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14. ABSTRACT The Origin Recognition Complex (ORC) is thought to function in the loading of replication proteins on the chromatin. In lower organisms, chromatin loading of ORC is required for replication, and thus, cell proliferation. In this funding period we report further progress on previous observations that low levels of Orc2 prevent activation of the Cyclin Dependent Kinase (CDK) cycle. By depleting Orc2 using siRNA, we showed that Cyclin E associated kinase activity was decreased. We have found that we can rescue this inhibition by depleting p27 and p21 along with Orc2. This restores CDK activity, and partially rescues BrDU incorporation. However, these rescued cells show increased apoptosis, indicating that the inhibition caused by Orc2 serves a protective role for the cells. We observed similar effects of low Orc2 levels on the CDK cycle in a stable cell line hypomorphic for Orc2. These cells show difficulty entering S-phase, and also have a Cyclin E associated kinase activity decrease. However, once these cells enter S-phase, cell cycle progression and replication fork firing appear to be normal. This indicates the primary role for Orc2 in mammalian cells may be activation of the CDK cycle.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6-7
References.....	7
Appendices.....	8

Introduction –

One of the hallmarks of human cancer is inappropriate progression through the cell cycle. Under normal circumstances, the cell has numerous checkpoints and mechanisms to ensure that cells do not divide until the genome is fully copied, and that a new cell does not begin copying its DNA until division has finished. DNA replication presents additional challenges, as the process must be tightly coordinated so that the genome is copied accurately, completely, and in a timely fashion.

To this end, cells must recruit a variety of factors to DNA before replication can begin. The first set of proteins to bind the DNA is the Origin Recognition Complex (ORC), a 6 member complex that is thought to recognize the actual replication origins, and then recruit necessary downstream factors. These downstream factors include Cdt1 and Cdc6, which in turn recruit the MCM2-7 complex (mini-chromosome maintenance). Once MCM2-7 (thought to be the replicative helicase) is recruited to DNA, a pre replication complex (pre-RC) is formed, and is ready to initiate S-phase pending CyclinE/cdk2 (cyclin dependent kinase 2) activation(1).

Our lab has been interested in the role the ORC plays in mammalian replication. We have previously reported that Orc2 RNA interference (RNAi), in addition to a decrease in chromatin loading of the pre-Replication complex (pre-RC) and overall replication, also causes a strong G1 arrest, and cyclin/cdk kinase inhibition. We believed this to be due to an increase in levels of the CDK inhibitor p27. This work had interesting implications for breast cancer: loss of this protective mechanism, in which Orc2 is required for cyclin/cdk kinase activity, and thus, cell cycle progression, may allow cells to replicate in the presence of low pre-RC levels, which may lead to genomic instability via a detrimental S-phase. We have further characterized this protective mechanism in breast cancer cells.

We have previously reported that an Orc2 hypomorphic cell line has a marginal effect on replication in a colon cancer cell line(2). While this mutation allows genomic replication, it does not allow replication of an Epstein Barr virus (EBV) plasmid. We hypothesize that while the reduction in Orc2 level was sufficient for EBV replication inhibition, a further, acute decrease in ORC proteins is required to affect cellular genomic replication. This funding cycle we have found that these cells do indeed have a subtle defect entering S-phase, similar to what we observe after Orc2 siRNA. However, once the cells enter S-phase, they proceed normally.

Body –

1. Characterize the membrane-permeable Cy motif containing peptides that selectively inhibit cyclin/cdk complexes.

Previously reported results on ORC and cell cycle were of continued high interest, so we focused on that work this funding period.

2. Investigate the Role of Replication Initiation proteins on growth of breast cancer.

We have previously reported that RNAi against Orc2 results in a G₁ arrest with low Cyclin E/Cdk2 activity. This work has been accepted for publication, and is included in the appendix.(3) Briefly, we found that in addition to p27, p21 levels are also increased. This p21 increase appears to be mediated by transcription, but is independent of p53. p27 on the other hand appears to be stabilized at the protein level. When we deplete these proteins together with Orc2, CDK inhibition is relieved. BrDU incorporation (a measure of replication) is also partly rescued, indicating that the CDK inhibition is causing a cell cycle arrest in response to low levels of Orc2. Finally, cells depleted of p21 and p27 with Orc2 show increased apoptosis, which suggests that the cell cycle inhibition is protecting the cells against a an insult, possibly DNA damage. Therefore, the role of Orc2 in CDK cycle activation seems to protect the cells, perhaps by preventing DNA damage resulting from a poor replication cycle with low Orc and pre-RC loading.

In addition to siRNA, we have used a previously generated Orc2 hypomorph cell line. Results in this system have recently been submitted for publication, and are currently undergoing revisions. This cell line expresses a slightly truncated (but active) form of Orc2 in greatly reduced amounts. We have previously shown that pre-RC loading is drastically decreased in these cells, and they have a proliferation defect. In this funding cycle we have further studied the effects of chronic low Orc2 levels on the cell cycle and on chromosomal replication.

Since the cell cycle is prolonged in the Orc2 hypomorph ($\Delta/-$) cells, we looked for checkpoint activation that may be arresting the cell cycle. We found that Chk2 was phosphorylated on threonine 68 in the $\Delta/-$ cells (Fig. 1a). Although Chk2 can target the Cdc25 family of proteins (phosphatases that activate CDKs), we saw no evidence of this pathway being stimulated. Cdc25c was not phosphorylated in $\Delta/-$ cells, nor was Cdc25A degraded. (Fig 1b.) These proteins usually act to remove inhibitory phosphates from CDK proteins. We examined inhibitory phosphorylation of CDK2, and found that phosphorylation was actually decreased (opposite what we would have expected if Cdc25a was being inhibited by Chk2.) (Fig 1c.) Another important target of Chk2 is p53. We have previously reported that p53 levels are undetectable in this cell line, at both message and protein levels. Although p53 loss was not caused by Orc2 decrease (p53 was lost during the cre-recombination stage, at which point Orc2 protein levels were still normal), the loss of p53 may have allowed the cells to survive in the presence of active Chk2.

Chk2 is usually phosphorylated by ATM (4) (and some cases by ATR). We used siRNA to deplete cells of ATM/ATR, and found that this decreased the level of Chk2 phosphorylation. (Fig 1d). As ATM/ATR are activated by forms of DNA damage, it is possible the $\Delta/-$ cells have higher levels of DNA damage due to low levels of Orc2.

We have previously observed an S-phase decrease in the $\Delta/-$ cells. To study the dynamics of nucleotide incorporation in these cells, we arrested them in nocodazole, and released them. At different timepoints we pulsed with radiolabeled thymidine, and assayed the amount of nucleotide incorporation. We found that the levels of incorporation are significantly less in the $\Delta/-$ cells. (Fig. 2a) The length of G_1 can be determined by the time it takes to reach half maximum incorporation. The $\Delta/-$ cells are also slightly delayed in entering S-phase.

The decrease in nucleotide incorporation could be caused by either difficulty during S-phase, or a failure in S-phase entry. We addressed this by assaying BrdU incorporation on a per-cell basis after nocodazole release using FACS analysis. The $\Delta/-$ cells show a distinct failure to increase the number of BrdU incorporating cells. (Fig. 2b) It appears that the majority of these cells do not enter S-phase. However, if cells are released from S-phase using thymidine/aphidicolin, they are able to incorporate nucleotide normally, which suggests that the cells have trouble moving from G_1 to S, but replicate normally once in S-phase.

Normal progression through G_1 is marked by phosphorylation of Rb, which relieves inhibition of E2F, a transcription factor responsible for upregulating many genes important for cell cycle progression. The $\Delta/-$ cells showed hypo-phosphorylated Rb after release from nocodazole. Further investigation revealed that Cdk2 kinase activity was also significantly decreased in $\Delta/-$ cells. (Fig. 3a) This failure to activate Cdk2 kinase activity could explain the defect in S-phase entry, as Cdk2 kinase activity is required for this transition. When cells are released from an S-phase arrest, Rb is phosphorylated normally, further supporting the notion that once cells can enter S-phase, they are able to proceed normally through the cell cycle. (Fig. 3b)

One of the main regulators of CDK activity is the Cyclin binding partner. In G_1 , Cyclin E levels increase, allowing increased Cyclin E/Cdk2 activity. However, Cyclin E levels in $\Delta/-$ cells do not increase as well as controls, which is likely the cause of low Cdk2 activity. (Fig. 3c). Cyclin E message levels at 3 and 6 hours are very similar to wild type cells, indicating this difference is probably post-transcriptional. (Fig. 3d) (The mRNA differences at 9 and 12 hours

are most likely due to increased E2F activity in the wild type cells, which is caused by Rb inactivation via phosphorylation.) Cyclin E levels could be low due to increased degradation. However, we did not see any decreased Cyclin E stability in the $\Delta/-$ cells, nor did we see any rescue of protein levels followed by inhibition of the proteasome using MG-132. (Fig. 4a,b) These results suggest that there is a decrease in translation of Cyclin E, although this remains to be tested.

We find it interesting that once $\Delta/-$ cells can enter S-phase, they are able to proceed through the cell cycle, even though Orc2 and pre-RC levels are low. We used DNA combing to further examine origin firing in these cells. Surprisingly, fork density is similar between the cell lines. (Fig. 5a) Additionally, replication progression is similar between $\Delta/-$ and wild type cells. (Fig. 5b) This suggests that pre-RC loading occurs in vast excess of amounts required for S-phase, supporting the earlier “Jesuit” model of origin firing in which many origins are called, but few are chosen. (5)

3. Increase my knowledge about the biology of breast cancer.

I have continued to learn much about the mechanics of breast cancer. I have read many papers, and attended many seminars dealing with the diseases. In addition, I will attend the Cold Spring Harbor “Eukaryotic DNA replication” meeting in September. As breast cancer and replication (specifically DNA damage repair) are often linked, this meeting will be very informative.

Key Research Accomplishments –

- Orc2 siRNA results in p21 and p27 protein increase.
- Reducing p21, p27 and Orc2 rescues Cdk2 inhibition and partially rescues BrDU incorporation.
- Reducing p21, p27 and Orc2 results in increased apoptosis.
- A checkpoint is active in Orc2 $\Delta/-$ cells, but may not be effective in halting the cell cycle.
- Orc2 $\Delta/-$ have less nucleotide incorporation, due to a large population of cells failing to enter S-phase.
- $\Delta/-$ cells show hypophosphorylated Rb in G_1 as well as reduced Cdk2 activity.
- Cyclin E levels are low in $\Delta/-$ cells in G_1 , although this decrease is not transcriptional or due to altered stability.
- Once $\Delta/-$ cells can enter S-phase, they appear to be able to proceed normally through the cell cycle, and appear to have normal replication fork dynamics.

Reportable Outcomes –

Investigate the Role of Replication Initiation Proteins on growth of Breast Cancer

1. The Orc2 siRNA results have recently been published in the Journal of Biological Chemistry (3).
2. Both stories will be presented as a research talk at the Cold Spring Harbor “Eukaryotic DNA Replication” meeting on Sept. 9-11, 2005.

Conclusions –

We have now observed a novel role for Orc2 in both breast cancer cells and colon cancer cells. Orc2 is not only required for pre-RC formation and replication, but it seems to be required for activation of Cyclin E/Cdk2 kinase, and therefore, the CDK cycle. In breast cancer cells, Orc2 siRNA reduces Orc2 level, and results in Cyclin E/Cdk2 kinase inhibition, which causes a G_1 arrest, preventing further cell cycling. This arrest can be overridden by co-silencing of p21

and p27 together with Orc2. Although these cells are no longer arrested in G₁, they show evidence of apoptosis. Under conditions of low Orc2, cells may suffer DNA damage during replication. However, the cell cycle inhibition caused by low Orc2 levels prevents cells from entering a potentially damaging period. This requirement for Orc2 in CDK cycle progression therefore may serve to protect cells, and prevent S-phase entry until Orc2, and thus pre-RC, levels are sufficient.

We have previously generated an Orc2 hypomorph cell line in colon cancer cells. We returned to this system to further study the role of Orc2 in CDK cycle progression. We found that although these cells are able to proliferate and survive, there is strong evidence that the same requirement for Orc2 exists. When cells are synchronized in G₁, we observe a failure to activate Cyclin E/Cdk2, which results in a large number of cells failing to enter S. Those cells that do enter S seem to proceed normally, despite low levels of Orc2 and pre-RC. This indicates that the primary role of Orc2 may be CDK cycle activation, which could serve to prevent replication with low pre-RC loading. When Orc2 levels are low, these cells have an activated DNA damage checkpoint, but it appears as if the checkpoint signal transduction is non-functional, as p53 is not expressed in these cells. This absence of p53 is likely important for the survival of these cells despite the active checkpoint. It is also interesting that a subpopulation of the cells is able to survive despite kinase inhibition. This could be due to higher basal levels of Cyclin E in the colon cancer cells. Although kinase activity is reduced, there may be enough residual Cyclin E/Cdk2 activity to allow some cells to enter S-phase, and continue to proliferate.

These results illustrate that breast cancer cells with normal levels of Cyclin E depend upon Orc2 for CDK cycle progression, and thus, cell cycle progression. However, our results suggest that if Cyclin E levels were elevated (as can happen in cancerous cells), this protective mechanism would no longer function to arrest the cell cycle. Although the cells may at that time activate a DNA damage checkpoint, mutations in such pathways are common (although the line we used is p53 wild type, several breast cancer cell lines are mutant for p53.) Without the DNA damage checkpoints, together with possible additional damage from low Orc2 replication, the mutagenic potential for such cells would be greatly increased, potentially leading to more dangerous forms of cancer.

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Appendix

Figure Legends –

Figure 1: (a,b) Western blots examining the Chk2 mediated checkpoint pathway. (c) IP for Cdk2 followed by Western. (d) Western blot after control or ATM and ATR co-siRNA.

Figure 2: (a) Measure of 3H thymidine incorporation after release from nocodazole arrest. (b,c) Percentage of cells incorporating BrDU after release from nocodazole (b) or thymidine/aphidicolin (c) using FACS.

Figure 3: (a,b,c) Western blots after release from indicated drug, and kinase assay (a, center). (d) RT-PCR indicating levels of Cyclin E mRNA.

Figure 4: Western blots after release from nocodazole arrest. (a) Cycloheximide was added 6 hours after release, and cells were harvested at the indicated time after addition. (b) MG-132 added 2 hours after release, and cells were harvested 6 hours after that.

Figure 5: Cells were released from thymidine/aphidicolin arrest, and genomic DNA was prepared and combed. (a) Fork density, as determined by forks per total DNA. (b) Replication progression as determined by replicated DNA divided by total DNA.

Figure 1.

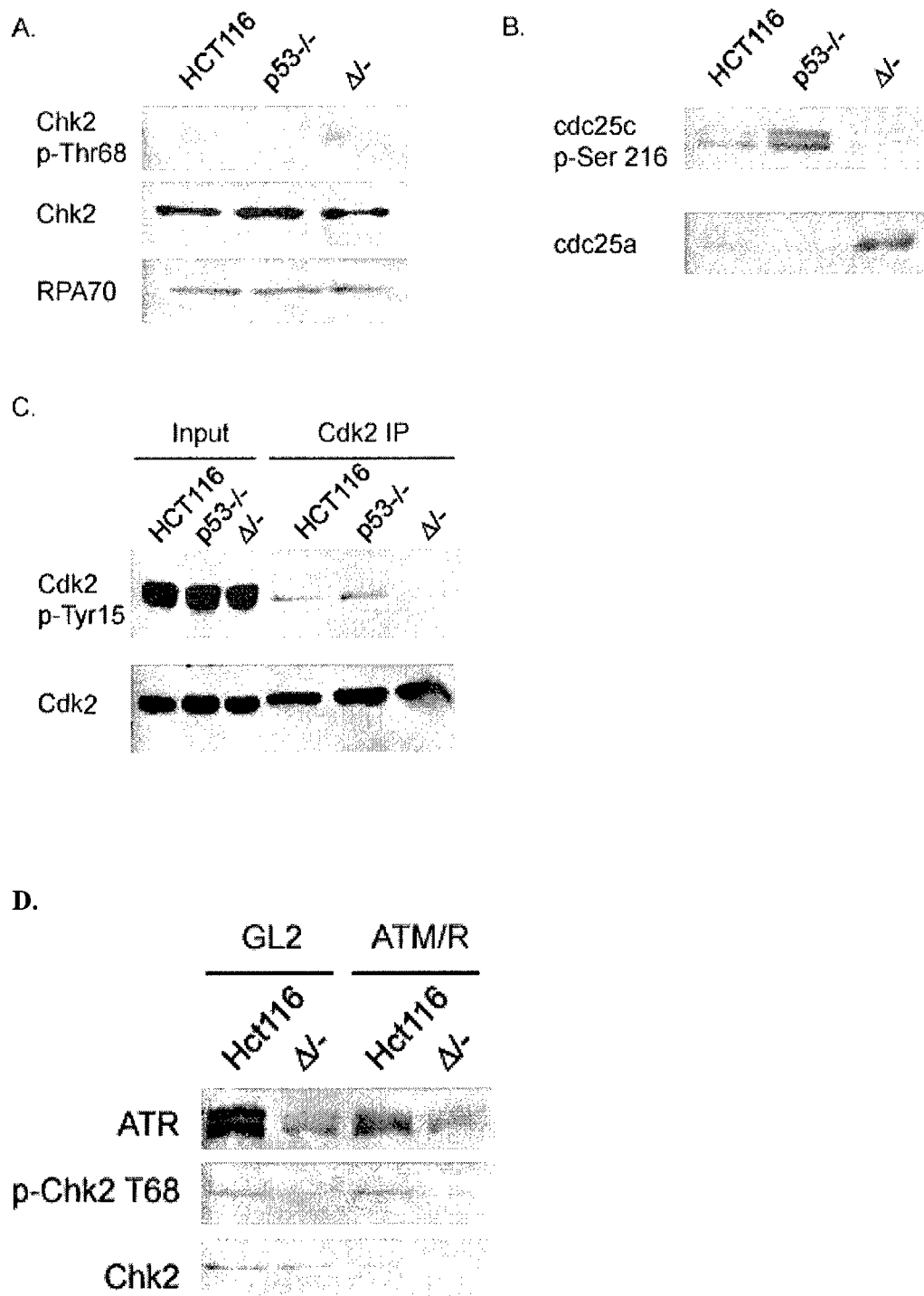
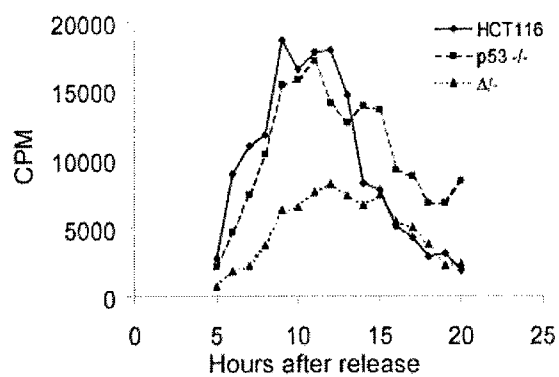
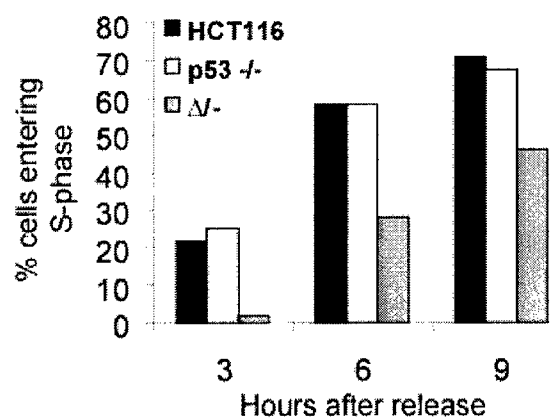


Figure 2.

A.



B.



C.

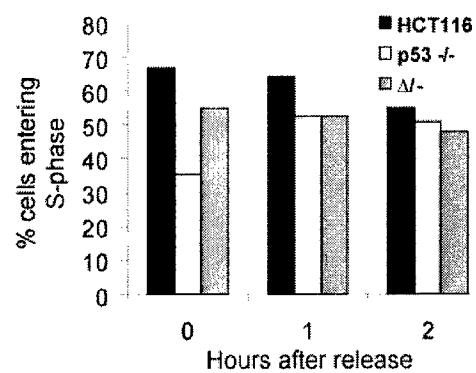
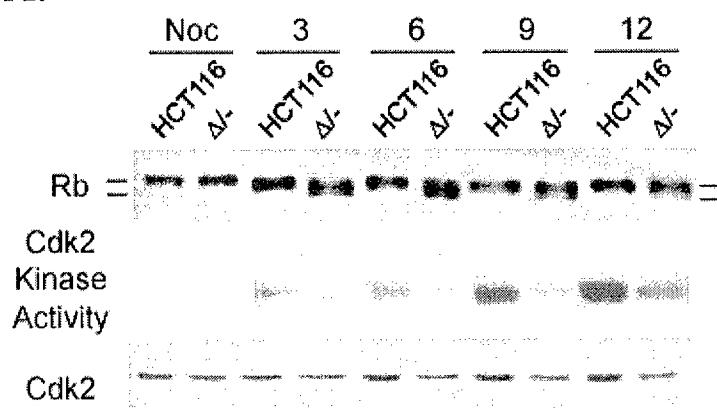
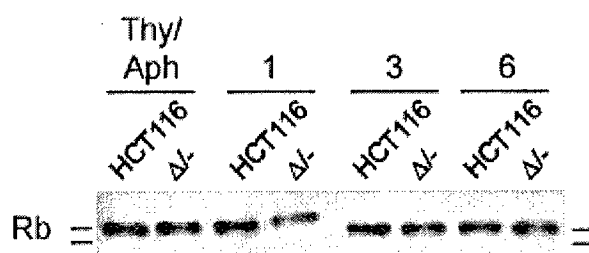


Figure 3

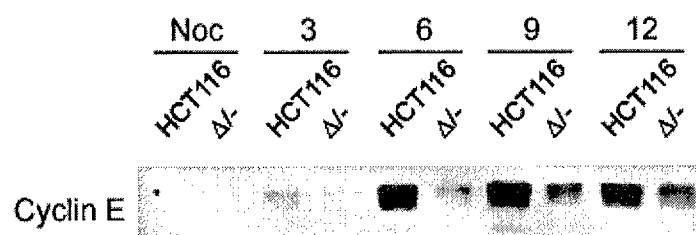
A.



B.



C.



D.

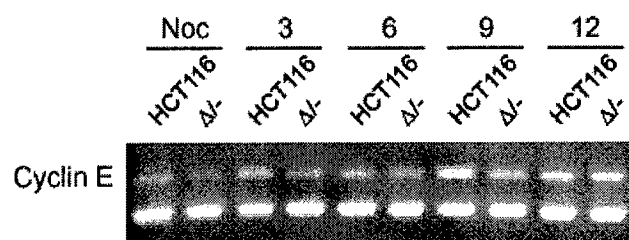
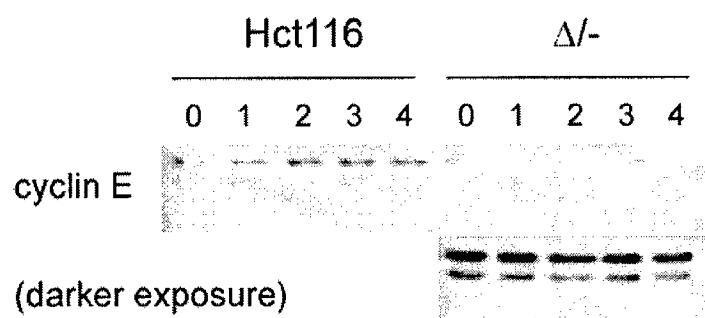


Figure 4

A.



B.

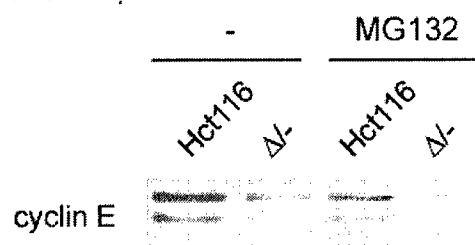
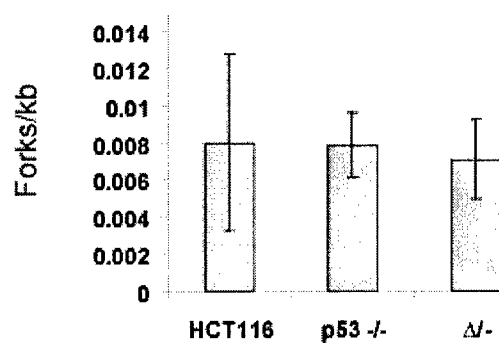
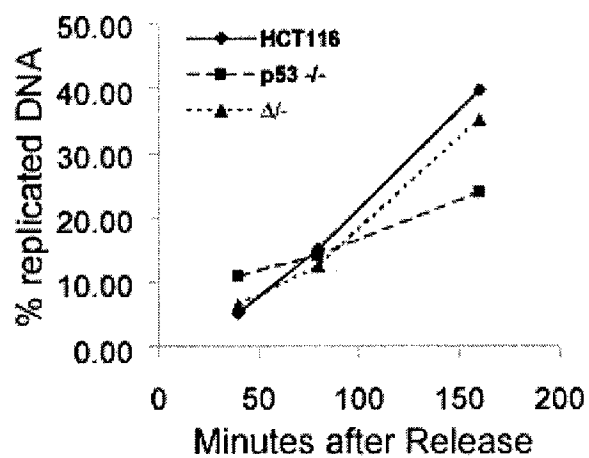


Figure 5

A.



B.



Acute Reduction of an Origin Recognition Complex (ORC) Subunit in Human Cells Reveals a Requirement of ORC for Cdk2 Activation*

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The origin recognition complex (ORC) is involved in formation of prereplicative complexes (pre-RCs) on replication origins in the G₁ phase. At the G₁/S transition, elevated cyclin E-CDK2 activity triggers DNA replication to enter S phase. The CDK cycle works as an engine that drives progression of cell cycle events by successive activation of different types of cyclin-CDK. However, how the CDK cycle is coordinated with replication initiation remains elusive. Here we report that acute depletion of ORC2 by RNA interference (RNAi) arrests cells with low cyclin E-CDK2 activity. This result suggests that loss of a replication initiation protein prevents progression of the CDK cycle in G₁. p27 and p21 proteins accumulate following ORC2 RNAi and are required for the CDK2 inhibition. Restoration of CDK activity by co-depletion of p27 and p21 allows many ORC2-depleted cells to enter S phase and go on to mitosis. However, in some cells the release of the CDK2 block caused catastrophic events like apoptosis. Therefore, the CDK2 inhibition observed following ORC2 RNAi seems to protect cells from premature S phase entry and crisis in DNA replication. These results demonstrate an unexpected role of ORC2 in CDK2 activation, a linkage that could be important for maintaining genomic stability.

The CDK cycle governs the sequence of cell cycle events by successively phosphorylating proteins that trigger essential processes of cell proliferation such as DNA replication and mitosis (1–3). In addition to binding to cyclins, the activity of CDK is regulated by its phosphorylation. Removal of the inhibitory phosphorylation of Thr¹⁴ and Tyr¹⁵ of CDK by Cdc25 phosphatase and phosphorylation of the T-loop by CDK-activating kinase are also required for CDK2 activity (4). Activities of G₁ and S phase cyclin-CDK complexes are also regulated by binding to CDK inhibitor proteins such as p27 and p21 (5). p27 protein levels are high in G₀ and G₁ phases to maintain low CDK activity. Proteasome-mediated degradation following ubiquitinylation by SCF^{Skp2} complex is responsible for the low levels of p27 protein during S-G₂ phases (6). p27 is also ubiquitinated by KPC1 and degraded by proteasome upon cell cycle entry (7). On the other hand, p21 plays an important role

in cell cycle arrest after DNA damage. p53 activation by checkpoint pathways induces p21 transcription to inhibit cell cycle progression, but there are several p53-independent pathways for inducing p21 mRNA (8).

Initiation of DNA replication first requires the assembly of prereplicative complexes (pre-RCs)¹ at origins of DNA replication during the G₁ phase (9). Origin recognition complex (ORC) is a six-subunit complex required to mark the origins of DNA replication. In late M and early G₁ phases, CDC6 and CDT1 are recruited to origins in an ORC-dependent fashion followed by chromatin-loading of MCM2–7 complexes to form pre-RCs (10). Elevated activity of CDK2 at G₁/S promotes loading of initiation proteins such as CDC45 on origins to initiate DNA replication (9). Activation of cyclin E-CDK2 is believed to be independent of the amount of replication initiator proteins. In this paper, however, we found that depletion of one of the replication initiation proteins results in G₁ arrest with low cyclin E-CDK2 activity. Cell cycle progression was halted by inhibition of cyclin E-CDK2 by p27 and p21 accumulation. These results suggest a requirement of replication proteins for CDK2 activation and provide a link between CDK2 and initiation proteins that couples cell cycle progression to replication initiation.

EXPERIMENTAL PROCEDURES

Cell Culture and RNAi—Cells were cultured under standard growth condition. For siRNA transfection, cells were grown at ~50% confluence in a 6-well plate and transfected at 24-h intervals with 0.24 nmol of annealed siRNA duplex (Dharmacon Research) using Oligofectamine (Invitrogen). Six hours after the first transfection, cells were split 1:2 to maintain the cells in log phase. Target sequences of oligonucleotides used were as follows: ORC2-A, AAGAAGGAGCGAGCGCAGCUU; ORC2-B, GAUCAGCUAGACUGGAUAGUA; p27-A, AAGGUUGCAUACUGACCAAG; p21-A, AACAUACUGGCCUGGACUGUU; p53-A, AAGACUCCAGUGGUAUUCUAC; control, AACGUACGCGGAUACUUCGA.

Western and Northern Blotting—ORC3, ORC4, and ORC6 antibodies were described earlier (11–13). Rabbit antibodies against ORC1 and ORC5 were raised using His₆-tagged recombinant proteins, ORC1 (220–831) and ORC5 (75–686). Anti-p27 (C-19), anti-p21 (C-19), anti-MCM7 (141.2), anti-cyclin E (HE12), anti-cyclin A (H-432), anti-cyclin B (H-433), and anti-CDK2 (M2) antibodies were purchased from Santa Cruz Biotechnology. Anti-ORC2 and anti-β-actin antibodies were purchased from BD Biosciences and Sigma, respectively. Anti-p53 antibody was obtained from Cell signaling technology. Anti-pRB antibody was the kind gift of Dr. E. Harlow. Cellular total RNA was purified using the RNeasy Midi kit (Qiagen).

Immunoprecipitation and Kinase Assay—Cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50

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¹ The abbreviations used are: pre-RC, prereplicative complex; Br-dUrd, 5-bromo-2'-deoxyuridine; CDK, cyclin-dependent kinase; ORC, origin recognition complex; RNAi, RNA interference; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; TRITC, tetramethylrhodamine isothiocyanate; MCM, minichromosome maintenance.

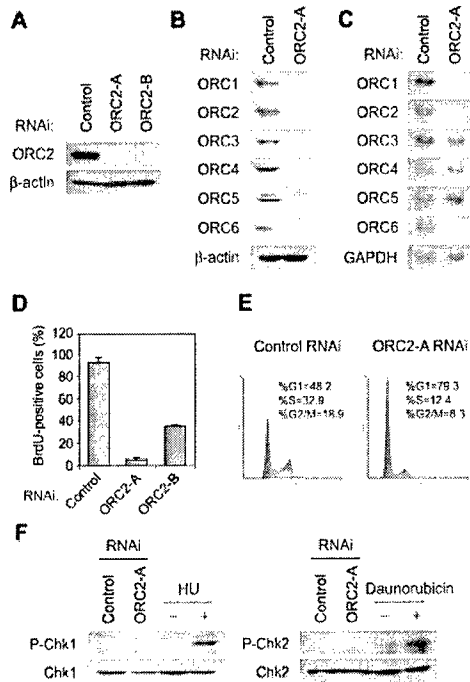


FIG. 1. ORC2 RNAi reduces other ORC subunits and decreases DNA replication. A, ORC2 levels after RNAi targeting ORC2. MCF10A cells were transfected with control or two different siRNA oligonucleotides targeting ORC2. Cell lysates were prepared after 72 h and analyzed by Western blotting with anti-ORC2 antibody. B, protein levels of ORC1–6 following ORC2-A RNAi. MCF10A cells were transfected with control or ORC2-A siRNAs for 72 h and expression of all six ORC subunits assessed by Western blotting. The blot was also probed with anti- β -actin antibody as a loading control. C, messenger RNA levels of ORC1–6 following ORC2-A RNAi. MCF10A cells were transfected with control or ORC2-A siRNAs for 72 h and mRNA levels of six ORC subunits analyzed by Northern blotting. The blot was also probed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. D, percentage of BrdUrd-positive cells after transfection of indicated siRNA oligonucleotides. MCF10A cells transfected with control and ORC2-A siRNAs for 72 h were treated with 10 μ M BrdUrd for the last 24 h of RNAi transfection and stained with 4',6-diamidino-2-phenylindole dihydrochloride and anti-BrdUrd antibody. BrdUrd-positive cells were counted. E, FACS analysis of ORC2-depleted cells. MCF10A cells were transfected with control and ORC2-A siRNAs. Cells were stained with propidium iodide after 72 h and then analyzed by flow cytometer. F, besides transfection with control or ORC2-A siRNAs, cells were also cultured with or without 10 mM hydroxyurea (HU) or 0.5 μ M daunorubicin for 5 h. Cell extracts were analyzed by Western blotting with antibodies against indicated proteins.

mm NaF, 1 mM Na_2VO_4 , and protease inhibitor mixture (Sigma). For kinase assay, equal amounts of cell lysates (100 μ g) were immunoprecipitated with anti-CDK4 (C-22), anti-cyclin E (HE111), anti-cyclin A (H-432), or anti-cyclin B1 (H-433) antibodies (Santa Cruz Biotechnology). *In vitro* kinase assay was carried out using recombinant pRB-C and histone H1 as substrates as described previously (14).

BrdUrd Immunostaining and FACS Analyses—Cells were treated with 10 μ M BrdUrd (Sigma), fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained with anti-BrdUrd antibody containing nuclease (Amersham Biosciences) diluted in 3% bovine serum albumin (1:2) for 45 min at room temperature. After rinsing with phosphate-buffered saline three times, cells were incubated with TRITC-conjugated anti-mouse IgG antibody (Pierce) diluted in 3% bovine serum albumin (1:500). Percentage of cells incorporating BrdUrd was calculated as an average of three samples. FACS analysis for DNA content was performed as described (15). Percentages of cells in G_1 , S, and G_2/M phases were calculated using ModFit.

RESULTS

DNA Synthesis Is Reduced in ORC2-depleted Cells—ORC2 is a part of the core of the origin recognition complex (15). Upon RNAi targeting ORC2 in MCF10A breast epithelial cells, ORC2 protein levels were decreased (Fig. 1A). Two siRNA oligonu-

cleotides against different parts of the ORC2 mRNA were used to guard against off-target activity. ORC2-A can reduce ORC2 protein more efficiently than ORC2-B. ORC2 RNAi also decreased levels of ORC1 and ORC3–6 proteins after 72 h (Fig. 1B). Messenger RNA levels of ORC3, ORC4, and ORC5 were unchanged in ORC2-depleted cells (Fig. 1C), suggesting that the other components of the ORC core subcomplex, ORC3–5, are unstable in the absence of ORC2 protein. This result is consistent with our previous result that ORC3 protein is decreased without change in mRNA level in an ORC2 hypomorphic mutant cell line (16). In contrast, the mRNA levels of ORC1 and ORC6 decreased significantly in ORC2-depleted cells. Since ORC1 is regulated by E2F (17), the decrease in ORC1 mRNA might be caused indirectly by the inhibition of CDK activity that we describe below. On the other hand, E2F-mediated regulation of ORC6 promoter has not been reported, and the genomic sequence upstream from the start site of the cDNA did not show any obvious E2F binding site (13).

Next, we tested whether ORC2 is decreased enough to impair DNA replication. We examined DNA synthesis after ORC2 RNAi. BrdUrd-positive cells were reduced by around 90% in cells treated with ORC2-A siRNA oligonucleotide (Fig. 1D). ORC2-B, which reduced ORC2 protein less efficiently, showed less effect on BrdUrd incorporation. Cells with G_1 DNA content accumulated and those with S phase DNA content decreased after 72-h treatment with ORC2 RNAi (Fig. 1E). Collectively these results indicate that depletion of ORC2 decreases several other ORC subunits and promotes the accumulation of cells with unreplicated DNA.

ORC2 Depletion Does Not Activate DNA Replication or DNA Damage Checkpoints—To test whether DNA damage or DNA replication checkpoint pathways were activated upon acute depletion of ORC2, phosphorylation of Chk1 on Ser³¹⁷ and Chk2 on Thr⁶⁸ were examined (Fig. 1F). Those residues are phosphorylated after activation of DNA replication or DNA damage checkpoints. Hydroxyurea-treated cells and daunorubicin-treated cells were used as a positive control for the activation of the two checkpoints. The results indicate that ORC2 depletion does not activate DNA replication or DNA damage checkpoints.

Cyclin E-associated Kinase Activity Is Inhibited in ORC2-depleted Cells—Activation of cyclin E-CDK2 at G_1/S was expected not to be affected by loss of an initiator protein. Surprisingly, *in vitro* kinase assays with immunoprecipitated proteins revealed that cyclin E-associated kinase was inhibited after 36-h treatment with ORC2-A RNAi (Fig. 2A). RNAi against p53 did not have such an effect, suggesting that the inhibition of cyclin E-CDK2 activity is not a nonspecific effect due to activation of RNA interference pathway. After 72 h, ORC2-depleted cells accumulate with 2N DNA content in the FACS analysis (Fig. 1E). We therefore examined where the CDK cycle is arrested in those cells. Examination of cyclin levels by immunoblotting showed that the cells are arrested with high cyclin D1 and cyclin E levels (Fig. 2B). *In vitro* kinase assays revealed that, although CDK4 kinase activity was unchanged, cyclin E-CDK2 activity is inhibited in these cells (Fig. 2C). Inhibition of cyclin E-CDK2 activity would account for the cyclin E accumulation seen in Fig. 2B due to a decrease in its autophosphorylation and ubiquitinylation (18, 19). Considering the accumulation of cyclin E protein, the decrease in the specific activity of cyclin E-associated kinase was even greater than the 70% seen in Fig. 2C. The increase of cyclin D1 protein after 72 h could be due to arrest of the CDK cycle in G_1 phase where the level of the protein is high. In contrast to G_1 cyclins, cyclin A and cyclin B1 levels were low in ORC2-depleted cells after 72 h (Fig. 2B), accounting for the low kinase activity in the

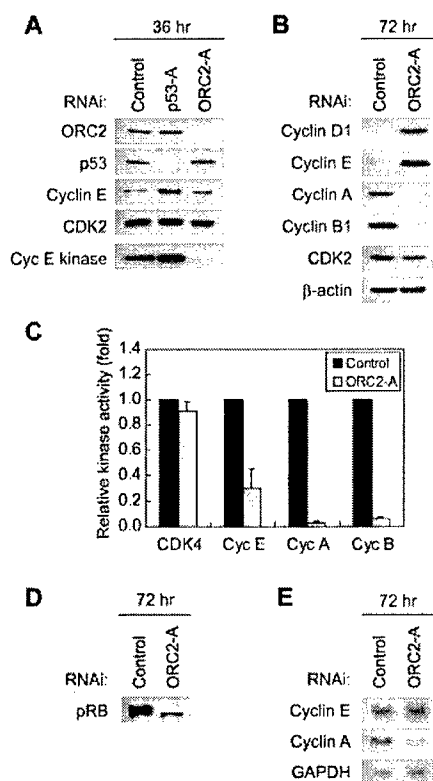


FIG. 2. Cyclin (Cyc) E-associated kinase activity is inhibited in ORC2-depleted cells. MCF10A cells were transfected with indicated siRNA oligos for the indicated time. **A**, cyclin E-associated kinase activity after RNAi with indicated siRNAs. Extracts were prepared from cells after 36 h and used for *in vitro* kinase assay using anti-cyclin E antibody and Western blot for indicated proteins. **B**, cyclin levels after ORC2-A RNAi (72 h). Cell extracts were prepared and analyzed by Western blotting for indicated proteins. **C**, kinase activities of cyclin-CDKs after ORC2-A RNAi (72 h). Equal amounts of cell extracts (100 μ g of proteins) were subjected to an *in vitro* kinase assay using antibodies against indicated proteins. Three independent *in vitro* kinase assays were quantitated by a PhosphorImager, and the average is shown. **D**, phosphorylation status of pRB in ORC2-depleted cells. Extracts of the cells transfected with indicated siRNAs (72 h) were analyzed by Western blotting for pRB. **E**, messenger RNA levels of cyclin E and cyclin A after ORC2-A RNAi (72 h). Total RNA was extracted, and expression of the indicated genes was analyzed by Northern blotting. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

in vitro kinase assay using anti-cyclin A and anti-cyclin B1 antibodies (Fig. 2C).

pRB was hypophosphorylated in cells transfected with ORC2 siRNAs, confirming that CDK2 is inhibited *in vivo* (Fig. 2D). The hypophosphorylated form of pRB binds E2F and works as a co-suppressor for E2F-regulated genes (20). It was reported that partial phosphorylation of pRB by cyclin D-CDK4/6 relieves the suppression of cyclin E promoter, allowing cyclin E expression. Consistent with the unchanged activity of cyclin D-CDK4, cyclin E mRNA was not decreased in ORC2 RNAi-treated cells (Fig. 2E). Further phosphorylation of pRB by cyclin E-CDK2 is required for relieving cyclin A promoter suppression by pRB (21, 22). Decrease in cyclin E-CDK2 activity therefore explains the decrease in cyclin A mRNA, cyclin A and B1 protein levels, and kinase activities (Fig. 2, B, C, and E). In summary, the CDK cycle is inhibited at the step of cyclin E-CDK2 activation in ORC2-depleted cells.

ORC2 Depletion Results in Accumulation of CDK Inhibitors p27 and p21—We next explored how cyclin E-CDK2 activity is inhibited in ORC2-depleted cells. Two CDK inhibitors, p27 and p21, were accumulated in MCF10A cells after decreasing ORC2 level by two different siRNA oligonucleotides (Fig. 3A). To

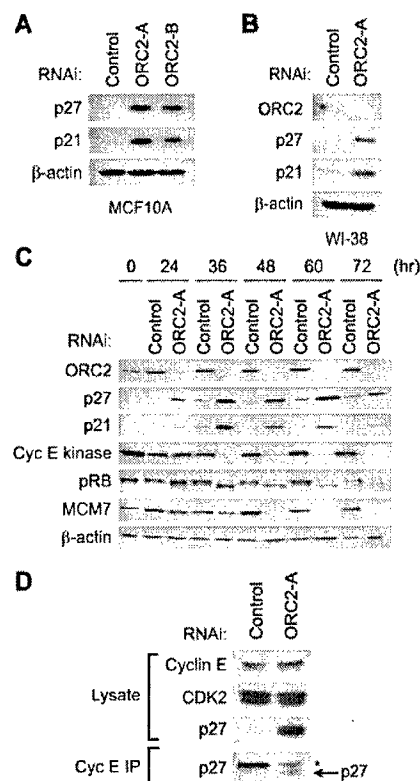


FIG. 3. p27 and p21 accumulate after ORC2 depletion. **A**, accumulation of p27 and p21 after ORC2 RNAi. MCF10A cells were transfected with the indicated siRNA duplexes for 72 h and p27 and p21 proteins analyzed by Western blotting. **B**, accumulation of p27 and p21 proteins in WI-38 following ORC2-A RNAi. Cells were transfected by control or ORC2-A siRNAs for 72 h and the indicated proteins analyzed by Western blotting. **C**, time course analysis of ORC2, p27, p21, cyclin E-associated kinase activity, pRB, and MCM7 after ORC2 RNAi. MCF10A cells were transfected with control or ORC2-A siRNA oligonucleotides. Cell extracts were prepared at the indicated times and analyzed by Western blotting for indicated proteins. Equal amounts of cell extracts (100 μ g of proteins) were also subjected to an *in vitro* kinase assay using anti-cyclin E antibody. **D**, cyclin E-associated p27 protein in ORC2-depleted cells. Extracts of the MCF10A cells transfected with control and ORC2-A siRNA were prepared after 36 h and subjected to immunoprecipitation (IP) using anti-cyclin E antibody. The amount of the indicated proteins in cell lysates and immunoprecipitation was assessed by Western blotting. The asterisk marks the Ig light chain. *Cyc*, cyclin.

ensure that the p27 and p21 responses were not unique to the breast epithelial cell line MCF10A, ORC2 was depleted in WI-38 primary lung fibroblasts. p27 and p21 accumulation was also observed in WI-38 (Fig. 3B).

Since p27 is targeted for degradation after phosphorylation by cyclin E-CDK2 (23, 24), we wanted to ensure that the p27 accumulation was not a secondary effect of CDK2 inhibition. A time course study was performed to determine whether p27 accumulation preceded or followed cyclin E-CDK2 inhibition. After ORC2-A RNAi p27 starts to accumulate as early as 24 h, in parallel with the decrease in ORC2 protein (Fig. 3C) and before the inhibition of cyclin E-CDK2 kinase activity or hypophosphorylation of RB. After 36 h, p27 levels increase further, and cyclin E-associated kinase activity decreases. At this time point, more p27 is associated with cyclin E (Fig. 3D, lane 2). Therefore, p27 accumulation precedes cyclin E-CDK2 inhibition and could be the cause rather than the effect of CDK2 inhibition. Consistent with the decrease in cyclin E-CDK2 activity, hypophosphorylated pRB appeared at the 36-h time point. MCM7, encoded by an E2F-regulated gene, starts to decrease after 48 h. These results suggest that p27 increase is an early response to ORC2 depletion and is not secondary to

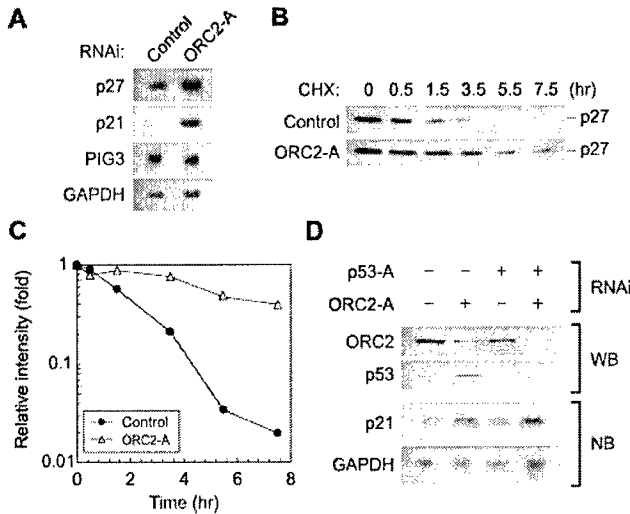


FIG. 4. ORC2 depletion causes p27 protein stabilization and p21 mRNA induction. *A*, MCF10A cells were transfected with control or ORC2-A siRNAs for 36 h and mRNA levels of p27, p21, and PIG3 were analyzed by Northern blotting. Blot was also probed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. *B*, stability of p27 protein following ORC2 depletion. MCF10A cells were transfected with control or ORC2-A siRNAs for 26 h. Cells were pretreated with cycloheximide (*CHX*; 50 μ g/ml) for 30 min, and the treatment was continued until cells were harvested at the indicated time points. p27 levels in proteins from control and ORC2 RNAi-treated cells (75 and 40 μ g, respectively) were examined by Western blotting. The blot for control RNAi was exposed two times longer than the one for ORC2 RNAi to get the same intensity for the p27 protein bands at the 0 h time point. *C*, quantitation of p27 protein levels after cycloheximide treatment. Intensity of p27 bands in *B* was quantitated, and the relative intensity to that seen at the 0 h time point is shown. *D*, p53-independent induction of p21 after ORC2 RNAi. MCF10A cells were transfected with control or p53 siRNAs for 48 h before transfection with control or ORC2-A siRNAs (48 h). Protein levels of ORC2 and p53 and mRNA levels of p21 were assessed by Western and Northern blotting, respectively.

the decrease in CDK2 kinase activity. Once CDK2 kinase activity is inhibited, suppression of E2F by pRB leads to further suppression of other S phase activators like MCM7 and cyclin A.

After ORC2 RNAi the p21 level also starts to increase at 24 h and reaches the maximum at 36 h time point, when cyclin E-CDK2 becomes inactive (Fig. 3C). This is much before the time point when secondary effects of the cell cycle arrest become evident as revealed by MCM decrease at 48 h, suggesting that p21 elevation could also be a cause (rather than effect) of cyclin E-CDK2 inhibition after ORC2 depletion.

p27 Protein Is Stabilized, and p21 mRNA Is Induced after ORC2 Depletion—We next sought the mechanism of p27 and p21 accumulation. p27 mRNA does not increase in the ORC-depleted cells suggesting that regulation of the protein is at the post-transcriptional level (Fig. 4A). After 26-h treatment of ORC2 RNAi, cycloheximide was added to block new protein synthesis and the rate of decrease of p27 protein examined (Fig. 4, B and C). Quantitation of p27 protein levels revealed that the half-life of p27 was increased from 1.4 h to 5.6 h in ORC2 RNAi-treated cells, indicating that p27 is stabilized after ORC2 RNAi.

In contrast to p27, the mRNA level of p21 was increased after 36-h treatment of ORC2 RNAi (Fig. 4A). p53 is a well known inducer of p21 in response to DNA damage. We tested involvement of p53 in the p21 induction by sequential transfection of siRNAs against p53 and ORC2. Although pretreatment of cells with p53 siRNA reduced p53 to lower than basal level, p21 mRNA was still increased by ORC2 RNAi (Fig. 4D). This result suggests that p21 expression is induced by a p53-independent

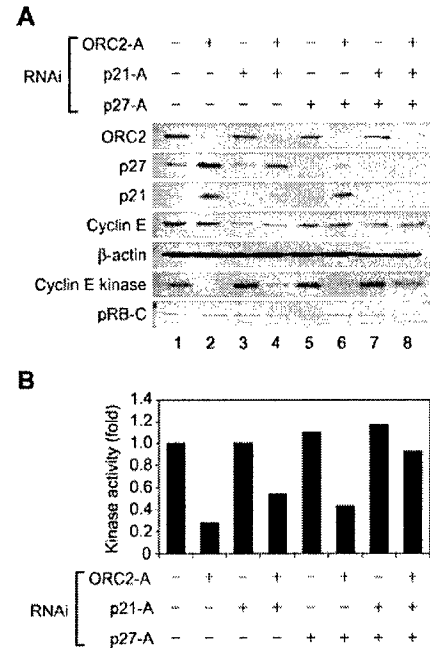


FIG. 5. p21 and p27 are responsible for CDK2 inhibition in ORC2-depleted cells. *A*, transfection of p21 and p27 siRNA oligonucleotides eliminates p21 and p27 accumulation after ORC2 RNAi. MCF10A cells were transfected with p21 and/or p27 siRNAs for 24 h before transfection with control or ORC2-A. After 48 h cell lysates were harvested and immunoblotted for indicated proteins. Equal amounts of cell extracts (100 μ g of proteins) were also used for immunoprecipitation and subjected to an *in vitro* kinase assay using pRB-C as a substrate. Coomassie Brilliant Blue staining of pRB-C protein is shown. *B*, relative levels of *in vitro* cyclin E-CDK2 kinase activity. The 32 P incorporated into the substrate in the *in vitro* kinase assay in *A* was quantitated by a PhosphorImager and was normalized to that seen with control cells treated with control siRNA oligonucleotides.

mechanism. Consistent with the idea that the general p53 pathway is not activated after ORC2 RNAi, another p53-regulated gene, *PIG3* (25), was not induced after ORC2 RNAi (Fig. 4A).

p27 and p21 Inhibit Cyclin E-CDK2 following ORC2 Depletion—To test whether p27 and p21 are responsible for the inhibition of cyclin E-CDK2 following ORC2 depletion, we performed RNAi against p27 and/or p21 prior to ORC2 RNAi. When p27 or p21 alone are depleted, cyclin E-CDK2 activity was only partially restored after ORC2 RNAi (Fig. 5A, lanes 4 and 6). However, when both p27 and p21 are depleted prior to ORC2 RNAi, the cyclin E-CDK2 activity was restored almost to the control level in ORC2-depleted cells (Fig. 5A, lane 8, and Fig. 5B). This result indicates that the induction of p21 and p27 is critical for inhibition of CDK2 after ORC2 depletion.

Inhibition of DNA Synthesis after ORC2 Depletion Is Caused Partly by CDK2 Inhibition—Restoration of cyclin E-CDK2 activity in ORC2-depleted cells by co-depletion of p21 and p27 prompted us to test if CDK2 inhibition contributes to inhibition of DNA synthesis after ORC2 depletion. Cells transfected with p21 and p27 siRNAs were treated with ORC2 RNAi and BrdUrd incorporation measured by immunostaining using anti-BrdUrd antibody. BrdUrd-positive cells decreased by 80% after ORC2 RNAi (Fig. 6A and 6B). Pretreatment with p21 and p27 siRNAs increased BrdUrd-positive cells after ORC2 RNAi, indicating that part of the inhibition of DNA synthesis following ORC2 depletion is caused by CDK2 inhibition but not by decrease in ORC2 protein levels. Furthermore, there is a 15-fold increase in the number of ORC2-depleted cells that enter mitosis when p27 and p21 are removed (Fig. 6C), supporting the notion that some ORC2-depleted cells are arrested by CDK

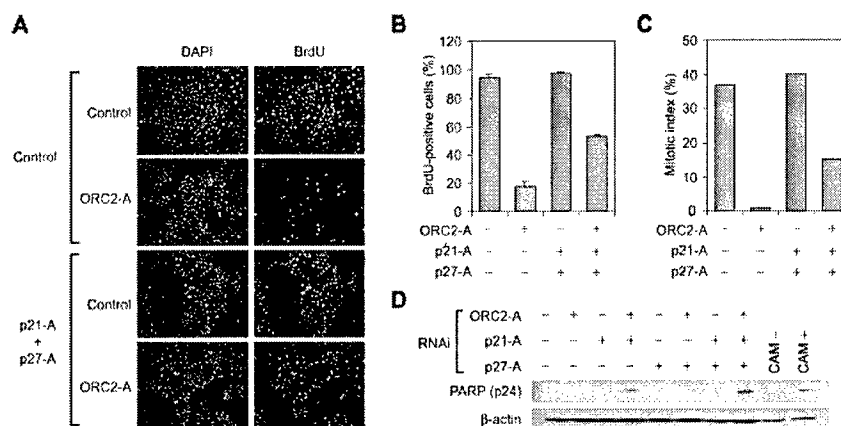


FIG. 6. Inhibition of DNA synthesis following ORC2 depletion is partly caused by CDK2 inhibition. MCF10A cells were transfected with control or p21 and p27 siRNAs for 24 h before transfection with control or ORC2-A siRNAs (48 h). **A**, BrdUrd staining of cells transfected with indicated siRNA oligonucleotides. Cells were incubated with BrdUrd (10 μ M) for 16 h and stained with anti-BrdUrd and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Pictures of the representative fields are shown. **B**, percentage of BrdUrd-positive cells after transfection of indicated siRNA oligos. BrdUrd-positive cells in the experiment in **A** were counted, and the average of three samples is shown. **C**, mitotic entry after transfection of indicated siRNA oligonucleotides. Mitotic cells were trapped by incubation with Nocodazole (40 ng/ml) for 16 h, and the mitotic index is shown. **D**, cell lysates were prepared and analyzed by Western blotting with anti-poly(ADP-ribose) polymerase (PARP) (p24) and anti- β -actin antibodies. Cells were also treated with 2 μ g/ml camptothecin (CAM) for 16 h as a positive control for apoptosis induction.

inhibition rather than inhibition of DNA replication. Therefore, in many cells CDK inhibition is seen at levels of ORC2 that is sufficient for DNA replication. The active CDK inhibition arrests these cells before S phase. Once CDK inhibition is relieved, these cells are actually capable of DNA replication and go into mitosis. We next tested whether apoptosis is induced in the ORC2-depleted cells when the CDK2 inhibition is abrogated. Poly(ADP-ribose) polymerase cleavage is a marker of apoptosis induction (26). Forced progression of ORC2-depleted cells into S phase by p27 and p21 RNAi induced apoptosis (Fig. 6D). Therefore inhibition of CDK2 by p27 and p21 prevents cells from initiating DNA synthesis with low levels of ORC and therefore protects cells from catastrophic events in the S phase.

DISCUSSION

In this study we discovered that progression of the CDK cycle is prevented after depletion of one of the replication initiation proteins, ORC2. ORC2-depleted cells are arrested in G₁ phase through inhibition of cyclin E-CDK2 (Fig. 7). Since initiation of DNA replication defines S phase (S for synthesis), one might say that cells are in G₁ phase when DNA replication is inhibited by ORC2 RNAi. Since CDK activity is low in the G₁ phase, one might therefore jump to the conclusion that CDK activity is low after ORC depletion simply as a reflection of the cell cycle stage. However, this logic is not correct because CDK activation at G₁/S is expected to occur regardless of ORC amount. For example, yeast Orc mutants activate CDK even when replication initiation fails, leading to activation of DNA damage checkpoint pathways or even premature entry into mitosis with unreplicated DNA (27–30). Therefore, the CDK2 inhibition that is seen following ORC2 depletion is unexpected and unique to higher eukaryotes. We suggest that the linkage between ORC function and CDK2 activation has evolved to prevent such premature progression of the mammalian cell cycle.

CDK inhibition following ORC2 RNAi is due to accumulation of p27 and p21. p27 and p21 accumulation is a cause of CDK inhibition rather than a result of G₁ arrest, since knock-down of p27 and p21 restores CDK activity in ORC2-depleted cells. RNAi decreases ORC2 in the entire population of cells to less than 10% of wild type levels, but there are bound to be variations in the extent of depletion between cells in the population. Indeed DNA synthesis was partially restored when CDK activity is recovered in ORC2-depleted cells. In many of the cells, DNA replication was normal enough to allow the cells to pro-

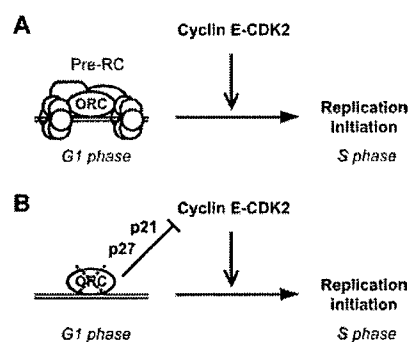


FIG. 7. The CDK cycle is regulated by replication initiators. **A**, in a normal cell cycle, pre-RC formation is completed during the G₁ phase, and cyclin E-CDK2 activation triggers replication initiation and S phase entry. **B**, in ORC2-depleted cells CDK activation at G₁/S is inhibited by both p27 and p21. Accumulation of p27 and p21 proteins could be the active checkpoint mechanism to prevent CDK activation in response to low levels of ORC or pre-RCs. Alternatively, accumulation of these proteins is due to an obligate role of ORC in the regulation of these proteins (see "Discussion" for details).

gress to mitosis, although we cannot distinguish whether the recovered DNA replication is from initiation at new origins or from elongation of pre-existing forks. Given that cancer cells can proliferate with less than 10% of ORC2 levels (16), we are not surprised to discover that many of the cells in the population have sufficient ORC2 for a normal S phase once the CDK cycle is de-repressed. However, apoptosis was observed in some of the cells, suggesting that CDK inhibition in ORC2-depleted cells can prevent catastrophic events due to premature S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of CDK2 activity, suggesting that in these cells the ORC2 levels were genuinely below a threshold sufficient for DNA replication. We think that differences in levels of residual ORC2 in various cells in the population account for the differences in the responses after de-repression of CDK2.

Checkpoint Activation or Obligate Role of ORC in the CDK Cycle—There are two possible interpretations for the inhibition of cyclin E-CDK2 activity in ORC-depleted cells. The first is that ORC is not normally required for CDK2 activation, but abnormally low levels of ORC activate emergency checkpoint pathways that prevent cyclin E-CDK2 activation and arrest the cell cycle in G₁. The second possibility is that ORC has an

obligate role in the normal activation of cyclin E-CDK2 even during the normal cell cycle. In this possibility, ORC-depleted cells fail to activate cyclin E-CDK2 resulting in cell cycle arrest in G₁. We cannot at present distinguish between these possibilities. Dissecting mechanisms linking p21 and p27 accumulation to ORC2 depletion will help determine whether this is an emergency checkpoint mechanism or an obligate role of ORC in p21 and p27 regulation.

Checkpoint-like Mechanism at G₁/S—Cell cycle checkpoint is defined as a mechanism preventing initiation of the next step until the previous step is complete (31). Since activation of cyclin E-CDK2 triggers initiation of DNA replication in the normal situation, the fact that cyclin E-CDK2 is inhibited in response to low levels of ORC seems to fit with the idea of a cell cycle checkpoint. Consistent with this, restoration of CDK2 activity in ORC2-depleted cells by p27 and p21 RNAi lets the cell cycle progress into S phase or induce apoptosis. p21 and p27 are therefore effectors in a checkpoint pathway that prevent initiation of DNA replication until G₁ events dependent on ORC function are complete. Alternatively, the checkpoint pathway might sense the ORC2 level directly. However, since ORC2 protein levels are unchanged during the cell cycle, we think it more likely that failure to complete G₁ events involving ORC is somehow sensed to prevent premature entry into S phase. Consistent with this, blocking pre-RC assembly by overexpression of a stable form of geminin in primary cells inhibits CDK leading to the appearance of hypophosphorylated pRB (32). Thus, it is possible that formation of pre-RCs is linked to CDK activation and allows cells to enter S phase once enough pre-RCs are formed. Normally p27 is transiently stabilized in early G₁ phase and degraded in late G₁ once CDK2 is active. Since this period of p27 stabilization overlaps with the time for pre-RC formation, we speculate that degradation of p27 is coupled to pre-RC formation.

ORC Function in p27 Degradation—Xic1, a *Xenopus* homolog of p27, is ubiquitinated by SCF^{Skp2} at origins of DNA replication in egg extracts and ORC depletion from extracts stabilizes Xic1 (33, 34). The stabilization of human p27 after ORC depletion is consistent with this result. However, at the earliest time point when p27 is stabilized (24–36 h), we did not see any decrease in the chromatin association of Skp2 (data not shown). Although the exact mechanism of p27 stabilization is unclear, we are currently entertaining two hypotheses. When cells exit from G₀, nuclear export of a small fraction of the p27 reduces the amount of p27 in nuclei and triggers activation of CDK2 (35, 36). Once activated, CDK2 accelerates the degradation of p27 by phosphorylating it and targeting it to the SCF complex. If a similar export of p27 is necessary to jump-start the CDK cycle in the G₁ phase of cycling cells, it is possible that such export is coupled to pre-RC formation. An alternative hypothesis is based on the role of ORC for formation of heterochromatin (37, 38). Insufficient levels of ORC2 could therefore lead to changes in chromatin structure that result in changes in expression of genes regulating p27 stability.

p21 Up-regulation after ORC2 Depletion—p21 was induced by a p53-independent pathway after ORC2 RNAi. Since overexpression of non-degradable form of geminin in primary cells also induces p21 in a p53-independent manner (32), p21 induction might be related to the failure to form pre-RCs. It has been reported that ORC is involved in gene silencing in *Saccharomyces cerevisiae* (39). In addition, *Drosophila* and human ORC2 associate with HP1, a protein in heterochromatin (37, 38). Therefore, as speculated above, ORC might regulate p21 mRNA levels by affecting gene expression.

ORC-dependent CDK2 Activation and Genomic Stability—The results reported in this paper establish for the first time

linkage between ORC and CDK2 activation in human cells. In retrospect, such a linkage seems essential for maintaining genomic stability. As a part of the mechanism to prevent re-replication, pre-RC formation is allowed only when CDK activity is low (40–42). Therefore premature activation of CDK2 before sufficient origins are licensed would further inhibit pre-RC formation and irreversibly push the cells into S phase. Replication initiation from fewer origins might lead to chromosomal damage as seen in the yeasts (43–45). Thus, ORC-dependent activation of CDK2 could have evolved to protect the mammalian cell from genetic instability resulting from premature activation of CDK2. ORC protein levels are low in quiescent tissues (46) so that cells emerging from quiescence have to accumulate ORC in preparation for the first S phase. It is interesting to speculate that by overriding the linkage between ORC abundance and cyclin E-CDK2 activity, overexpression of cyclin E is particularly deleterious to cells as they emerge from quiescence. Consistent with this, overexpression of cyclin E induces chromosomal instability in mammalian cells (47), and many human tumors overexpress cyclin E (48). Recently, it was shown that pre-RC formation was severely impaired in cells expressing cyclin E at high levels (49), suggesting the importance of proper regulation of cyclin E-CDK2 activity for chromosome stability.

The recent creation of viable mice with CDK2 deletion raises questions as to whether CDK2 has an essential role in mammalian cells (50, 51). The yeast CDK is, however, essential for G₁/S transition and plays a critical role in maintaining genomic stability by preventing re-replication in one cell cycle. We therefore favor the hypothesis that the firing of origins and the prevention of re-replication both require CDK2 in mammals, but these actions are executed by one of the other mammalian CDKs in the CDK2 null mice. Thus, we believe that the requirement of ORC for the proper activation of CDK2 is likely to be an important feature of cell cycle control and genomic stability for mammalian cells *in vivo*.

Finally, recent studies (37, 52, 53) have implicated ORC in mitotic functions like chromosome condensation and segregation. The results reported here add to the cellular pathways and functions that are dependent on ORC and extend the interconnection between ORC and other players in the cell cycle machinery.

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