AD_____

Award Number: DAMD17-99-1-9138

TITLE: Investigation of the Causes of Breast Cancer at the Cellular Level: Isolation of In Vivo Binding Sites of the Human Origin Recognition Complex

PRINCIPAL INVESTIGATOR: Juan Mendez, Ph.D.
Doctor Bruce Stillman

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 2002 | Annual Summary (1 Jul 99 - 1 Jul 02) |

**4. TITLE AND SUBTITLE**
Investigation of the Causes of Breast Cancer at the Cellular Level: Isolation of In Vivo Binding Sites of the Human Origin Recognition Complex

**5. FUNDING NUMBERS**
DAMD17-99-1-9138

**6. AUTHOR(S)**
Juan Mendez, Ph.D.
Doctor Bruce Stillman

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

**E-Mail:** mendezj@cshl.org

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**



**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

We study the process of DNA replication in proliferating human cells. Our efforts are directed to the identification and characterization of proteins that promote DNA replication ("initiators") as well as the DNA sequences recognized by them ("replicators"). We have focused in a group of initiator proteins called Origin Recognition Complex (ORC), Cdc6, and the Mini-Chromosome Maintenance (MCM) proteins. hOrc1p, the largest subunit of ORC, appears to be a critical factor for the coordination of DNA replication with the cell division cycle. hOrc1p levels are higher between the exit of mitosis and the end of G1, the stage at which initiator proteins are assembled at the origins of replication. As cells enter S phase, hOrc1p is polyubiquitinated on chromatin and degraded by the proteasome. hOrc1p destruction is signaled in part by the $SCF^{Skp2}$ ubiquitin conjugating machinery. The controlled destruction of hOrc1p likely contributes to avoid DNA overreplication, a common phenomenon in cancer cells. Our efforts to identify DNA replicator sequences using chromatin immunoprecipitation (ChIP) assays support the hypothesis that human origins of replication may not be defined by short specific DNA sequences but rather by higher-order chromatin structures.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| breast cancer, origin recognition complex (ORC), DNA replication | 25 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION

Understanding the basic mechanisms of the cell division cycle is the first step in our fight against cancer. Each time a normal cell divides, the genomic information contained in its chromosomes is precisely duplicated and distributed between the two daughter cells. Accuracy in the transmission of genetic information is essential. Otherwise, our cells would quickly accumulate genetic lesions (mutations, deletions, chromosome reorganizations), and undergo "programmed cell death" as a defense mechanism to prevent further damage. Occasionally the genetic damage is directed to the key genes that regulate cellular proliferation, and the affected cells start to divide without control, resulting in a tumor. Many genes participate in the regulation of the cell cycle, including those that control the process of DNA replication. Our laboratory studies the function of several human genes, such as the Origin Recognition Complex (ORC), Cdc6, Cdt1 and Mini-Chromosome Maintenance (MCM) that regulate initiation of DNA replication (1). Based in previous studies with model systems, it has been postulated that ORC proteins recognize and bind to specific "replicator" DNA sequences, located in the vicinity of, or overlapping with, the actual origins of replication. The replicator sequences serve as the assembly point of multiprotein structures called pre-replicative complexes (pre-RCs) that include initiator proteins ORC, Cdc6, Cdt1 and MCMs. The assembly and disassembly of pre-RCs is regulated in the cell cycle to ensure that the genome is replicated precisely once before cell division. The main goals of this project were the characterization of human pre-replicative complexes, the generation of specific antibodies against human initiator proteins, and the design and optimization of a chromatin immunoprecipitation experimental procedure to identify human replicator sequences. Some of our results were unexpected and led to new ways of looking at the molecular events that promote DNA replication. We report a novel mechanism that likely contributes to prevent genome overreplication, a phenomenon leading to genome instability, one of the hallmarks of cancer cells.

## BODY

Our initial experiments were designed to characterize human origins of replication using chromatin immuno-precipitation (ChIP) assays (Task 1). First, an *in vivo* chromatin cross-linking procedure was optimized (Task 1, SOW 1). Treatment of tissue cultured cells with 1% formaldehyde for 10 minutes resulted in efficient protein-DNA crosslinking. Nuclear extracts were prepared and sonicated to break the crosslinked chromatin in small molecules (0.5-2 Kb) suitable for immunoprecipitation assays. In addition, crosslinked DNA-protein complexes could be separated from the free protein and chromatin by ultracentrifugation in CsCl gradients (Task 1, SOW 2). Several antibodies were generated against the different subunits of the human ORC, Cdc6 and the MCM protein complex (2-3). After the crosslinking proccess, antibodies anti-hOrc2p, anti-Cdc6 or anti-MCM subunits efficiently immunoprecipitated their target proteins associated to the chromatin (Task 1, SOW 3). Upon reversal of the chemical crosslinks, the co-precipitated DNA sequences were analyzed by qualitative as well as quantitative PCR methods (Task 1, SOW 4). As a positive control for the ChIP procedure we used antibodies directed against SNAPc190, a transcription factor whose DNA binding site had been characterized (4). In several independent experiments, the concentration of SNAPc190 binding sites in the precipitated DNA was 8- to 10-fold higher than the concentration of a control DNA sequence located upstream to the binding site.

We subsequently used this procedure to try to identify DNA binding sequences for the human initiator proteins, hORC in particular. Most of the experiments were performed with a monoclonal antibody anti-hOrc2p that was shown to immunoprecipitate hOrc2p, as well as other hORC subunits. Given the high complexity of the human genome, we focused our analysis in two specific regions known to contain active origins of replication: the loci containing the β-globin and the lamin B2 genes (5). Although origins of replication had been mapped within these regions by different methods, their binding sites for human initiator proteins remained to be elucidated (6).

Multiple DNA primer pairs were designed to amplify fragments (400-500 bp in length) across these two loci. However, the ChIP experiments failed to reveal any preferential binding sites for hORC across the 5 Kb of the β-globin locus or the 7 Kb of

the lamin B2 locus analyzed. On the contrary, all the DNA regions seemed to be equally represented in the immunoprecipitated DNA. This observation was reproduced under different conditions and confirmed with antibodies raised against other components of the pre-replication complex, such as Orc6, Cdc6 and MCM proteins. This somehow puzzling result could indicate that hORC is evenly distributed across these two loci. To date, no other laboratory has reported the identification of specific ORC, Cdc6 or MCM binding sites within these two regions. However, very recently, the ChIP approach led to the identification of a new ORC-containing origin of replication in the MCM4-PRKDC intergenic region (7), but no specific ORC binding sequences were identified. Our results, combined with those of other laboratories, support the notion that human origins of replication, unlike *Saccharomyces cerevisiae* origins, may not be defined by short, specific DNA sequences but rather by more complex chromatin structures that could be epigenetically inherited (8).

The results described above prompted us to reevaluate Task 2 in the original project, which involved developing a fluorescence *in situ* hybridization technique to compare in vivo ORC binding sites with putative origins of replication. An interesting idea emerged while trying to understand the lack of well-defined hORC binding sites: hORC proteins could be unstable, or their association with the chromatin could be regulated. To test this, the abundance of hORC proteins in the cell cycle was evaluated in cell lines synchronized by centrifugal elutriation. Interestingly, we found that the levels of hOrc1p, the largest subunit of hORC, accumulated during G1, decreased significantly after the G1-S transition and were very low during the S and G2 phases (3). In contrast, the levels of other hORC subunits were constant across the cell cycle. This new result implied that hOrc1p could have a regulatory role in the activation of origins of replication and the prevention of re-replication in human cells.

The levels of key cell cycle regulator proteins, such as cyclins and cyclin-CDK inhibitors, are controlled by ubiquitin-mediated proteolysis (9). The Skp1-cullin-F-box protein complex (SCF) is an ubiquitin-conjugating complex that regulates several proteolytic events that drive the cells through the G1-S transition. It is composed of Skp1, Cul1 and Rbx1, and an F-box protein that recognizes specific substrates (9). Skp2 is an F-box protein that participates at least in the degradation of cyclin-CDK inhibitor $p27^{Kip1}$ (10,

11) and transcription factor E2F-1 (12). We found that Skp2 also interacts with hOrc1p, both *in vivo* and *in vitro* (3), suggesting that SCF/Skp2 and the 26S proteasome may regulate the levels of hOrc1p by ubiquitin-mediated proteolysis. The role of Skp2 in hOrc1p turnover was further confirmed by downregulating its activity. When a dominant-negative version of Skp2 was introduced in HeLa cells, or small interfering RNAs silenced Skp2 expression, hOrc1p was stabilized (3). The steady-state levels of hOrc1p were also increased when HeLa cells were treated with proteasome inhibitors MG132 and β-lactone, whereas the levels of hOrc2p were not affected (3). Besides its regulation in the cell cycle, hOrc1p also became limiting when HeLa cells were treated with the chemotherapeutic agent adriamycin, which produces double stranded DNA breaks. The treatment with adriamycin greatly reduced the amount of chromatin-associated hOrc1p, likely to prevent further events of initiation of DNA replication.

Polyubiquitination of hOrc1p occurred on the chromatin (3) as it has been reported for Xenopus CDK inhibitor $p27^{Xic1}$ (13). Our data suggests that in human cells, hOrc1p could recruit the SCF/Skp2 complex to the chromatin in the vicinity of the origins of replication. An attractive possibility is that ubiquitination of hOrc1p serves as a dual signal, activating the protein for its function in DNA replication and at the same time promoting its degradation. This "double switch" mechanism would be similar to the one described for transcription factors carrying the VP16 activation domain (14) and would guarantee that each origin is activated once and only once in each cell cycle.

Finally, we have found that the levels of initiator proteins are elevated in tumor cell lines compared to normal, diploid cells (see Appendix, Figure 1). This observation opens the exciting possibility of using initiator proteins as targets for new cancer diagnosis tools.

## KEY RESEARCH ACCOMPLISHMENTS

1. Preparation of specific antibodies against hORC, hCdc6 and hMCM proteins. Most of these antibodies are functional in immunoblotting, immunoprecipitation and immunofluorescence applications.

2. Design and optimization of an experimental procedure to immunoprecipitate DNA sequences after *in vivo* chromatin crosslinking.

3. Design and optimization of a quantitative PCR procedure to analyze the DNA sequences corresponding to the human β-globin and lamin B2 loci.

4. Identification of a novel regulation of hOrc1p in the cell cycle by ubiquitin-mediated proteolysis.

5. Identification of SCKF/Skp2 as the ubiquitin-conjugating machinery responsible for the degradation of hOrc1p.

6. Proposal of a new mechanism based on controlled degradation of protein hOrc1p that contributes to prevent DNA re-replication.

7. Identification of antibodies anti-hCDC6 and anti-MCMs as potential tools in tumor diagnosis.

## REPORTABLE OUTCOMES

1. Communication to the Cold Spring Harbor Laboratory Meeting on "The cell cycle" (Cold Spring Harbor, New York, May 2000). Abstract follows:

REGULATION OF HUMAN ORC1, CDC6 AND MCM PROTEINS DURING THE CELL CYCLE

Juan Méndez, X. Helena Zou-Yang and Bruce Stillman. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724.

Evidence obtained from yeast and *Xenopus* indicate the initiation of DNA replication to be a multistep process. The origin recognition complex (ORC), Cdc6p and minichromosome maintenance (MCM) proteins are required for establishing pre-replicative complexes, upon which initiation is triggered by the activation of cyclin-dependent kinases (CDKs) and the Dbf4p-dependent kinase Cdc7p. The identification of human homologues of these replication proteins allows investigation of S phase regulation in mammalian cells. Using centrifugal elutriation of several human cell lines, we demonstrate that whereas hOrc2p and hMcm protein levels are constant throughout the cell cycle, hOrc1p and hCdc6p levels vary. hCdc6p is almost absent in early G1 and accumulates until cells enter mitosis. hOrc1p levels, on the other hand, decrease after the G1/S transition. Both proteins can be polyubiquitinated *in vivo* and are stabilized by proteasome inhibitors. $p45^{Skp2}$, a subunit of the ubiquitin-protein ligase SCF that is required for S phase progression, is involved in the proteolysis of hOrc1p. Because hOrc1p is likely the critical ATPase subunit of hORC, this result suggests a novel cellular mechanism by which re-initiation of DNA replication is prevented. Using biochemical fractionation of human cells, we show that a fraction of hCdc6p is present on chromatin throughout the cell cycle, whereas hMcm proteins alternate between soluble and chromatin-bound forms. Loading of hMcm proteins onto chromatin occurs in late mitosis concomitant to the destruction of cyclin B, suggesting that the mitotic kinase activity inhibits pre-replicative complex formation in human cells.

2. Communication to the Salk Institute Meeting on "The cell cycle". (La Jolla, California, June 2001). Abstract follows:

HUMAN ORIGIN RECOGNITION COMPLEX LARGEST SUBUNIT IS DEGRADED BY UBIQUITIN-MEDIATED PROTEOLYSIS AFTER INITIATION OF DNA REPLICATION

Juan Méndez, Helena Zou-Yang, Masumi Hidaka and Bruce Stillman. Cold Spring Harbor Laboratory. 1 Bungtown Rd. Cold Spring Harbor, New York 11724.

Eukaryotic cells display overlapping mechanisms to ensure that DNA replication is restricted to the S phase of the cell cycle. We find that the levels of hOrc1p, the largest subunit of the human origin recognition complex, are tightly controlled in the cell cycle. During mitosis hOrc1p chromatin association is prevented.

The protein is expressed and targeted to chromatin in early G1, when pre-replicative complexes are formed at origins of DNA replication. In contrast, hOrc2p is stable throughout the cell cycle, and 50% remains bound to chromatin during mitosis. Unlike yeast Orc1p, hOrc1p levels decrease abruptly as cells enter S phase and cyclin A accumulates. Cyclin A-CDK2 interacted with hORC and phosphorylated hOrc1p and hOrc2p. HOrc1p levels were controlled by its polyubiquitination and destruction by the 26S proteasome. hOrc1p interacted with the F-box protein Skp2, suggesting the involvement of the Skp1-Cullin-F-box protein (SCF) ubiquitin conjugating machinery. Specific degradation of hOrc1p was also observed upon induced DNA damage. This novel regulation of hOrc1p can contribute to the maintenance of ploidy in human cells.

## 3. Communication to the Jacques Monod Conference "The cell cycle and its checkpoints" (Roscoff, France, September 2002). Abstract follows:

FORMATION OF PRE-REPLICATIVE COMPLEXES IN HUMAN CELLS DURING LATE MITOSIS AND EARLY G1

Juan Méndez[1], Susanna Ekholm-Reed[2], Helena Zou-Yang[1], So-Young Kim[1], William P. Tansey[1], Steve Reed[2] and Bruce Stillman[1]. [1]Cold Spring Harbor Laboratory. 1 Bungtown Rd. Cold Spring Harbor, New York. [2]The Scripps Research Institute, La Jolla, California.

To initiate DNA replication in eukaryotic cells, DNA replication origins are licensed by the assembly of pre-replicative complexes (pre-RCs) consisting of the Origin Recognition Complex (ORC), Cdc6, Cdt1 and the Mini-Chromosome Maintenance (MCM) family of proteins. Pre-RCs are formed at the M/G1 transition, during the period of low cyclin-dependent kinase activity that follows the proteolytic degradation of mitotic cyclins. We asked whether the premature expression of cyclin E during mitosis and G1, which results in genome instability and impaired S-phase progression in human cells, could affect the assembly of functional pre-RCs. A recombinant adenovirus was used to constitutively express cyclin E in human cells, and the abundance of initiator proteins on the chromatin was analyzed by deconvolution microscopy and biochemical fractionation. The association of hMcm4p with the chromatin in early G1 cells was severely impaired. In contrast, cyclin E did not interfere with the loading of other initiator proteins such as Orc1p, Cdc6p or Mcm2p. hMcm4, that contains several consensus sites for CDK phosphorylation, is likely the direct target of an inhibitory phosphorylation signal by cyclin E-CDK2.

hOrc1p, the largest subunit of the human ORC, appears to be another critical regulator for the timely formation of pre-RCs in the cell cycle. In rapidly proliferating cells, hOrc1p levels are higher from the exit of mitosis to the end of G1, when pre-RCs are assembled. As cells enter S phase, hOrc1p is polyubiquitinated on chromatin and then degraded by the 26S proteasome. hOrc1p destruction is signaled in part by the $SCF^{Skp2}$ ubiquitin conjugating machinery. HOrc1p was also degraded upon induced DNA damage. The regulated degradation of hOrc1p and the activation of cyclin E at the G1/S transition likely

cooperate to prevent the formation of pre-RCs during the S, G2, and early M phases of the cell cycle, thus contributing to prevent DNA re-replication and maintain the ploidy of human cells.

4. Communication to the CNIO Meeting on "The cell cycle and cancer". (Madrid, Spain, September 2002). Abstract follows:

DEREGULATED EXPRESSION OF CYCLIN E INTERFERES WITH THE FORMATION OF PRE-REPLICATIVE COMPLEXES IN HUMAN CELLS DURING EARLY G1

Juan Méndez[1], Susanna Ekholm-Reed[2], Helena Zou-Yang[1], So-Young Kim[1], William P. Tansey[1], Steve Reed[2] and Bruce Stillman[1]. [1]Cold Spring Harbor Laboratory. 1 Bungtown Rd. Cold Spring Harbor, New York. [2]The Scripps Research Institute, La Jolla, California.

In eukaryotic cells, DNA replication origins are licensed during G1 by the assembly of pre-replicative complexes (pre-RCs) consisting of the Origin Recognition Complex (ORC), Cdc6, Cdt1 and the Mini-Chromosome Maintenance (MCM) family of proteins. hOrc1p, the largest subunit of the human ORC, appears to be a critical regulator for the timely formation of pre-RCs in the cell cycle. In rapidly proliferating cells, hOrc1p levels are higher from the exit of mitosis to the end of G1, when pre-RCs are assembled. As cells enter S phase, hOrc1p is polyubiquitinated on chromatin and then degraded by the 26S proteasome. hOrc1p destruction is signaled in part by the $SCF^{Skp2}$ ubiquitin conjugating machinery.

Pre-RCs are formed during the period of low cyclin-dependent kinase activity that follows the proteolytic degradation of mitotic cyclins.We find that premature expression of cyclin E during mitosis and G1, which results in genome instability and impaired S-phase progression, affects the assembly of functional pre-RCs. Cyclin E was constitutively expressed in human cells using a recombinant adenovirus, and the abundance of initiator proteins on the chromatin was analyzed by deconvolution microscopy and biochemical fractionation. The association of hMcm4p with the chromatin in early G1 cells was severely impaired. In contrast, cyclin E did not interfere with the loading of other initiator proteins such as Orc1p, Cdc6p or Mcm2p. hMcm4, that contains several consensus sites for CDK phosphorylation, is likely the direct target of an inhibitory phosphorylation signal by cyclin E-CDK2.

The regulated degradation of hOrc1p and the activation of cyclin E at the G1/S transition likely cooperate to prevent the formation of pre-RCs during the S, G2, and early M phases of the cell cycle, thus contributing to prevent DNA re-replication and maintain the ploidy of human cells.

5. Published research article: J. Méndez, X. Helena Zou-Yang, S.-Y. Kim, M. Hidaka, W. P. Tansey and B. Stillman. 2002. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Mol Cell* **9:** 481-491. See Appendix: attached reprint of the article.

## CONCLUSIONS

The original statements of work have been attempted and partially completed. An efficient chromatin immunoprecipitation assay was developed and several anti-hORC, anti-hCdc6p, and anti-hMCM antibodies were raised and characterized. During the period of activity (1999-2002) we learnt that our predictions about the nature of human origins of replication needed to be refined. Multiple hORC immunoprecipitations did not reveal any short, specific DNA sequences that could be ORC binding sites, even when analyzing genomic regions that contain active origins of replication. In human cells, DNA replication origins are likely defined by chromosomal context and epigenetic elements, that facilitate the coordination of DNA replication with other important chromosomal functions, such as transcription, chromatin assembly and the establishment of sister chromatid cohesion.

We found that the large subunit of the human Origin Recognition Complex is unstable in the cell cycle. The SCF/Skp2 ubiquitin-ligase machinery is responsible for the control of hOrc1p levels. Skp2 is an oncogenic protein overexpressed in human cancers (15). $Skp2^{-/-}$ murine cells display high levels of $p27^{Kip1}$, cyclin E and polyploidy (16). Indeed, in human cells we find that suppression of Skp2 activity also leads to hOrc1p stabilization. The inability to degrade hOrc1p could be one of the factors leading to genome overreplication.

Finally, the antibodies generated for this project have been used to compare the levels of initiator proteins in normal and transformed cell lines. Because initiator proteins are much more abundant in tumor cells, we expect these antibodies to have applications in the diagnosis of cancer in the immediate future.

## REFERENCES


1) Bell, S.P. and Dutta, A. (2002). DNA replication in eukaryotic cells. Ann. Rev. Biochem. *71*, 333-74.
2) Méndez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol *20*, 8602-12.
3) Méndez, J., Zou-Yang, X.H., Kim, S.Y., Hidaka, M., Tansey,W. P. and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol. Cell *9*, 481-91.
4) Zhao, X.Y., Pendergrast, S. and Hernandez, N. (2001). A positioned nucleosome on the human U6 promoter allows recruitment of SNAPc by the Oct-1 POU domain. Mol. Cell *7*, 539-49.
5) DePamphilis, M. L. (1999). Replication origins in metazoan chromosomes: fact or fiction? Bioessays *21*, 5-16.
6) Gilbert, D.M. (2001). Making sense of eukaryotic DNA replication origins. Science *294*, 96-100.
7) Ladenburger, E.M., Keller, C. and Knippers, R. (2002). Identification of a binding region for human origin recognition complex proteins 1 and 2 that coincides with an origin of DNA replication. Mol Cell Biol. *22*,1036-48.
8) Mechali, M. (2001). DNA replication origins: form sequence specificity to epigenetics. Nature Reviews Genetics *2*, 640-5.
9) Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-79.
10) Carrano, A.C., Eytan, E., Hershko, A. and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol. *1*, 193-9.
11) Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U. and Krek, W. (1999). P45Skp2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat Cell Biol *1*, 207-14.
12) Marti, A., Wirbelauer, C., Scheffner, M. and Krek, W. (1999). Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol. *1*, 14-19.
13) Furstenthal, L., Swanson, C., Kaiser, B.K., Eldridge, A.G. and Jackson, P.K. (2001). Triggering ubiquitination of a CDK inhibitor at origins of DNA replication. Nat Cell Biol *3*, 715-22.
14) Salghetti, S.E., Caudy, A.A., Chenoweth, J.G. and Tansey, W.P. (2001). Regulation of transcriptional activation domain function by ubiquitin. Science *293*, 1651-3.
15) Gstaiger, M., Jordan, R., Lim, M., Catzavelos, C., Mestan, J., Slingerland, J., and Krek, W. (2001). Skp2 is oncogenic and overexpressed in human cancers. Proc Natl Acad Sci U S A *98*, 5043-8.
16) Nakayama, K., Nagahama, H., Minamishima, Y. A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., Kitagawa, M., and Hatakeyama, S. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. Embo J *19*, 2069-81.

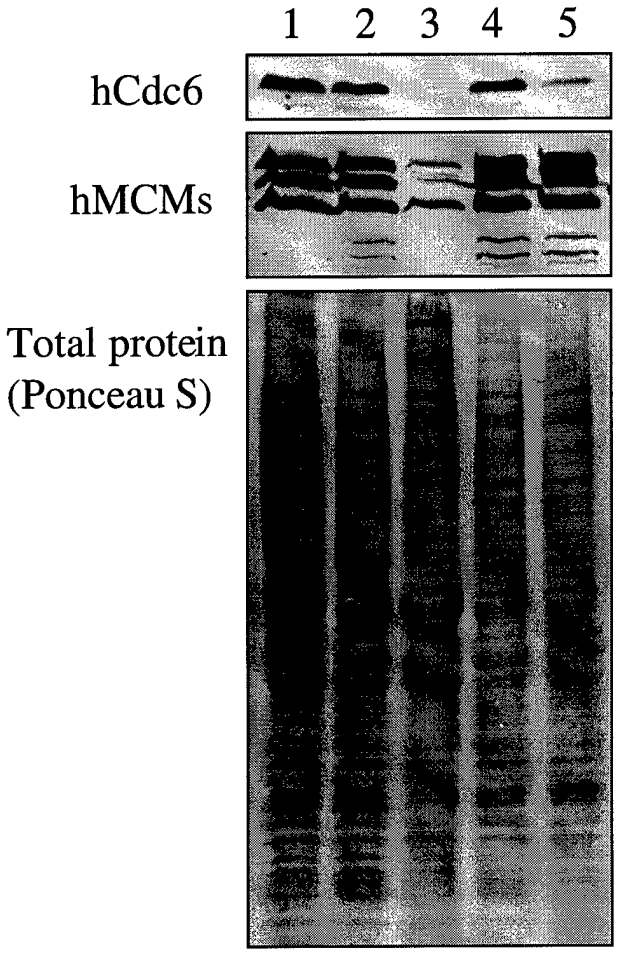


**Figure 1**. Human initiator proteins are overexpressed in tumor cell lines.Total cell extracts were prepared from: (1) HeLa (a cervical tumor cell line), (2) 293 (fibroblasts transformed with the adenoviral oncogene E1A), (3) IMR90 (normal diploid fibroblasts), (4) Manca (a non-Burkitt lymphoma cell line), (5) Raji (a Burkitt lymphoma cell line). The presence of Cdc6p and MCM proteins was analyzed by immunoblots with specific antibodies. The total protein level in each lane is shown by staining with Ponceau S. Note that the levels of Cdc6 and MCM proteins are much lower in normal fibroblasts (lane 3).

Molecular Cell, Vol. 9, 481–491, March, 2002, Copyright ©2002 by Cell Press

# Human Origin Recognition Complex Large Subunit Is Degraded by Ubiquitin-Mediated Proteolysis after Initiation of DNA Replication

Juan Méndez,[2] X. Helena Zou-Yang,[2,3]
So-Young Kim, Masumi Hidaka,[4]
William P. Tansey, and Bruce Stillman[1]
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

## Summary

**Eukaryotic cells possess overlapping mechanisms to ensure that DNA replication is restricted to the S phase of the cell cycle. The levels of hOrc1p, the largest subunit of the human origin recognition complex, vary during the cell division cycle. In rapidly proliferating cells, hOrc1p is expressed and targeted to chromatin as cells exit mitosis and prereplicative complexes are formed. Later, as cyclin A accumulates and cells enter S phase, hOrc1p is ubiquitinated on chromatin and then degraded. hOrc1p destruction occurs through the proteasome and is signaled in part by the $SCF^{Skp2}$ ubiquitin-ligase complex. Other hORC subunits are stable throughout the cell cycle. The regulation of hOrc1p may be an important mechanism in maintaining the ploidy in human cells.**

## Introduction

The molecular mechanisms that prevent genome overreplication were first studied in the yeast *Saccharomyces cerevisiae*. Before initiation of DNA replication, the six-subunit origin recognition complex (ORC) and Cdc6p are strictly required to load minichromosome maintenance (MCM) proteins onto chromatin, defining a prereplicative (pre-RC) state at the origins of replication. Entry into S phase requires the activation of Clb-CDK and Dbf4-Cdc7 kinases. Cdc6p is rapidly degraded upon the commitment to cell division, and the bulk of MCM proteins are displaced from chromatin during S phase. At this time, the origins switch to a postreplicative (post-RC) state in a temporally regulated manner (reviewed by Stillman, 1996; Dutta and Bell, 1997; Kelly and Brown, 2000). The absence of Cdc6p on chromatin during the remainder of the cell cycle is one control mechanism for preventing reestablishment of pre-RCs. Active CDKs also inhibit pre-RC formation through overlapping mechanisms (Nguyen et al., 2001).

Although the nature of the origins of replication in higher eukaryotes remains an open question (reviewed by Gilbert, 2001), it is striking that the six-subunit ORC, as well as other known initiator proteins, has been conserved in evolution. In human cells, however, hCdc6p is not degraded when cells enter S phase. On the contrary, hCdc6p levels remain at fairly constant levels across the cell cycle (Williams et al., 1997; Saha et al., 1998). Although it has been proposed that the nuclear exclusion of hCdc6p after the G1/S transition is functionally equivalent to the degradation of Cdc6 in yeast (Saha et al., 1998; Petersen et al., 1999; Jiang et al., 1999), a significant fraction of hCdc6p remains associated with chromatin throughout later stages of the cell cycle (Méndez and Stillman, 2000; Coverley et al., 2000). The persistence of hCdc6p on chromatin may facilitate subsequent reloading of MCM proteins onto chromatin, as occurs in *Schizosaccharomyces pombe* (Yanow et al., 2001). It is therefore likely that human cells possess additional mechanisms to prevent pre-RC formation following the G1-S transition.

These mechanisms could involve the specific regulation of ORC. Yeast ORC is stably associated with the chromatin across the cell cycle, and its levels do not fluctuate (Aparicio et al., 1997; Liang and Stillman, 1997). In contrast, *Xenopus laevis* Orc1p association with chromatin is strong in early interphase, but it is destabilized after incorporation of the MCM proteins (Rowles et al., 1999). After this "licensing" point, XlORC can be removed from chromatin by exposure to high cyclin A levels without affecting DNA replication (Hua and Newport, 1998). In *Drosophila melanogaster*, Orc1p abundance is developmentally regulated (Asano and Wharton, 1999). In the embryo, it accumulates mostly in proliferating cells, and it becomes cell cycle regulated in the eye imaginal disc, being abundant from late G1 until the end of S phase. In the ovary, DmOrc1p is directed to subnuclear foci at the time of the switch from endoreplication to amplification of the chorion gene clusters (Asano and Wharton, 1999).

In this work, we report the regulation of hOrc1p, the largest of the six subunits of the human ORC that have been identified and partially characterized (Gavin et al., 1995; Ishiai et al., 1997; Quintana et al., 1997, 1998; Tugal et al., 1998; Dhar and Dutta, 2000). These six proteins form a complex when expressed in insect cells using recombinant baculoviruses or when translated in vitro using reticulocyte lysates (Vashee et al., 2001; X.H.Z.-Y. and B.S., unpublished data). We show that hOrc1p levels are low in mitosis, accumulate during G1, and are reduced as cells progress into S phase. hOrc1p is phosphorylated by S phase CDKs and interacts with the F box protein Skp2, a substrate recognition component of the Skp1-cullin-F box (SCF) ubiquitin-ligase complex. Moreover, hOrc1p is polyubiquitinated in vivo and accumulates when Skp2 expression is reduced or proteasome activity is blocked. Together, these data suggest that hOrc1p is targeted for destruction by an $SCF^{Skp2}$ complex during S phase. The resemblance of hOrc1p regulation to that of yeast Cdc6p suggests that the ORC1 and CDC6 genes, highly conserved at the sequence level, have partially switched functions during evolutionary divergence of yeast and mammalian cells.

[1]Correspondence: stillman@cshl.org
[2]These authors contributed equally to this work.
[3]Present address: Iris Pharmaceuticals, Carlsbad, California 92008.
[4]Present address: National Institute for Basic Biology, Okazaki 444, Japan.

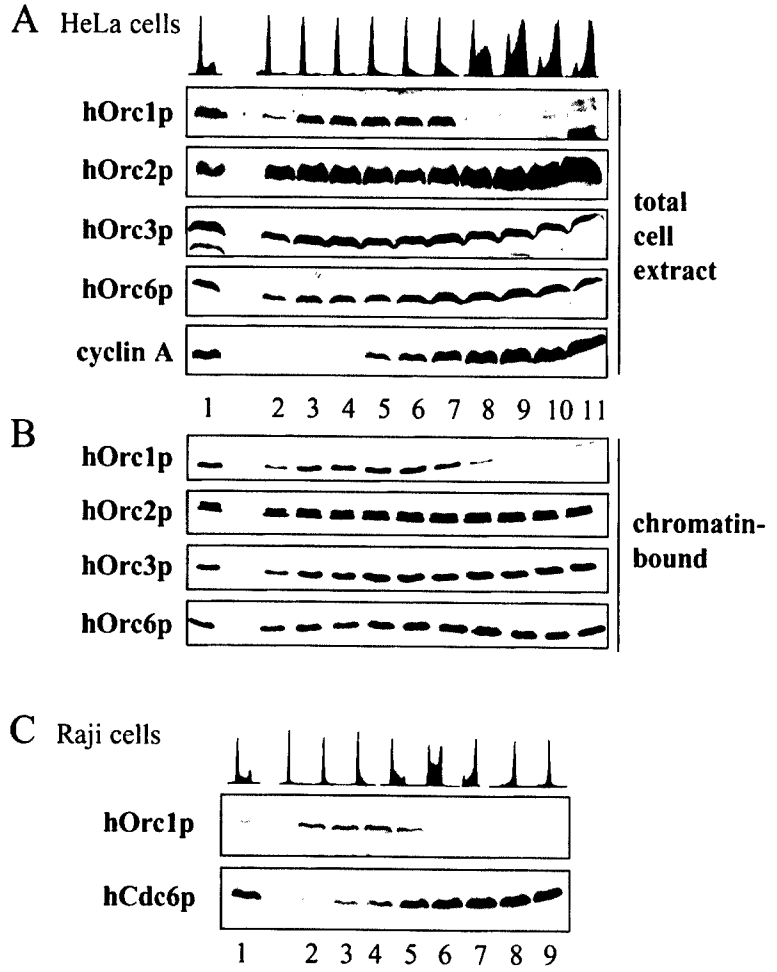


Figure 1. hOrc1p Is Regulated during the Cell Cycle

(A) HeLa cells were separated in different stages of the cell cycle by centrifugal elutriation. The top panel shows the DNA content for the cells in each fraction, as determined by flow cytometry after propidium iodide staining. Equivalent amounts of total cell extracts (normalized by cell number) were subjected to SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

(B) The chromatin fraction was purified from the elutriated HeLa cells, and the presence of the indicated proteins in each fraction was analyzed as indicated above.

(C) Levels of hOrc1p and hCdc6p in the cell cycle in elutriated Raji cells. Total cell extracts were prepared, and the presence of hOrc1p and hCdc6p was analyzed as above. The hCdc6p immunoblot has been taken from Méndez and Stillman (2000) and is reproduced here solely for comparison with the new hOrc1p data.

## Results

### HOrc1p Levels Are Regulated during the Cell Cycle

The relative abundance of different subunits of hORC was first measured in HeLa cells isolated at different stages of the cell cycle by centrifugal elutriation (Figure 1A). The different hORC subunits were detected in total cell extracts by immunoblotting with specific, affinity-purified antibodies. hOrc2p, hOrc3p, or hOrc6p subunits were present at constant levels across the cell cycle. In contrast, hOrc1p accumulated during G1, but its levels decreased significantly after the G1-S transition, being very low during S and G2 (Figure 1A, lanes 8–11). The degradation of hOrc1p was concomitant with the expression of cyclin A. To analyze the chromatin association of the different hORC subunits in the cell cycle, aliquots of the same elutriated HeLa cells were subjected to a simple biochemical fractionation that separates soluble proteins from the proteins associated with chromatin and/or the nuclear matrix (see schematic in Figure 2B). The levels of hOrc1p on chromatin were significantly reduced during S and G2/M (Figure 1B, lanes 8–11), consistent with the fluctuation of hOrc1p in total cell extracts (Figure 1A). A small fraction of soluble hOrc1p was detected in all fractions (not shown). A similar regulation of hOrc1p was observed in elutriated Raji cells (Figure 1C). Interestingly, the cell cycle regulation of hOrc1p is opposite to that of hCdc6p (Figure 1C; Méndez and Stillman, 2000; Petersen et al., 2000).

To further confirm these observations, HeLa cells were synchronized in early S phase with hydroxyurea (HU) or in early mitosis with nocodazole (NOC). The amount of hOrc1p in total cell extracts was significantly reduced in HU- or NOC-arrested cells (Figure 2A). Longer exposures of the immunoblot revealed a small fraction of hOrc1p molecules that escaped degradation (see below). The levels of hOrc2p or a control protein (cytosolic kinase MEK2) remained unchanged.

Aliquots of the asynchronously growing cells, as well as the HU- or NOC-synchronized cells, were fractionated as outlined in Figure 2B. In the asynchronous culture, hOrc1p and hOrc2p were found mainly in the insoluble fraction P3, although a small fraction of soluble hOrc2p could be detected (Figure 2C, lanes 1–3). The small amounts of hOrc1p detected in cells synchronized in early S phase or early mitosis with HU or NOC (by long exposure of the blot to film) were equally distributed between the soluble and the chromatin bound fractions (Figure 2C, lanes 4–9). A similar distribution was observed for hOrc2p, suggesting that a fraction of the hORC complex may dissociate from chromatin during S phase, at least upon treatment with the synchronizing drugs. The cytosolic kinase MEK2 was detected exclusively in the soluble cytoplasmic fraction (S2) in each case, confirming that the extraction of soluble proteins was complete. The combination of the centrifugal elutriation and the drug synchronization experiments strongly suggests that hOrc1p is a labile subunit within the hORC

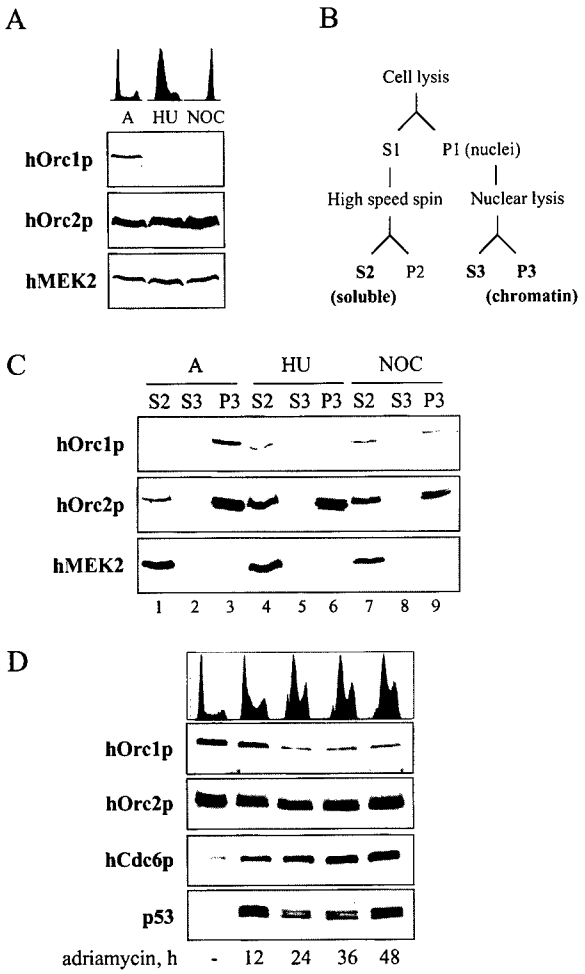


Figure 2. Release of hOrc1p from Chromatin during S phase and following DNA damage

(A) Total cell extracts were prepared from an asynchronous culture of HeLa cells (A), cells synchronized with hydroxyurea (HU), or cells synchronized with nocodazole (NOC). Equivalent amounts of total cell extracts, normalized by cell number, were subjected to SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (lanes 1–3).

(B) Schematic of the biochemical fractionation of cells (see Experimental Procedures for details).

(C) A fraction of each cell population was subjected to the biochemical fractionation outlined in (B), and the relative abundance of hOrc1p and hOrc2p in the different fractions was tested by immunoblotting. S2, soluble cytosolic fraction; S3, soluble nuclear fraction; P3, chromatin/nuclear matrix fraction. Cytosolic kinase MEK2 is shown as a control.

(D) hOrc1p is degraded after induced DNA damage. HeLa cells were incubated with adriamycin at a concentration of 0.5 ng/ml for the indicated amount of time. The top panel shows the DNA content of each cellular population, analyzed by flow cytometry after propidium iodide staining. The levels of hOrc1p, hOrc2p, hCdc6p, and p53 in the chromatin-enriched fraction were analyzed by immunoblotting.

and it might be a limiting factor for initiation of DNA replication.

### hOrc1p Is Degraded in Response to Induced DNA Damage

To test whether hOrc1p is also limiting under a situation of cellular stress, HeLa cells were treated with the chemotherapeutic agent adriamycin, which produces double-stranded DNA breaks and eventually leads to p53-induced apoptosis. The effect of this drug on the cellular DNA content was measured by flow cytometry analysis after propidium iodide staining (Figure 2D, top panel). The accumulation of cells with a DNA content between 1C and 2C reflects the severe interference of adriamycin with cellular DNA replication. The presence of hOrc1p, hOrc2p, hCdc6p, and p53 on chromatin was evaluated by immunoblotting (Figure 2D). After 24 hr of adriamycin treatment, the levels of hOrc1p were significantly reduced, as p53 was induced and targeted to the chromatin. In contrast, hOrc2p was not affected by adriamycin. Interestingly, hCdc6p accumulated on chromatin as hOrc1p was released, revealing again a symmetry in the regulation of these two initiator proteins. No soluble hOrc1p was detected in this experiment (not shown), suggesting that hOrc1p was either directly degraded on the chromatin or first released and then quickly degraded.

### Cyclin A/CDK2 Interacts with hORC and Can Phosphorylate hOrc1p and hOrc2p Subunits at Multiple Sites

Considering that the decrease in hOrc1p levels correlated with the accumulation of cyclin A (Figure 1A), we tested whether cyclin A/CDK2 could participate directly in the regulation of hOrc1p. When endogenous hOrc1p was immunoprecipitated from nuclear cell extracts with an affinity-purified anti-hOrc1p antibody, it coprecipitated cyclin A and CDK2 in addition to hOrc2p (Figure 3A, lane 2). The coimmunoprecipitation was specifically inhibited by the peptide used to generate the anti-hOrc1p antibody but not by an unrelated peptide (Figure 3A, lanes 3–4). In a separate immunoprecipitation experiment, a specific anti-hOrc2p antibody also coprecipitated cyclin A, in addition to hOrc1p (Figure 3B). Next, we tested whether hOrc1p could be phosphorylated in vitro by purified cyclin A/CDK2. hOrc1p contains three (S/T)Px(K/R) consensus sites for CDK phosphorylation (Ser258, Ser273, and Thr375) and up to twelve additional sites with relaxed consensus sequences (Figure 3C). Two derivatives of hOrc1p were constructed: a triple mutant in which amino acids Ser258, Ser273, and Thr375 were changed into Ala (hOrc1p$^{\Delta CDK1,2,3}$) and a truncated version lacking the N-terminal 387 aa that eliminates 12 out of the 15 potential CDK sites (hOrc1p$^{\Delta N387}$; Figure 3C). This truncated hOrc1p still contains the conserved domains characteristic of the AAA$^+$ superfamily of ATPases, including the ATP binding pocket (Neuwald et al., 1999). Wild-type hOrc1p, as well as the ΔCDK1,2,3 and ΔN387 derivatives, were overproduced in *E. coli* and partially purified as GST fusions (Figure 3D, left panel). When incubated with purified cycA-CDK2, both GST-hOrc1p and the ΔCDK1,2,3 mutant were efficiently phosphorylated in vitro, whereas the ΔN387 derivative was phosphorylated to a much lower extent (Figure 3D, middle panel). The average of three independent experiments indicated that the phosphorylation efficiency of GST-hOrc1p$^{\Delta N387}$ was less than 15% of that of the full-length protein. Under the same conditions, control GST protein was not phosphorylated (not shown). Interestingly, purified cyclin E/CDK2 also phosphorylated GST-hOrc1p and, to a lesser extent, the ΔCDK1,2,3 mutant, whereas the ΔN387 form was only residually phosphory-

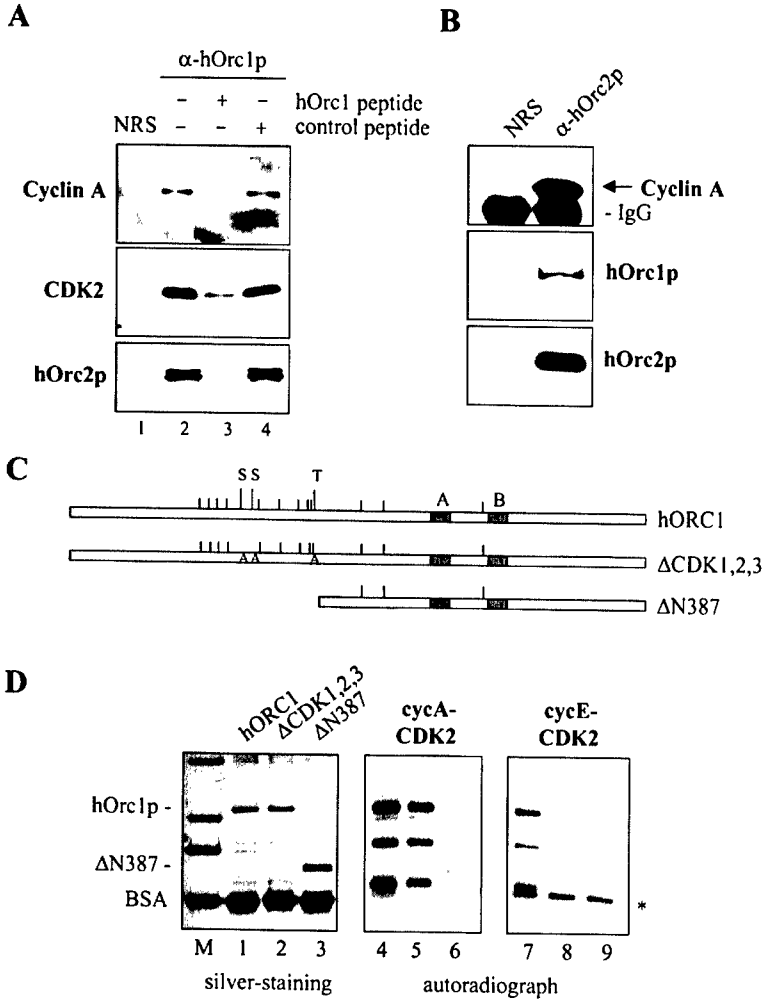


Figure 3. hORC Interacts with Cyclin A/CDK2

(A) Immunoprecipitation with α-hOrc1p antibody from human nuclear extracts (293 cells), followed by immunoblotting with the indicated antibodies. NRS, normal rabbit serum. See text for details.

(B) Immunoprecipitation with α-hOrc2p antibody from human 293 nuclear extracts and immunoblotting with the indicated antibodies.

(C) Schematic of hORC1p potential CDK phosphorylation sites. Long bars indicate the position of the three perfect consensus CDK sites. Short bars represent relaxed consensus sites. Boxes A and B indicate the position of the Walker A (P loop) and B (DExD box) motifs.

(D) In vitro kinase assays. GST-hOrc1p, GST-hOrc1p$^{\Delta CDK1,2,3}$, and GST-hOrc1$^{\Delta N387}$ were expressed and partially purified in *E. coli*. The input amounts of the three substrate proteins are shown by silver staining (left panel). M, molecular weight markers (from the top, 200, 116, 97, and 66 kDa). The next two panels show autoradiographs after in vitro kinase assays with 1 ng of purified cyclin A/CDK2 or cyclin E/CDK2. The labeled protein marked with an asterisk is likely autophosphorylated cyclin E.

lated (Figure 4D, right panel). These results indicate that hOrc1p can be phosphorylated in multiple sites, most of them located in the N-terminal half of the protein. We also found that hOrc2p is phosphorylated in vitro by cyclin A/CDK2, specifically at residues Thr116 and Thr226 (not shown). Because phosphorylation of substrates is a common but not essential signal for ubiquitin-mediated proteolysis (see below), we tested whether an ubiquitin-ligase and the proteasome were involved in hOrc1p turnover.

### hOrc1p Is Polyubiquitinated In Vivo and Stabilized by Inhibition of the Proteasome

We used an in vivo ubiquitination assay that has been useful to detect polyubiquitinated intermediates of other proteins such as c-Jun (Treier et al., 1994), c-Myc (Salghetti et al., 1999), and hCdc6p (Méndez and Stillman, 2000). HeLa cells were cotransfected with a plasmid expressing T7-tagged hOrc1p and a plasmid expressing $(His)_6$-tagged ubiquitin (His-Ubi). Twenty-four hours posttransfection, cell extracts were prepared and ubiquitinated proteins purified by affinity chromatography. When cells expressed both T7-hOrc1p and His-Ubi, a smear of high MW products was detected, likely corresponding to polyubiquitinated hOrc1p intermediates (Figure 4A, lanes 6–8). None of these products were detected in the absence of His-Ubi (Figure 4A, lane 5). Interestingly, hOrc1p$^{\Delta N387}$, whose phosphorylation efficiency was much lower than that of the full-length protein (Figure 3D), was still ubiquitinated very efficiently in vivo (Figure 4B).

To test if polyubiquitination occurred while hOrc1p was associated with chromatin, cells expressing T7-hOrc1p or T7-hOrc1p$^{\Delta N387}$ were cotransfected with a plasmid expressing His-Ubi and then subjected to the biochemical fractionation described above (Figure 2B). The majority of the ubiquitinated products were recovered in the chromatin-enriched fractions (Figure 4C, lanes 4 and 8).

To determine if the 26S proteasome is involved in the regulated destruction of hOrc1p, HeLa cells were treated with proteasome inhibitors MG132 and β-lactone. The steady-state levels of endogenous hOrc1p were increased over 7-fold by MG132 and approximately 5-fold by β-lactone. LLM, an inhibitor of the calpains, stabilized hOrc1p to a lesser extent (Figure 4D). Treatment of the cells with the same inhibitors did not have any effect on hOrc2p levels. hOrc1p was also significantly stabilized by specific proteasome inhibitor LLnL in HeLa and other cell lines such as U2-OS or IMR90 (not shown). These results reinforce the idea that hOrc1p levels are controlled by polyubiquitination and proteasome degradation.

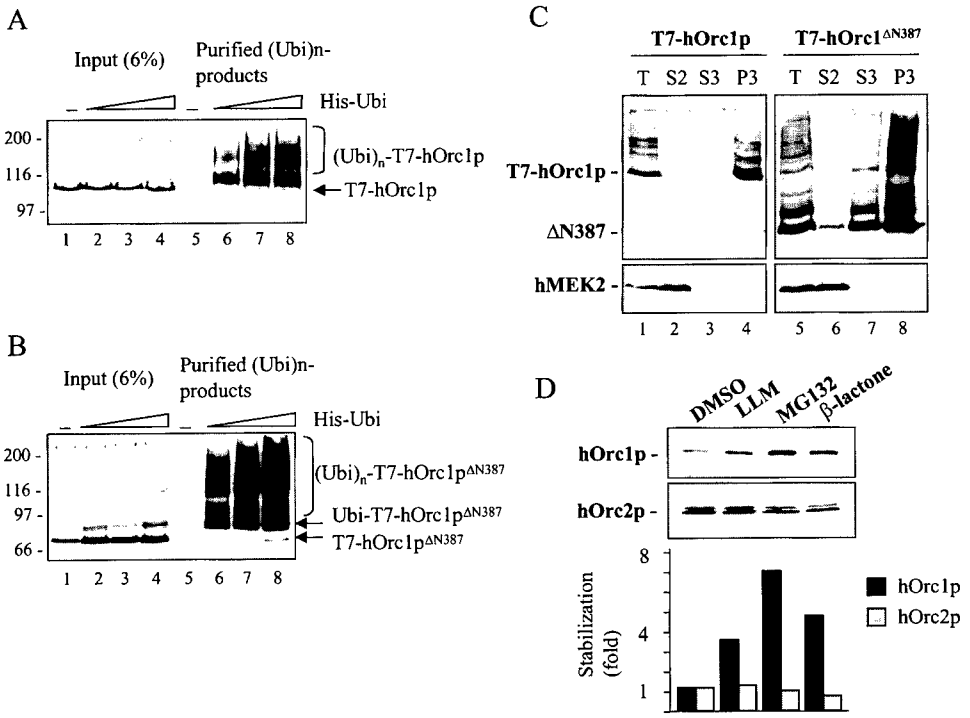


Figure 4. In Vivo Polyubiquitination of hOrc1p

(A) HeLa cells were transiently transfected with 0.5 μg pKG28 (expressing T7-hOrc1p) in the absence or in the presence of increasing amounts (0.05, 0.2, 0.5 μg) of pMT107 (expressing His-tagged ubiquitin). After 24 hr, cells were harvested and lysed. An aliquot was kept for input analysis (lanes 1–4). The ubiquitinated proteins were purified from the extract in a Ni-NTA affinity column. Purified proteins were subjected to SDS-PAGE and analyzed by immunoblotting with anti-T7 antibody. Polyubiquitinated proteins are detected as a smear of high-MW products (lanes 5–8).

(B) Same as above, but HeLa cells were transfected with 0.5 μg of pCGT-hOrc1$^{\Delta N387}$ instead of pKG28.

(C) Ubiquitination of hOrc1p occurs on the chromatin. Cells were transfected with 0.5 μg of plasmids expressing T7-hOrc1p (lanes 1–4) and T7-hOrc1p$^{\Delta N387}$ (lanes 5–8) plus 0.5 μg of pMT107 (His-Ubi). After 24 hr, cells were harvested and fractionated as described in Figure 2B. The presence of ubiquitinated T7-hOrc1p or T7-hOrc1p$^{\Delta N387}$ in the total cell extracts (T), as well as soluble (S2/S3) and chromatin bound (P3) fractions, was analyzed by immunoblots with anti-T7 antibody. Cytosolic kinase MEK2 is shown as a control.

(D) hOrc1p is stabilized by inhibition of the proteasome. HeLa cells were incubated for 6 hr in regular medium supplemented with DMSO (control), LLM, MG132, or β-lactone. Total cell extracts were prepared, and the presence of hOrc1p and hOrc2p was analyzed by immunoblotting. The exposed films were scanned for quantitation.

## Skp2 Associates with hOrc1p and Regulates Its Stability

The SCF protein complex regulates the proteolytic events that drive cells through the G1-S transition. It is composed of Skp1, Cul1, and Rbx1, as well as a variable component called the F box protein, which provides substrate specificity (reviewed by Hershko and Ciechanover, 1998). Skp2 is the F box protein responsible for the ubiquitination of the CDK inhibitor p27$^{Kip1}$ (Carrano et al., 1999; Sutterluty et al., 1999) and E2F-1 (Marti et al., 1999). Skp2 was originally identified as a cyclin A/CDK interacting protein that is expressed preferentially during S phase (Zhang et al., 1995). Considering the fluctuation of hOrc1p in the cell cycle (Figures 1 and 2), the hOrc1p-cyclin A interaction (Figure 3), and the polyubiquitination of hOrc1p (Figure 4), we tested whether Skp2 could also interact with hOrc1p. Recombinant GST-Skp2 protein, expressed and purified from *E. coli*, pulled down in vitro translated hOrc1p (Figure 5A, lanes 1–3). This interaction was specific for hOrc1p, as GST-Skp2 did not pull down in vitro translated hOrc2p (Figure 5A, lanes 4–6). The hOrc1p-Skp2 interaction was confirmed by immunoprecipitation from whole-cell extracts transiently expressing T7-hOrc1p. Anti-Skp2 antibodies but not a control preimmune serum coimmunoprecipitated a fraction of the T7-hOrc1p protein present in the extract (Figure 5B). We found that two regions of hOrc1p contact Skp2, one in the N-terminal half of the protein (aa 276–474) and the other one in the C-terminal region (aa 560–861; data not shown).

The hOrc1p-Skp2 interaction suggested that Skp2 might participate in hOrc1p ubiquitination. To test this hypothesis, the expression of Skp2 in HeLa cells was silenced using small interfering RNA (siRNA; Experimental Procedures). Transient transfection of a 21 bp siRNA corresponding to Skp2 reduced the steady-state levels of Skp2 to less than 5% of their normal levels and at the same time led to the accumulation of endogenous hOrc1p, compared to control cells transfected with siRNAs directed against firefly luciferase (Figure 5C). p27$^{Kip1}$, a known target of Skp2, was also stabilized, whereas hOrc2p was not. The participation of Skp2 in hOrc1p turnover was further confirmed by using a dominant-negative form of Skp2 (Skp2$^{\Delta F}$) that retains the substrate recognition motif but lacks an F box domain. Expression of Skp2$^{\Delta F}$ in HeLa cells increased the steady-state levels of T7-hOrc1p approximately 5-fold (Figure 5D, lanes 1–2). Interestingly, T7-hOrc1p$^{\Delta N387}$ was repro-

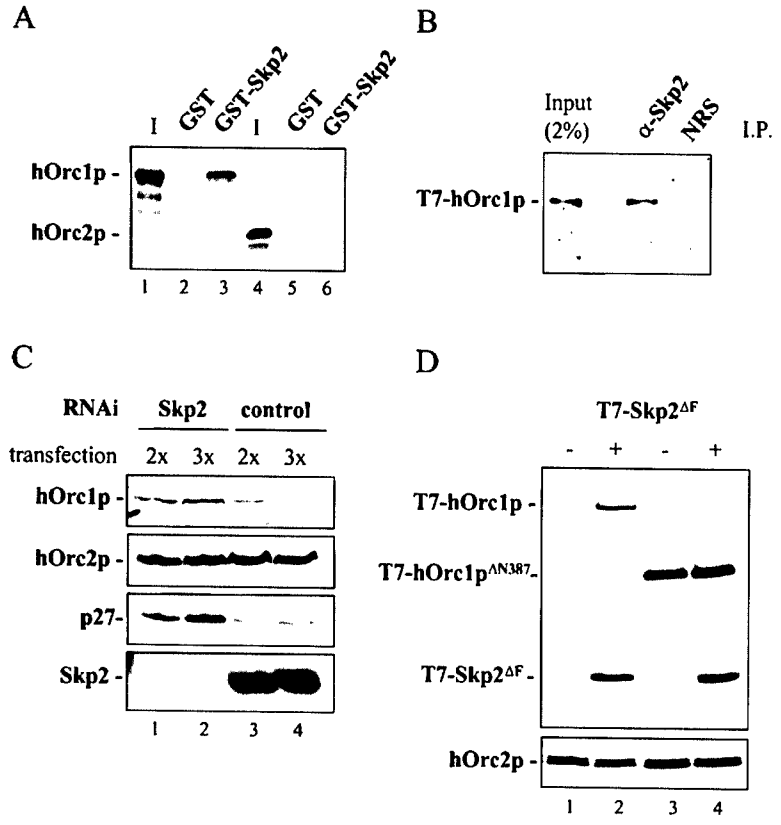


Figure 5. Skp2 Is Involved in hOrc1p Regulation

(A) In vitro GST pull-down assays. In vitro translated hOrc1p but not hOrc2p was pulled down by purified GST-Skp2.

(B) In vivo immunoprecipitations. U2-OS cells were transiently transfected with pKG28 (expressing T7-hOrc1p). Total cell extracts were prepared and used for immunoprecipitation with a control rabbit serum (NRS) or anti-Skp2 antibody. The immunoprecipitates, along with 2% of the amount of extract used for the immunoprecipitation, were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-T7 antibody.

(C) Silencing of Skp2 expression results in stabilization of hOrc1p and $p27^{Kip1}$. HeLa cells were transfected every 24 hr, either two or three times with siRNA molecules corresponding to Skp2 or firefly luciferase as control (Experimental Procedures). Cells were harvested and lysed 24 hr after the last transfection, and the levels of hOrc1p, hOrc2p, $p27^{Kip1}$, and Skp2 in total cell extracts were analyzed by immunoblots.

(D) $Skp2^{\Delta F}$ stabilizes hOrc1p but not $hOrc1^{\Delta N387}$. HeLa cells were transfected with plasmids expressing T7-hOrc1 or T7-$hOrc1^{\Delta N387}$, in the absence or in the presence of pCGT-$Skp2^{\Delta F}$, expressing a dominant-negative version of Skp2 that lacks the F box domain. The cells were harvested 24 hr posttransfection, and the chromatin fraction was purified (Figure 2B) and subjected to SDS-PAGE. The abundance of hOrc1p, $hOrc1^{\Delta N387}$, and $Skp2^{\Delta F}$ was determined by immunoblots with anti-T7 antibody. The levels of cellular hOrc2p are shown as loading control.

ducibly expressed at higher levels than the full-length protein, and its abundance was not influenced by $Skp2^{\Delta F}$. This effect was not observed by deletion of the C-terminal region (aa 650–861; data not shown). These data combined strongly suggest that Skp2 promotes hOrc1p turnover and that the N-terminal domain of hOrc1p, containing most of the phosphorylation sites and overlapping with one of the Skp2-interacting domains, is a regulatory element for hOrc1p stability.

### Simultaneous Chromatin Association of hOrc1p and Degradation of hCdc6p during Early G1

hOrc1p is most abundant during G1, when hORC likely participates in the assembly of pre-RCs in mammalian cells. It has been reported that CgOrc1, the hamster homolog of hOrc1p, does not associate with chromatin during mitosis but becomes stably bound a few hours after cells have entered G1 (Natale et al., 2000). To analyze the transition between mitosis and G1 in human cells, a HeLa cell culture was synchronized in metaphase with nocodazole. After 24 hr, cells were released from the block and collected at different times. As expected, cyclin B was degraded as the cells exited mitosis, whereas cyclin E was only detected 8 hr postrelease, as cells reached the G1-S transition (Figure 6A, bottom panels). The presence of initiator proteins on chromatin was analyzed (Figure 6A, top panels). The level of hOrc1p on chromatin increased sharply at 3 hr postrelease, coinciding with hCdc6p degradation (see also Figure 6B). Insignificant amounts of soluble hOrc1p were detected (not shown). hCdc6p levels were very low at the M/G1 transition, probably due to APC-mediated ubiquitination and proteolysis (Méndez and Stillman, 2000; Petersen et al., 2000). Geminin, an inhibitor of Mcm protein loading (McGarry and Kirschner, 1998), was degraded at the same time as hCdc6p, whereas the levels of hOrc2p on chromatin remained unchanged. Interestingly, the loading of hMcm3 and hMcm4 proteins occurred in parallel with that of hOrc1p at a point when hCdc6p levels decreased. Therefore, hOrc1p might be the limiting factor for pre-RC formation at this stage of the cell cycle.

## Discussion

ORC1 is emerging as the key player in origin control in higher eukaryotes (see review by Cimbora and Groudine, 2001). Here we report that the levels of hOrc1p, the largest subunit of human ORC, were regulated in proliferating cells by ubiquitin-mediated proteolysis. Using both centrifugal elutriation and drug synchronization experiments, we demonstrated that hOrc1p was abundant in G1 but its levels were significantly reduced after the cells entered S phase, whereas other hORC subunits were constant across the cell cycle. The instability of

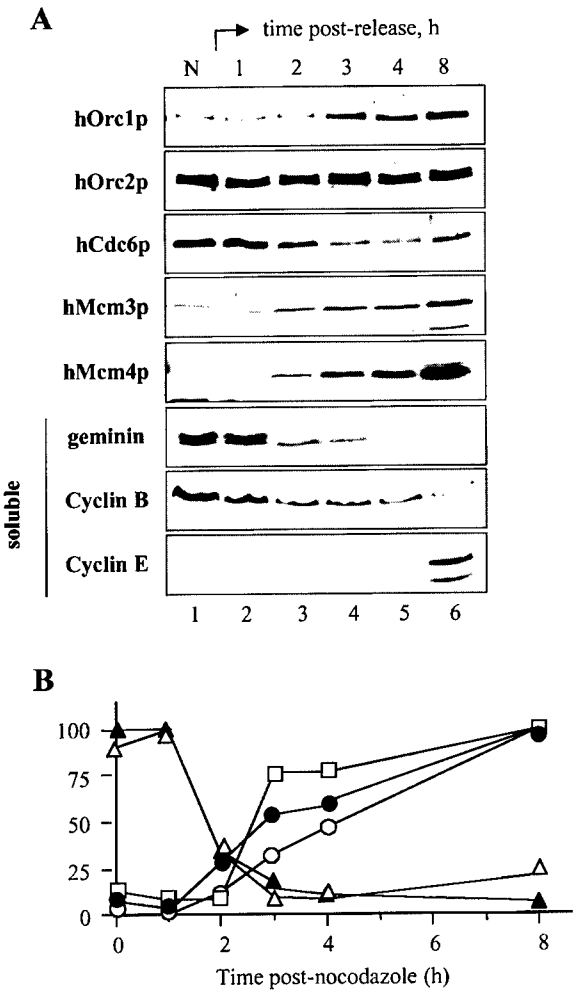


Figure 6. Association of Initiation Factors with Chromatin during M/G1

(A) HeLa cells were synchronized with nocodazole for 24 hr and then released back into regular medium (5% FBS-DMEM). Cells were harvested at different times postrelease and subjected to the biochemical fractionation procedure described in Figure 2B. The levels of hOrc1p, hOrc2p, hCdc6p, hMcm3p, and hMcm4p in the chromatin-bound fraction (P3) were analyzed by immunoblotting with the corresponding antibodies. The levels of geminin, cyclin B, and cyclin E were analyzed in the soluble (S2) fractions.

(B) Quantitation of the fluctuation of hOrc1p (open squares), hCdc6p (open triangles), hMcm3p (filled circles), hMcm4p (open circles), and geminin (filled triangles) levels in the course of the experiment. The *y* axis represents the percentage of the maximum signal in each curve.

hOrc1p suggests that it might be the limiting component in the active hORC.

Consistent with the data presented in this paper, Kreitz et al. (2001) recently reported a significant reduction in chromatin-associated hOrc1p during S phase and G2, using HeLa cultures synchronized by double-thymidine block and release. It is worth noting that hOrc1p regulation may be different between actively proliferating cells and cells that exit from quiescence because no significant fluctuation in hOrc1p levels at the G1/S transition was observed in primary cells synchronized in G0 by serum starvation and released back into the proliferative cycle (Tatsumi et al., 2000). A recent report suggests that hamster Orc1p levels associate with the chromatin during the whole-cell cycle, including S phase (Okuno et al., 2001). Besides the possibility that human and hamster Orc1p may not be regulated in the same way, the discrepancy could be explained by the different synchronization methods used. Okuno et al. (2001) synchronized a cell population using drug "block and release" steps, and every fraction collected contained a significant amount of cells in early or mid S phase. Even if CgOrc1p was targeted for degradation by the SCF, these cell populations could contain detectable levels of CgOrc1p.

The significance of hOrc1p destruction was further reinforced by the fact that hOrc1p was specifically degraded when cells were treated with the chemotoxic agent adriamycin. This observation suggested that hORC could be a direct target of the DNA-damage checkpoint pathways. Alternatively, the reduction in hOrc1p levels could be a consequence of the S phase arrest observed in HeLa cells upon the adriamycin treatment. Regardless of the precise mechanism, the degradation of hOrc1p in a situation of DNA damage is likely to prevent further initiation of DNA replication.

Our paper demonstrates that hOrc1p was polyubiquitinated in vivo, and it was stabilized by inhibition of the 26S proteasome. The degradation of hOrc1p after the G1/S transition suggested the involvement of the SCF ubiquitin-ligase that is responsible in yeast for the degradation of Cdc6p in late G1/early S phase (Perkins et al., 2001). Indeed, hOrc1p interacted with the F box protein Skp2 both in vivo and in vitro. Downregulation of Skp2 expression by siRNA stabilized endogenous hOrc1p but not hOrc2p. In addition, transient expression of $Skp2^{\Delta F}$ in HeLa cells increased the steady-state levels of cotransfected T7-hOrc1p. $Skp2^{\Delta F}$ is a mutant version of Skp2 lacking the F box region that probably interferes with the function of the endogenous Skp2 in a dominant-negative fashion. The link between hOrc1p and Skp2 is intriguing, as Skp2 is oncogenic and overexpressed in some human cancers (Gstaiger et al., 2001). Targeted disruption of Skp2 in mice results in high levels of $p27^{Kip1}$, cyclin E, and polyploidy in some cell types (Nakayama et al., 2000). Interestingly, hepatocytes from the $Skp2^{-/-}$ mice can accumulate up to 16C DNA content. This could be explained by abnormal $p27^{Kip1}$-mediated inhibition of Cdc2 impeding progression through mitosis and the accumulation of Orc1p leading to genome overreplication. However, whether mouse Orc1p is regulated in the same way as its human counterpart remains to be tested.

hOrc1p N-terminal domain contains the majority of the CDK phosphorylation sites and one of the two regions that can interact with Skp2. Interestingly, the levels of $T7\text{-}hOrc1p^{\Delta N387}$ were not affected by the expression of the dominant-negative $Skp2^{\Delta F}$, suggesting that the hOrc1p N terminus contains the degron for Skp2-dependent turnover. Still, $T7\text{-}hOrc1p^{\Delta N387}$ was efficiently ubiquitinated in vivo. This could readily be explained by the participation of other F box proteins that also promote hOrc1p polyubiquitination, such as the recently characterized homolog of yeast Cdc4 (Koepp et al., 2001; Strohmaier et al., 2001).

Polyubiquitination of hOrc1p occurred on the chroma-

tin. This resembles the case of *Xenopus* CDK inhibitor $p27^{Xic1}$ (Furstenthal et al., 2001b). $p27^{Xic1}$ polyubiquitination occurs in the vicinity of the origins of replication, with ORC and Cdc6 acting as chromatin-associated receptors for $p27^{Xic1}$-cyclin E/Cdk2 and the SCF ubiquitin-ligase activity (Furstenthal et al., 2001a, 2001b). In mammalian cells, the $SCF^{Skp2}$ ubiquitin-ligase could also be recruited to the prereplicative complexes through its interaction with hOrc1p. A very interesting possibility is that ubiquitination of hOrc1p works as a dual signal, both activating the protein for a function in initiation of DNA replication and then promoting its degradation. This "double-switch" mechanism would guarantee that each origin is activated only once in each cell cycle. In yeast, transcription factors carrying the VP16 activation domain are activated by ubiquitination, and at the same time they are labeled for degradation (Salghetti et al., 2001). Other initiator proteins may be regulated by a similar mechanism. HCdt1, the human homolog of the novel *S. pombe* and *Xenopus* Cdt1 proteins (Nishitani et al., 2000; Maiorano et al., 2000; Wohlschlegel et al., 2000), is also destabilized after entry into S phase, likely by ubiquitin-mediated proteolysis (Nishitani et al., 2001).

We have also analyzed in detail the loading of different initiator proteins onto chromatin during the exit from mitosis and progression through G1, a critical period of the cell cycle in which pre-RCs are assembled and the replication timing of chromosomal domains is established (Dimitrova and Gilbert, 1999). hOrc1p association with chromatin was weak during mitosis and increased as the cells progressed into G1. Even though Cdc6p is an MCM-loading factor in yeast and *Xenopus*, hMCM proteins started to associate with the chromatin when hOrc1p was very abundant on chromatin and the bulk of hCdc6p was degraded (Figure 6). hMCM chromatin association occurred as cyclin B was degraded and before the induction of cyclin E, in agreement with the notion that CDK activity inhibits pre-RC formation (Nguyen et al., 2001). The *Drosophila* early embryonic cell cycles would be an exception to this rule, as MCM proteins associate with mitotic chromosomes in the presence of constant levels of cyclin E or stabilized cyclin B (Su and O'Farrell, 1997).

The overall regulation of hOrc1p resembles that of yeast Cdc6p. ORC1 and CDC6 have most likely evolved from a common ancestor. Indeed, ORC1 and CDC6 from various species have significant sequence similarity (Bell et al., 1995; Tugal et al., 1998). Both genes are regulated by E2F transcription factors (Ohtani et al., 1996, Yan et al., 1998), and their patterns of expression in human tissues are virtually identical (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/3/481/DC1). In fact, Archeal organisms only contain one ORC1/CDC6 gene with the exception of *Methanobacterium thermoautotrophicum*, which has two (Liu et al., 2000). If, during evolution, hOrc1p turned out to be the functional equivalent of yeast Cdc6p, why is hCdc6p conserved? One possibility is that hCdc6p recruited a second subset of MCM proteins and/or other proteins to the origins during late G1. Alternatively, hCdc6p could modify the structural organization of the pre-RCs, perhaps exposing hOrc1p to S phase CDKs. At least in yeast, Cdc6p can modulate the structure of ORC (Mizushima et al., 2000). Another attractive possibility is that the chromatin bound hCdc6p contributes to stabilize the hOrc2-6 complex at origins of replication from S phase to mitosis when hOrc1p is absent.

Finally, it is worth noting that the observed association of hOrc1p with chromatin during G1 followed by its degradation at S phase correlates remarkably well with the in vivo footprint analysis at the lamin B2 human origin of replication. No footprinting was observed during mitosis, but a region spanning 100 bp was protected during G1, which shrinks to 70 bp after the cells entered S phase (Abdurashidova et al., 1998). One of the next critical questions will be to determine whether hOrc1p or other factors such as MCM proteins are responsible for these dynamic protein-DNA interactions at the lamin B2 region or other potential human origins of replication.

## Experimental Procedures

### Antibodies

To generate antibodies to different human ORC subunits, synthetic peptides corresponding to aa 849–861 (hOrc1p), 193–208 (hOrc3p), and 176–192 (hOrc6p) were conjugated to maleimide-activated carrier protein KLH (Pierce) and used to immunize NZW rabbits (Covance). Polyclonal antibodies anti-hOrc2p, hCdc6p, and hMCM proteins have been described before (Gavin et al., 1995; Méndez and Stillman, 2000). Anti-geminin was raised in rabbits using a KLH-coupled peptide corresponding to aa 1–13. The anti-Skp2 antibody used for immunoprecipitation was a gift from G. Hannon and D. Beach (Cold Spring Harbor Laboratory). The following antibodies were from commercial sources: anti-T7 tag (Novagen), anti-MEK2 and anti-cyclin B (Transduction Laboratories), anti-cyclin A and anti-CDK2 (Santa Cruz Biotechnology), anti-cyclin E (Upstate Biotechnology INC), anti-p53 (Novocastra), and anti-Skp2 (Zymed).

### Expression Plasmids

pKG28, a plasmid expressing hOrc1p fused to a single copy of the T7 epitope under the control of a CMV promoter, has been described before (Gavin et al., 1995). The DNA sequence corresponding to aa 388–861 of hOrc1p was PCR-amplified and subcloned into pCGT, the parent vector for plasmid pKG28, generating plasmid pCGT.hOrc1ΔN387. To create the mammalian cell expression vector for T7 epitope-tagged $Skp2^{\Delta F}$, sequences encoding residues 154–435 of Skp2 were PCR-amplified from the full-length Skp2 coding sequence and subcloned into the same parent vector. For in vitro transcription-translation of hOrc1p or hOrc2p, vectors pNCITE. hOrc1p (Iizuka and Stillman, 1999) or pKS-hORC2F were used, respectively. pKS-hORC2F was generated by subcloning the cDNA encoding full-length hOrc2p into pBluescript-KS (Stratagene). The cDNA encoding full-length hOrc1p was subcloned into a variant of pET11c (Novagen) that carries a glutathione-S-transferase (GST) moiety, resulting in plasmid pGST-hOrc1. Plasmids pGST-hOrc1ΔCDK1,2,3 and pGST-hOrc1ΔN387 were generated by Quikchange mutagenesis (Stratagene). A plasmid expressing GST-Skp2 was a gift from G. Hannon and D. Beach (Cold Spring Harbor Laboratory).

### Cell Manipulations and Centrifugal Elutriation

Logarithmically growing HeLa cells were arrested in early S phase by adding 0.5 mM hydroxyurea (Sigma) to the regular medium (DMEM-5% FBS) and incubating for 24 hr. To synchronize the cell population at mitosis, the cells were cultured for 24 hr in the presence of 50 ng/ml nocodazole (Sigma), and rounded, mitotic cells were shaken off the dishes. To induce DNA damage in HeLa cells, regular medium was supplemented with adriamycin (a.k.a. doxorubicin; Sigma) at 0.5 μg/ml for the indicated period of time. HeLa or Raji cell cultures were fractionated into distinct cell cycle phases by centrifugal elutriation in a Beckman J2-21 M centrifuge and a JE-6B rotor with a large (40 ml) separation chamber, as previously described (Méndez and Stillman, 2000). For cytofluorometric analyses, an aliquot of $10^6$ cells was fixed in ethanol and incubated for 30 min at 37°C in 0.5 ml staining solution (25 μg/ml propidium iodide

and 10 μg/ml RNase in PBS). Stained cells were analyzed on a Becton-Dickinson FACScan.

### Silencing by Small Interfering RNA

Logarithmically growing HeLa cells were seeded at a density of $2.5 \times 10^5$ cells/6 cm dish and transfected at 24 and 48 hr (2×) or 24, 48, and 72 hr (3×), using Oligofectamine (Invitrogen) as described (Elbashir et al., 2001). Twenty-four hours after the last transfection, lysates were prepared and analyzed by SDS-PAGE and immunoblotting. The siRNA used for Skp2 silencing was a 21 bp synthetic molecule corresponding to nt 847–867 of the Skp2 coding region. A 21 nt siRNA duplex corresponding to the firefly luciferase gene was used as control.

### Total Cell Extracts, Nuclear Extracts, and Chromatin Isolation

To prepare total cell extracts (TCE), tissue-cultured cells were harvested by centrifugation, washed in PBS, and directly resuspended in Laemmli buffer, followed by sonication for 10 s in a Tekmar CV26 sonicator set at 25% amplitude. Preparation of nuclear extracts and the biochemical fractionation to separate soluble and chromatin bound proteins were performed exactly as described in Méndez and Stillman (2000). Typically, in this fractionation protocol of HeLa cells, approximately 70%–75% of the cellular protein content is recovered in the soluble cytosolic (S2) fraction, approximately 5%–10% in the soluble nuclear (S3) fraction, and the remaining 20% in the chromatin/nuclear matrix fraction (P3). The proteins in the latter fraction that are associated with the DNA can be readily solubilized by micrococcal nuclease treatment (Méndez and Stillman, 2000).

### In Vivo Ubiquitination Assays and Proteasome Inhibition

Ubiquitinated intermediates in human cells were detected with the $(His)_6$-tagged Ubi method of Treier et al. (1994), as used by Salghetti et al. (1999). $1 \times 10^6$ HeLa cells were transfected with 0.5 μg of pKG28 (which encodes T7-tagged hOrc1p) or pCGT.hOrc1pΔN387, either in the absence or in the presence of increasing amounts (0.05, 0.2, and 0.5 μg) of plasmid pMT107, which encodes His-tagged ubiquitin. Cells were harvested 36 hr posttransfection, and His-tagged (therefore ubiquitinated) proteins were purified on Ni-NTA-agarose and subjected to SDS-PAGE. T7-tagged proteins were detected by immunoblot with anti-T7 monoclonal antibody. To inhibit the proteasome, $1 \times 10^6$ HeLa cells were treated for 6 hr with LLM, MG132, β-lactone, or LLnL (CalBiochem) at a concentration of 25 μM. Cells were then harvested and total cell extracts prepared as described above.

### In Vitro Transcription-Translation, GST Pull-Down Assays, and Phosphorylation Assays

In vitro transcription-translation reactions were performed using the TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. GST pull-down assays were performed essentially as described (Kaelin et al., 1991). For in vitro phosphorylation assays, approximately 0.1 μg of partially purified GST, GST-hOrc1p, GST-hOrc1$^{\Delta CDK1,2,3}$, GST-hOrc1p$^{\Delta N387}$, or GST-hOrc2p was incubated in 40 μl of kinase buffer (50 mM HEPES [pH 7.0], 10 mM MgCl2, 4 mM MnCl2, 1 mM DTT, 0.1 mg/ml BSA, 2 μCi of [γ-32P]ATP, and 6000Ci/mmol) for 30 min at 30°C in the presence of 1 ng of purified cyclin A/CDK2 or purified cyclin E-CDK2. Reactions were stopped with Laemmli buffer, subjected to SDS-PAGE, and autoradiographed.

### Immunoprecipitation, Immunoblotting, and Northern blots

Standard protocols for immunoblotting and immunoprecipitation were used, with the modifications noted in Méndez and Stillman (2000). To measure the expression of hORC1, hORC2, or β-actin in different human tissues (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/3/481/DC1), the corresponding cDNAs were radioactively labeled by random priming and used as probes for RNA detection in MTN blots (multiple tissue Northern; Clontech), using the ExpressHyb hybridization solution and protocol (Clontech).

### Acknowledgments

We thank S. Reed for providing purified cyclin E/CDK2, S. Waga for the cyclin A/CDK2 purification protocol, G. Hannon and D. Beach for Skp2 cDNA and antibodies, members of the S. Lowe lab for advice with the DNA damage experiments, P. Wendel and K. Cronin for technical assistance, W. Herr for helpful suggestions, and A. Losada for critically reading the manuscript. This work was supported by grant CA13106 from the National Cancer Institute. J.M. was the recipient of a postdoctoral fellowship from the U.S. Department of Defense (Breast Cancer Program). W.P.T. is a Leukemia and Lymphoma Society Scholar. This paper is dedicated to our friend and colleague Dr. Kaetrin Simpson, who was involved in human ORC research at Cold Spring Harbor Laboratory until her death due to cancer in January, 2000.

Received May 23, 2001; revised January 14, 2002.

### References

Abdurashidova, G., Riva, S., Biamonti, G., Giacca, M., and Falaschi, A. (1998). Cell cycle modulation of protein-DNA interactions at a human replication origin. EMBO J. *17*, 2961–2969.

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. (1997). Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. Cell *91*, 59–69.

Asano, M., and Wharton, R.P. (1999). E2F mediates developmental and cell cycle regulation of ORC1 in Drosophila. EMBO J. *18*, 2435–2448.

Bell, S.P., Mitchell, J., Leber, J., Kobayashi, R., and Stillman, B. (1995). The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell *83*, 563–568.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Cimbora, D.M., and Groudine, M. (2001). The control of mammalian DNA replication: a brief history of space and timing. Cell *104*, 643–646.

Coverley, D., Pelizon, C., Trewick, S., and Laskey, R.A. (2000). Chromatin-bound Cdc6 persists in S and G2 phases in human cells, while soluble Cdc6 is destroyed in a cyclin A-cdk2 dependent process. J. Cell Sci. *113*, 1929–1938.

Dhar, S.K., and Dutta, A. (2000). Identification and characterization of the human ORC6 homolog. J. Biol. Chem. *275*, 34983–34988.

Dimitrova, D.S., and Gilbert, D.M. (1999). The spatial position and replication timing of chromosomal domains are both established in early G1 phase. Mol. Cell *4*, 983–993.

Dutta, A., and Bell, S.P. (1997). Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell Dev. Biol. *13*, 293–332.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Furstenthal, L., Kaiser, B.K., Swanson, C., and Jackson, P.K. (2001a). Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. J. Cell Biol. *152*, 1267–1278.

Furstenthal, L., Swanson, C., Kaiser, B.K., Eldridge, A.G., and Jackson, P.K. (2001b). Triggering ubiquitination of a CDK inhibitor at origins of DNA replication. Nat. Cell Biol. *3*, 715–722.

Gavin, K.A., Hidaka, M., and Stillman, B. (1995). Conserved initiator proteins in eukaryotes. Science *270*, 1667–1671.

Gilbert, D.M. (2001). Making sense of eukaryotic DNA replication origins. Science *294*, 96–100.

Gstaiger, M., Jordan, R., Lim, M., Catzavelos, C., Mestan, J., Slingerland, J., and Krek, W. (2001). Skp2 is oncogenic and overexpressed in human cancers. Proc. Natl. Acad. Sci. USA *98*, 5043–5048.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hua, X.H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. J. Cell Biol. *140*, 271–281.

Iizuka, M., and Stillman, B. (1999). Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J. Biol. Chem. *274*, 23027–23034.

Ishiai, M., Dean, F.B., Okumura, K., Abe, M., Moon, K.Y., Amin, A.A., Kagotani, K., Taguchi, H., Murakami, Y., Hanaoka, F., et al. (1997). Isolation of human and fission yeast homologues of the budding yeast origin recognition complex subunit ORC5: human homologue (ORC5L) maps to 7q22. Genomics *46*, 294–298.

Jiang, W., Wells, N.J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc. Natl. Acad. Sci. USA *96*, 6193–6198.

Kaelin, W.G., Jr., Pallas, D.C., DeCaprio, J.A., Kaye, F.J., and Livingston, D.M. (1991). Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell *64*, 521–532.

Kelly, T.J., and Brown, G.W. (2000). Regulation of chromosome replication. Annu. Rev. Biochem. *69*, 829–880.

Koepp, D.M., Schaefer, L.K., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. Science *294*, 173–177.

Kreitz, S., Ritzi, M., Baack, M., and Knippers, R. (2001). The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. J. Biol. Chem. *276*, 6337–6342.

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. *11*, 3375–3386.

Liu, J., Smith, C.L., DeRyckere, D., DeAngelis, K., Martin, G.S., and Berger, J.M. (2000). Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. Mol. Cell *6*, 637–648.

Maiorano, D., Moreau, J., and Mechali, M. (2000). XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. Nature *404*, 622–625.

Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. (1999). Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat. Cell Biol. *1*, 14–19.

McGarry, T.J., and Kirschner, M.W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell *93*, 1043–1053.

Méndez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. *20*, 8602–8612.

Mizushima, T., Takahashi, N., and Stillman, B. (2000). Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes Dev. *14*, 1631–1641.

Nakayama, K., Nagahama, H., Minamishima, Y.A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., et al. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. *19*, 2069–2081.

Natale, D.A., Li, C.J., Sun, W.H., and DePamphilis, M.L. (2000). Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals. EMBO J. *19*, 2728–2738.

Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V. (1999). AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. *9*, 27–43.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068–1073.

Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. Nature *404*, 625–628.

Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001). The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. J. Biol. Chem. *276*, 44905–44911.

Ohtani, K., DeGregori, J., Leone, G., Herendeen, D.R., Kelly, T.J., and Nevins, J.R. (1996). Expression of the HsOrc1 gene, a human ORC1 homolog, is regulated by cell proliferation via the E2F transcription factor. Mol. Cell. Biol. *16*, 6977–6984.

Okuno, Y., McNairn, A.J., den Elzen, N., Pines, J., and Gilbert, D.M. (2001). Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. EMBO J. *20*, 4263–4277.

Perkins, G., Drury, L.S., and Diffley, J.F. (2001). Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. EMBO J. *20*, 4836–4845.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J. *18*, 396–410.

Petersen, B.O., Wagener, C., Marinoni, F., Kramer, E.R., Melixetian, M., Denchi, E.L., Gieffers, C., Matteucci, C., Peters, J.M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev. *14*, 2330–2343.

Quintana, D.G., Hou, Z., Thome, K.C., Hendricks, M., Saha, P., and Dutta, A. (1997). Identification of HsORC4, a member of the human origin of replication recognition complex. J. Biol. Chem. *272*, 28247–28251.

Quintana, D.G., Thome, K.C., Hou, Z.H., Ligon, A.H., Morton, C.C., and Dutta, A. (1998). ORC5L, a new member of the human origin recognition complex, is deleted in uterine leiomyomas and malignant myeloid diseases. J. Biol. Chem. *273*, 27137–27145.

Rowles, A., Tada, S., and Blow, J.J. (1999). Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. J. Cell Sci. *112*, 2011–2018.

Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol. Cell. Biol. *18*, 2758–2767.

Salghetti, S.E., Kim, S.Y., and Tansey, W.P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J. *18*, 717–726.

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G., and Tansey, W.P. (2001). Regulation of transcriptional activation domain function by ubiquitin. Science *293*, 1651–1653.

Stillman, B. (1996). Cell cycle control of DNA replication. Science *274*, 1659–1664.

Strohmaier, H., Spruck, C.H., Kaiser, P., Won, K.A., Sangfelt, O., and Reed, S.I. (2001). Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature *413*, 316–322.

Su, T.T., and O'Farrell, P.H. (1997). Chromosome association of minichromosome maintenance proteins in Drosophila mitotic cycles. J. Cell Biol. *139*, 13–21.

Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999). p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. *1*, 207–214.

Tatsumi, Y., Tsurimoto, T., Shirahige, K., Yoshikawa, H., and Obuse, C. (2000). Association of human origin recognition complex 1 with chromatin DNA and nuclease-resistant nuclear structures. J. Biol. Chem. *275*, 5904–5910.

Treier, M., Staszewski, L.M., and Bohmann, D. (1994). Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell *78*, 787–798.

Tugal, T., Zou-Yang, X.H., Gavin, K., Pappin, D., Canas, B., Kobayashi, R., Hunt, T., and Stillman, B. (1998). The Orc4p and Orc5p subunits of the Xenopus and human origin recognition complex are related to Orc1p and Cdc6p. J. Biol. Chem. *273*, 32421–32429.

Vashee, S., Simancek, P., Challberg, M.D., and Kelly, T.J. (2001).

Assembly of the human origin recognition complex. J. Biol. Chem. *276*, 26666–26673.

Williams, R.S., Shohet, R.V., and Stillman, B. (1997). A human protein related to yeast Cdc6p. Proc. Natl. Acad. Sci. USA *94*, 142–147.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science *290*, 2309–2312.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J.R., and Williams, R.S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proc. Natl. Acad. Sci. USA *95*, 3603–3608.

Yanow, S.K., Lygerou, Z., and Nurse, P. (2001). Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. EMBO J. *20*, 4648–4656.

Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995). p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. Cell *82*, 915–925.