1/S transition, and this checkpoint is deficient in cancer cells. In the latter scenario, the attempted S phase by the cancer cells ultimately results in catastrophic DNA damage, which then triggers the observed apoptosis.

Given the potential danger of entering S phase before licensing is complete, it would be advantageous for cells to delay entry into S phase until at least a minimum number of origins have been licensed. As mentioned previously, the existence of such a "licensing checkpoint" was first hypothesized from the observation that licensing inhibition in normal cells induced an apparent G1 arrest, but tumor-derived cells activated a robust apoptotic response (56,124,181). Presumably, genetic alterations in cancer cells can inactivate a regulatory link between origin licensing and S phase entry that protects normal cells from attempting an S phase that is doomed to fail. However, the mechanisms that prevent S phase entry in normal cells with insufficiently licensed origins have not been determined.

The focus of the work presented in this dissertation has been on dissecting some of the molecular networks involved in a potential "licensing checkpoint". In this chapter, evidence is provided for a link between replication licensing in G1 and cyclin/Cdk activation in normal cells. Results demonstrate that insufficient licensing results in a loss of cyclin/Cdk activity, loss of Rb phosphorylation, and accumulation of cells in G1.

Materials and Methods

Cell culture and siRNA transfection

Normal human foreskin fibroblasts immortalized with human telomerase (NHF1-hTert and NHF10-hTert) (78) and non-immortalized WI-38 lung fibroblasts obtained from (ATCC) were cultured in minimum Eagle's medium (MEM, Gibco) containing 10% fetal bovine serum (Sigma), 1X non-essential amino acids (Gibco) and 2 mM L-glutamine (Sigma). Cancer cell lines (HeLa and U2OS) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Sigma) and 2 mM L-glutamine (Sigma). All cultures were maintained in a humidified incubator at 37 ºC and 5% CO_2.

Log phase normal fibroblasts were plated at a density of 5 x 10^5 per 10-cm dish 24 h prior to transfection. SiRNA oligonucleotides were synthesized by Invitrogen (GFP control, 5'-GGCUACGUCCAGGAGCGCACCTT-3'; Cdc6 siRNA-2144, 5'-UCUAGCCAAUGUGCUUGCAAGUGUA-3'; Cdc6 siRNA-2534, 5'-CACCAUGCUCAGCCAUUAAGGUAUU-3'; Cdt1 siRNA, 5'-CCUACGUCAAG CUGGACAATT -3'; Orc2 siRNA, 5'- GAGCUAAACUGGAUCAGCAAACUUU-3'). Transfections were performed with a total of 100 nM siRNA duplex using Dharmafect 1 reagent (Dharmacon), according to manufacturer's guidelines. In the case of Cdc6 siRNA transfection, 50 nM of 2534 and 50 nM of 2144 were used. Twenty-four hours after siRNA transfection, cells were re-plated and cultures were typically incubated for an additional 48 h. Due to the stability of Orc2 protein, efficient depletion was achieved by using the following protocol. Log phase normal fibroblasts were seeded at 80% confluence in 6-cm dishes and transfected with 100

nM Orc2 or GFP control siRNA the following day. Cells were incubated for 24 h, and then split into 10-cm dishes and transfected again 24 h later. Cells were collected 48 h after the second siRNA transfection.

Cell synchronization

Asynchronously growing fibroblasts were plated at a density of 5×10^5 cells per 10-cm dish and siRNA transfected 24 h later, as described above. Twelve hours after siRNA transfection, transfection solution was removed and replaced with medium containing 0.1% FBS. Cells were stimulated 72 h after transfection by the addition of 10% FBS and collected at the indicated times.

Immunoblot analysis

Total cell lysates were prepared by resuspending pellets in lysis buffer containing 0.1% Triton X-100, 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM orthovanadate, 1 mM β-glycerolphosphate, 5 μg/ml phosvitin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin (Sigma) in phosphate-buffered saline (PBS); protein concentrations were determined by the Bradford assay (Bio-Rad). Chromatin fractions were prepared as described in (33). Samples containing equal amounts of protein were combined with Laemmli sample buffer (10% glycerol, 0.05 M Tris (pH 6.8), 0.1% bromophenol blue, 1% SDS) containing 10% beta-mercaptoethanol and boiled. Samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore) which were probed with appropriate antibodies to detect the following proteins: Cdc6 (sc-9964), cyclin H (sc-609), Mat1 (sc-13142), and cdk2 (M2) from Santa Cruz Biotechnology; phospho-Rb (S807/811), phospho-Cdc2/Cdk2 (Y15), and phosho-Cdk2 (T160) from Cell

Signaling Technologies; MCM2 (BM-28), ORC2, Cdc25A, and Rb from BD

Pharmingen, phospho-RNA Pol II (S5) from Covance; tubulin (DM1A) from Sigma;

and Cdk2, p27, p21 (Ab-10), Cdk7 (MO-1.1), and cyclin E (HE12) from Neomarkers.

Antibodies detecting p27 and cyclin E were generous gifts from Dr. Yue Xiong

(University of North Carolina at Chapel Hill). Anti-Cdt1 antiserum has been

described (32).

Cell Cycle and Cleaved Caspase-3 Analysis

Cells to be analyzed by flow cytometry were labeled with 10 µM BrdU for 1 h

prior to trypsinization and ethanol fixation. Nuclei were stained with fluorescein

isothiocyanate (FITC)-labeled anti-BrdU antibody (BD Biosciences) and

counterstained with propidium iodide. Nuclei were analyzed using the CyAn

software (DakoCytomation), and cell cycle distributions were determined using

Summit v4.3 software (DakoCytomation). The fraction of apoptotic cells was

determined using an active caspase-3 antibody kit (BD Pharmingen), according to

the manufacturer's guidelines.

Kinase assays

Cells were lysed for 30 min at 4° C in RIPA (50 mM Tris-HCL, pH 8.0, 200

mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 50 µg/ml of AEBSF, 10 µg/ml aprotinin,

20 mM NaF, 0.1 mM sodium orthovanadate). Lysates were clarified and subjected

to immunoprecipitation with anti-cyclin E antiserum and protein A-agarose for a total

of 3 h. Beads were washed twice with wash buffer A (20 mM Tris-HCL, pH 8.0, 250

mM NaCl, 1 mM EDTA, 0.5% NP-40), twice in buffer B (buffer A but containing 100

mM NaCl), and then once in kinase buffer (50 mM Tris-HCL, pH 7.5, 10 mM MgCl$_2$,

and 1 mM DTT). Kinase reactions were carried out in 25 µl kinase buffer containing

5 µg of histone H1 (Sigma), 1 µM ATP, and 5 µCi ^{32}P-γATP (Perkin Elmer) and

incubated at 30°C for 30 min. Reactions were stopped with Laemmli sample buffer,

boiled and separated on 10% SDS-PAGE. Gels were washed 3 times by soaking

them for 15 min each in buffer C (20 mM Tris base, 200 mM glycine, 0.1% SDS,

10% glycerol, 1% sodium pyrophosphate), dried and autoradiographed. Relative

phosphorylation of histone H1 was determined using the ImageJ program (Rasband,

W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

http://rsb.info.nih.gov/ij/, 1997-2006).

Results

Cdc6 depletion induces apoptosis in cancer cells, but not in normal human fibroblasts.

 We investigated the relationship between origin licensing and S phase entry in two well-characterized cancer cell lines, HeLa and U2OS, and a cell line derived from diploid fibroblasts immortalized by constitutive telomerase expression, NHF1-hTert (hereafter abbreviated as NHF1) (78). We disrupted origin licensing by transfecting these cells with siRNA targeting the essential licensing factor Cdc6, which resulted in substantial depletion of Cdc6 protein to levels nearly undetectable by immunoblotting (Figure 3.1A). We observed morphological changes associated with cytotoxicity and a substantial number of detached cells in the cancer cell cultures, but not in those from normal fibroblasts, following transfection with Cdc6 siRNA and comparing to transfection with control siRNA targeting GFP. Flow cytometric analysis of DNA content detected a substantial increase in the fraction of cells with sub-G1 DNA content in the Cdc6-siRNA treated HeLa (7% to 16%) and U2OS (12% to 28%) cells, but not in NHF1 (1.1% to 1.2%) (Figure 3.1B). To verify that the sub-G1 population of cells represented those undergoing apoptosis, we examined the activation of caspase-3. As expected, Cdc6 siRNA-treated HeLa and U2OS cells displayed activation of cleaved caspase-3, confirming that the sub-G1 population represented cells undergoing apoptosis (Figure 3.1C).

 It had been previously observed that a variety of cancer cells, but not normal cells, also initiate apoptosis after depletion of Mcm2 or Cdc6, or through over-expression of the Cdt1 inhibitor geminin (56,181). Consistent with previous
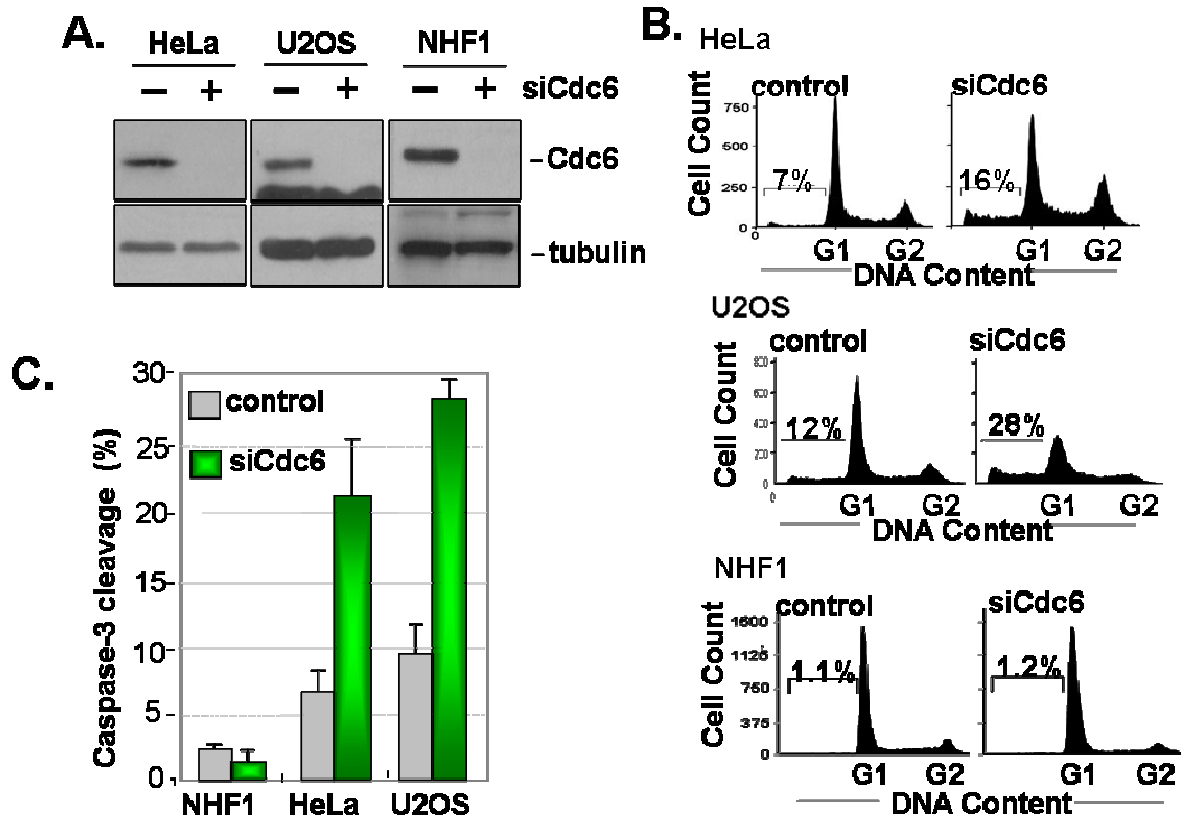
Figure 3.1: Cancer cells, but not normal cells, undergo apoptosis following depletion of Cdc6. (A) HeLa, U2OS and NHF1-hTert (NHF1) cells were transfected with a total of 100 nM of control siRNA (targeting GFP) or Cdc6 siRNA, incubated for 72 h (NHF1) or 96 h (HeLa and U2OS). Cells were collected and subjected to immunoblot analysis of Cdc6 and tubulin. (B) A portion of the cells from A were analyzed by flow cytometry with propidium iodide for DNA content. (C) HeLa, U2OS, and NHF1 were transfected as in A, collected and stained for cleaved caspase-3 according to the manufacturer's protocol.

observations, Cdc6-depleted NHF1 showed no evidence of cleaved caspase-3, unlike the HeLa and U2OS cells (Figure 3.1C). We noted that the population of cells with S phase DNA content was markedly reduced in Cdc6-depleted NHF1 cells, but not in the Cdc6-depleted HeLa or U2OS cells (Fig 3.1B). This observation suggested that Cdc6-depleted normal cells undergo a cell cycle arrest (or long delay in G1) prior to S phase entry, and we sought to determine the mechanisms underlying this response.

NHF1 cells were transfected with control (targeting GFP), Cdc6, or Cdt1 siRNA; 48 and 72 h after transfection, they were pulse-labeled for 1 h with BrdU and analyzed by flow cytometry. Depletion of Cdc6 and Cdt1 protein was detected as early as 48 h after transfection (Figure 3.2A). Given that Cdc6 and Cdt1 are essential DNA replication factors, it was not surprising that Cdc6 and Cdt1 depletion resulted in a marked decrease in the number of S phase cells (Figure 3.2B) and a concomitant increase in the number of G1 cells (not shown) 72h after transfection. (The ~10% of cells incorporating BrdU after Cdc6 and Cdt1 siRNA transfection presumably represents cells with residual Cdc6 and Cdt1 protein). In addition, we noted the loss of detectable Cdc6 in the Cdt1 siRNA treated cells. Levels of Cdc6 protein are very low in G1 therefore the most likely explanation for the loss of Cdc6 is that Cdt1 depleted cells are arrested in G1.

We confirmed the licensing defect caused by Cdc6 depletion in NHF1 cells by monitoring the chromatin association of an MCM subunit, Mcm2, after using an established chromatin fractionation protocol (33,136). As expected, Cdc6 depletion
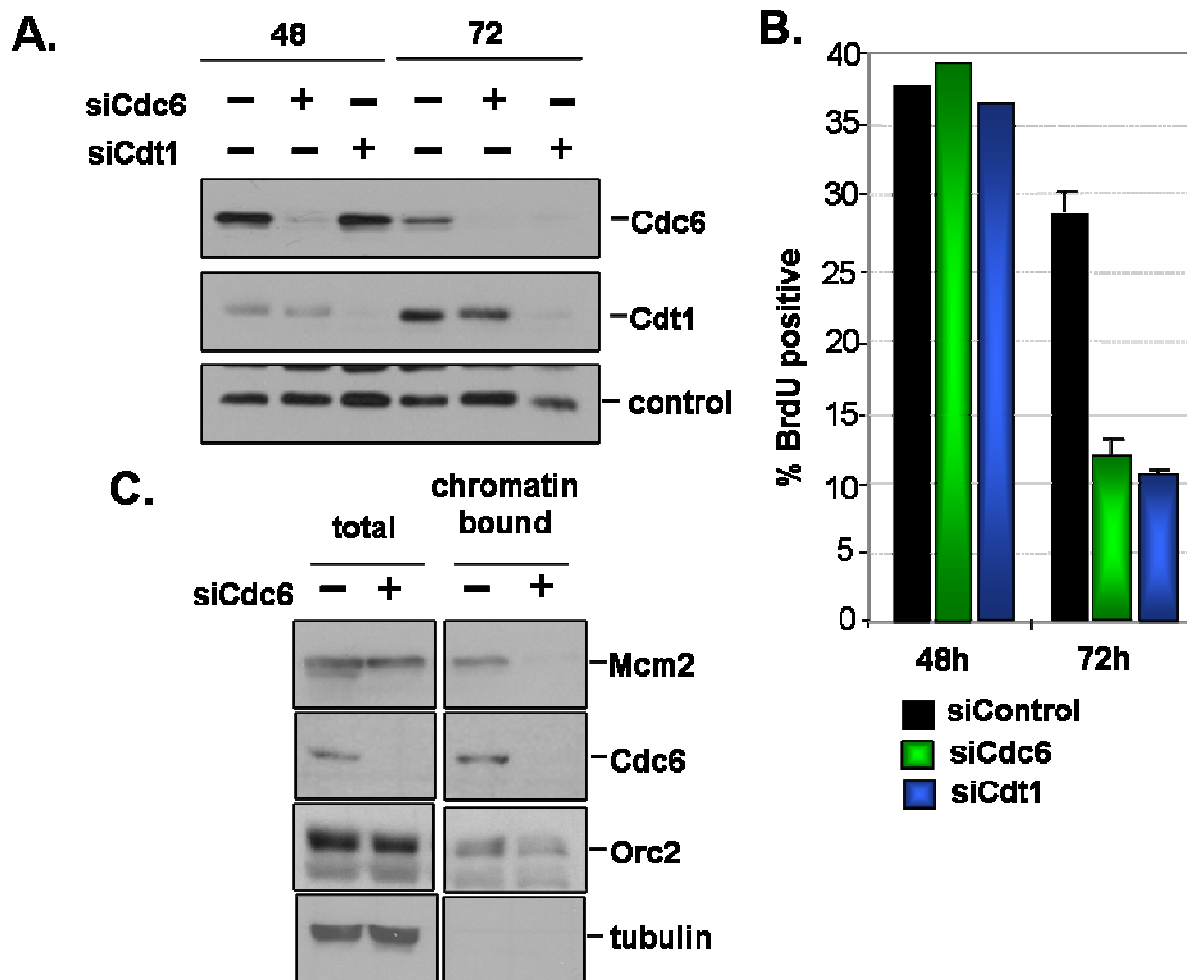
Figure 3.2: Insufficient origin licensing results in a G1 delay in normal human fibroblasts. NHF1 cells were transfected with a total of 100 nM of control (targeting GFP), Cdc6, or Cdt1 siRNA, incubated for 48 or 72 h, and then labeled with BrdU for 1 h prior to collection. A portion of the cells were subject to immunoblot analysis (A) and the remaining sample was analyzed by flow cytometry with anti-BrdU antibody to detect DNA synthesis and with propidium iodide for DNA content (B). The histogram shows the percentage of BrdU-positive, S phase cells at 48 and 72 h after transfection with the indicated siRNAs. (C) Whole cell extracts and chromatin-bound fractions from cells treated as in A were evaluated for Mcm2, Cdc6, Orc2, and tubulin by immunoblotting.

resulted in the reduction of chromatin-bound Mcm2, demonstrating a deficiency in origin licensing (Figure 3.2C).

<u>Cdc6 depletion in normal human fibroblasts results in an arrest of cells in the G1 phase.</u>

The loss of S phase cells after Cdc6-depletion could have been due to effects on the G1 to S phase transition, or it could have been due to secondary effects that occurred after entry into S phase. To distinguish between these possibilities, we synchronized siRNA-transfected cells by serum starvation/re-stimulation, and analyzed origin licensing and progression into S phase. Cdc6 protein was undetectable in serum-starved cells (t=0 h), but serum stimulation induced Cdc6 protein and its chromatin association beginning at 6 h after serum addition (Figure 3.3A). By 10 h, the increase in Cdc6 protein levels was accompanied by elevated Mcm2 chromatin association. As expected, cells depleted of Cdc6 were deficient in MCM chromatin loading (Figure 3.3A, right panel). Cells depleted of Cdc6 and re-stimulated to enter the cell cycle spent more time in G1 than control cells and delayed S phase entry by four hours, as determined by BrdU incorporation (Figure 3.3B).

The defect in G1 progression could have been a consequence specific only to loss of Cdc6 protein, or it could have been due to the licensing defect caused by loss of Cdc6 function. To distinguish between these possibilities, we depleted NHF1 cells of Cdt1 or Orc2. Cdt1 and Orc2 depletion also caused a reduction in the
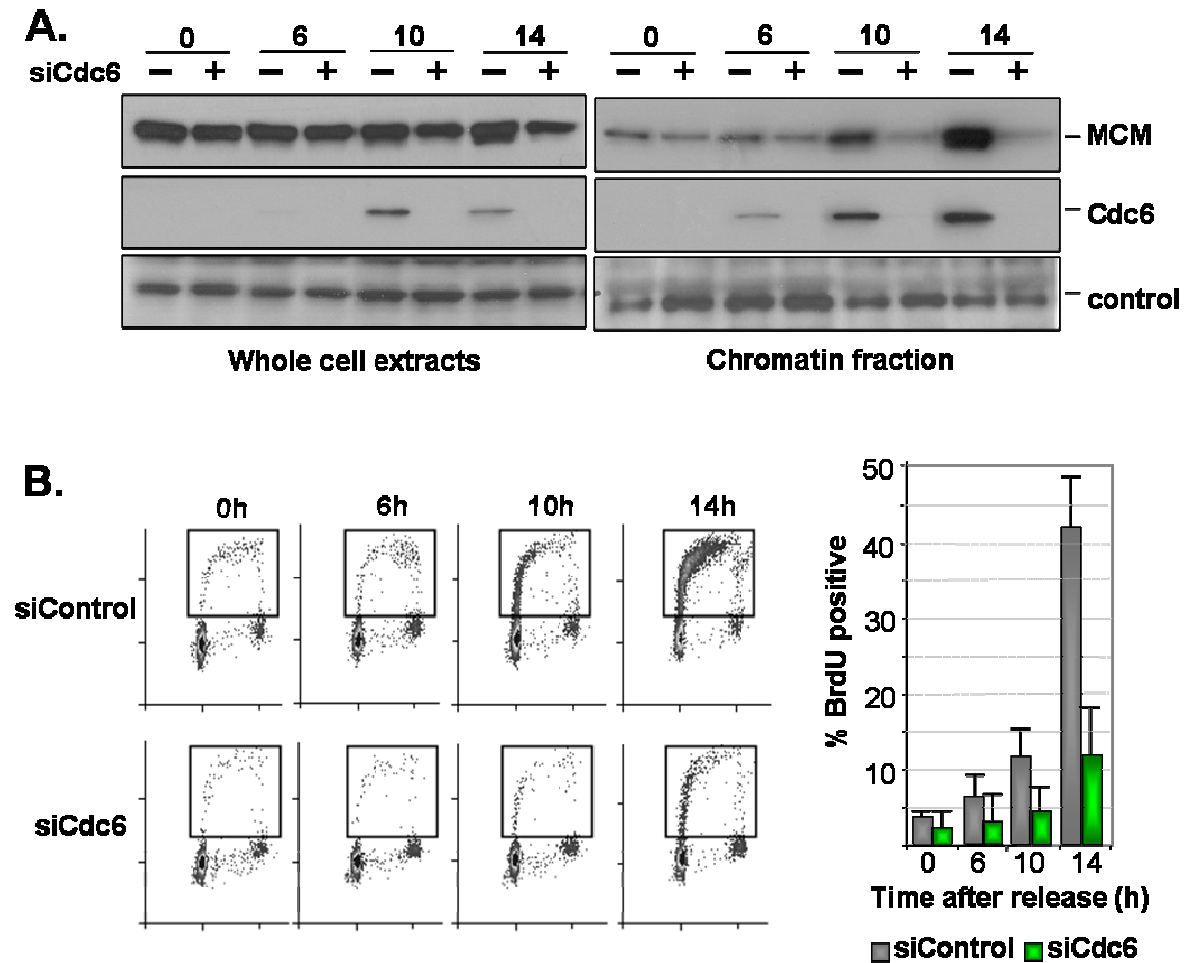
Figure 3.3: **Serum-stimulated, Cdc6-depleted NHF1 cells delay S phase entry.** (A) NHF1 cells were transfected with 100 nM control siRNA or Cdc6 siRNA, incubated for 12 h, the medium was changed to medium containing 0.5% FBS and incubated an additional 60h. Cells were re-stimulated into the cell cycle by addition of 10% FBS, and labeled for 1 h prior to collections at 0, 6, 10, and 14 h. Whole cell extracts and chromatin-bound extracts were processed for immunoblot analysis of MCM and Cdc6. A non-specific band serves as the loading control. (B) A portion of the cells transfected in A were analyzed by flow cytometry. Bar graph represents the percentage of BrdU positive cells in three independent experiments.
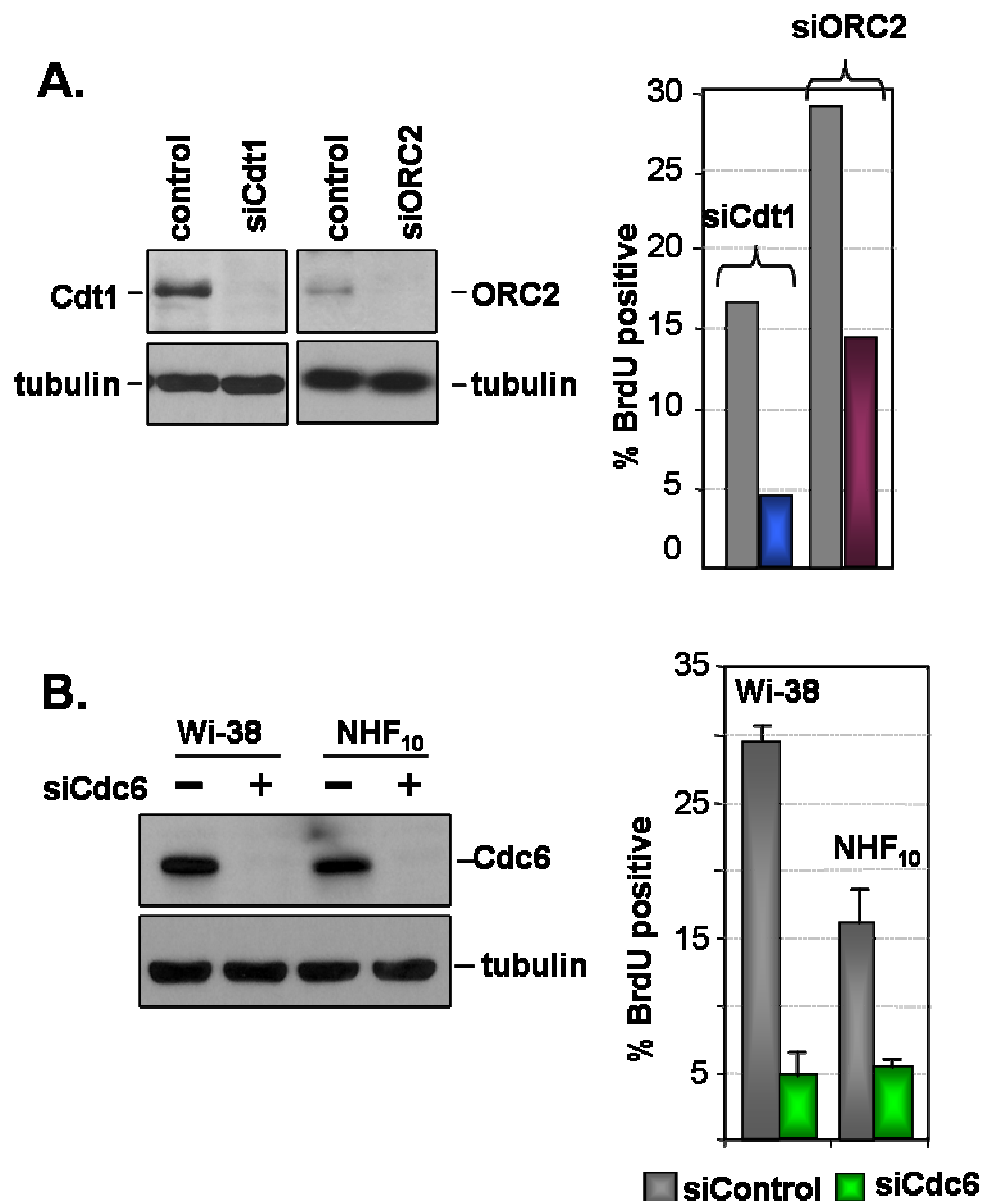
Figure 3.4: G1 arrest is not cell type or a Cdc6-specific response. (A) NHF1 cells were transfected with Cdt1 siRNA as in Figure 3.2. NHF1 cells were transfected with 100 nM siRNA targeting Orc2, split 24 h later, transfected a second time with 100 nM 24 h later, then collected 48 h after final transfection. A portion of the cells were subject to immunoblot analysis (left panel) the remaining sample was analyzed by flow cytometry (left panel) with anti-BrdU antibody to detect DNA synthesis and with propidium iodide for DNA content. The percentage of BrdU-positive, S phase cells profiles were graphed. (B) WI-38 and NHF10 cells were transfected with 100 nM control and Cdc6 siRNA, labeled, collected and processed as in Figure 3.2.

number of BrdU positive cells (Figure 3.4A, bar graph) with no signs of apoptosis (data not shown). In addition, the effects of Cdc6 depletion were not cell line specific because other normal cell lines, NHF10 and WI-38, behaved identically to the NHF1 cells (Figure 3.4B). Taken together, these data suggest that defects in origin licensing during G1 profoundly delay the G1 to S phase transition in normal cells.

We were not satisfied with BrdU labeling as the only measure of cell cycle progression in licensing-defective cells. Because origin licensing is a prerequisite for origin firing and DNA synthesis, failure to incorporate BrdU could be due to an active cell cycle response or it could be simply a consequence of the inhibition of DNA synthesis due to the depletion of an essential DNA replication factor. To address this question, we examined the phosphorylation status of the retinoblastoma protein (Rb), which is required for the G1/S transition but precedes origin firing. We examined the phosphorylation state of Rb by SDS-PAGE mobility and with a phosphospecific antibody. In comparison to control cells, Cdc6 depletion displayed an increase in the gel mobility of total Rb protein, indicating a generally hypophosphorylated state (Figure 3.5A). In addition, examination of Rb using a phosphospecific antibody demonstrated a decrease in the specific phosphorylation of Rb at serines 807 and 811 (Figure 3.5B). In its active unphosphorylated state, Rb inhibits cell cycle progression by binding to the transcription factor E2F, which is involved in the transcriptional activation of genes required for DNA replication. Therefore, we reasoned that changes in Rb phosphorylation status might be due to defects in the kinases that are responsible for the phosphorylation of this protein.
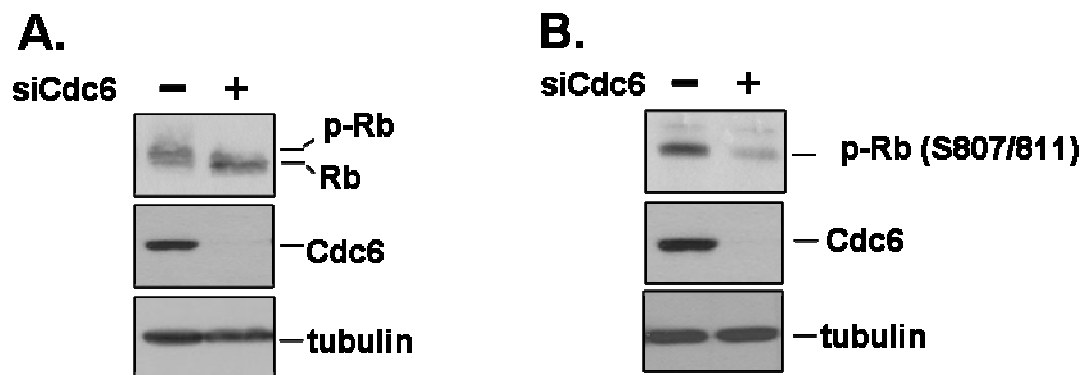
Figure 3.5: Depletion of Cdc6 results in loss of Rb phosphorylation. Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies to (A) total Rb or (B) with a phosphospecific antibody that recognizes Rb phosphorylated at S807 and S811.

Insufficient origin licensing affects cyclin/Cdk expression and activity.

As the cells traverse through G1, Rb becomes partially phosphorylated by cyclin D-Cdk4/6, which amongst other things, allows for the transactivation of *cyclin E*, an E2F target (52,73,145). Cyclin E/Cdk2 relieves the remaining inhibitory effects of Rb on E2F and also phosphorylates E2F, which allows for cyclin E to stimulate its own transcription (143). Therefore, we examined the effects of Cdc6 depletion on both cyclin D-Cdk4/6 and cyclin E-Cdk2 status.

Effects of Insufficient Origin Licensing on Cyclin D-Cdk4/6

We examined whether deficiencies in origin licensing had any effect on cyclin D levels. There are three isoforms of cyclin D (D1, D2, and D3), and their expression is cell-type specific. Depletion of Cdc6 in NHF1 resulted in a decrease in the levels of cyclin D1 and cyclin D3 protein (Figure 3.6A, left panel). WI-38 cells depleted of Cdc6 also displayed decreased levels of cyclin D3 (Figure 3.6A, right panel). Loss of cyclin D did not have any impact on the protein level of one of its catalytic partners, Cdk4 (Figure 3.6B). These findings suggested an overall deregulation of cyclin D in the presence of insufficient origin licensing.

As previously discussed (Chapter 2), cyclin D is regulated on multiple levels, one of which is transcriptional activation by growth factors; the other is post-translational modifications that result in its degradation (Fig 2.3). Growth factors stimulate a MAP kinase pathway, ultimately activating ERK1 and ERK2, which then results in the transcriptional control of cyclin D. If reduction of Cdc6 interferes with
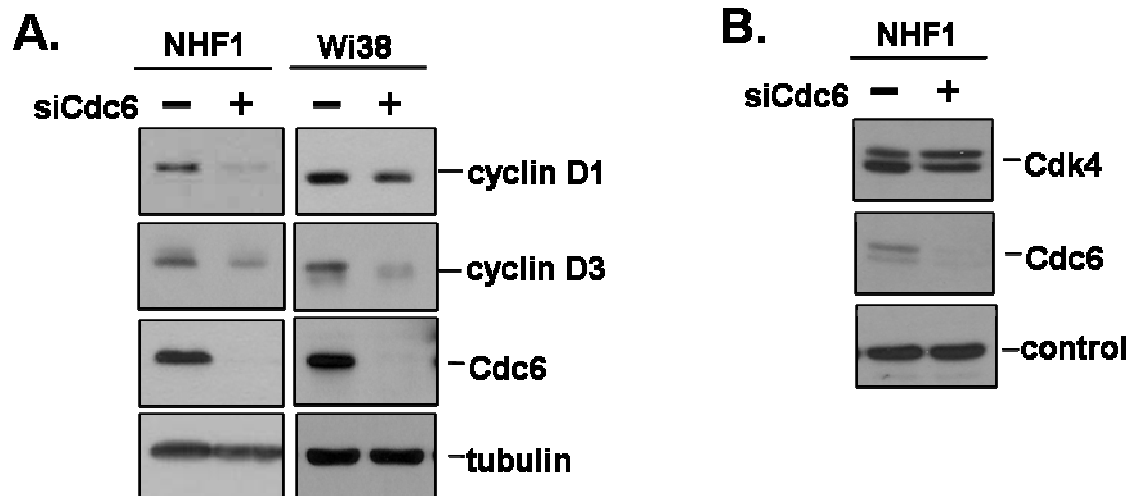
Figure 3.6: Depletion of Cdc6 results in loss of cyclin D but not Cdk4 protein. Whole cell extracts from NHF1 and WI-38 cells were transfected with control or Cdc6 siRNA and probed with antibodies to (A) cyclin D1, cyclin D3, Cdc6 and tubulin or (B) Cdk4 and Cdc6. A non-specific band serves as a loading control.

the activation of the MAP kinase pathway, this would result in down-regulation of cyclin D message and a subsequent loss of protein. No differences in the levels of phospho-ERK were detected when Cdc6-depleted cells were compared to the control cells (Figure 3.7). Another possible explanation for the loss of cyclin D protein is post-transcriptional regulation. In this case, Ras activates Akt to inhibit GSK-3β, allowing for the accumulation of cyclin D protein (see Fig 2.3). If this pathway were involved in regulating cyclin D levels, then defects would be apparent by the loss of phosphorylated Akt and up-regulation of PTEN, an inhibitor of this pathway. However, upon Cdc6 depletion there were no changes in PTEN levels or in the levels of p-AKT when compared to control cells (Figure 3.7). These results suggest a much more complex regulation of cyclin D protein.

Recent evidence supports our findings that insufficient origin licensing results in down-regulation of cyclin D levels. The cause of this down-regulation was shown to be caused by reduction of cyclin D1 mRNA that was attributed to a decrease in RNA Pol II on the cyclin D1 promoter (119). Surprisingly, this group demonstrated that over-expression of cyclin D in origin licensing deficient cells did not allow cells to enter S phase, suggesting there may be multiple mechanisms to safeguard entry of cells into S phase in the presence of insufficient licensed origins. Therefore, we examined the other kinase required for G1/S phase progression, cyclin E/Cdk2.

Effects of Insufficient Origin Licensing on Cyclin E/Cdk2

Cyclin E/Cdk2 is the major kinase responsible for stimulating S phase entry. (Cdk1 can substitute as a partner for cyclin E, but only in the absence of Cdk2)
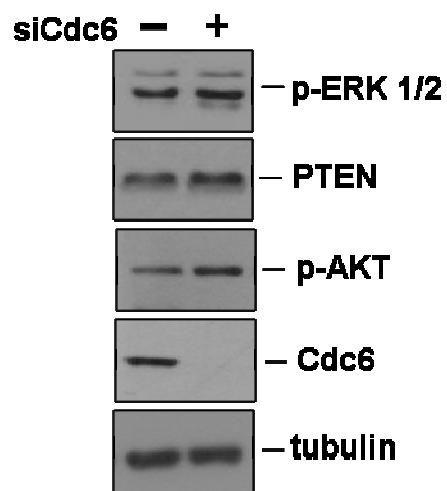
Figure 3.7: Loss of cyclin D protein is not due to inhibition of the MAP-kinase pathways. Whole cell extracts from NHF1 were transfected with control or Cdc6 siRNA and probed with antibodies to p-ERK 1/2, PTEN, p-AKT, Cdc6 and tubulin.

(81,176). We compared histone H1 kinase activity in cyclin E immunoprecipitates from lysates of control and Cdc6-depleted cells. By this measurement, Cdc6 depletion caused on average a 2.5-fold reduction in cyclin E-associated kinase activity (Figure 3.8A). We further examined the cyclin E-associated kinase activity in siRNA transfected NHF1 cells synchronously progressing through G1. Cdc6-depleted cells contained substantially less cyclin E-associated kinase activity compared to control cells, and this difference was detectable by 6 h after serum stimulation (Figure 3.8B), well before the G1/S transition is observed in the control cells (at approximately 10 h) (Figure 3.3B, bar graph).

Loss of cyclin E/Cdk2 activity is not caused by interaction with known cyclin-dependent kinase inhibitors (CKIs).

Cyclin E/Cdk2 activity is controlled by a number of mechanisms, and we sought to determine which of these might be responsible for the reduced cyclin E-dependent activity in Cdc6-depleted cells. First we examined total cyclin E levels and found that Cdc6 depletion did not alter the levels of cyclin E compared to control (Figure 3.9A), suggesting that loss of cyclin E/Cdk2 kinase activity was not caused by loss of cyclin E protein. Cyclin E/Cdk2 kinase activity is blocked when bound to cyclin-dependent kinase inhibitors (CKI). In extracts from Cdc6-depleted cells we observed no up-regulation of either p21 or p27 CKI proteins (Figure 3.9B), nor did we observe any changes in p21 or p27 binding to the cyclin E/Cdk2 complex (Figure 3.9C; no differences in the ratio of p21 to cyclin E after immunoprecipitation with cyclin E antibodies). A previous study used immortalized cells to investigate the

Figure 3.8: Reduction in the abundance of Cdc6 results in inhibition of cyclin E-dependent kinase activity. (A) Extracts of NHF1 cells transfected with control or Cdc6 siRNA were subjected to immunoprecipitation with normal rabbit serum or with anti-cyclin E antibody. The precipitates were divided and analyzed by immunocomplex kinase assay with purified histone H1 and [γ-32P] ATP, followed by SDS-PAGE (top row) or analyzed for cyclin E protein by immunoblotting (bottom row). The bar graph reports cyclin E-associated H1-kinase activity in cells depleted of Cdc6 relative to the control in three independent experiments. (B) Extracts from transfected and serum-stimulated NHF1 cells (refer to Figure 3.3 for protocol) were subjected to cyclin E kinase assay as in A.
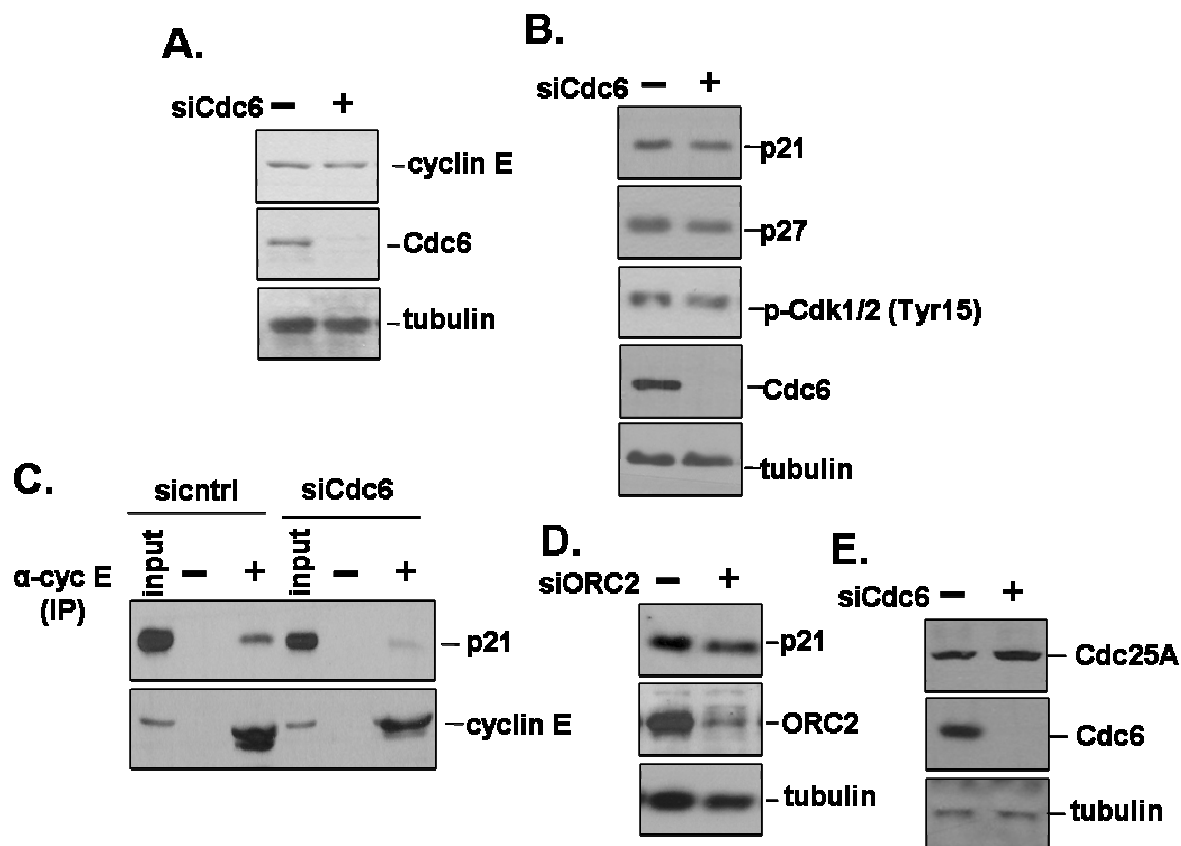
Figure 3.9: Loss of cyclin E/Cdk2 kinase activity is not due to induction or binding of CKI's. Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies to recognize the following endogenous proteins: (A) cyclin E, Cdc6, and tubulin; and (B) p21, p27, phospho-Cdk (Y15), Cdc6, and tubulin. (C) Extracts of siRNA-transfected NHF1 cells were subjected to immunoprecipitation with normal rabbit serum [control extract (-)] or with anti-cyclin E antibody (+). Inputs (5% of whole cell extracts) and immunoprecipitates were analyzed for p21 and cyclin E protein by immunoblotting. (D) Whole cell extracts from NHF1 cells transfected with control or Orc2 siRNA were probed with antibodies to detect p21, Orc2 and tubulin. (E) Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies for Cdc25A, Cdc6, and tubulin.

effects of Orc2 depletion, which also blocks origin licensing, on Cdk2 activity and concluded that licensing inhibition induced p21 and p27 accumulation (124). We performed similar experiments in NHF1 cells and did not detect any increase in p21 protein levels, despite effective depletion of Orc2 (Figure 3.9D). These differing results could be attributed to cell type variations, for Machida *et al.* examined the effects of Orc2 depletion in MCF10A cells. Therefore, the reduced cyclin E-associated kinase activity in Cdc6-depleted NHF1 cells was not due to any of the most commonly studied mechanisms of inhibition of this cell cycle regulator.

Cyclin E/Cdk2 is also regulated by two inhibitory phosphorylations on Cdk2 at threonine 14 (T14) and tyrosine 15 (Y15), and retention of phosphorylation at these sites blocks S phase entry (35,55,126). We probed lysates of siRNA-transfected cells with an antibody that recognizes both Cdk1 and Cdk2 phosphorylated at Y15. We found no increase in the level of this inhibitory phosphorylation in Cdc6-depleted cells compared to control cells (Figure 3.9B), nor did we detect changes in abundance of the Cdc25A phosphatase responsible for activating Cdk2 (Figure 3.9E).

It has been suggested that in the absence of an active cyclin E/Cdk2 complex that cyclin A can bind with Cdk2 and carry out the functions of cyclin E/Cdk2. To address whether Cdk2 was functioning in conjunction with cyclin A, we examined the histone H1 kinase activity in Cdk2 immunoprecipitates in the same manner as mentioned above. Cdk2 immunoprecipitates from Cdc6-depleted cells showed an even greater reduction in kinase activity (Figure 3.10A) with no increases in p21 or p27 bound to the kinase (Figure 3.10B). These findings demonstrate that there is an
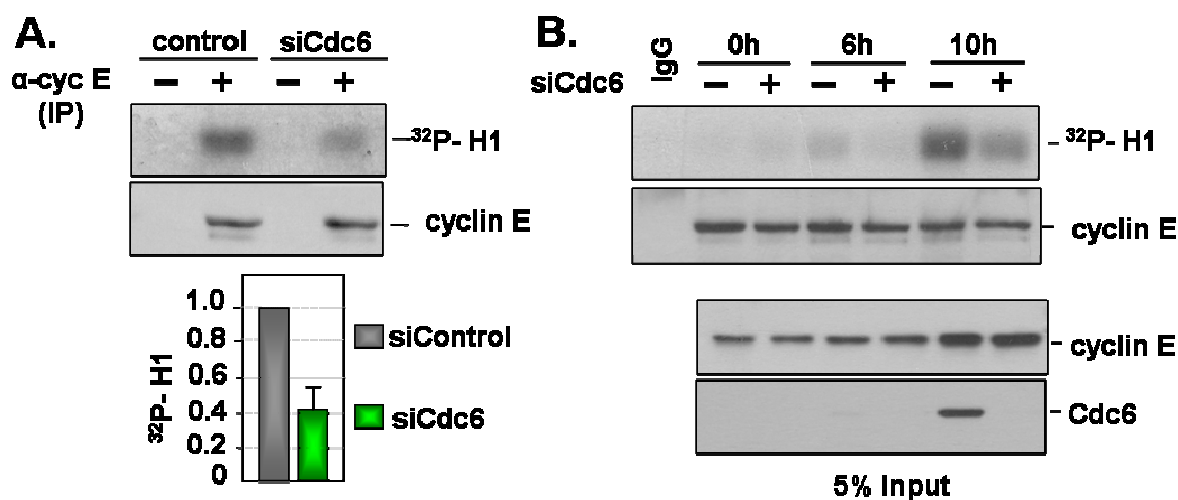
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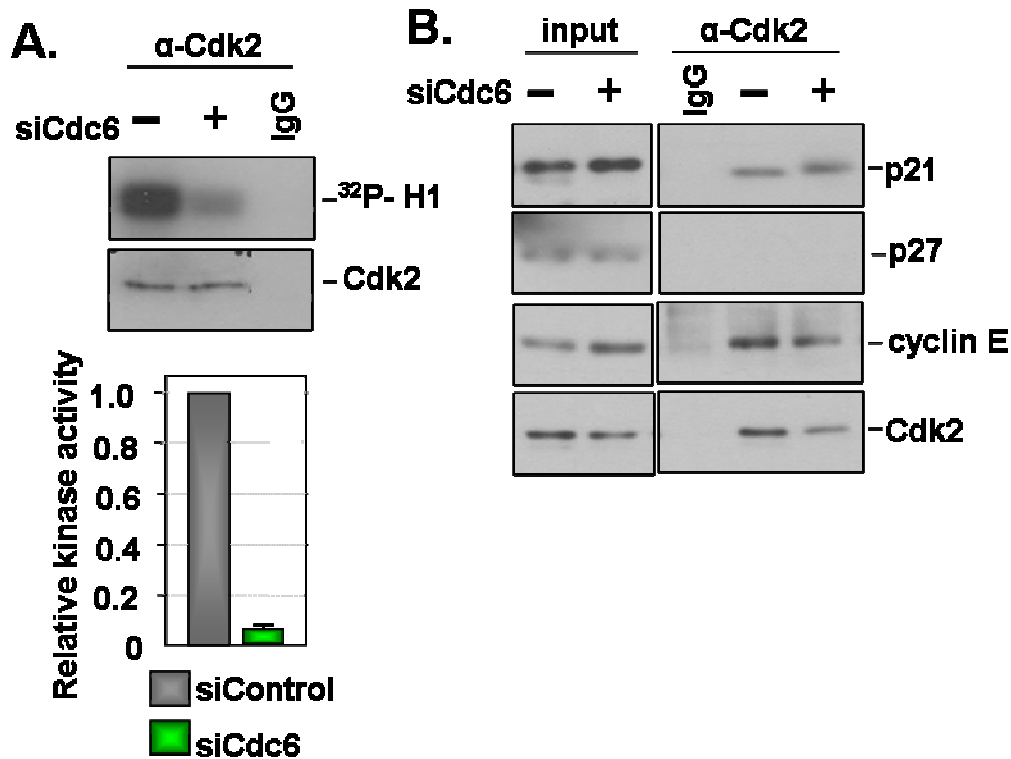
Figure 3.10: Reduction in Cdc6 abundance results in inhibition of Cdk2-dependent kinase activity. (A) Extracts of NHF1 cells transfected with control or Cdc6 siRNA for 48 h were subjected to immunoprecipitation with normal rabbit serum or with anti-Cdk2 antibody. The precipitates were divided and analyzed by immunocomplex kinase assay with purified histone H1 and [γ-32P] ATP, followed by SDS-PAGE (top row) or analyzed for Cdk2 protein by immunoblotting (bottom row). The bar graph reports cyclin E-associated H1-kinase activity in cells depleted of Cdc6 relative to the control in three independent experiments. (B) Extracts of NHF1 cells transfected with control or Cdc6 siRNA for 48 h were subjected to immunoprecipitation with anti-Cdk2 antibody. Immunoprecipitates were subjected to immunoblot analysis for p21, p27, cyclin E, and Cdk2.

inherent loss of Cdk2 kinase activity that is not dependent on cyclin E or cyclin A and is not the result of CKI binding.

Insufficient origin licensing inhibits CAK-dependent activation of cyclin E/Cdk2.

In human cells, the activating phosphorylation of all Cdks is primarily attributed to Cdk activating kinase (CAK), a heterotrimeric complex of Cdk7, cyclin H, and Mat1 (182). We examined the status of the activating phosphorylation on T160 Cdk2 in Cdc6-depleted cells and found that insufficient origin licensing resulted in the loss of T160 phosphorylation on Cdk2 (Figure 3.11A). We observed similar results upon Cdc6 depletion in WI-38 (Figure 3.11B) and upon depletion of Cdt1 in NHF1 cells (Figure 3.11C). We wondered if the loss in T160 phosphorylation could explain the loss of cyclin E/Cdk2 activity, so we decided to examine whether pre-incubation with purified CAK would have any effect on the activity of Cdk2 in the Cdc6-depleted cells. Cdk2 immunoprecipitates from lysates of control and Cdc6-depleted cells were incubated with or without purified CAK for 30 minutes. Immunoprecipitates were washed and histone H1 kinase activity of Cdk2 was determined. As expected, in the absence of CAK, Cdc6-depleted NHF1 displayed a dramatic reduction in the activity of Cdk2 (Figure 3.11D, left panel) with no changes in Cdk2 protein levels (Figure 3.11E). Pre-incubation of Cdk2 immunoprecipitates with purified CAK partially restored Cdk2 activity (Figure 3.11D, right panel). These findings suggest that cyclin E/Cdk2 in immunoprecipitates of Cdc6-depleted cells can be activated by CAK, further demonstrating that the inhibition of the kinase
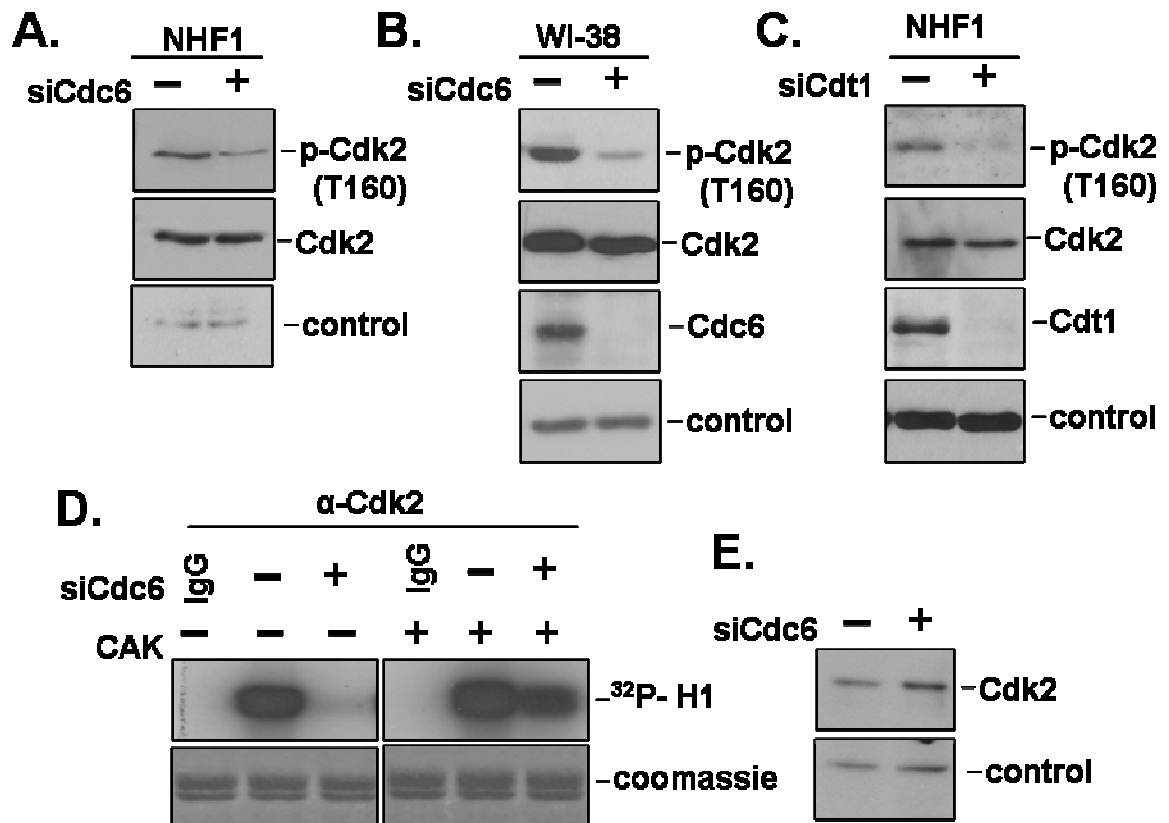
Figure 3.11: Insufficient origin licensing results in a defect in Cdk2 activation. Whole cell extracts from (A) NHF1 or (B) WI-38 cells transfected with control or Cdc6 siRNA were probed with antibodies to recognize the following endogenous proteins: phospho-Cdk2 (T160), Cdk2, and Cdc6. A non-specific band served as a loading control. (C) NHF1 cells were transfected with control or Cdt1 siRNA and their extracts immunoblotted for the indicated proteins. (D) Extracts of NHF1 cells transfected with control or Cdc6 siRNA for 48 h were subjected to immunoprecipitation with normal rabbit serum or with anti-Cdk2 antibody. Immunoprecipitates were divided, and pre-incubated for 30 min at room temperature with or without purified CAK. Immunoprecipitates were washed once, then analyzed by immunocomplex kinase assay with purified histone H1 and [γ-32P] ATP, followed by SDS-PAGE (top row). Coomassie stain of the gel prior to drying serves as a loading control. (E) Extracts from D were subject to immnoblot analysis for Cdk2. Non-specific band serves as a loading control.

activity is not due to unidentified inhibitor(s), but most likely to the loss of phosphorylation on Cdk2 T160.

Next we investigated the status of CAK activity in the cultured fibroblasts. In addition to activation of Cdk2, CAK is also responsible for phosphorylation of serine 5 in the RNA Pol II C-terminal domain, CTD (57,74,93,178). We reasoned that if CAK activity were low in Cdc6-depleted cells, then phosphorylation of the RNA Pol II CTD would be reduced also. Indeed, Cdc6-depleted cells reproducibly display a reduction in CTD phosphorylation on S5 (Fig 3.12A). We next examined whether any of the components of the CAK complex were deregulated. In spite of seeing loss of CTD phosphorylation and Cdk2 T160 phosphorylation in Cdc6-depleted cells, there were no changes in the protein levels of Cdk7, cyclin H or Mat1 (Figure 3.12B); or in the *in vitro* kinase activity of CAK (Figure 3.12C). These findings suggest that the loss of T160 phosphorylation in Cdk2 is more complicated than the simple loss of CAK kinase activity.

Two cancer cell lines fail to inhibit Cdk activation in response to insufficient origin licensing.

Previous reports that virtually all transformed cell lines die by apoptosis when origin licensing is blocked suggested that these cells might be unable to induce the same G1 arrest described in the preceding sections. We demonstrated above that both HeLa cells and U2OS cells showed clear evidence of apoptosis after transfection with siRNA targeting Cdc6 (Figure 3.1A and B). To determine if these cells were capable of down-regulating both Rb phosphorylation and Cdk2 T160
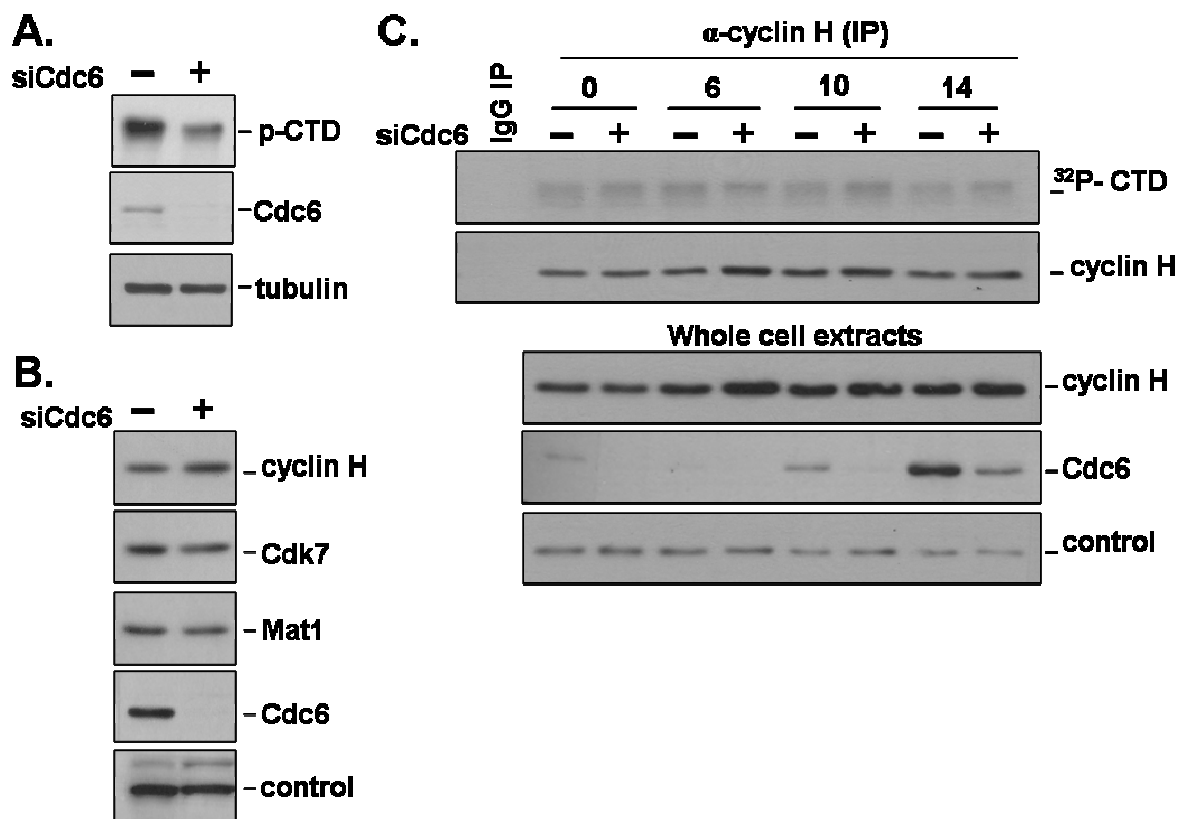
Figure 3.12: Depletion of Cdc6 results in defective CAK-dependent functions.
Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies to recognize the following endogenous proteins: (A) phospho-RNA Pol II (S5, CTD), Cdc6 and tubulin; and (B) cyclin H, Cdk7, Mat1, and Cdc6; a non-specific band serves as a loading control. (C) Extracts from transfected and serum-stimulated NHF1 cells (refer to Figure 3.3 for protocol) were subjected to cyclin H kinase assay as in Figure 3.8A.

phosphorylation, we probed for these markers by immunoblotting. Despite effective

Cdc6 depletion, neither HeLa nor U2OS cells showed the same loss of Rb and Cdk2

phosphorylation that we observed in the non-transformed NHF1 or WI-38 cells

(Figure 3.13A and B). These findings suggest that cancer cells may undergo

apoptosis as a result of an inability to down-regulate the activating phosphorylation

of Cdk2, thus allowing cells to enter a doomed S phase.
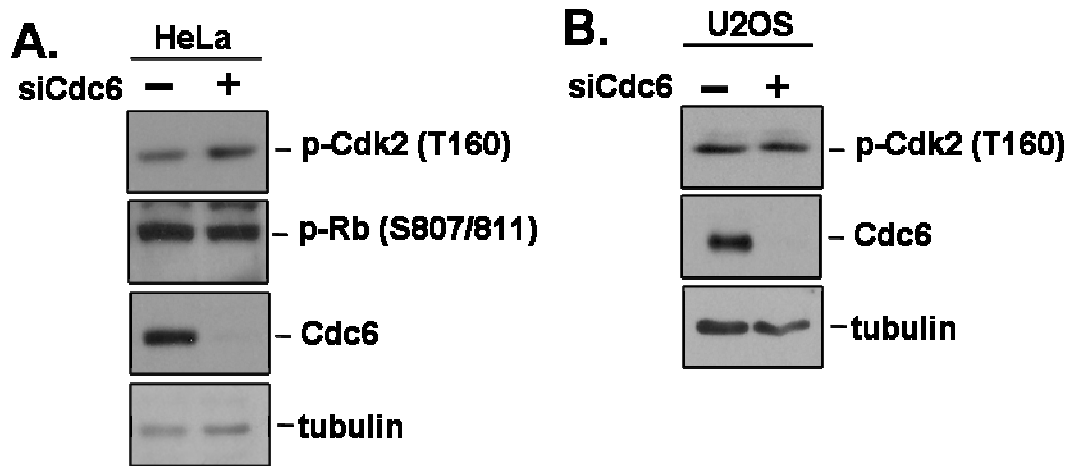
Figure 3.13: Cancer cells do not down-regulate the phosphorylation on Cdk2. (A) HeLa and (B) U2OS cells were transfected with control or Cdc6 siRNA, incubated 96 h, and whole cell extracts subjected to immunoblot analysis with antibodies to detect phospho-Cdk2 (T160), phospho-Rb (S807 and S811), Cdc6 and tubulin.

Discussion

In this chapter, we demonstrate that insufficient origin licensing activates a G1 delay and we provide mechanistic links connecting this response to the inhibition of cyclin E/Cdk2. We show evidence that the G1 delay is not simply due to loss of essential DNA replication factors, but instead it constitutes an active response to insufficient origin licensing. Similar Cdk2 down-regulation was observed in both Cdt1-depleted cells, which express Cdc6, and in Cdc6-depleted cells, which express Cdt1. The disruption common to both Cdc6 and Cdt1 depletion is the failure to fully load MCM complexes at origins, indicating that it is origin licensing itself that is required for Cdk2 activation.

We also observed loss of cyclin D1 and D3 protein in Cdc6-depleted cells (Fig 3.6) which is consistent with what has been observed by others (120). Loss of cyclin D protein cannot fully explain the G1 arrest as over-expression of cyclin D in cells depleted of Mcm7 was not enough to allow cells to enter S phase (120). Given our evidence for Cdk2 inhibition, the findings of Liu at al. (2009) are not surprising. Over-expression of cyclin E can drive quiescent cells into S phase but over-expression of cyclin D cannot (115). This suggests that there may be multiple mechanisms to prevent S phase entry upon insufficient origin licensing.

The low activity of cyclin E/Cdk2 in Cdc6-depleted fibroblasts could be explained by the failure to phosphorylate Cdk2 on T160 (Figure 3.11) since no other major Cdk regulatory mechanisms (e.g., inhibition by p21 or p27) were perturbed in these cells (Figure 3.9). This activating phosphorylation is mediated by Cdk-activating kinase (CAK), and we find that phosphorylation of the other major CAK

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substrate, RNA Pol II was similarly reduced (Figure 3.12). We were also able to demonstrate that pre-incubation with purified CAK can partially rescue the Cdk2 kinase activity *in vitro* (Figure 3.11). Studies of the regulation of CAK-dependent Cdk activation are limited, so the finding that this event is responsive to licensing inhibition represents a novel aspect of Cdk control. Expression of CAK subunits was not inhibited in licensing-deficient cells (Figure 3.12B), and our preliminary evaluation of CAK activity *in vitro* revealed no biochemical defects in kinase specific activity using the CTD of RNA Pol II or Cdk2 as a substrate (Figure 3.12C).

In actively cycling cells (as opposed to cells re-entering the cycle from G_0), MCM complexes can be detected on chromatin as early as telophase and persist throughout G1(47). If Cdk activation is dependent on a minimum amount of licensing, as our data indicate, then that minimum level of licensing may be reached very early in G1. Under such circumstances, Cdk activation would normally be quite rapid, and only when licensing is strongly delayed is the dependence of Cdk activation on licensing revealed. Since Cdk activity inhibits origin licensing through inactivation of individual preRC components, it is important to maintain a window of low Cdk activity in these early G1 phases to allow sufficient licensing before S phase begins (45,54). Coordinating Cdk activation with replication licensing would ensure that this window of opportunity is maintained.

Recent evidence from budding yeast suggests that even in cells with standard G1 phases, competence to assemble preRCs must be actively maintained throughout G1. Depletion of yeast Orc6 after MCM complexes are loaded results in decay of MCM chromatin association over time (180). This finding may be

particularly relevant in G1 cells exposed to any of a variety of stresses or genotoxic insults. Ionizing radiation, ultraviolet light, and chemical modification of DNA bases induce the ubiquitination and degradation of Cdt1 and Cdc6 (14,70,79,84,151), as well as phosphorylation of MCM and ORC subunits (34,209). Moreover, hypertonic stress in G1 prevents MCM loading (87). Once the inhibition of MCM loading is removed (i.e., DNA is repaired, adaptation to the stress has been induced, etc.) cells should still ensure that origins are licensed before proceeding into S phase.

Previous studies had noted that cancer cell lines die by apoptosis when licensing is inhibited whereas normal cells arrest in G1, and we have recapitulated those observations here using HeLa and U2OS cells. We have further extended these findings to demonstrate that these cancer cell lines fail to induce the same down regulation of Cdk2 phosphorylation on T160 or the same hypophosphorylation of Rb. We note that a majority of cancer cells have lesions in both the p53 and Rb pathways; in the case of HeLa cells these disruptions include expression of the HPV E6 and E7 proteins, and in U2OS cells it is overexpression of the p53 ubiquitin ligase, Mdm2 (107) and loss of the p16 Cdk4 inhibitor (37). The dependence of Cdk2 phosphorylation on origin licensing during G1 might have not been previously observed in HeLa cells (and presumably in most cancer cell lines) due to the wide variations in expression and regulation of preRC components. It may be that coordination of Cdk2 activation and origin licensing can only occur in cells with normal, rather than hyperactivated Cdks and low levels of E2F-regulated gene products, rather than the overproduced levels associated with oncogenic transformation.

It has long been appreciated that Cdk activity regulates preRC assembly positively by promoting transcription of genes for preRC proteins and negatively by phosphorylating preRC components themselves. In this study we provide evidence that preRC assembly itself regulates Cdk activation. Important questions remain as to the molecular nature of the interactions between origin licensing components, CAK, and Cdk2. Among the possibilities for future testing are recruitment of Cdk2 to chromatin by licensed origins to promote nuclear retention and/or recruitment of both Cdk2 and CAK to loaded MCM complexes to facilitate their interaction. The fact that many preRC components are substrates of Cdks plus an intriguing report that Mcm7 associates with the Mat1 subunit of CAK support the idea that Cdk2 and CAK could associate with chromatin-bound MCM complexes (199). The ultimate outcome of these investigations will define how origin licensing in G1 is ensured before S phase entry.

CHAPTER 4

EVIDENCE FOR A REPLICAITON LICENSING CHECKPOINT

Background

Cell cycle checkpoints are pathways that slow or arrest progression through the cell cycle in response to a variety of insults or until important cellular events are complete. The most common and best understood of these checkpoints are the DNA damage checkpoints. These are activated in response to various forms of DNA damage, from double strand breaks to bulky adducts on the DNA. In addition to the DNA damage checkpoints there are also surveillance checkpoints which include the decatenation checkpoint and the spindle checkpoint. Surveillance checkpoints are not activated in response to damage but instead by the incompletion of a cellular process. For example, the spindle checkpoint initiates a "wait" signal that inhibits the anaphase promoting complex (APC/C) until all chromosomes attach to the microtubules and align at the metaphase plate (171,172). Once all sister chromatin-microtubule-kinetochore attachments have been made the checkpoint is turned off and APC is activated [reviewed in (95)]. Another cellular event that would be important to monitor, so as to preserve the faithful duplication of the genome, is the licensing of an adequate number of origins of replication before cells commit to the S phase of the cell cycle.

In order to define a cell cycle delay as a checkpoint as opposed to interference of a metabolic process, one must be able to abrogate the response by genetic manipulation (160). For example, in response to ultraviolet light, the cell activates an intra-S checkpoint, inhibiting fork progression and preventing new origin firing (27). If this delay in S phase is the result of a checkpoint then abrogation should be accomplished by depletion of an essential factor within the pathway. And this was exactly what has been demonstrated, removing Chk1 or ATR using siRNA (of which both are required) attenuates the checkpoint response in response to UV (179). Therefore, in order to demonstrate that depletion of Cdc6 or Cdt1 results in the activation of a checkpoint, regulators must be identified by demonstrating that upon their physical or functional removal the original response is abrogated (i.e. cell cycle progression resumes without the correction of the activating event).

It was demonstrated in Chapter 3 that depletion of Cdc6 or Cdt1 resulted in the loss of Rb phosphorylation, cyclin E/Cdk2 kinase activity and a G1 delay. A variety of proteins that are involved in already characterized checkpoints have been shown to regulate one of the above mentioned events. Activation of p53 by serine 15 phosphorylation results in the stabilization and accumulation of p53 and the transcriptional up-regulation of p21 (191). Additionally, p21, a cyclin-dependent kinase inhibitor, binds to and inactivates the cyclin E/Cdk2 complex, preventing hyperphosphorylation of Rb and causing cells to arrest in the G1 phase of the cell cycle (147).

The involvement of Chk1 in DNA damage responses and in fork stabilization during unperturbed DNA replication has been demonstrated (31,161,162). In

addition to p53, Chk1 also phosphorylates Cdc25-A, -B, and -C; and along with other post-translational modifications, signals for the degradation of the Cdc25 family of proteins (49). The Cdc25 family of phosphatases are required to remove the inhibitory phosphorylation on Cdc2 (same as Cdk1) and Cdk2. Through the combined actions of a Chk1-mediated, p53-dependent up-regulation of p21 and inactivation of Cdc2 and Cdk2, cells are unable to enter S phase or start mitosis (Figure 4.1).

Though it is unlikely that these exact pathways are the mechanisms for inhibition of Cdk2 in response to insufficient origin licensing (refer to Chapter 3 Figure 3.9B- no up-regulation of p21, and Figure 3.9E- no changes in Cdc25), there is evidence connecting p53 and Chk1 to Cdk2 activity. For instance, inhibition of Chk1 kinase activity or depletion of Chk1 *via* siRNA in an unperturbed cell cycle increases Cdk2 activity, resulting in an increase in origin firing (188). Though the exact mechanism of how Chk1 alters Cdk2 activity in an unperturbed cell cycle is not known, it establishes a connection between the two kinases.
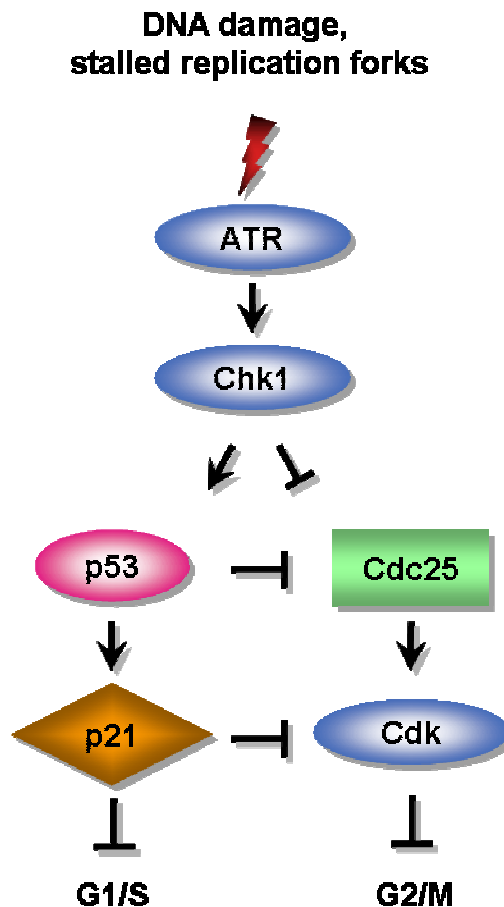
Figure 4.1: Schematic of ATR dependent cell cycle arrests. In response to various forms of DNA damage, ATR becomes activated, which in turn activates Chk1 through phosphorylations on S317 and S345. The activation of Chk1 can halt cells in both the G1/S and the G2/M transitions through activation of p53 and inhibition of Cdc25. These effects result in upregulation of p21 or accumulation of the inhibitory phosphorylation on Cdk, respectively. Both of these events result in the inhibition of cyclin/Cdk complexes halting cells in their current phase of the cell cycle.

Materials and Methods

Cell culture and siRNA transfection

Cells were cultured and maintained as stated in Chapter 3 with the following modifications. NFH1 cells were treated with 2 mM hydroxyurea for 24 h and collected to serve as a positive control for activation of the DNA damage response markers. SiRNA oligonucleotides were synthesized by Invitrogen (p53 siRNA, 5'-AAGGAA GACUCCAGUGGUAAU-3'; Chk1 siRNA, 5'- GCGUGCCGUAGACUGU CCA-3'; and ATR siRNA 5'-AACCUCCGUGAUGUUGCUUGA-3'). Transfections were performed with a total of 100 nM siRNA duplex using Dharmafect 1 reagent (Dharmacon), according to manufacturer's guidelines. In the dual knockdowns, p53 siRNA was transfected 24 h after Cdc6 siRNA, while Chk1 and ATR siRNA were transfected simultaneously with Cdc6 siRNA. Cells were then examined 72 h after initial transfection. When evaluating the co-depleted cells for activation of caspase-3 cleavage, NHF1 cells were examined 96 h after the initial transfection.

Cell synchronization

Serum starvation and re-stimulation protocols were performed as described in Chapter 3, with simultaneous transfection of Cdc6 and p53 siRNA.

Immunoblot analysis

Whole cell extracts were processed as described in Chapter 3 and immunoblot analysis was performed using these additional antibodies: p53 (sc-126), ATR, and Chk1 from Santa Cruz Biotechnology and phospho-Chk1 (S345), phospho-Chk2 (Th68), and phosph-p53 (S15) from Cell Signaling Technologies

Results

The G1 delay in response to insufficient origin licensing requires p53 and Chk1.

The differential response of non-transformed and transformed cells to licensing inhibition suggested that a commonly mutated pathway in cancer cells is involved in coordinating origin licensing with cyclin E/Cdk2 activation. The p53 tumor suppressor pathway is deregulated in the vast majority of human cancers, and p53 is capable of inducing a G1 arrest in response to a variety of perturbations. We have consistently observed a modest increase in p53 protein levels in Cdc6-depleted cells compared to control cells (Figure 4.2). For these reasons, we considered p53 a candidate for participation in the cell cycle effects of origin licensing inhibition. To determine if p53 status affects the ability of NHF1 cells to remain in G1 when origin licensing is inhibited, we performed co-depletion experiments. As observed before, transfection with Cdc6 siRNA alone reduced the number of S phase cells (Figure 4.3A, siCdc6). Depletion of p53 alone had little effect on the cell cycle profile of normal cells under these conditions (Figure 4.3A, si-p53). However, co-depletion of p53 24 h after transfection with Cdc6 siRNA completely prevented the cell cycle effects of Cdc6 depletion, fully restoring the number of S phase cells to control levels (Figure 4.3A, siCdc6/p53). Notably, the low intensity of the BrdU staining in the co-depleted cells (note the y-axis) suggested that even though the co-depleted cells entered S phase, replication was still very inefficient. Depletion of p53 did not rescue Cdc6 expression (Figure 4.3B), which likely accounts for the inefficient DNA synthesis.
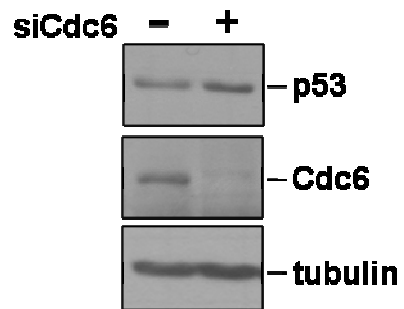
Figure 4.2: Cdc6 depletion results in a modest increase in p53 levels. Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies to p53, Cdc6 and tubulin.
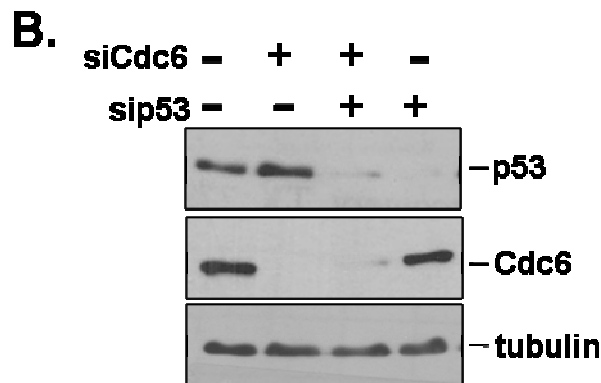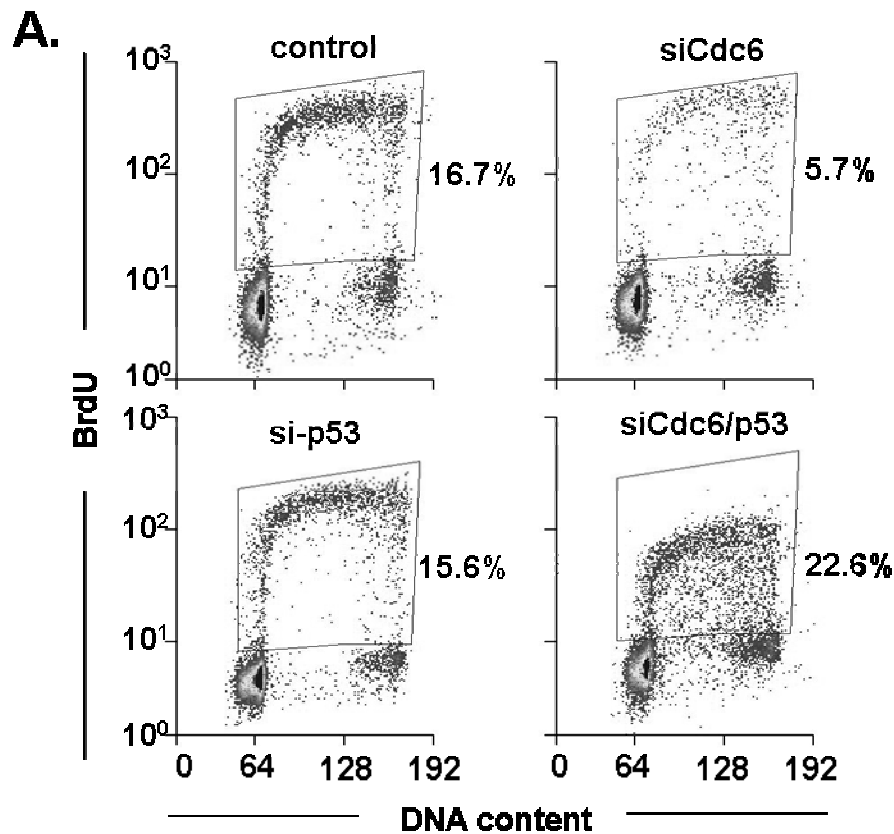
Figure 4.3: Co-depletion of Cdc6 and p53 abrogates the G1 arrest. NHF1 cells were transfected with a total of 100 nM of control (targeting GFP) or Cdc6 siRNA, and 24 h later transfected with p53 siRNA. Cells were incubated for a total of 72 h, and then labeled with BrdU for 1 h prior to collection. (A) A portion of the cells were analyzed by flow cytometry with anti-BrdU antibody to detect DNA synthesis and with propidium iodide for DNA content and (B) the remaining sample was subjected to immunoblot analysis for p53, Cdc6 and tubulin.

The requirement for p53 implicated components of the DNA damage checkpoint pathway in the G1 arrest induced by licensing inhibition. We thus examined cells co-depleted for Cdc6 and upstream signaling components of the DNA damage response. Co-depletion of Chk1 completely reversed the arrest, just as effectively as p53 co-depletion (Figure 4.4A, siCdc6/Chk1). Depletion of Chk1 alone had a mild effect on the number of S phase cells (11.8% compared to 16.3% in the control), but reduced the amount of BrdU incorporation per cell in keeping with a role for Chk1 in replication fork progression (27,161,162). On the other hand, co-depletion with Chk1 restored the number of S phase cells from a low of 4.3% in the population transfected with Cdc6 siRNA alone to 14% in Chk1 and Cdc6 co-depleted cells. Despite the fact that Chk1 is required for the arrest in G1 of Cdc6-depleted cells, we have frequently noted that Cdc6-depleted cells have reduced levels of Chk1 compared to control cells (Figure 4.4B) possibly due to Rb-mediated repression of the *CHK1* promoter (67).

Virtually identical results were obtained when p53 or Chk1 was co-depleted with Cdt1 (Figure 4.5A and 4.5B, respectively), indicating that p53 and Chk1 are required for the cell cycle effects of the licensing defect (i.e. loss of Cdc6 and Cdt1 functions) rather than the loss of Cdc6 or Cdt1 protein. Cdc6 and Cdt1 are essential replication factors, so we attribute the replication in cells co-depleted of p53 and Cdc6, p53 and Cdt1, Chk1 and Cdc6, or Chk1 and Cdt1 to the very low levels of origin licensing promoted by the scant Cdc6 or Cdt1 protein in these experiments. Importantly, the rescue of the S phase population when p53 or Chk1 was co-

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Figure 4.4: Co-depletion of Cdc6 and Chk1 abrogates the G1 arrest. NHF1 cells were transfected with a total of 100 nM of control (targeting GFP) or Cdc6 siRNA together with 100 nM of Chk1 siRNA. Cells were incubated for a total of 72 h, and then labeled with BrdU for 1 h prior to collection. (A) A portion of the cells were analyzed by flow cytometry with anti-BrdU antibody to detect DNA synthesis and with propidium iodide for DNA content (and (B) the remaining sample was subject to immunoblot analysis for Chk1, Cdc6 and tubulin.
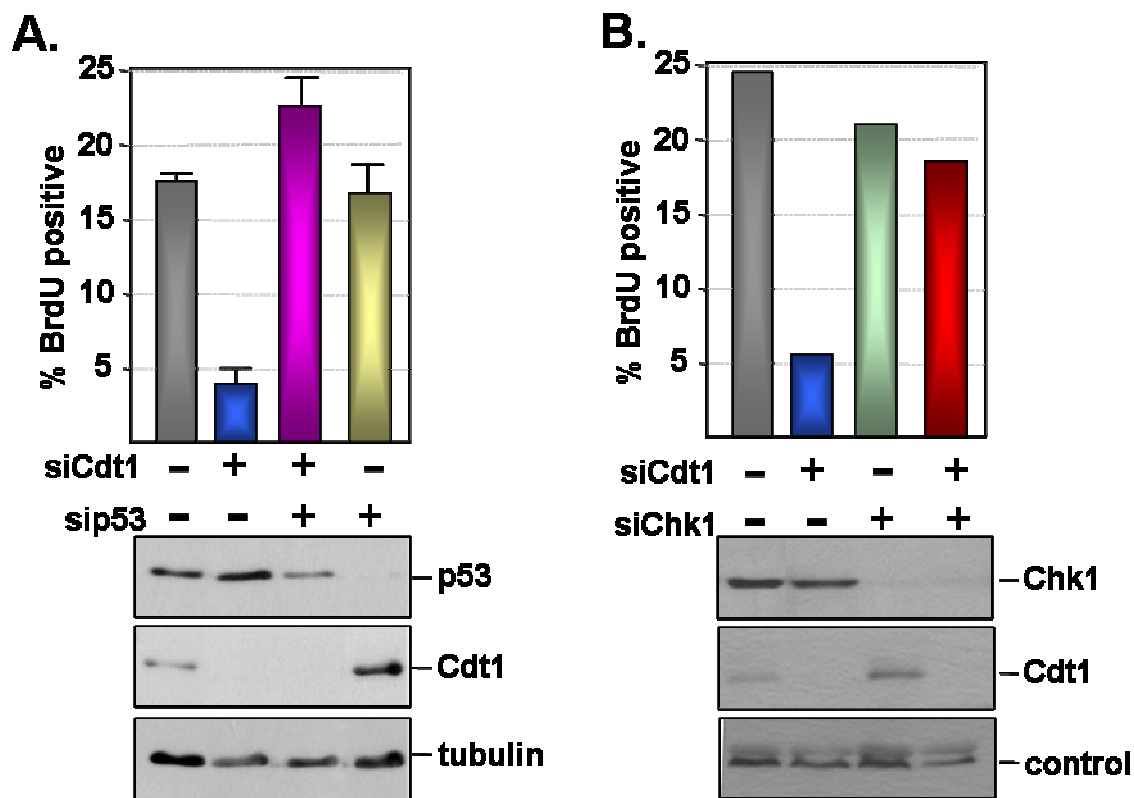
Figure 4.5: Co-depletion of Cdt1 with p53 or Chk1 abrogates the G1 arrest.
(A) NHF1 cells were transfected with control (targeting GFP), Cdt1, or p53 siRNA and processed as in Figure 4.3. (B) NHF1 cells were transfected with control, Cdt1 or Chk1 siRNA and processed as in Figure 4.4.

depleted implies that in the cells transfected with just Cdc6 or Cdt1 siRNA, there were a small number of licensed origins present, but these origins did not fire.

We also performed the p53 co-depletion experiments in synchronized cells following the same procedure as in Figure 3.3. Cdc6 expression was undetectable in quiescent cells but was induced in control cells by serum stimulation, and p53 depletion did not affect either of these events (Figure 4.6A). Consistent with the result in log phase cells (Figure 4.2), Cdc6-depleted cells exhibited a slight induction of p53 even in quiescent cells (Figure 4.6A). As expected, Cdc6-depleted NHF1 cells markedly delayed entry into S phase compared to control cells (Figure 4.6B, gray and green bars). NHF1 cells depleted of p53 alone, had only a slight S phase entry delay when compared to control (Figure 4.6B, yellow bar), but more importantly, cells co-depleted of Cdc6 and p53 entered S phase in similar numbers as control and p53-depleted cells (Figure 4.6B, orange bar).

Importantly, the ability of Cdc6- and Cdt1-depleted cells to enter S phase when co-depleted for p53 or Chk1 demonstrates that the reduction of Cdc6 does not render cells incapable of S phase entry. The suppression of the G1 arrest by p53 or Chk1 reduction strongly implies that normal cells with low levels of Cdc6 or Cdt1 do not enter S phase with a small number of licensed origins because they are restrained by Chk1 and p53.

Finally, the requirement of p53 and Chk1 in the G1 arrest implied that ATR may be important for the G1 arrest also. Co-depletion experiments with
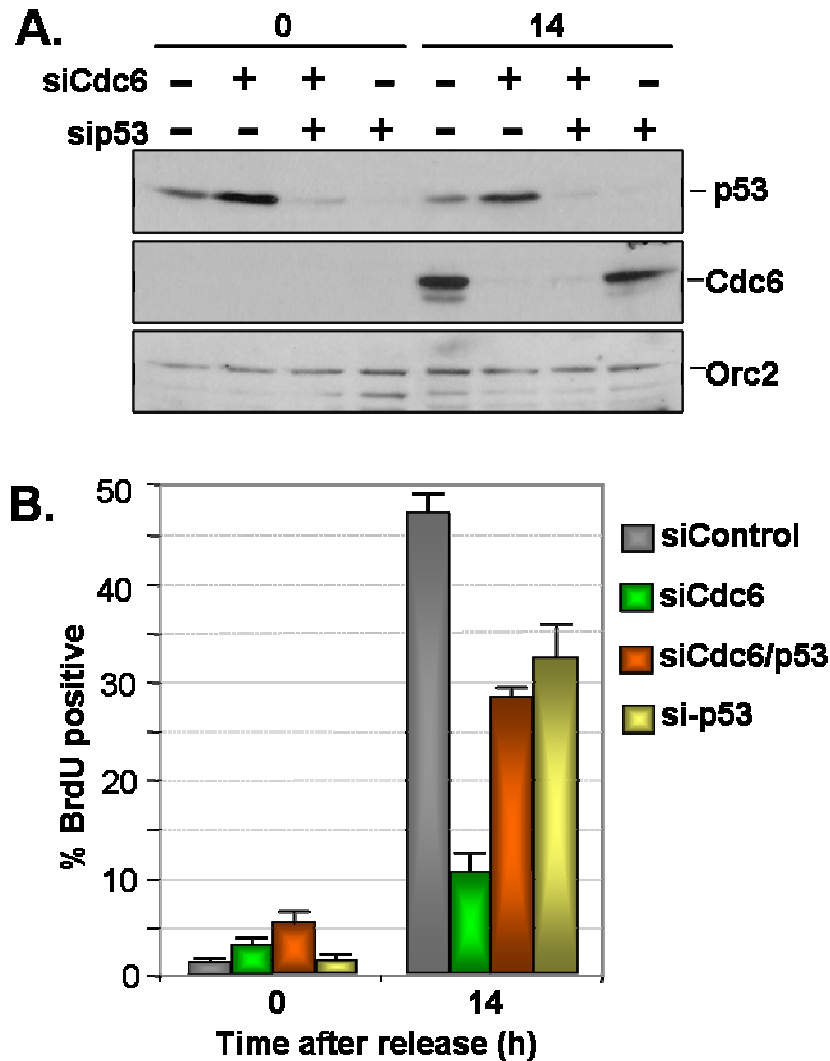
Figure 4.6: Co-depletion of Cdc6 with p53 abrogates the G1 delay in synchronized cells. (A) NHF1 cells were transfected with 100 nM control siRNA, Cdc6 siRNA, p53 siRNA, or both, incubated for 12 h, the medium was changed to medium containing 0.5% FBS and incubated an additional 60 h. Cells were re-stimulated into the cell cycle by addition of 10% FBS, and labeled for 1 h prior to collections at 0, 6, 10, and 14 h and Whole cell extracts were analyzed by immunoblot analysis for p53, Cdc6, and Orc2. (B) A portion of the sample from A was analyzed by flow cytometry. Bar graphs represent the percentage of BrdU positive cells (S phase cells) in three independent experiments.

simultaneous transfection of ATR and Cdc6 siRNA were performed. Depletion of

ATR in NHF1 did not appear to have any cell cycle effect for the percentage of cells

in S phase was similar to control cells (Figure 4.7, charcoal bar). Co-depletion of

Cdc6 and ATR reversed the G1 arrest (Figure 4.7, purple bar). Although these

findings are interesting and exciting, they represent the results of a single

experiment and their validation awaits further experimental verification.

<u>Cdc6 depletion does not induce a canonical DNA damage response.</u>

The involvement of p53 and Chk1 in the G1 arrest of Cdc6-depleted cells

raised the possibility that licensing inhibition generates DNA damage, which then

activates Chk1 and p53. In response to various forms of DNA damage, including

replication stress, the ATR kinase phosphorylates and activates Chk1 to induce

Cdc25A degradation, which results in the accumulation of Y15 phosphorylated Cdk2

and Cdk1 (126). In addition, p53 is phosphorylated and stabilized to promote the

transcriptional upregulation of p21 (Figure 4.1) (3,96,175). However, several

observations suggested that the DNA damage checkpoint was not strongly activated

by Cdc6 depletion. Cdc6 siRNA transfection was associated with modest

accumulation of p53 (Figures 4.3B, 4.5A, 4.6A, 4.9, 4.10B) but more importantly, this

small change was not followed by induction of p21 (Figures 3.9B and 4.10B).

Moreover, Cdc6 depletion did not lead to increased Y15 Cdk phosphorylation

(Figure 3.9B). Even so, we sought to thoroughly assess the status of the DNA
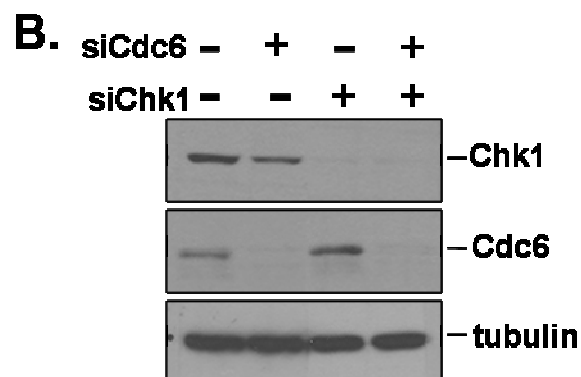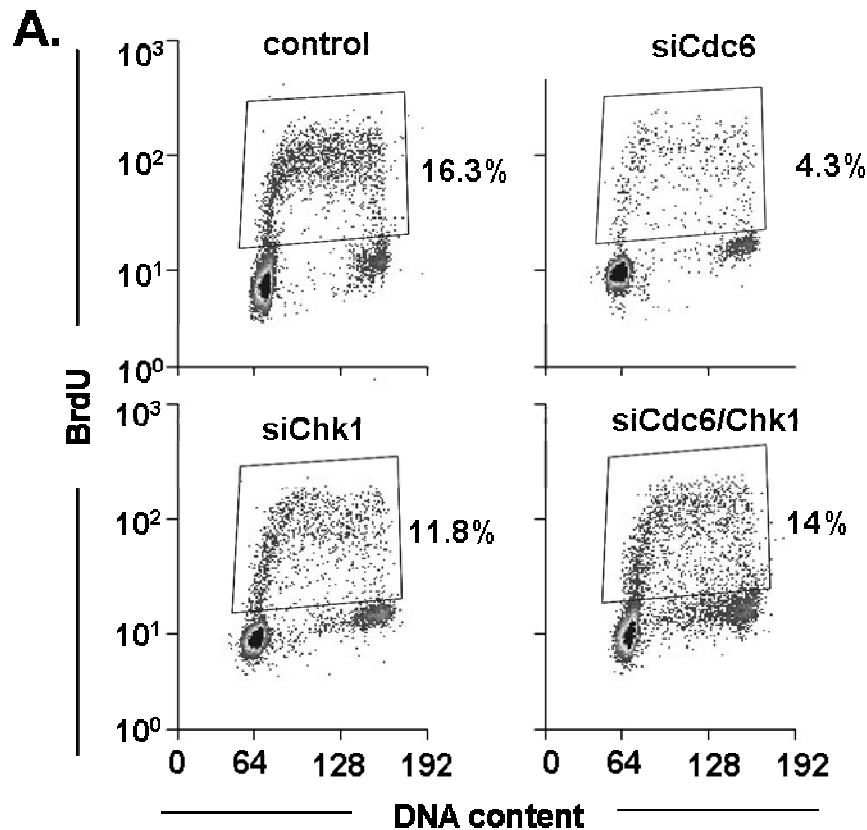
damage pathway in Cdc6 depleted cells.
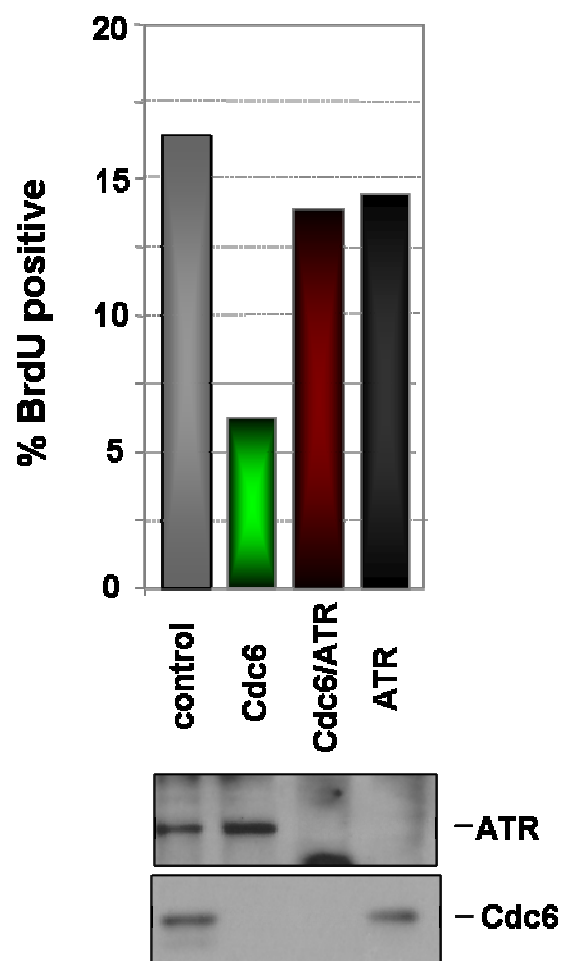
Figure 4.7: Co-depletion of ATR appears to abrogate the G1 arrest. NHF1 cells were transfected with a total of 100 nM of control (targeting GFP), Cdc6, ATR, or both siRNA. Cells were incubated for a total of 72 h, and then labeled with BrdU for 1 h prior to collection. A portion of the cells were analyzed by flow cytometry with anti-BrdU antibody to detect DNA synthesis and with propidium iodide for DNA content and the remaining sample was subjected to immunoblot analysis for ATR and Cdc6. Bar graph represents only one experiment.

We probed Cdc6-depleted cell extracts with antibodies to the phosphorylated and activated forms of the DNA damage checkpoint proteins; Chk1, Chk2, and p53. As a positive control for activation of the DNA damage checkpoint we treated NHF1 cells with hydroxyurea (HU) for 24 h. Although phosphorylation of Chk1, Chk2, and p53 were readily detectable in HU-treated cells (Figure 4.8, lane 1), we observed no induction of these events in Cdc6-depleted cells (Figure 4.8, compare lanes 2 and 3). We further evaluated cells from 24 h to 72 h after transfection with Cdc6 siRNA to determine if a transient increase in DNA damage checkpoint signaling could be detected, but we found no such evidence (data not shown). We thus concluded that the roles of p53 and Chk1 in the G1 arrest induced by Cdc6 depletion are distinct from their roles in the DNA damage response.

Co-depletion with p53 or Chk1 rescues the molecular phenotypes associated with insufficient origin licensing.

The observation that p53 and Chk1 are required for the G1 delay in licensing-deficient cells, coupled with our earlier demonstration that Cdc6 depletion led to both reduced phosphorylation of Rb and loss of cyclinE/Cdk2 activity, prompted us to test whether co-depletion of Cdc6 with p53 or Chk1 similarly suppressed these molecular phenotypes. We probed lysates of cells transfected with siRNAs targeting Cdc6, p53, or Chk1 either singly or in combination for phosphorylation of Rb and Cdk2 (T160). As we had observed before, Cdc6-depleted cells had substantially diminished phosphorylation of Rb and Cdk2 T160 (Figure 4.9). Strikingly, co-depletion of p53 with Cdc6, or Chk1 with Cdc6, largely restored Rb and Cdk2 (T160)
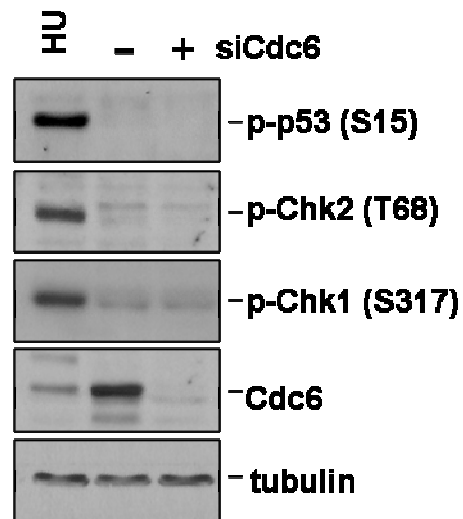
Figure 4.8: Cdc6 depletion does not activate a canonical DNA damage response. Whole cell extracts from NHF1 cells transfected with control or Cdc6 siRNA were probed with antibodies to phospho-p53 (S15), phospho-Chk2 (T68), phospho-Chk1 (S317), Cdc6 and tubulin.
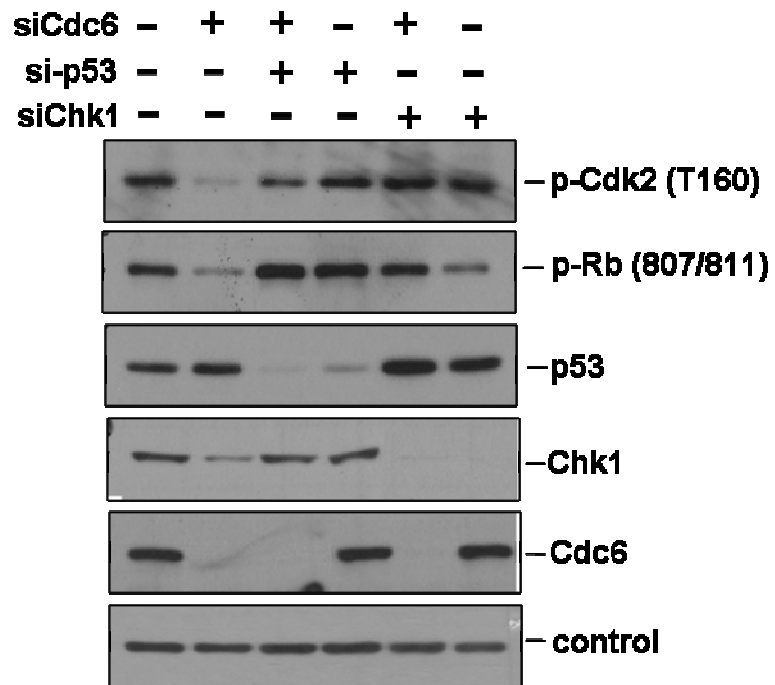
Figure 4.9: Co-depletion of Cdc6 with p53 or Chk1 rescues Rb and Cdk2 T160 phosphorylation. Whole cell extracts from NHF1 cells transfected with control, Cdc6, p53 or Chk1 siRNA were probed with antibodies to phospho-Cdk2 (T160), phospho-Rb (S807/811), p53, Chk1, and Cdc6. A non-specific band serves as a loading control.

phosphorylation (Figure 4.9), in keeping with the near complete rescue of the S phase population (Figure 4.3A and 4.4A, respectively).

Next, we examined whether co-depletion altered the kinase activity of Cdk2. We measured the histone H1 kinase activity in cyclin E immunoprecipitates from lysates of p53 and Cdc6-deficient cells. Cdc6 depletion caused an approximate 5-fold reduction in cyclin E-associated kinase activity (Figure 4.10A, green bar). In contrast, co-depletion of Cdc6 and p53 maintained cyclin E kinase activity at a level similar to that measured in the control cells (Figure 4.10A, red bar). Thus, p53 is required not only for the cell cycle effects of licensing inhibition, but also for the effects of licensing inhibition on Rb phosphorylation and presumably Cdk2 activation.

In the course of these experiments, we further noted that the cyclin E-associated kinase activity in cells transfected with just the p53 siRNA was almost 2-fold higher than the kinase activity in control cells (Figure 4.10A). We considered the possibility that a general increase in Cdk activity could account for suppression of the effects of licensing inhibition. The p53 tumor suppressor is required not only for induced expression of the p21 Cdk inhibitor but also basal expression of p21 in unperturbed cells. We reasoned that loss of p21 expression in p53-depleted cells could at least partially explain the requirement for p53 in the cell cycle delay imposed by licensing inhibition. Indeed, when lysates of cells treated with Cdc6 or p53 siRNA were probed for endogenous p21, it was clear that depletion of p53 resulted in marked loss of p21 protein (Figure 4.10B). We cannot, however, attribute
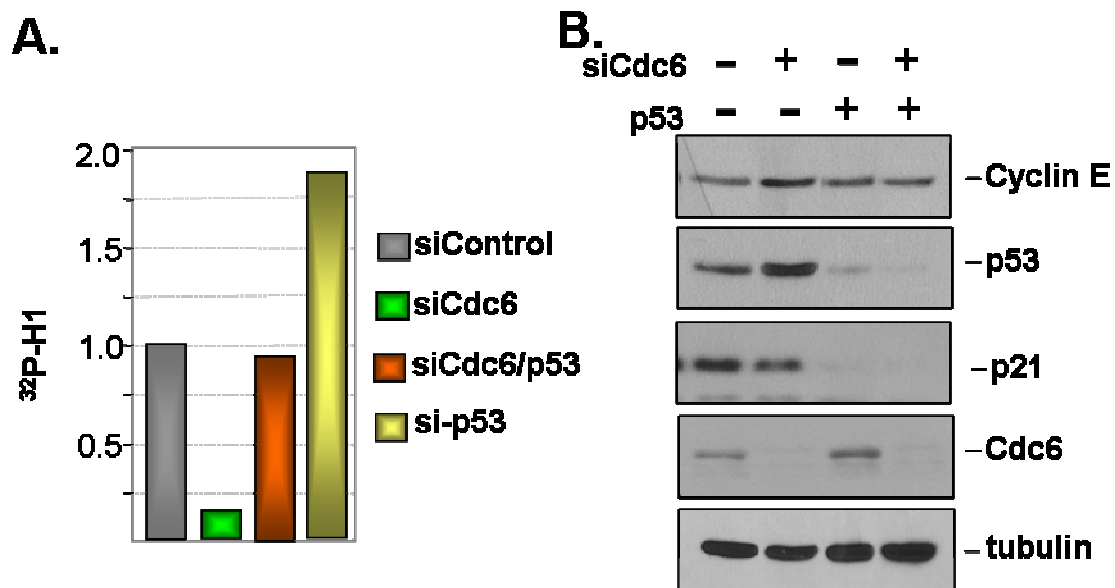
Figure 4.10: Co-depletion of Cdc6 with p53 rescues cyclin E/Cdk2 kinase activity. (A) Extracts of NHF1 cells transfected with control, Cdc6 or p53 siRNA were subjected to immunoprecipitation with normal rabbit serum or with anti-cyclin E antibody and analyzed by immunocomplex kinase assay with purified histone H1 and [γ-32P] ATP. (B) A portion of the same extracts were analyzed for cyclin E, p53, p21, Cdc6 and tubulin protein by immunoblotting.

all of the effects of p53 in licensing-deficient cells to expression of p21. We reasoned that if the role of p53 in the G1 arrest were partially through p21, then depletion of p21 should also suppress the cell cycle arrest induced by Cdc6 depletion. Experiments utilizing p21 siRNA did not demonstrate any abrogation of the G1 arrest induced by Cdc6 depletion and a p21 -/- fibroblast cell line arrested in G1 similarly to the wild-type fibroblasts when Cdc6 expression was knocked down (data not shown). These observations suggest that additional p53 target genes might also be required for the cell cycle effects of Cdc6 depletion, or p53 may play a non-transcriptional role in this system that has yet to be discovered.

Bypass of the G1 arrest induces apoptosis in Cdc6-depleted cells.

At the outset of these experiments, we hypothesized that S phase entry with insufficient numbers of licensed origins would be detrimental to genome stability. Replication forks from sparsely distributed active origins might not be able to merge and ultimately may collapse and generate double-strand breaks. One prediction of this hypothesis is that both cancer cells with documented lesions in the p53 pathway and normal cells transfected with p53 siRNA would accumulate double-strand breaks when origin licensing is inhibited and cells still enter S phase. In support of this model, we observed that Cdc6 depletion in U2OS cells (which overproduce the p53 ubiquitin ligase, Mdm2) or HeLa cells (which express the p53 ubiquitin ligase adaptor E6) had elevated levels of phosphorylated Chk2, a marker of double-strand breaks (Figure 4.11A). Importantly, these cells also failed to down-regulate Cdk2 (T160) or Rb phosphorylation (Figure 3.11), suggesting that they were incapable of
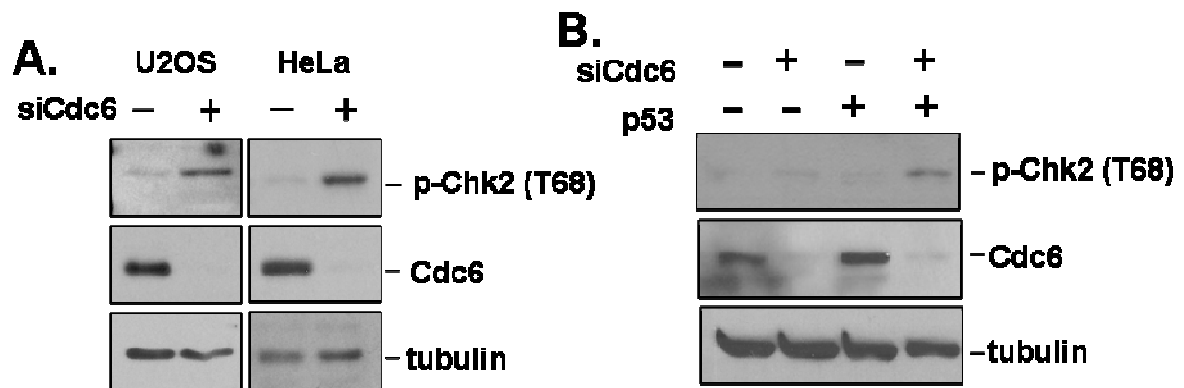
Figure 4.11: Co-depletion of Cdc6 with p53 display markers of double strand breaks. (A) Whole cell extracts from U2OS and HeLa cells transfected with control or Cdc6 siRNA were probed for antibodies to phospho-Chk2 (T68), Cdc6, and tubulin. (B) Whole cell extracts from NHF1 cells were transfected with control, Cdc6, or p53 siRNA and probed with antibodies to phospho-Chk2 (T68), Cdc6, and tubulin.

mounting the same cell cycle response to origin licensing inhibition that the normal cells did.

To determine if DNA damage was also generated in the licensing-deficient normal cells forced into S phase by p53 depletion, we probed lysates of these cells for phosphorylated Chk2. Strikingly, phospho-Chk2 was specifically elevated in p53- and Cdc6-depleted cells, compared to control cells, whereas neither Cdc6 depletion nor p53 depletion alone induced Chk2 phosphorylation (Figure 4.11B). These findings suggest that p53 is required to prevent licensing deficient cells from entering a doomed S phase.

In Chk1-depleted NHF1 cells we noted the appearance of a small population of cells with an S phase DNA content but little to no BrdU incorporation (Figure 4.4, siCdc6/Chk1). We termed this population of cells the "collapsed S phase" population. Co-depletion of Chk1 with Cdc6 greatly enhanced the number of collapsed S phase cells in both NHF1 and WI38 cells (Figure 4.12A). One explanation for this enhancement would be inappropriate firing of widely spaced origins, thus creating a greater need for the function of Chk1 at replication forks over great distances or over long periods of time. Chk1 has important roles in unperturbed DNA replication that include regulation of replication fork movement and replication fork stability (27,130,148,162). In the absence of Chk1, these forks frequently collapse leaving cells trapped in S phase.

If indeed the depletion of Cdc6 and Chk1 increased the likelihood that replication forks collapse, then we expected to find evidence of double-strand breaks
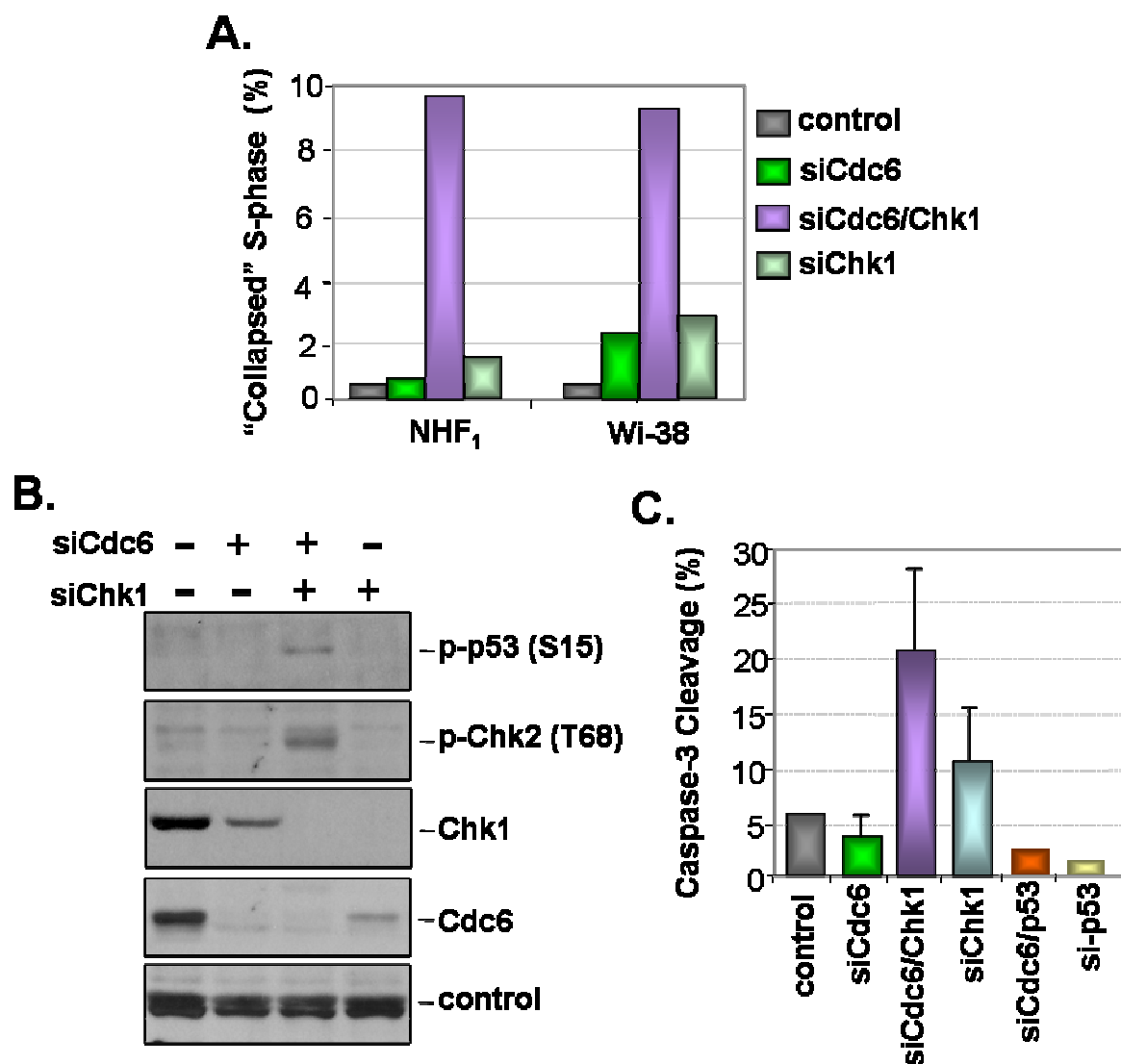
Figure 4.12: Co-depletion of Cdc6 with Chk1 (but not with p53) results in the activation of apoptosis. (A) The percentage of S phase cells not incorporating BrdU ("collapsed") was determined in NHF1 and WI-38 cells transfected with control, Cdc6, or Chk1 siRNA according to the legend to Figure 4.4. (A) Whole cell extracts from NHF1 cells transfected with control, Cdc6, or Chk1 siRNA and incubated for 96 h were probed with antibodies to phospho-p53 (S15), phospho-Chk2 (T68), Chk1, and Cdc6. A non-specific band serves as loading control. (C) NHF1 cells were transfected with control, Cdc6, Chkt1, or p53 siRNA as in Figures 4.3 and 4.4 but analyzed 96 h after transfection. Cells were collected and stained for cleaved caspase-3 according to the manufacturer's protocol.

in the co-depleted cells. We probed extracts of NHF1 cells treated with Cdc6 and

Chk1 siRNA either alone or in combination for phosphorylation of Chk2 at T68 and

phosphorylation of p53 at S15. Both of these phosphorylations are mediated by the

ATM kinase in response to double strand breaks (104,165,175). Neither Cdc6 nor

Chk1 depletion alone were sufficient to induce detectable Chk2 or p53

phosphorylation (Figure 4.12B). In contrast, co-depletion of Cdc6 and Chk1 induced

detectable phosphorylation of both p53 and Chk2 (Figure 4.12B). Moreover, by 96 h

post-transfection significant numbers of cells depleted of both Cdc6 and Chk1 had

committed to apoptosis as evidenced by the appearance of cleaved caspase-3

(Figure 4.12C).

Discussion

In this study we demonstrated that Chk1 and p53 are required for cell cycle arrest in cells with insufficient origin licensing through down regulation of both Rb and Cdk2 T160 phosphorylation. Despite the fact that both Chk1 and p53 are required for the arrest, we find that Cdc6-depleted cells have not activated the canonical DNA damage checkpoint since neither upstream markers (phosphorylation of Chk1 and Chk2) nor downstream targets (p53, p21, Cdc25A, Cdk Y15 phosphorylation) are altered by Cdc6 depletion. Our conclusion then is that the roles of Chk1 and p53 in blocking S phase entry are different from their functions in the DNA damage response.

We showed that in the cells co-depleted of Cdc6 and p53 the measured cyclin E/Cdk2 kinase activity was at control levels (Figure 4.10A). One possible explanation for this observation is that reduction of p53 alone increases the basal activity of cyclin E/Cdk2 and when cells are co-depleted of Cdc6 the attendant reduction in kinase activity brings it to the level of control cells (Figure 4.13). On the other hand, if this was the entire contribution of p53 in modulating the G1 arrest, than co-depletion of p53 would not be expected to rescue Rb and Cdk2 T160 phosphorylation (Figure 4.9).

Importantly, we have demonstrated a strong correlation between the ability of Cdc6 and Cdt1-depleted cells to enforce a G1 arrest with their ability to avoid a fatal S phase. Co-depletion of Chk1 and Cdc6, or Chk1 and Cdt1, abrogated the G1 arrest, sending cells into S phase without the resources to completely replicate their DNA. Ultimately, these cells accumulated markers of double strand breaks and died

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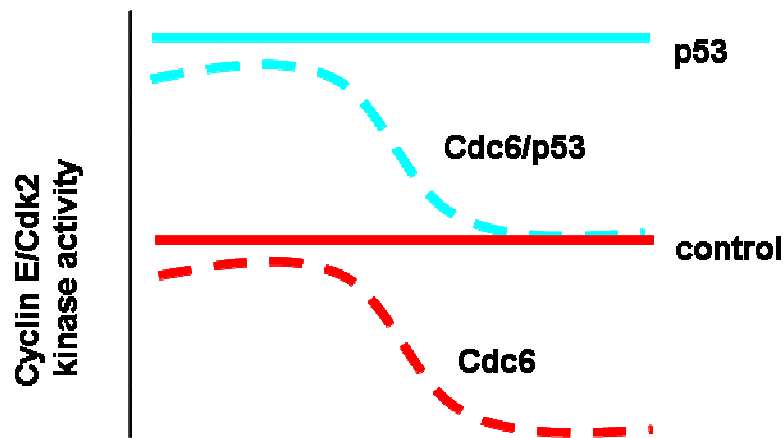
Figure 4.13: Schematic illustrating possible mechanisms for p53-dependent abrogation of the G1 arrest. Normal cells going through late G1 phase of the cell cycle maintain a steady level of cyclin E/Cdk2 kinase activity to carry out G1/S functions (solid red bar). Upon insufficient origin licensing brought about by Cdc6 depletion, cyclin E/Cdk2 kinase activity is inhibited preventing S phase entry (dashed red line). Depletion of p53 alone, elevates the cyclin E/Cdk2 kinase activity almost 2-fold over control cells (solid blue bar), and upon insufficient origin licensing in the Cdc6/p53 co-depleted cells the kinase activity is only inhibited down to the level in control cells, still allowing for entry into S phase (dashed blue line).

by apoptosis. Co-depletion of p53 had the same effect on the G1 arrest but did not lead to a robust apoptotic response, in all likelihood due to a specific requirement for p53 in apoptosis. In HeLa cells, which are sensitive to apoptosis induced by depletion of Cdc6 alone, we also observed markers of double strand breaks even without Chk1 depletion. One explanation for this result is that HeLa cells have a disruption in the normal function of Chk1 that disconnects replication licensing from S phase entry. The nature of this difference is not yet clear, but could provide an opportunity for therapeutic intervention in the treatment of at least some cancers.

Our finding that Chk1 is required for Cdk inactivation in Cdc6-depleted cells suggests a possible role for Chk1 in G1. As stated in the introduction, Chk1 depletion was shown to increase the kinase activity of Cdk2 in S phase. It is possible that this same mechanism holds true for G1 phase. To date, the only confirmed substrates of Chk1 are p53, Cdc25 isoforms, and Tlk1 (tousled-like kinase 1). In Cdc6-depleted cells we find no evidence for p53 phosphorylation that would lead to stabilization or p21 induction. Additionally, Cdc25 is not phosphorylated since Cdc25A is not degraded in Cdc6-depleted cells, nor is the target of Cdc25 isoforms, Y15-phosphorylated Cdk, increased. The Tlk1 kinase is only phosphorylated in S phase where it activates the Asf1 chromatin assembly factor (69), making it unlikely that Tlk1 is a critical substrate for the G1 arrest imposed by Cdc6 depletion. Although we cannot rule out the possibility that Chk1-dependent p53 and Cdc25 phosphorylations are extremely transient or below the level of detection in Cdc6-depleted cells, we consider it more likely that Chk1 has a unique substrate or interaction partner that connects licensing to Cdk activation. The

requirement for p53 in preventing Cdk2 activation and maintaining the G1 arrest, even when Chk1 is present, suggests that the target of Chk1 may be an interaction with (or phosphorylation of) p53 that is distinct from the relationship of Chk1 and p53 in the DNA damage response. In that regard, this novel requirement for Chk1 in an early G1 arrest adds to the growing list of Chk1 functions that includes replication fork progression, fork stability, and coordinating mitotic entry with S phase completion.

Chapter 5

Conclusions and Perspectives

A. Conclusions

The work presented in this dissertation provides evidence for a novel DNA replication licensing checkpoint that protects cells from entry into S phase with insufficient licensed origins. Prior to the work presented here, other groups had shown that reduction of various preRC components (Orc2, Mcm7, and over-expression of Geminin) resulted in a G1 delay in normal cells while apoptosis was activated in a variety of cancer cells (56,124,181). The present study contributes to our understanding of particular events that are required to maintain normal cells arrested in G1 until an optimal number of licensed origins are reached.

We demonstrated that the G1 arrest in response to insufficient origin licensing in normal human fibroblasts (NHF1, NHF10, and WI38) is characterized by loss of Rb phosphorylation and loss of cyclin E/Cdk2 kinase activity. The loss of cyclin E/Cdk2 kinase activity is most likely attributed to loss of Cdk2 T160 phosphorylation. In addition, we find that both p53 and Chk1 are required to maintain the G1 arrest and reducing the abundance of one or the other protein rescues the phosphorylation of both Rb and Cdk2 T160.

B. Possible Mechanisms for the Loss of Cdk2 T160 Phosphorylation

Cdk-Activating Kinase (CAK)

Little is known about the mechanisms that regulate the activity of Cdk2 through phosphorylation of the T160 residue. Phosphorylation of this site appears to be regulated only in cells coming out of quiescence (114), with very little being known about its regulation throughout the cell cycle. The Cdk-activating kinase, or CAK complex, is the only known kinase responsible for the activating phosphorylation on the T160 residue of Cdk2 (182). Inhibition of CAK could explain the defect in Cdk2 T160 phosphorylation. As shown in Figure 3.12, we observe no changes in Cdk7, cyclin H or Mat1 protein levels or in CAK activity in Cdc6-depleted fibroblasts. This suggests that, at least *in vitro*, there is no defect in CAK kinase activity. However, this does not discount the possibility that there is a defect in CAK *in vivo.* It is possible that an additional factor is bound to CAK preventing it from being able to bind to cyclin E/Cdk2 and therefore preventing the activation of Cdk2.

Phosphatase Activation

Another possible explanation for the loss in Cdk2 T160 phosphorylation that has yet to be explored is the activation of a phosphatase (Figure 5.1). The potential role of phosphatases in G1 progression and Cdk2 activation should be considered. KAP, or the Cdk-associated protein phosphatase, has been identified as the enzyme that dephosphorylates T160 of Cdk2 (72). Most KAP studies have examined its role in regulating the cyclin A-Cdk2 interaction. It has been shown that KAP prefers to dephosphorylate monomeric Cdk2, which prevents cyclin A binding and indirectly promotes degradation of cyclin A (167). The lack of evidence for a similar activity on
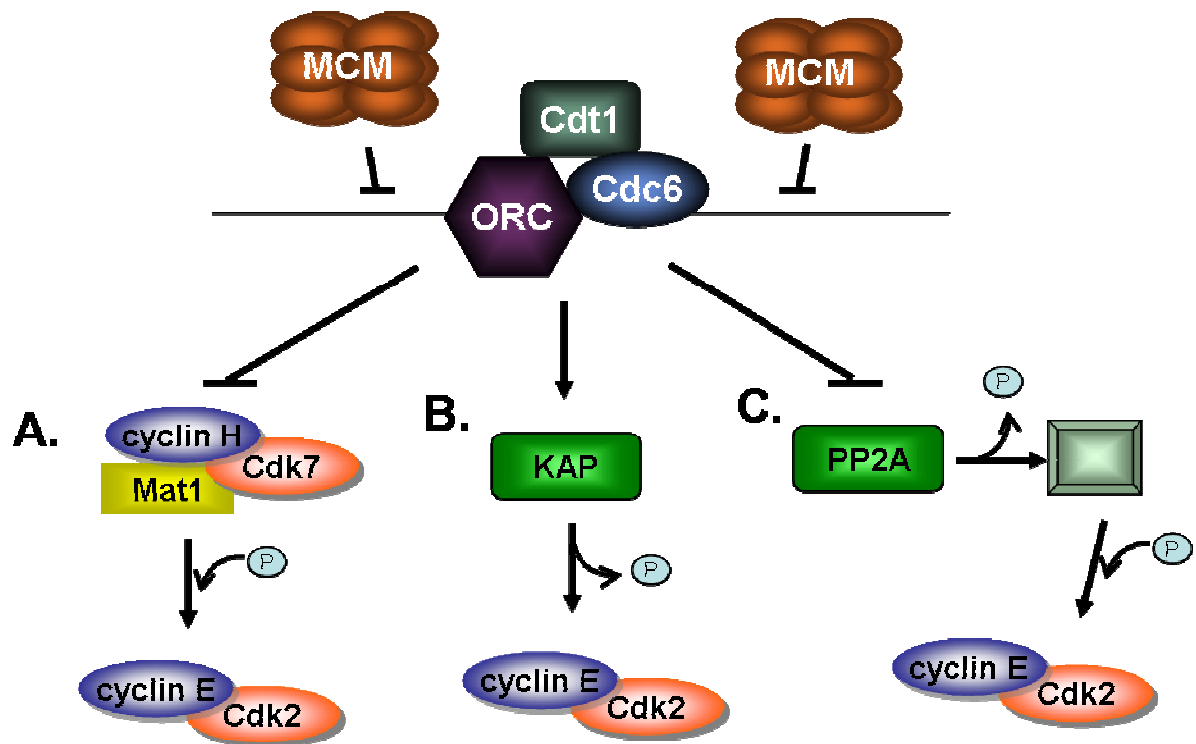
Figure 5.1: Possible Mechanisms for Inhibition of Cyclin E/Cdk2. Insufficient origin licensing (A) inhibits CAK preventing Cdk2 T160 phosphorylation, (B) activates Cdk-activating phosphatase (KAP) dephosphorylating Cdk2 T160, or (C) inhibits protein phosphatase 2A (PP2A) which is required to dephosphorylate a substrate required for the phosphorylation of Cdk2 T160.

cyclin E-Cdk2 could suggest that KAP is only active in S phase. If KAP (or another phosphatase) functioned in the same manner with cyclin E-Cdk2, one would expect to see loss of cyclin E protein, which is not observed in response to insufficient origin licensing.

Alternatively, it is possible that the activity of a phosphatase is an indirect requirement for the phosphorylation on T160 of Cdk2. For example, inhibition of protein phosphatase 2A (PP2A) has been shown to inhibit the activation of Cdk2, Cdk4 and Cdk6, and the phosphorylation of Rb (206). This suggests that PP2A might remove an inhibitory phosphorylation on a yet unidentified protein (i.e. a kinase or a required cofactor), thus allowing for the activation of Cdk2. Recent evidence in *Xenopus* demonstrated that dephosphorylation by PP2A of a unknown soluble substrate was required for the loading of Cdc45 onto the chromatin (29). This brings together an interesting model for the involvement of PP2A in origin licensing (Figure 5.2). As mentioned in Chapter 2, loading of Cdc45 onto chromatin requires the phosphorylation of MCM by cyclin E/Cdk2 and Cdc7/Dbf4. Both the loading of Cdc45 and activation of cyclin E/Cdk2 require the dephosphorylation by PP2A of a yet to be identified substrate in order for these events to occur. The identity of this substrate is unknown at this time, but it could hold the key to understanding how a deficit in Cdk2 T160 phosphorylation would be linked to insufficient origin licensing and would prevent cells from entering S phase.

Cdk2 Localization

It has been demonstrated that when changes in Cdk activating phosphorylation are observed, they are often accompanied by changes in Cdk2
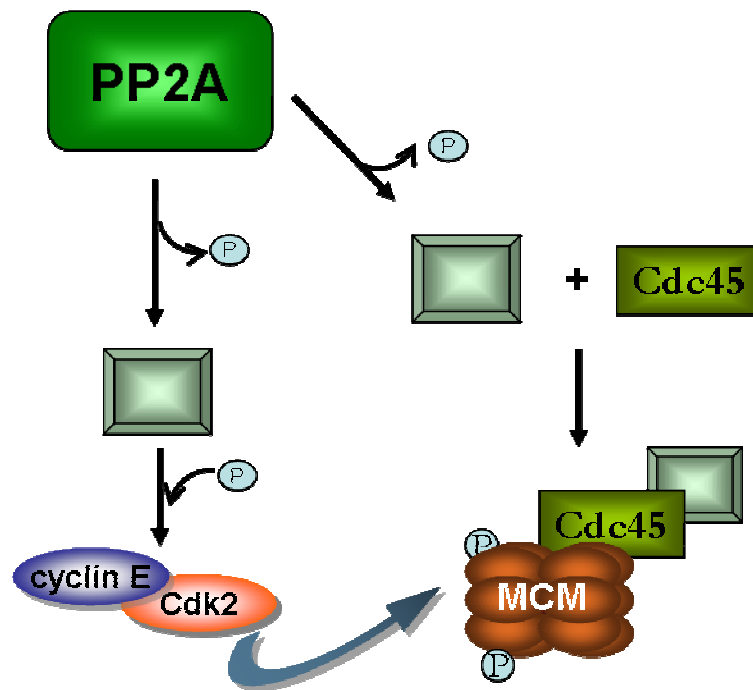
Figure 5.2: Speculation for the involvement of PP2A in Origin Licensing Checkpoint. Loading of Cdc45 onto chromatin requires the phosphorylation of MCM by cyclin E/Cdk2 and Cdc7/Dbf4. Both the loading of Cdc45 and activation of cyclin E/Cdk2 require the dephosphorylation by PP2A of a yet to be identified substrate in order for these events to occur. The identity of this substrate is unknown at this time, but it could hold the key to understanding how a deficit in Cdk2 T160 phosphorylation would be linked to insufficient origin licensing and would prevent cells from entering S phase.

localization. In quiescent fibroblasts, Cdk2 resides primarily in the cytoplasm, but late in G1 it translocates to the nucleus by a mechanism that is still poorly understood (20-22,44,97). Since all subunits of CAK are constitutively expressed in the nucleus (190), the increase in Cdk2 phosphorylation on T160 in late G1 has been attributed at least in part to its relocalization. We have analyzed nuclear and cytosolic extracts from serum-stimulated Cdc6-depleted cells and observed a loss of Cdk2 in the nuclear fraction (Figure 5.3A). Although this is an intriguing finding, there are some concerns about the loss of Cdk2 in the nucleus as being the explanation for loss of the phosphorylation of Cdk2 on residue T160. First, in the synchronized experiments where we observed loss of Cdk2 protein in the nucleus, we also observed an overall loss of total Cdk2 protein in the whole cell extract (Figure 5.3B). This is not consistent with what is observed in the log phase cells, where loss of phosphorylation on residue T160 is decreased but no changes in total Cdk2 are observed (Figure 3.11). Further examination of Cdk2 localization in the log phase experiments would give further validity to the synchronized experiments. Second, generation of a cell line stably expressing Cdk2 fused to a constitutive nuclear localization signal (Cdk2-NLS) did not abrogate the G1 delay in response to Cdc6 depletion (Figure 5.3C).

C. Possible Explanations for p53 in the Maintenance of the G1 Arrest

We demonstrated that p53 is required to maintain the G1 arrest in response to insufficient origin licensing. We consistently observed a modest induction of p53 upon insufficient origin licensing, but did not observe any up-regulation of p21 or
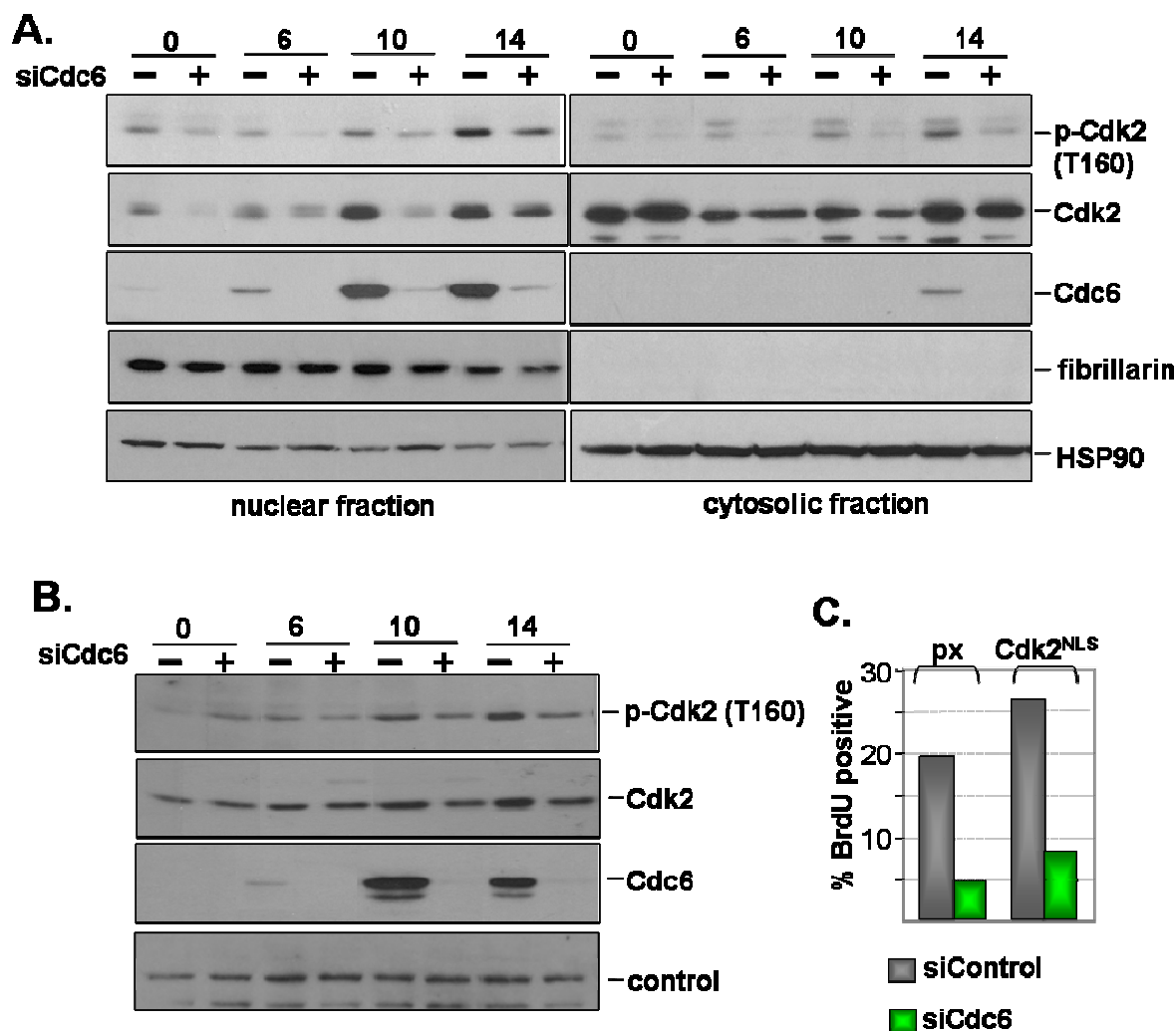
Figure 5.3: Insufficient origin licensing may lead to re-localization of Cdk2.
(A) NHF1 cells were transfected with 100 nM control siRNA or Cdc6, incubated for 12 h, the medium was changed to medium containing 0.5% FBS and incubated an additional 60 h. Cells were re-stimulated into the cell cycle by addition of 10% FBS, and collectioned at 0, 6, 10, and 14 h. Cells were fractionated by hypotonic lysis into cytosolic and nuclear fractions and subject to immunoblot analysis. (B) Whole extracts treated as in A, were subject to immunoblot analysis. (C). NHF1-pLXSN cells and NHF1-pLXSN-Cdk2^{NLS} cells were transfected with 100 nM control or Cdc6 siRNA and incubated 72 h. BrdU incorporation was evaluated by flow cytometry.

activation of markers of DNA damage, such as p53 phosphorylation at S15 or S20. The modest induction of p53 raises the possibility that it has become more stabilized, possibly through phosphorylation on residues that have yet to be investigated. P53 is phosphorylated on a number of sites (S6, S9, S33, S37, S46, S315, and S392); so, it is possible that one of these phosphorylations may play a role in maintaining the G1 arrest. Of these, the most interesting is the phosphorylation of p53 at S33. *In vitro* work has demonstrated that the phosphorylation on S33 is carried out by the CAK complex (100) and provides a possible link between p53 and cyclin E/Cdk2 activation.

Another alternative, but less exciting explanation would be an indirect role for p53 in the G1 arrest. We cannot rule out the possibility that the only role for p53 is in regulating the basal levels of cyclin E/Cdk2 kinase activity. We observed in both control and Cdc6-depleted cells a small amount of p21 bound to cyclin E/Cdk2 complexes. This amount of p21 may serve as a check and balance to maintain the correct level of kinase activity. Therefore, depletion of p53 results in loss of p21 expression, which leads to an increase in cyclin E/Cdk2 activity.

D. Other Possible Explanations for the G1 Arrest

We cannot ignore the possibility that there are other mechanisms responsible for inhibiting cyclin E/Cdk2 kinase activity besides the loss of Cdk2 T160 phosphorylation. We demonstrated that depletion of p53 elevates the Cdk2 kinase activity two-fold over control. When we then co-depleted Cdc6 and p53, cells recovered the T160 phosphorylation and entered S phase, but we still observed an

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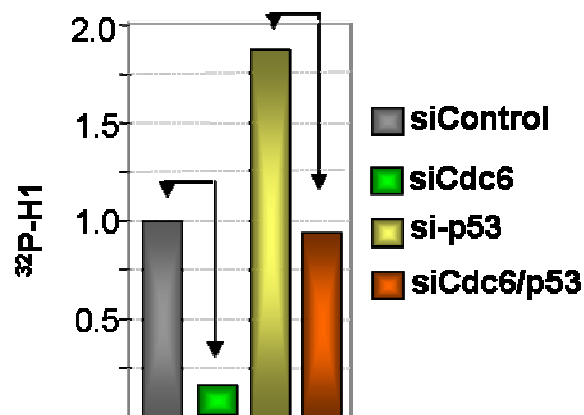
Figure 5.4: Diagram displaying the inhibition of cyclin E/Cdk2 kinase activity. Activity of cyclin E/Cdk2 kinase is strongly inhibited in extracts from cells transfected with Cdc6 siRNA. Depletion of p53 elevates the kinase activity of cyclin E/Cdk2 almost 2-fold over control cells. Co-depletion of Cdc6 and p53, abrogates the G1 arrest and cyclin E/Cdk2 kinase activity returns to control levels. However, Cdc6 depletion in cells also depleted of p53 still inhibit activity (arrows).

inhibition of cyclin E/Cdk2 kinase activity relative to the matched control, i.e. depletion of p53 only (Figure 5.4). This would suggest that there are additional mechanisms involved in inhibiting cyclin E/Cdk2 activity.

E. Future Directions

The work presented here only began to address what is occurring in response to insufficient origin licensing. There are many aspects of this pathway that still need to be investigated. Questions that need further investigation are: (1) how is insufficient origin licensing determined by the cell, (2) what are the mechanisms linking loss of MCM loading with inhibition of Cdk2 activation, and (3) what is preventing the few licensed origins from firing?

How is insufficient origin licensing sensed by the cell?

How is the cell able to know whether or not an appropriate number of origins have been licensed to complete S phase? Does origin licensing (loading of the MCM complex) recruit/release factors to/from the chromatin? One possibility is that mammalian origins are marked by "indicator" proteins that upon origin licensing are released from the chromatin. Once the level of these indicators reaches a certain threshold, that is a signal to the cell that enough origins have been licensed and is safe to proceed into S phase (Figure 5.5A). A reciprocal model is another possibility. In this case, loading of the MCM complex onto chromatin recruits the
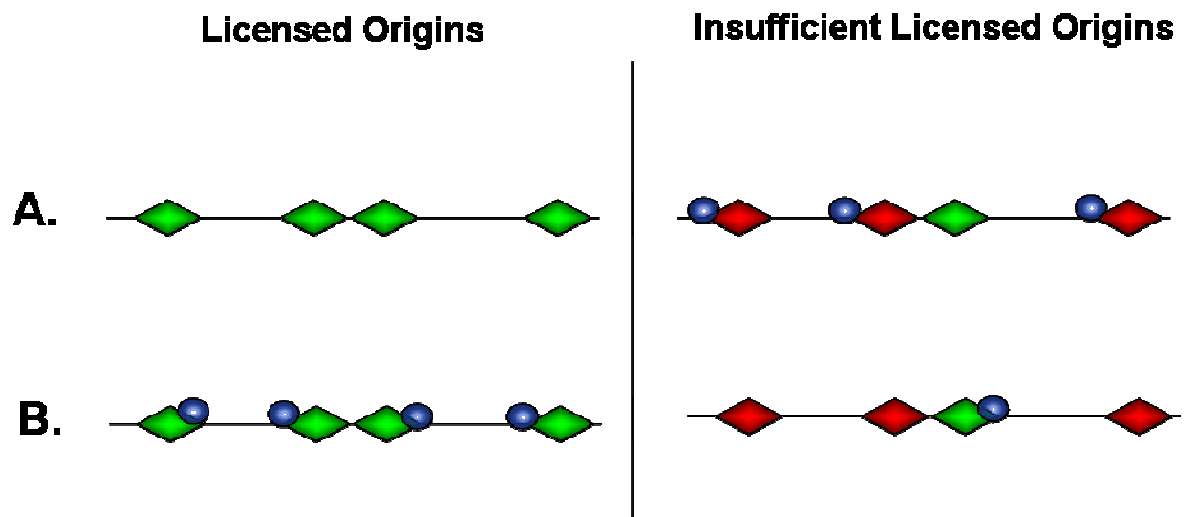
Figure 5.5: Origin Licensing recruits/releases a protein to/from the chromatin. (A) In this model, in the presence of origin licensing (green triangles) the indicator protein (blue circles) is released from the chromatin (left side) while it remains chromatin bound when insufficient origin licensing occurs (red triangles-right side). (B) In this model, origin licensing recruits the indicator protein to the chromatin; insufficient licensing results in low chromatin bound levels of the indicator protein.

"indicator" protein and it is the loss of the soluble fraction or gain of the chromatin fraction that signals to the cell to proceed into S phase (Figure 5.5B).

What links origin licensing to Cdk2 activation?

We demonstrate that in the absence of origin licensing Cdk2 does not become activated. Is it possible that chromatin serves as a platform for Cdk2 and CAK to encounter one another and this is facilitated by MCM loading? It has been shown that Cdk2 localization to chromatin is dependent on origin licensing (62) but it is not known whether this chromatin association occurs at origins in G1. To investigate this question one could perform chromatin immunoprecipitation (ChIP) using Cdk2 or CAK and examine origin occupancy at known origins such as *c-myc* and *lamin B2*. This technique would allow for the examination of Cdk2 and CAK at origins and determine if defects in origin licensing alter this interaction.

What is preventing the few licensed origins from firing when cells are depleted of an origin licensing factor?

The use of siRNA results in significant reduction of the target protein but not complete loss of the protein. The small amount of protein that is still present in the cell, allows for a small number of origins to be licensed. What is preventing these origins from firing? This dissertation has focused primarily on the events that occur at the origin, i.e. origin licensing. In order to determine what is preventing these few licensed origins from firing, examination of the pre-initiation complex might be insightful. It may be that p53 is functioning not in signaling to the cell that there are

an insufficient number of licensed origins, but instead at the pre-initiation complex

preventing the origin from firing. Or it may simply be that the few minimally licensed

origins do not fire because Cdk2 activity is low.

Dissecting the role of Chk1 in maintaining the G1 arrest

We can only speculate what role Chk1 has in the G1 arrest at this point.

There is some concern that the cells co-depleted with Chk1 siRNA are not actually

displaying an abrogation of the G1 arrest, but instead a compounding phenotype

due to Chk1 role in S phase. The argument can be made that since cells

transfected with Chk1 siRNA alone display problems with S phase progression, it is

possible that upon co-depletion of Chk1 and Cdc6 the cells transit through the cell

cycle too slowly to exhibit a G1 arrest.

One approach to addressing this concern would be the use of Chk1 kinase

inhibitors, SB218078 and Gö6976. Both of these compounds are indolocarbazoles

and have been found to have better selectivity and lower toxicity than the commonly

used Chk1 kinase inhibitor, UCN-01 (90,101,212). To date we have been

unsuccessful in using these two Chk1 inhibitors in experiments with log phase cells.

One possibility would be to utilize the Chk1 inhibitors in cells that have been

synchronized by the serum starvation/re-stimulation protocol. This would shorten

the time the cells are exposed to the inhibitor, since control cells enter S phase

within 10-14 h after re-stimulation. Evaluation of the rate of entry into S phase of

Cdc6-depleted cells treated with either of the inhibitors would provide further

evidence for the involvement of Chk1 in the G1 arrest.

Establishing that in fact Chk1 is involved in the G1 arrest would be a novel and exciting finding since there is very limited evidence of involvement of Chk1 in G1. Determining whether the kinase activity of Chk1 or the presence of Chk1 protein itself is required would help validate the role of Chk1 in G1 phase. In response to most forms of DNA damage, Chk1 becomes activated by phosphorylation on serine residues 317 and 345, but in the case of insufficient origin licensing we do not observe phosphorylation on either residue. It is possible that there are other sites on Chk1 that activate its kinase activity. Measuring the kinase activity of Chk1 would give us insight into whether there is an additional residue on Chk1 that is being modified to activate its kinase activity.

We could also utilize a kinase dead version of Chk1 to determine if it is the presence of the protein that is required and not necessarily the kinase activity of Chk1. There have been some technical difficulties with the kinase-dead and wild-type Chk1 adenovirus, in that over-expression of wild-type Chk1 in cells coming out of quiescence interferes with control cells entering S phase. These are preliminary results and further optimization would be needed to determine if this is an appropriate approach.

In addition to examining the kinase activity of Chk1, it would also be interesting to examine the status of cyclin E/Cdk2 kinase activity in the Chk1 co-depleted cells. Does depletion of Chk1 result in increased activity of cyclin E/Cdk2, as has been demonstrated by others (188)?

F. The Checkpoint as a Whole

      With the elucidation of the mechanisms of this checkpoint still in its infancy we can only speculate what is happening. The model that makes most sense with the data presented here is that, similar to the spindle checkpoint, the origin licensing checkpoint is always on and, upon sufficient origin licensing, gets turned off (Figure 5.6). In this model, as cells enter G1 phase cyclin E/Cdk2 remains inhibited and p53 is involved in inhibiting origins from firing as they are being licensed. Once sufficient origin licensing has been achieved, cyclin E/Cdk2 becomes active and p53 is no longer needed to inhibit origin licensing.
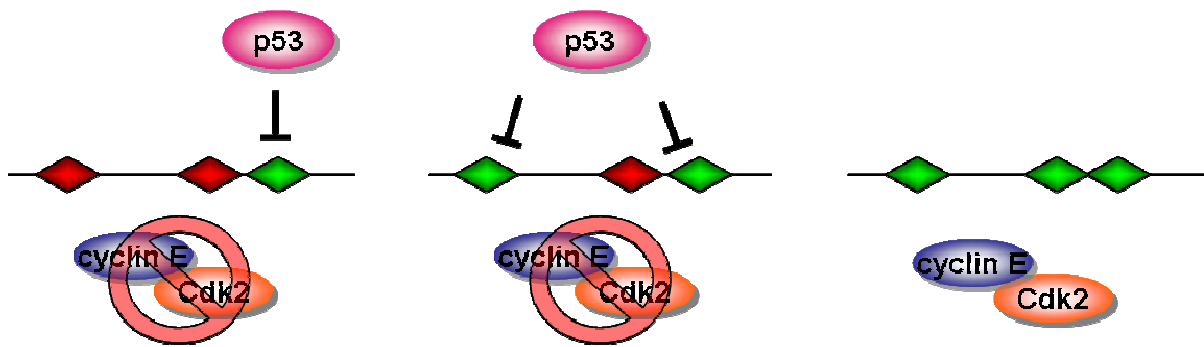
Figure 5.6: Model for an Origin Licensing Checkpoint. In this model, the cell enters S phase and cyclin E/Cdk2 kinase activity remains inactivated and p53 is involved in preventing firing from origins that have been licensed. Once adequate numbers of origins are licensed, cyclin E/Cdk2 becomes activated and p53 is no longer needed to inhibit origin firing.

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