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 G\textsubscript{1} phase. At the G\textsubscript{1}/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 G\textsubscript{1}, 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 Thr\textsuperscript{14} and Tyr\textsuperscript{15} of CDK by Cdc25 activating kinase are also required for CDK2 activity (4). Activities of G\textsubscript{1} 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 G\textsubscript{0} and G\textsubscript{1} phases to maintain low CDK activity. Proteasome-mediated degradation following ubiquitylation by SCF\textsuperscript{Csk2} complex is responsible for the low levels of p27 protein during S-G\textsubscript{2} phases (6). p27 is also ubiquitinylated 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)\textsuperscript{1} at origins of DNA replication during the G\textsubscript{1} phase (9). Origin recognition complex (ORC) is a six-subunit complex required to mark the origins of DNA replication. In late M and early G\textsubscript{1} 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 G\textsubscript{1}/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 deple- tion of one of the replication initiation proteins results in G\textsubscript{1} 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 conditions. 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; p21-A, AAGGUUGCAUGACUGGAUAGUA; p21-B, AAGGUUGCAUGACUGGAUAGUA; control, AAGGUGCAUGACUGGAUAGUA.

Western and Northern Blotting—ORC3, ORC4, and ORC6 antibodies were described earlier (11–13). Rabbit antibodies against ORC1 and ORC5 were raised using His\textsubscript{6}-tagged recombinant proteins, ORC1 (220–351) and ORC5 (75–266). Anti-p27 (C-19), anti-p21 (C-19), anti-cyclin E (HE12), anti-cyclin A (E-12), anti-cyclin B (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 Assays—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; BrdUrd, 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.

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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 oligonucleotides 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 G1 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 was 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 G1/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 accumulated 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 ubiquitylation (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 G1 phase where the level of the protein is high. In contrast to G1 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
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 vitro (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 renews 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 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
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 ORC2-depleted cells suggesting that regulation of the protein is at the post-transcriptional level (Fig. 4A). After 24-h treatment of ORC2 RNAi, cycloheximide was added to block new protein synthesis and the rate of decrease of p27 protein examined (Fig. 4B 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 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 Parity 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
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 progress 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 S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins. In nearly 50% of the cells DNA synthesis was not restored despite the restoration of S phase entry with fewer functional origins.

**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).
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 phase 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 prevents 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 ubiquitinylated by SCF<sup>Skp2</sup> 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 the 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 chromosomal 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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**REFERENCES**

1. van den Heuvel, S., and Harlow, E. (1993) Science 262, 2050–2054
2. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995) Mol. Cell. Biol. 15, 2612–2624
3. Pines, J., and Hunter, T. (1989) Cell 58, 833–846
4. Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992) EMBO J. 11, 3995–4005
5. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
6. Har, T., Kamura, T., Nakayama, K., Oshikawa, K., and Hatakeyama, S. (2001) J. Biol. Chem. 276, 48897–48903
7. Kamura, T., Har, T., Matsumoto, M., Ishida, N., Okumura, P., Hatakeyama, S., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2004) Nat. Cell Biol. 6, 1229–1235
8. MacLeod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995) Genes Dev. 9, 935–944
9. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
10. Mendez, J., and Stillman, B. (2000) Mol. Cell. Biol. 20, 8602–8612
11. Pinto, S., Quintana, D. G., Smith, P., Mihaele, R. M., Hou, Z. H., Boynton, S., Jones, C. J., Hendrick, M., Velinzon, K., Wohlschlegel, J. A., Austin, R. J., Lane, W. S., Tully, T., and Dutta, A. (1999) Neuron 23, 45–54
12. Quintana, D. G. Hou, Z., Tully, T., Khan, K. C., Hendrick, M., Saha, P., and Dutta, A. (1997) J. Biol. Chem. 272, 28247–28251
13. Dharm, S. K., and Dutta, A. (2000) J. Biol. Chem. 275, 34983–34988
14. Chen, J., Saha, P., Kernbah, S., Dynlacht, B. D., and Dutta, A. (1996) Mol. Cell. Biol. 16, 4675–4682
15. Dharm, S. K., and Dutta, A. (2001) J. Biol. Chem. 276, 29067–29071
16. Dharm, S. K., Yoshida, A., Machida, Y., Khanjra, P., Chauhuri, B., Wohlschlegel, J. A., Leffak, M., Yates, J., and Dutta, A. (2001) Cell 106, 287–296
17. Ohtani, K., DeGregori, J., Leone, G., Herenden, D. R., Kelly, T. J., and Nevins, J. R. (1996) Mol. Cell. Biol. 16, 4977–4984
18. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. (1996) *Genes Dev.* 10, 1979–1990
19. Won, K. A., and Reed, S. I. (1996) *EMBO J.* 15, 4182–4193
20. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev.* 14, 2393–2409
21. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) *Cell* 98, 850–869
22. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., Harbour, J. W., and Dean, D. C. (2000) *Genes Dev.* 14, 2393–2409
23. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) *Cell* 98, 859–869
24. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., Harbour, J. W., and Dean, D. C. (2000) *Cell* 101, 79–89
25. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) *Curr. Biol.* 9, 661–664
26. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) *Genes Dev.* 13, 1181–1189
27. Flatt, P. M., Polyak, K., Tang, L. J., Scatena, C. D., Westfall, M. D., Rubinstein, L. A., Yu, J., Kinzler, K. W., Vogelstein, B., Hill, D. E., and Pietenpol, J. A. (2000) *Cell* 101, 79–89
28. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* 371, 346–347
29. Hofmann, J. F., and Beach, D. (1994) *EMBO J.* 13, 425–434
30. Labib, K., Kearsey, S. E., and Diffley, J. F. X. (2001) *Mol. Biol. Cell* 12, 3658–3667
31. Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993) *Cell* 74, 371–382
32. Piatti, S., Lengauer, C., and Nasmyth, K. (1995) *EMBO J.* 14, 3788–3799
33. Hartwell, L. H., and Weinert, T. A. (1989) *Science* 246, 629–634
34. Shreeram, S., Sparks, A., Lane, D. P., and Blow, J. J. (2002) *Oncogene* 21, 6624–6632
35. Forster, L., Swanson, C., Kaiser, B. K., Eldridge, A. G., and Jackson, P. K. (2001) *Nat. Cell Biol.* 3, 715–722
36. You, Z., Harvey, K., Kong, L., and Newport, J. (2002) *Cell* 101, 381–394
37. Prasanth, S. G., Prasanth, K. V., Siddiqui, K., Spector, D. L., and Stillman, B. (2004) *EMBO J.* 23, 2651–2663
38. Pak, D. T., Pflumm, M., Chesnokov, I., Huang, D. W., Kellum, R., Barr, J., Romanowski, P., and Botchan, M. R. (1997) *Cell* 91, 311–323
39. Foss, M., McNally, F. J., Lauretson, P., and Rine, J. (1993) *Science* 262, 1838–1844
40. Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995) *Curr. Biol.* 5, 1257–1269
41. Hua, X. H., Yan, H., and Newport, J. (1997) *Cell* 101, 1257–1269
42. Detweiler, C. S., and Li, J. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2384–2389
43. Lengronne, A., and Schwob, E. (2002) *Mol. Cell* 9, 1067–1078
44. Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995) *Curr. Biol.* 5, 1257–1269
45. Hua, X. H., Yan, H., and Newport, J. (1997) *Cell* 89, 6624–6632
46. Pak, D. T., Pflumm, M., Chesnokov, I., Huang, D. W., Kellum, R., Barr, J., Romanowski, P., and Botchan, M. R. (1997) *Cell* 91, 311–323
47. Foss, M., McNally, F. J., Lauretson, P., and Rine, J. (1993) *Science* 262, 1838–1844
48. Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995) *Curr. Biol.* 5, 1257–1269