RB Loss Promotes Aberrant Ploidy by Deregulating Levels and Activity of DNA Replication Factors*

Seetha V. Srinivasan1, Christopher N. Mayhew1, Sandy Schwemberger1, William Zagorski1, and Erik S. Knudsen1,2

From the 1Department of Cell and Cancer Biology, Vontz Center for Molecular Studies, 2Shriners Hospital for Children, Department of Surgery, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267

The retinoblastoma tumor suppressor (RB)3 is a critical regulator of cell cycle and cellular proliferation, and its functional inactivation occurs in a variety of human cancers (1–3). In quiescent, senescent, or differentiated cells, RB is hypophosphorylated, wherein it interacts with a host of cellular proteins to repress transcription of genes required for cell cycle progression (4). Specifically, RB can interact with the E2F family of transcription factors and either directly inhibit transcription of cell cycle genes or mediate transcriptional repression through the recruitment of co-repressors (e.g. HDAC1 or SWI/SNF) (5, 6). During progression into the cell cycle, RB is inactivated by phosphorylation, thereby disrupting the RB-mediated transcriptional repressor complexes (7). Thus, it is widely accepted that RB functions to regulate cell cycle transitions, particularly relating to entry into S phase.

Recent studies have identified roles for RB in modulating DNA replication during the S phase of the cell cycle (8–11). DNA replication requires the sequential assembly of a prereplication complex (pre-RC) that comprises multiple proteins, including the origin recognition complex, Cdc6 (cell division cycle 6) protein, Cdt1 (cell division transition 1 protein), and the minichromosome maintenance family of proteins (MCM2 to -7) (12–14). Together, assembly of the prereplication complex on chromatin specifies origins that are competent for the initiation of DNA replication (15, 16). The regulation of pre-RC assembly is tightly controlled and is a critical means through which DNA replication is restricted to a single round per cell cycle (17–19). Subsequent to origin activation, additional factors are recruited to initiate DNA replication (e.g. Replication Protein A and primase) (20). As replication forks mature, DNA polymerases (e.g. DNA polymerase delta) and associated processivity factors (e.g. proliferating cell nuclear antigen (PCNA)) are recruited to facilitate replication of the genome (21, 22). The influence of RB on the functional activity of the pre-RC remains unclear. Studies utilizing overexpression of active alleles of RB demonstrate that RB does not a priori inhibit pre-RC assembly. Rather, it functions downstream to actively inhibit the retention of PCNA within the replication machinery (23). Additionally, other studies have identified a role for RB in the maintenance of genome integrity. The impact of RB loss on genomic instability was initially observed in HPV-infected fibroblasts, where E7-mediated inactivation of RB led to impaired chromosomal integrity (24). Subsequent studies show that cellular stresses, such as DNA damage or exposure to cytotoxic compounds, coupled with RB loss also elevate cell ploidy (25–29). Polyploidy is a likely precursor of aneuploidy, which in turn is a hallmark of most tumors (30–32), and loss of RB can lead to poly- and aneuploidy (29, 33–35).

Although several studies report the effect of RB loss on cell proliferation and its impact on cancer, few studies document the consequence of RB deficiency on DNA replication control and its subsequent effect on cancer. It is known that loss of RB enables aberrant DNA replication in the presence of DNA dam-

* This study was supported by NCI, National Institutes of Health, Grant CA 106471 (to E. S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIEHS, National Institutes of Health, Grant T32 CA117846-01A1 and the Robert and Emma Lou Cardell Fellowship.

2 To whom correspondence should be addressed: Dept. of Cell Biology, University of Cincinnati, Vontz Center for Molecular Studies, 3125 Eden Ave., Cincinnati, OH 45267-0521. Tel.: 513-558-8885; Fax: 513-558-4454; E-mail: Erik.knudsen@uc.edu.

3 The abbreviations used are: RB, retinoblastoma protein; GFP, green fluorescent protein; Ad, adenovirus; GFP cells, Ad-GFP vector-infected cells that are RB-proficient; GFP-CRE or CRE cells, Ad-GFP-CRE-infected cells that are RB-deficient; PCNA, proliferating cell nuclear antigen; pre-RC, prereplication complex; BrdUrd, bromodeoxyuridine; PI, propidium iodide; PIPES, 1,4-piperazinediethanesulfonic acid; CSK, cytoskeletal.
RB Loss Compromises Genome Integrity

age or under conditions of mitotic blockade (36, 37). Such aberrant replication is linked with secondary forms of DNA damage and has been hypothesized to compromise genome integrity (27, 29, 38). Whether these effects involve a direct action of RB on chromatin or indirect effects in transcriptional control have yet to be conclusively determined. Furthermore, the mechanisms through which RB loss bypasses normal controls over replication, the critical effectors in this process, and how they contribute to tumorigenesis are unknown. Given that RB controls replication, which in turn determines cell ploidy, it is critical to determine the consequences of RB loss on these phenomena. In the present study, we investigated the impact of RB loss on the DNA replication machinery and show that RB deficiency has profound effects on the establishment of the prereplication complex and enables inappropriate rounds of DNA replication. Furthermore, the consequences of RB loss on cell ploidy can be phenocopied by exogenous E2F expression in RB-proficient cells. The current findings indicate that transcriptional control is important in the deregulation of DNA replication that is observed upon RB loss and that deregulation of DNA synthesis resulting from loss of RB contributes to genomic imbalance.

EXPERIMENTAL PROCEDURES

Isolation of Primary Rb<sup>loxP/loxP</sup> Mouse Adult Fibroblasts (MAFs), Cell Culture, and Adenoviral Infections—Primary fibroblasts were isolated from the peritoneal fascia of Rb floxed mice and cultured as previously described (29). All cells used in this study were between passages 2 and 4 unless indicated otherwise. Primary Rb<sup>loxP/loxP</sup> MAFs were infected with adenoviruses expressing the control GFP vector alone (Ad-GFP) or GFP and Cre recombinase (Ad-GFP-CRE). The Ad-GFP-CRE infection rendered conditional RB knock-out cells due to the activity of the Cre recombinase. After infection, cells were cultured at least 3 days before experimentation.

Detection of Recombination and RB Knockdown in MAFs—Genomic DNA was isolated from either GFP (RB-proficient) or GFP-CRE (RB-deficient) MAFs using the Dneasy kit (Qiagen). To detect Cre-mediated recombination, PCR analysis of Rb exon 19 was done as described previously (39). The efficiency of the Rb deletion was also confirmed by Western blot analysis of target protein deregulation.

**FIGURE 1.** RB loss elevates cell ploidy and deregulates the expression of several DNA replication factors. A, primary mouse adult fibroblasts isolated from the peritoneal fascia of Rb<sup>loxP/loxP</sup> mice were infected with adenoviral

GFP or adenoviral GFP-CRE. Forty-eight hours postinfection, genomic DNA was isolated from the cells, and PCR using primer sequences that flank either end of Rb exon 19 was performed to evaluate recombination at the floxed Rb locus. B, asynchronously growing RB-proficient (GFP) and RB-deficient (CRE) MAFs were harvested after 48 h, fixed in 70% ethanol, and stained with propidium iodide. The stained cells were processed by flow cytometric analysis to identify cell populations with 2, 4, and 8N DNA content. C, GFP and CRE MAFs were fixed and stained with propidium iodide for flow cytometry. The number of cells with >4N DNA content was determined by ModFit software and plotted as a percentage of the total gated population. Values indicate the average of three experiments.

D, asynchronously proliferating RB-proficient and RB-deficient cells were labeled with BrdUrd for 2 h and harvested by trypsinization. Cells were fixed in ethanol and stained with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and propidium iodide. Samples were then subjected to flow cytometric analysis. The boxed regions indicate BrdUrd<sup>−</sup> cells with >4N DNA. E, Ad-GFP- and Ad-GFP-CRE-infected MAFs were lysed, and 60 μg of total protein was resolved by SDS-PAGE electrophoresis. The membranes were blotted using antibodies against the indicated proteins. The Cdk4 protein served as a loading control.
BrdUrd/Propidium Iodide Flow Cytometry Analysis

Staining—RB-proficient and RB-deficient MAFs were plated (2 \times 10^6 cells/10-cm² dish) in 10-cm² dishes. Eighty percent confluent cells were treated with 100 ng/ml nocodazole or Me₃SO control. After 24 h, the cells were washed and labeled with BrdUrd (Amersham Biosciences) for 2 h, trypsinized, and fixed in 70% ethanol. Fixed cells were labeled with PI and stained with propidium iodide (50 \mu g/ml) and RNase A (80 \mu g/ml). Cells were then processed for flow cytometry analysis.

Flow Cytometry—RB-proficient and RB-deficient MAFs were plated as described above. Two days post-plating, cells were treated with either Me₃SO or 100 ng/ml nocodazole (Calbiochem) for 24 h. Cells were then trypsinized and fixed with 70% ethanol. Fixed cells were treated with RNase (80 \mu g/ml) and stained with propidium iodide (50 \mu g/ml). Cells were processed for flow cytometric analysis as previously described (39). Modfit software was used to determine the percent of cells with >4 N DNA content.

Preparation of Cell Lysates and Western Blotting—RB-proficient (GFP) and RB-deficient (GFP-CRE or CRE) MAFs were plated (2 \times 10^6/10-cm² dish). Cells were then lysed in RIPA buffer supplemented with protease inhibitors, sonicated, and quantified (Pierce). Lysates were boiled and subjected to SDS-PAGE electrophoresis. Specific proteins MCM7, PCNA, Cdc6, lamin B, Cdk4, cyclin A, cyclin E, Replication Protein A (Santa Cruz Biotechnology), and Cdt1 (a gift from Dr. Jeanne Cook) were detected by Western blotting.

BrdUrd Incorporation Assay—Cells were plated on coverslips and treated with Me₃SO or nocodazole (100 ng/ml) for 24 h. Cells were washed and labeled with BrdUrd (Amersham Biosciences) for 6 h to detect DNA synthesis. Labeled cells were probed with anti-BrdUrd antibody as described previously (40).

Biochemical Fractionation to Isolate Chromatin-bound Proteins—The isolation of chromatin-associated proteins was performed as described previously (41). Briefly, cells were treated with Me₃SO or nocodazole (100 ng/ml), washed with PBS, and trypsinized. The cell pellet was resuspended in ice-cold cytoskeletal (CSK) buffer (10 mM PIPES, pH 6.8, 100 mM Tween 20 in PBS. Anti-BrdUrd-fluorescein isothiocyanate (BD Pharmingen) was added to the cell suspension and incubated for 30 min. Propidium iodide (50 \mu g/ml) and RNase A (80 \mu g/ml) were added to the cell suspension and allowed to stain overnight at 4 °C. Samples were analyzed for BrdUrd/propidium iodide (PI) by bivariate flow cytometry analysis.
NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100). Total cell lysates were generated by lysing one-third of the cells in CSK buffer. The remaining cells were extracted twice with cold CSK buffer on ice. The suspension was then centrifuged, and the pellet was resuspended in CSK buffer. All samples were quantified and processed for blotting of the indicated proteins.

**Immunofluorescence Assay to Visualize Chromatin-bound Replication Factors**—Cells were plated on coverslips, and one set was utilized to detect total proteins. The second set was subjected to CSK extraction to retain only the chromatin-bound proteins, as previously described (41). All cells were methanol-fixed and probed with the indicated antibodies. Cells were visualized using a fluorescent microscope.

**Ectopic Expression of Ad-Cdc6, Ad-Cdt1, Ad-E2F1, and Ad-E2F2 in RB-proficient Cells and Dominant Negative E2F-A/B in RB-deficient Cells**—GFP cells (RB-proficient) were plated and infected with virus, at least 1 × 10¹¹ plaque-forming units/10-cm² dish for 24–30 h, as previously described (41). Cells were then treated with Me₂SO control or nocodazole and processed for flow cytometry, CSK extraction, and Western blotting as described above. RB-deficient cells were plated (2 × 10⁶/10-cm² dish) and transfected with either H2B-GFP plasmid or H2B-GFP and E2F-A/B using Fugene transfection agent. Forty-eight hours later, cells were treated with Me₂SO control or nocodazole (100 ng/ml). Twenty-four hours after treatment, cells were labeled with BrdUrd for 6 h and processed for BrdUrd incorporation as described above.

**Microscopy and Digital Image Acquisition**—A DIX digital camera (Nikon) attached to a Microphot FXA upright microscope (Nikon) with a 63 or 100 dippable Zeiss objective was used to visualize and photograph cells. NikonView5 software was used to transfer images. For the immunofluorescence images, an ORCA-ER Hamamatsu Avioplan 2 imaging microscope was used with a 100 dippable Zeiss objective and Hoechst and Rhodamine Zeiss filters. Metamorph software was used to acquire images.

**RESULTS**

**RB Loss Compromises Genome Integrity**

*FIGURE 3.* RB-deficient cells bypass nocodazole-induced cell cycle arrest, continue DNA rereplication, accumulate >4 N cells, and show increased micronuclei formation. A, proliferating RB-proficient (GFP) and RB-deficient (CRE) MAFs were treated with either Me₂SO or nocodazole (100 ng/ml) for 24 h. Cells were fixed and stained by propidium iodide for flow cytometric analysis. The percentage of >4 N cells was determined by ModFit software and averaged as the percentage of the total gated cell population. The values are averaged from three experiments. B, Ad-GFP and Ad-GFP-CRE MAFs were plated on coverslips, treated with Me₂SO (control) or 100 ng/ml nocodazole (Noc) for 24 h, and then labeled with BrdUrd (BrdU) for 6 h. Cells were fixed with formaldehyde and stained with primary anti-BrdUrd and rhodamine-conjugated secondary antibody. Cells were also stained with the nuclear Hoechst dye. Stained cells were visualized on a fluorescent microscope. Bottom, cells plated, stained, and fixed as above were counted in multiple fields. The BrdUrd-positive cells are plotted as a percentage of total cell number.

Values are averaged from three independent experiments. C, Ad-GFP and Ad-GFP-CRE cells were plated on coverslips and treated with Me₂SO (control) or nocodazole (100 ng/ml) for 24 h. Cells were then fixed and stained as described above. The micronuclei were visualized using a fluorescent microscope and counted in multiple fields. The data are presented as a percentage of total cell number in untreated and nocodazole-treated cell populations and represent the average of three independent experiments.
RB Loss Compromises Genome Integrity

AUGUST 17, 2007 • VOLUME 282 • NUMBER 33

JOURNAL OF BIOLOGICAL CHEMISTRY

RB Loss Is Associated with Increased Chromatin Association of DNA Replication Factors—The preceding results indicate that the total protein levels of several DNA replication factors are enhanced with RB loss. Therefore, we next investigated the extent to which these proteins engage in DNA replication. Most DNA replication proteins are considered active when they are associated with chromatin. For instance, it has been determined that the chromatin-bound fraction of MCM2 to -7 denotes the presence of a functional prereplication complex (42–44). Therefore, we assessed the relative levels of chromatin-bound replication factors in RB-proficient versus RB-deficient cells, since they represent the functional components of the replication machinery. Western blot analysis of replication factors, such as MCM7, PCNA, and Cdc6, demonstrated increased total protein levels (Fig. 2A, compare lanes 1 and 2). In addition, levels of other S phase-associated factors, such as PCNA, cyclin A, cyclin E, and Replication Protein A were also elevated (Fig. 1E). These results suggest that the elevated levels of replication factors may functionally contribute to the aberrant ploidy observed with the loss of RB.

CB

RB Loss Compromises Genome Integrity

FIGURE 4. RB loss results in deregulated S phase control and compromised genome integrity. A, RB-proficient (GFP) and RB-deficient (CRE) MAFs were treated with nocodazole (Noc) as described in the legend to Fig. 3. Cells were then lysed for total protein and chromatin-associated protein by a CSK fractionation procedure. Cell lysates were immunoblotted for the indicated proteins. B and C, Ad-GFP and Ad-GFP-CRE MAFs were plated on coverslips and treated with nocodazole for 24 h. Treated and untreated cells were subjected to the CSK fractionation procedure to retain chromatin-tethered MCM7 or PCNA proteins before methanol fixation. A parallel control set was directly fixed in methanol after nocodazole treatment. Both sets of cells were stained with MCM7 (B) or PCNA (C) and also stained with the nuclear Hoechst dye. Cells were visualized on a fluorescent microscope and counted for MCM7- and PCNA-positive cells. The number of positive cells is plotted as a percentage of total cell number. Values are averaged from three independent experiments. D, Ad-GFP and Ad-GFP-CRE cells were plated on coverslips and treated with Me2SO (control) or nocodazole (100 ng/ml) for 24 h. Cells were then methanol-fixed and stained with MCM7 antibody. The micronuclei were visualized and photographed using a fluorescent microscope.

Consistent with previous observations (29), the acute loss of RB results in a 7-fold increase in the percentage of cells that exhibit >4 N DNA content (Fig. 1, B (flow cytometry trace) and C (combined average from three experiments)). Coordinate analyses of DNA replication (via BrdUrd incorporation) and DNA content reveal that the 2 N population exhibits pronounced BrdUrd incorporation (Fig. 1D). Since the majority of these cells are cycling, diploid cells, there is a high degree of BrdUrd incorporation in the 2 N fraction. However, a small fraction of these cells have begun to acquire >4 N DNA content (Fig. 1D, boxed region); among these cells, a greater proportion of RB-deficient cells are BrdUrd-positive when compared with RB-proficient cells (Fig. 1D, boxed regions, compare BrdUrd-positive cells with >4 N DNA in GFP and CRE populations). This result, coupled with the discrete peaks at 4 and 8 N (Fig. 1B), suggests that the increased ploidy is the result of a complete round of DNA replication. To determine if aberrant DNA replication in RB-deficient cells was associated with changes in DNA replication factors, the levels of these factors were determined by immunoblotting. In RB-deficient cells, components of the prereplicative complex, such as Cdc6, Cdt1, and MCM7 were greatly increased as compared with RB-proficient cells (Fig. 1E, compare lanes 1 and 2). In addition, levels of other S phase-associated factors, such as PCNA, cyclin A, cyclin E, and Replication Protein A were also elevated (Fig. 1E). These results suggest that the elevated levels of replication factors may functionally contribute to the aberrant ploidy observed with the loss of RB.
that the increased tethering of the replication factors is specific to RB loss. Since the accumulation of pre-RC proteins in the chromatin fraction was pronounced in RB-deficient cells, this suggests that assembly of the prereplication complex is deregulated with the loss of RB. These results were further validated by immunofluorescence, wherein cells were subjected to in situ extraction to remove soluble cytosolic/nucleoplasmic proteins and retain only chromatin-tethered proteins. They were probed with antibodies to MCM7, PCNA, cyclin A, Cdk1 (negative control for tethering), and lamin B (additional control) and evaluated by fluorescence microscopy (Fig. 2, B–F). RB-deficient cells exhibited higher levels of total and tethered replication proteins. Data from fractionation and immunofluorescence experiments thus demonstrate enhanced chromatin retention of replication proteins, suggesting that RB loss influences DNA replication control by altering the level and activity of components of the DNA replication machinery.

**RB Loss Facilitates DNA Rereplication in the Presence of a Mitotic Block**—Although deregulation of DNA replication processes could contribute to alterations in genome copy, similar effects can occur as a result of high frequency mitotic failures (45). Therefore, we sought to distinguish between the contribution of DNA replication and mitotic progression to the increased ploidy of RB-deficient cells. To this end, we used the microtubule-destabilizing agent nocodazole to block mitotic progression in RB-proficient and RB-deficient cells. In this manner, any changes in ploidy by definition would be the specific consequence of reinitiation of DNA replication. Under this condition, nocodazole significantly inhibits DNA replication in RB-proficient cells as detected by flow cytometry, and cells accumulate with 4N DNA as expected, with minimal cells harboring >4N DNA content (Fig. 3A). In contrast, RB-deficient cells failed
to arrest following exposure to nocodazole. Furthermore, in the RB-deficient condition, ~20% of cells harbored >4N DNA content (Fig. 3A), indicating that DNA rereplication does occur in these cells and enhances cell ploidy. Additionally, nocodazole-treated RB-proficient cells showed a decrease in BrdUrd incorporation, indicating cessation of DNA synthesis, whereas the RB-deficient cells bypassed the mitotic block and exhibited continued BrdUrd incorporation, indicating active, ongoing DNA synthesis (Fig. 3B, top). Determination of the percentage of BrdUrd-positive cells demonstrated that treatment with nocodazole resulted in a 3-fold decrease in the BrdUrd incorporation in RB-proficient cells, whereas the RB-deficient cells retained comparable levels of BrdUrd-positive cells either in the absence or presence of nocodazole (Fig. 3B, bottom). The aberrant ploidy occurring in RB-deficient cells was accompanied by a significant increase in micronuclei that were largely positive for BrdUrd incorporation (Fig. 3B, compare nuclei in nocodazole-treated GFP and CRE images). The numerical increase in micronuclei seen upon RB loss was further exacerbated by nocodazole treatment (Fig. 3C). Thus, RB deficiency enables DNA replication to proceed under inappropriate conditions and contributes to increased ploidy.

To determine the mechanistic basis for the observed DNA rereplication, we evaluated the influence of nocodazole on the levels of DNA replication factors in RB-proficient and RB-deficient cells. In cells containing functional RB, treatment with nocodazole resulted in diminished levels of MCM7 and PCNA in both total and chromatin-associated fractions (Fig. 4A, compare lane 1 with lane 2 and lane 5 with lane 6). In contrast, the chromatin-tethered levels of MCM7 and PCNA in RB-deficient cells were retained at high levels relative to RB-proficient cells (Fig. 4A, compare lane 2 with lane 4 and lane 6 with lane 8). Additionally, nocodazole treatment did not diminish levels of these proteins in RB-deficient cells (compare lane 3 with lane 4 and lane 7 with lane 8). Evaluation of MCM7 and PCNA levels and their association with chromatin by fluorescence microscopy revealed that nocodazole-treated RB-deficient cells show decreased levels of total and tethered replication proteins (GFP in Fig. 4, B and C). However, RB loss resulted in the retention of chromatin association of these proteins even in the presence of nocodazole (CRE in Fig. 4, B and C). These results concur well with the biochemical fractionation experiments shown in Fig. 4A.

Another feature of nocodazole-treated RB-deficient cells was the increased presence of micronuclei. Analysis of such cells by immunofluorescence demonstrated that they also show significant levels of MCM7 (Fig. 4D). This finding illustrates the functional consequence of RB loss on genomic integrity. Collectively, these data indicate that RB deficiency results in the abrogation of nocodazole-induced cell cycle arrest and inappropriate retention of DNA replication factors onto chromatin. Consequently, RB-deficient cells undergo DNA synthesis under nonpermissive conditions, thus leading to aberrant increases in cell ploidy and genome instability.

**Ectopic Cdc6 or Cdt1 Expression Does Not Recapitulate the Effects of RB Loss**—The loading of MCMs onto chromatin to assemble a functional prereplication complex is dependent on the activity of Cdc6 and Cdt1, since these two proteins recruit the MCMs to sites of DNA replication (46). As shown in Fig. 1E, the levels of these proteins are significantly elevated in RB-deficient cells. These factors have previously been shown to drive aberrant DNA replication when overexpressed (47, 48), suggesting that these proteins could be largely responsible for the observed effects on pre-RC formation. Therefore, we used recombinant adenoviruses to ectopically express Cdc6 in RB-proficient cells to levels similar to those observed in RB-deficient cells (Fig. 5A, compare lanes 1 and 2 with lanes 5 and 6). The overexpression of Cdc6 had minimal effect on cells with >4N DNA content (Fig. 5B). Similarly, overexpression of Cdt1 in RB-proficient cells (Fig. 5C, lanes 1 and 2) was not sufficient to drive cells to >4N DNA content in a manner consistent with RB loss (Fig. 5D); the flow cytometry traces (Fig. 5E) clearly demonstrate this, and RB-proficient cells overexpressing either one or both of these factors do not show elevated >4N DNA (Fig. 5E). In order to verify the activity of the adenoviral Cdc6 and Cdt1, we used H1299 lung cancer cells, wherein the expression of Cdt1 is known to drive rereplication, and the combined expression of Cdc6 and Cdt1 significantly enhances DNA rereplication in a synergistic manner (49). Cdt1 expression in H1299 cells induced rereplication that was significantly enhanced by co-expression with Cdc6 (Fig. 5F), thereby demonstrating that the adenoviruses produce active Cdc6 and Cdt1 proteins. Together, these data indicate that expression of Cdc6 or Cdt1 is not sufficient to phenocopy the influence of RB loss in elevating ploidy in primary mouse fibroblasts.

**Expression of E2Fs Recapitulates the Effects of RB Loss on DNA Rereplication**—The results observed with exogenous expression of Cdc6 or Cdt1 indicated that expression of these two factors in RB-proficient cells was not sufficient to alter DNA replication control in a manner analogous to RB loss. As shown in Fig. 1E, RB controls the expression of a large number of replication factors that are transcriptionally regulated by the E2F family of transcription factors. Therefore, we next evaluated the influence of elevated E2F activity on replication con-

---

**FIGURE 5.**  **Exogenous expression of Cdc6 or Cdt1 in RB-proficient cells is not sufficient to elevate cell ploidy.** A, Ad-GFP-, Ad-GFP-CRE-, and Ad-Cdc6-infected MAFs were treated with Me2SO control or 100 ng/ml nocodazole (Noc) for 24 h. Cells were then harvested, and lysates were immunoblotted for the indicated proteins. Lamin B was used as a loading control. B, Ad-GFP-, Ad-GFP-CRE-, and Ad-Cdc6-infected MAFs were treated with Me2SO control or nocodazole (100 ng/ml) for 24 h. Cells were then harvested, fixed, and labeled with PI for flow cytometric analysis. The data are represented as a percentage of cells with >4N DNA content from a total gated population of PI-positive cells. Results represent the average of three independent experiments. C, Ad-GFP-, Ad-GFP-CRE-, and Ad-Cdt1-infected cells were treated with Me2SO control or 100 ng/ml nocodazole for 24 h. Cells were then harvested and fixed in ethanol before PI staining and flow cytometric analysis. The graph represents the average of three independent experiments and shows the percentage of cells with >4N DNA content from a total gated population of PI-positive cells. E, MAFs were infected with adenoviruses expressing GFP, CRE, Cdt1, Cdc6, or Cdt1 + Cdc6 as indicated and processed for flow cytometry as described earlier. Flow traces show populations of cells with 2, 4, and 8N DNA content. F, H1299 cells were infected with the indicated viruses for 48 h before being harvested for flow cytometry. The cytometry traces show populations of cells with 2, 4, and 8N DNA content.
RB Loss Compromises Genome Integrity

A

|            | Total protein | Tethered protein |
|------------|---------------|------------------|
| MCM7       |               |                  |
| PCNA       |               |                  |
| Lamin B    |               |                  |
| 1          | 2             | 3               |
| 4          | 5             | 6               |
| 7          | 8             |                 |

B

C

D

|            | E2F1 | GFP | CRE | E2F2 |
|------------|------|-----|-----|------|
| MCM7       | -    | +   | -   | +    |
| PCNA       | -    | +   | -   | +    |
| Lamin B    | -    | +   | -   | +    |

E

|            | E2F1 | GFP | CRE | E2F2 |
|------------|------|-----|-----|------|
| MCM7       | -    | -   | +   | -    |
| PCNA       | -    | -   | +   | -    |
| Lamin B    | -    | -   | +   | -    |

F

G

|            | H2B-GFP | BrdU | Hoechst | H2B-GFP | BrdU |
|------------|---------|------|---------|---------|------|
| H2B-GFP    |         |      |         |         |      |
| E2F-A/B    |         |      |         |         |      |

- Noc(0ng/ml) + Noc(100ng/ml)

CRE MAFs

% BrdU-positive cells
RB Loss Compromises Genome Integrity

The RB tumor suppressor plays a central role in controlling cell cycle progression in multiple settings. Here we investigated the specific influence of RB loss on regulation of DNA replication and the corresponding effects on genome integrity. We found that RB is a critical regulator of both the expression and intrinsic activity of DNA replication factors. This activity was sufficient to maintain DNA replication even under conditions that abrogate mitotic progression. As a result, RB deficiency resulted in aberrant ploidy and an increased number of cells with micronuclei. Interestingly, this function of RB was not recapitulated by ectopic expression of the licensing factors Cdt1 and Cdc6. Rather, it was the deregulation of E2F activity that resulted in both aberrant regulation of the DNA replication machinery and corresponding alterations in DNA content.

RB Loss Deregulates Replication Control—The S phase of the cell cycle is tightly regulated to ensure that the entire genome is duplicated only once per cycle and in a manner that is both timely and error-free (18, 19). S phase initiation is regulated by the cell cycle machinery and checkpoint pathways (18). One aspect of this regulation is transcriptional coordination by the RB/E2F pathway (52). Elevation of E2F is known to drive cells into S phase, whereas its repression inhibits S phase entry and increases the proportion of cells in G1 (53). Microarray studies coupled with E2F knock-out data indicate that E2Fs regulate genes involved in DNA replication (54, 55). The E2F family is known to control multiple classes of genes that differentially influence DNA replication, such as cyclin E, cyclin A, and cdk2, which are involved in S phase entry (56); components of the prereplication complex, including the origin recognition complex proteins Cdc6, Cdt1, and MCMs (57); and genes required for synthesis of DNA, such as ribonucleotide reductase, thymidine synthase, and DNA polymerase α (9). More recently, studies have identified distinct roles for RB in regulating DNA replication (8, 9, 41). For instance, constitutively active RB is known to inhibit S phase progression and completion (58). RB is required to elicit proper DNA damage response, and RB loss under such conditions is known to result in deregulated replication control and inappropriate replication (59). Furthermore, RB deficiency can lead to ectopic DNA rereplication, which, in the absence of cell division, results in increased ploidy (27). Given these emerging roles for RB in replication control, it is important to assess the impact of RB loss on specific DNA replication proteins. Our current study, in addition to confirming the elevation of several replication proteins upon RB loss, also shows that loss of RB increases the levels of chromatin-bound replication factors. This is especially significant, since the chromatin-tethered fraction of replication proteins represents their functional, “active” state (42–44), and their increased retention could facilitate increased replication. Also, our immunofluorescence data show that RB-deficient cells have a higher density

FIGURE 6. Exogenous expression of E2F1/E2F2 in RB-proficient cells followed by nocodazole treatment results in elevated ploidy and increased total and chromatin-tethered MCM7 and PCNA levels that are comparable with cells harboring RB loss; disruption of endogenous E2Fs in RB-deficient cells diminishes the RB negative phenotype. A, Ad-GFP-, Ad-GFP-CRE-, and Ad-E2F1/E2F2-infected MAFs were lysed for total and tethered proteins by CSK fractionation. Cell lysates were then subjected to immunoblotting and probed for the indicated proteins. Lamin B serves as the loading control. B, Ad-GFP-, Ad-GFP-CRE-, and Ad-E2F1/E2F2-infected MAFs were treated with MeSO or nocodazole (Noc; 100 ng/ml) for 24 h. Cells were then processed for PI staining and fluorescence-activated cell sorting analysis. The cytometric traces show the cells with 2, 4, and 8 N DNA content in untreated and nocodazole-treated cell populations. C, Ad-GFP-, Ad-GFP-CRE-, and Ad-E2F1/E2F2-infected cells were fixed in ethanol and stained with propidium iodide before flow cytometric analysis. The graph represents the average of three independent experiments and shows the percentage of cells with >4 N DNA content. D and E, Ad-GFP-, Ad-GFP-CRE-, and Ad-E2F1/E2F2-infected MAFs were lysed for total (D) and tethered (E) proteins by CSK fractionation. Cell lysates were then subjected to immunoblottting and probed for the indicated proteins. Lamin B serves as the loading control. F, RB-deficient MAFs (CRE) were transfected with H2B-GFP alone or co-transfected with H2B-GFP and E2F-A/B plasmids. Forty-eight hours later, cells were treated with nocodazole for 24 h, labeled with BrdUrd, and processed for immunofluorescence microscopy using anti-BrdUrd antibody. G, RB-deficient cells (CRE) were transfected and processed as described above (F), and H2B-GFP-positive cells were scored as BrdUrd+ or BrdUrd− cells. The data are presented as the percentage of BrdUrd-positive cells among the transfected cell population and represent the average of three experiments.
of MCM7 immunofluorescence, suggesting the intriguing possibility that loss of RB can lead to increased functional pre-RC density. Recently, studies show that excessive pre-RC formation can contribute to checkpoint bypass and facilitate the formation of DNA lesions (60, 61). Presumably, such a mechanism also contributes to aberrant ploidy, wherein loss of RB allows the reinitiation of DNA synthesis, thereby resulting in cells with increased DNA content.

RB Loss Allows DNA Rereplication under Nonpermissive Conditions—The DNA replication machinery is typically subjected to multifaceted coordination to ensure that DNA synthesis only initiates under the appropriate conditions (13, 21, 62). Under nonpermissive conditions, such as DNA damage or exposure to cytotoxic agents, DNA replication is inhibited through a variety of mechanisms, such as inhibition of pre-RC formation through Cdt1 degradation, inhibition of replication licensing, and destabilization of components of pre-RC through altered activity of cyclin-dependent kinases (17). Tumor suppressors, such as p53 and RB, are required to prevent rereplication in response to cytotoxic chemicals (27). Specifically, RB is required to install the checkpoint response upon DNA damage and exposure to chemotherapeutic agents (29, 38), and RB loss can result in elevated ploidy and genomic instability (26, 39, 63). In addition, RB is known to be required for the G1 tetraploidy/aneuploidy checkpoints (63), and loss of RB interaction with histone-modifying enzymes is also known to cause aneuploidy (64). Typically, ploidy increase can occur through loss of S phase control and/or loss of G2/M control and mitotic failure (45). To distinguish between these two possible mechanisms that lead to increased ploidy, we employed a microtubule inhibitor, nocodazole, to induce cell cycle arrest. In RB-proficient cells, this resulted in decreased BrdUrd incorporation and diminished levels of replication proteins. It should be noted that even in RB-deficient cells, a slight reduction in the levels of replication proteins was observed. This suggests that negative signals can still impinge on the replication machinery in RB-deficient cells. Nevertheless, the retained levels were (a) considerably higher than those in RB-proficient cells and (b) apparently sufficient to ensure additional rounds of DNA replication. The experiments with nocodazole also suggest that RB loss uncouples DNA replication from cell cycle progression, resulting in deregulated S phase and elevated ploidy.

To further interrogate the mechanism by which RB controls ploidy, we challenged RB-proficient cells to recapitulate the molecular aspects of RB-deficient cells. It is well established that Cdc6 and Cdt1 are recruited to the replicative origins (46, 65) and facilitate loading of MCMs on the pre-RC (66). These factors are elevated in RB-deficient cells and thus were speculated to contribute to or mediate the observed deregulation of replication even in nonpermissive conditions, such as a mitotic block. Furthermore, aberrant expression of these factors is known to facilitate aberrant DNA replication initiation (60), especially in tumor cell lines (49). However, in our primary MAF cultures, exogenous expression of Cdc6 or and Cdt1 did not elevate the levels of chromatin-tethered MCM7 and failed to significantly augment cell ploidy. It is possible that the concurrent overexpression of several factors is required in primary cell cultures, since these cells do not harbor inherent mutations other than the targeted loss of RB. In fact, abrogation of the RB/E2F pathway is known to deregulate ~150 genes, many of which participate in DNA replication (67). Consistent with this idea, overexpression of E2F protein was sufficient to stimulate both the expression and chromatin retention of replication factors in addition to phenocopying the ploidy increase observed in RB-deficient cells. Interestingly, loss of RB increased ploidy but did not result in apoptotic death; however, consistent with previous studies (68), the expression of E2F1 caused both elevated ploidy and significant cell death, as indicated by the elevated sub-G1 population of cells. Additionally, suppression of endogenous E2Fs in RB-deficient cells by the dominant negative E2F-A/B mutant diminished the RB-negative phenotype. These results indicate that transcriptional control of DNA replication genes by the E2F/RB pathway is important for the observed changes in the control of their cell cycle.

Tumor-suppressive functions of RB have classically been attributed to its role in limiting cell proliferation by controlling the G1/S checkpoint. However, recent data allude to a broader functional repertoire for RB in phenomena that include other phases of the cell cycle (35, 69), DNA replication (23, 70, 71), centrosome amplification (72–74), global chromatin remodeling (75, 76), and genome stability (34, 35, 69). Furthermore, work from our laboratory and others have shown that loss of RB can lead to elevated ploidy (39, 74), suggesting that RB loss compromises genome integrity. Here, we show that RB loss leads to increased DNA replication through enhanced engagement of DNA replication factors onto chromatin, culminating in elevated ploidy. Furthermore, we show that deregulation of the E2F pathway in RB-proficient cells is sufficient to phenocopy the effects of RB loss and also confer elevated ploidy. Such changes in ploidy are consistent with relaxed control over cell cycle transitions and are associated with aneuploidy that is commonly observed in solid tumors (77). Importantly, deregulation of RB/E2F-regulated genes occur with high frequency in cancer and are speculated to directly contribute to chromosome instability (5, 33, 35, 69, 73). The findings here clearly support deregulation of the replication machinery via RB loss and aberrant E2F activity as two means of compromising genome integrity.

Acknowledgments—We thank Dr. Jeanne Cook for providing the AdCdc6 and AdCdt1 viruses and the anti-Cdt1 antibody. We thank Drs. Birgit Ehmer, Nancy Kleene, and Sejal Fox for expert microscopy guidance. We are grateful to Drs. Karen Knudsen, Chris Mayhew, Emily Bosco, A. Kathleen McClendon, Clay Comstock, and Wes Braden for critical reading of the manuscript and all members of both Knudsen laboratories for helpful suggestions and guidance.

REFERENCES
1. Weinberg, R. A. (1992) Cancer Surv. 12, 43–57
2. Kaelin, W. G., Jr. (1997) Ann. N. Y. Acad. Sci. 833, 29–33
3. Bartek, J., Bartkova, J., and Lukas, J. (1997) Exp. Cell Res. 237, 1–6
4. Bartek, J., Bartkova, J., and Lukas, J. (1996) Curr. Opin. Cell. Biol. 8, 805–814
5. Classon, M., and Harlow, E. (2002) Nat. Rev. Cancer 2, 910–917
6. Giacinti, C., and Giordano, A. (2006) Oncogene 25, 5220–5227
7. Ma, D., Zhou, P., and Harbour, J. W. (2003) J. Biol. Chem. 278, 19358–19366
