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Distinct Action of the Retinoblastoma Pathway on the DNA Replication Machinery Defines Specific Roles for Cyclin-Dependent Kinase Complexes in Prereplication Complex Assembly and S-Phase Progression▽

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The retinoblastoma (RB) and p16ink4a tumor suppressors are believed to function in a linear pathway that is functionally inactivated in a large fraction of human cancers. Recent studies have shown that RB plays a critical role in regulating S phase as a means for suppressing aberrant proliferation and controlling genome stability. Here, we demonstrate a novel role for p16ink4a in replication control that is distinct from that of RB. Specifically, p16ink4a disrupts prereplication complex assembly by inhibiting mini-chromosome maintenance (MCM) protein loading in G_1, while RB was found to disrupt replication in S phase through attenuation of PCNA function. This influence of p16ink4a on the prereplication complex was dependent on the presence of RB and the downregulation of cyclin-dependent kinase (CDK) activity. Strikingly, the inhibition of CDK2 activity was not sufficient to prevent the loading of MCM proteins onto chromatin, which supports a model wherein the composite action of multiple G_1 CDK complexes regulates prereplication complex assembly. Additionally, p16ink4a attenuated the levels of the assembly factors Cdt1 and Cdc6. The enforced expression of these two licensing factors was sufficient to restore the assembly of the prereplication complex yet failed to promote S-phase progression due to the continued absence of PCNA function. Combined, these data reveal that RB and p16ink4a function through distinct pathways to inhibit the replication machinery and provide evidence that stepwise regulation of CDK activity interfaces with the replication machinery at two discrete execution points.

Due to their profound influence on tumorigenesis, substantial effort has been directed at determining the function of the p16ink4a and retinoblastoma (RB) tumor suppressors in cell cycle control. Both tumor suppressors are inactivated in human tumors, as achieved by genetic mutation, gene silencing, or functional inactivation (6, 53, 63, 64). Biochemical and genetic evidence demonstrate a functional connection between the two tumor suppressors as part of a growth inhibitory network that is disrupted in the majority of cancers.

p16ink4a is a cyclin-dependent kinase (CDK) inhibitor, which arrests cells in G_1 (38, 48) and is dependent on inhibition of CDK4 and CDK2-associated activity. The influence of p16ink4a on CDK4 is mediated by direct binding and involves the disruption of CDK4/cyclin D interactions (57, 63). This function of p16ink4a is required for its ability to inhibit cell cycle progression and is disrupted in tumors which express elevated levels of CDK4 (thus titrating p16ink4a) or in tumors that harbor specific CDK4 mutations that compromise p16ink4a association (28, 70). In addition, p16ink4a-mediated disruption of the CDK4/cyclin D complex releases p27Kip1 and p21Cip1, leading to inhibition of CDK2 complexes (31, 65). This action is similarly required for p16ink4a-mediated cell cycle inhibition and therefore differentiates the function of p16ink4a from the genetic loss of CDK4 alone. A principle target of CDK4 and CDK2 kinase complexes is RB, which is inactivated by CDK-mediated phosphorylation in mid-G_1 (6, 53, 62, 63, 65). The significance of RB as a downstream target of p16ink4a was demonstrated by the finding that cells deficient in RB function are resistant to p16ink4a-mediated cell cycle inhibition (38, 44). Together, these studies suggest the existence of a linear pathway wherein p16ink4a functions upstream of RB to regulate proliferation and tumorigenesis. This model is supported by studies of human cancer wherein the loss of RB and p16ink4a are mutually exclusive events (49, 52, 53, 64).

RB is the target of functional inactivation in a multitude of tumors through a variety of different regulatory mechanisms. For example, genetic mutation of RB is found in retinoblastoma, small-cell lung cancer, and osteosarcoma, while the HPV-E7 protein targets RB function in cervical cancer (64). RB functions as a transcriptional corepressor, which inhibits the expression of critical gene products required for cell cycle progression (11, 24). Paramount among these targets is cyclin A, which has an exceedingly short half-life in the presence of RB and is required for cell cycle progression (2, 39). The repressive action of RB is manifest through interactions with the E2F family of transcription factors and the recruitment of

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additional corepressors (e.g., histone deacetylase, SWI/SNF) that facilitate the repression of E2F target genes (24). The inactivation of RB via phosphorylation disrupts transcriptional repression and enables the expression of E2F-regulated genes. In this regard, loss of p16ink4a function is believed to deregulate G_1 CDK activity, thus leading to aberrant phosphorylation/inactivation of RB. E2F-regulated genes are involved in the activation of CDK2 (e.g., cyclin A) or DNA replication (e.g., mini-chromosome maintenance protein 2 [MCM2]) (30, 56), and it is believed that E2F/RB activity serves a critical role in coordinating entry into S phase (11).

DNA replication is a highly complex process containing multiple control mechanisms to ensure proper genomic duplication (7, 9, 18). As cells enter G_1 from mitosis, Cdc6 and Cdt1 are independently recruited to a six-subunit origin recognition complex (ORC), which is believed to be bound to origins throughout the cell cycle (21, 67). These factors serve to recruit MCMs 2 to 7, the members of the MCM hexameric complex that load onto the origin (18, 19, 45, 50). The MCM complex is necessary for replication initiation, and the disruption of any one of the MCM family members results in the cessation of DNA replication (36). MCM binding renders the origin competent for the initiation of DNA replication and is termed the prereplicative complex (preRC) (17–19, 45, 67). As cells progress through G_1 into early S, the preRC matures to facilitate the recruitment of functional replication complexes that harbor DNA polymerases and associated processivity factors (e.g., proliferating cell nuclear antigen [PCNA]) (17). As these factors replicate the genome, the preRC is disrupted and reassembly is prevented by a variety of mechanisms which largely target the activity of Cdc6 and Cdt1 (9, 17, 18). In this manner, replication initiation at a given origin is restricted to once per cell cycle. The coordination of these events is believed to be reliant on the activation state of CDK complexes that have both positive and negative roles in DNA replication and the synthesis/degradation of critical regulatory factors (16, 23, 40, 46).

Recent studies have shown that RB plays a specific role in regulating DNA replication. Initially, it was observed that expression of constitutively active alleles of RB inhibits progression through S phase (3, 12, 60). Additional studies have shown that loss of RB deregulates replication control, enabling DNA replication to occur under inappropriate conditions (e.g., following DNA damage) (5, 34). The mechanism through which RB mediates replication control remains a subject of intense study, as both direct and indirect effects of RB on the replication machinery in S phase have been reported (10, 26, 32, 54, 66, 68). However, the activation of endogenous RB or the introduction of constitutively active alleles of RB attenuates CDK2 activity (through the downregulation of cyclin A) and induces a loss of PCNA activity in S phase (3, 47, 60). Although these studies connect RB to replication control, the influence of p16ink4a on replication control has not been addressed. Such an action by p16ink4a is highly relevant, since p16ink4a is required for response to DNA damage and for mediating cell cycle exit as a function of stresses that are associated with aberrant cellular proliferation. Here, we demonstrate a distinct effect of p16ink4a that influences the preRC at the level of MCM loading, which is dependent on multiple G_1 CDK activities. Together, these studies provide a new role for p16ink4a and G_1 CDK activity in regulating DNA replication, via a mechanism not recapitulated by RB.

TABLE 1. Summary of cell lines used in this study[a]

Cell line	RB status	p16ink4a status
A2-4 (+DOX)	Wild type	ND
A2-4 (−DOX)	PSM-RB induced	ND
U2OS	Wild type	Null
Saos-2	Null	Wild type
CV-1	Wild type	ND
MCF-7 (+shDonor)	Wild type	Null
MCF-7 (+shRB)	Knockdown	Null

[a] ND, not detectable; Null, gene deletion/silencing/mutant; +DOX, cultured in the presence of DOX; −DOX, cultured in the absence of DOX; +shDonor, containing shDonor; +shRB, containing shRB.

MATERIALS AND METHODS

Cell culture, transfections, and synchronizations. The following cell lines were used based on their RB and p16ink4a statuses as summarized in Table 1. A2-4 cells express an active allele of RB, PSM-RB, under the control of doxycycline (DOX) (60). U2OS human osteosarcoma cells express wild-type RB and CDK4 but are null for p16ink4a. Saos-2 human osteosarcoma cells express a mutant nonfunctional RB but also express endogenous p16ink4a (1, 38). CV-1 cells express wild-type RB, but p16ink4a is not detectable. MCF-7 breast carcinoma cells express wild-type RB and are p16ink4a null (49). These cells were transfected with plasmids encoding shRB or shDonor, and stable cells lines containing an effective knockdown of RB expression were created. A2-4, U2OS, CV-1, Saos-2, and MCF-7 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin-streptomycin, and 2 mM L-glutamine at 37°C in 5% CO_2. A2-4 cells harboring inducible expression of PSM-RB were supplemented with 1 mg/ml doxycycline. To activate the PSM-RB allele, cells were thoroughly washed three times in phosphate-buffered saline (PBS) and cultured in medium lacking DOX. Cells treated with roscovitine (Calbiochem) were incubated for 24 h at a concentration of 10 μM. The creation of shRB MCF-7 cells was described by Bosco et al. (submitted for publication).

To synchronize cells in early S phase, 5 μg/ml aphidicolin (APH; Calbiochem) or 1 mM hydroxyurea (Sigma) was added to the medium for 16 h. To release cells from the aphidicolin or hydroxyurea block, cells were washed three times with PBS and fresh medium was added. To activate PSM-RB expression, A2-4 cells were washed thoroughly with PBS to remove DOX and immediately placed in fresh medium containing APH. Cells synchronized in mitosis were treated with 50 ng/ml nocodazole (NOC; Calbiochem) for 16 h. To release the nocodazole block, cells were washed three times with PBS and cultured in fresh medium.

Protein stability was determined by transducing cells with the indicated adenoviruses (see below), followed by cycloheximide addition at 24 h postinfection. Cells were then subjected to Western blot analysis and bands quantitated by both densitometry and the Odyssey infrared imaging system (Li-COR Biosciences). Relative intensities were averaged and half-life values calculated. In addition to using cycloheximide to determine protein half-lives, pulse chase labeling was performed as previously described (2) (data not shown). Hemagglutinin (HA)-Cdt1 was resolved by immunoprecipitation with anti-HA antibody.

U2OS and Saos-2 cells were transfected using plasmids encoding pCDNA (vector-negative control), p16ink4a, CDK4, cyclin D1a, CDK4-R24C, NBCE, or cyclin E. Histone H2B-green fluorescent protein (GFP) expression plasmid was cotransfected to mark transfected cells. Transfections were performed using FuGene (Roche) according to the manufacturer's protocol. At 48 h posttransfection, cells were harvested by trypsinization and fixed using 3.7% formaldehyde in PBS or methanol at room temperature for 15 min.

Adenoviral infections. A2-4, CV-1, Saos-2, or U2OS cells (10^5) were seeded on coverslips or in 10-cm dishes and infected with either Ad-GFP, Ad-p16ink4a, Ad-GFP-Cdc6, Ad-HA-Cdt1, Ad-p21Cip1, or Ad-RBΔCDK at an approximate multiplicity of infection of 10 for 24 h. The HA-tagged Cdt1 adenoviral construct was generated by cloning a double-stranded oligonucleotide encoding two tandem copies of the HA tag at the 5′ end of the human Cdt1 cDNA in the pENTR3C vector. Recombination with pAd/CMV/V5-DEST (Invitrogen) and subsequent packaging in 293A cells were performed according to the manufacturer's instructions. The RBΔCDK cDNA was excised from PCDNA-RBΔCDK

(15) and cloned into the pShuttleCMV vector (Qbiogene, Inc., Irvine, CA). Recombination and packaging in HEK293 cells was carried out with standard protocols. Viruses were purified by double cesium chloride gradient and viral titers determined by standard approaches. In aphidicolin synchronization experiments, cells were treated with aphidicolin (as described above) for 16 h before infection with adenovirus for 24 h. In mitotic synchronizations, cells were treated with NOC minutes before adenoviral infection. Cells were cultured for 16 h prior to PBS wash and addition of fresh medium to initiate NOC release. Infection efficiency was visualized by GFP fluorescence at 90 to 95%.

Biochemical fractionation and Western blotting. To isolate chromatin-bound proteins, the protocol of Fujita et al. (25) was employed. Briefly, cells were cultured in 15-cm plates, washed three times with PBS, trypsinized, and centrifuged at 5,000 rpm for 5 min at 4°C. Soluble proteins were then extracted with ice-cold CSK buffer {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride} supplemented with 0.1% Triton X-100 for 15 min at 4°C. Extracted cells were centrifuged at 5,000 rpm for 5 min at 4°C, and the supernatant was removed. The pellet was then reextracted and suspended in CSK. Total cell extracts were harvested as previously described (3). Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with the following antibodies: anti-cyclin A (C19 mouse, H432 human; Santa Cruz), anti-β-tubulin (D10; Santa Cruz), anti-PCNA (PC10; Santa Cruz), anti-MCM7 (141.2; Santa Cruz), anti-ORC3 (1D6; Santa Cruz), anti-p16 (H156; Santa Cruz), anti-Lamin B (M20; Santa Cruz), anti-Cdc6 (180.2; Santa Cruz), anti-MCM5 (C18; Santa Cruz), anti-CDK4 (C22; Santa Cruz), and antigeminin (Fl209; Santa Cruz). Antibodies against RB (J. Wang), RPA (M. Wold), Cdt1 (J. Cook), and MCM2 (I. Todorov) were provided by the indicated investigators.

Immunofluorescence microscopy. For PCNA, MCM2, and MCM7 staining, approximately 10^5 cells were seeded onto coverslips, washed with ice-cold PBS, and fixed with methanol for 5 min. To visualize chromatin-tethered proteins, coverslips were washed three times with PBS and extracted with a modified CSK buffer as described above. Coverslips were washed with PBS and fixed in ice-cold methanol for 5 min. The following antibodies were used for immunofluorescence: PCNA (PC-10; Santa Cruz) and MCM7 (141.2; Santa Cruz). Immunofluorescence data are from three independent experiments, with at least 200 cells scored per experiment. BrdU incorporation assays were performed as previously described (33).

RT-PCR. RNA was harvested from U2OS cell cultures by using TRIzol according to the manufacturer's suggested protocols. Superscript reverse transcriptase (RT; Invitrogen) and 5 μg of total RNA were used to generate cDNAs with random hexamer primers. PCR was carried out using the following primers: 5′-GCG ATG ACA ACC TAT GCA ACA CTC-3′ and 5′-GAT GAC ATC CAT CTC CCT TTC CCT-3′ (human Cdc6), 5′-AGG TCA GAT TAC CAG CTC ACC ATC-3′ and 5′-ATT GAC TCA AGG CCT TCT CCA TCC-3′ (human Cdt1), and 5′-GGT CAT CAA TGG GAA ACC CAT CAC-3′ and 5′-AGT ACT GGT GTC AGG TAC GGT AGT-3′ (for glyceraldheyde-3-phosphate dehydrogenase).

Flow cytometry. Cells were harvested by trypsinization, fixed with ethanol, and incubated with propidium iodide (3). Histograms represent 10,000 events.

RESULTS

p16ink4a and RB exert distinct effects on the DNA replication machinery. To investigate the coordinate action of the RB and p16ink4a tumor suppressors in replication control, a previously characterized rat fibroblast cell line (A2-4) was utilized (60). This cell line harbors the inducible expression of PSM-RB, a phosphorylation site mutant allele of RB that is constitutively active. As expected, the removal of doxycycline from the medium resulted in the induced expression of PSM-RB, and correspondingly, specific targets of RB-mediated transcriptional repression, such as cyclin A, were downregulated at the protein level (Fig. 1A). Under these same conditions, the protein levels of PCNA were not attenuated, although cell cycle progression was inhibited (Fig. 1A). To analyze the influence of PSM-RB on the replication machinery, a well-characterized fractionation method was utilized, wherein actively engaged replication proteins are tethered to chromatin, while replication proteins not involved in replication are in a soluble nucleoplasmic pool (25). As shown in Fig. 1B, the induction of PSM-RB (via removal of doxycycline) had no influence on the percentage of cells expressing PCNA (unextracted), which is consistent with immunoblot analyses (Fig. 1A, left panel). However, PSM-RB expression significantly attenuated the chromatin-associated (extracted) pool of PCNA (Fig. 1B, left panel, and Fig. 1E, lanes 3 and 4). In contrast, PSM-RB expression had minimal influence on both total and chromatin-associated pools of MCM7 (Fig. 1B, right panel, and Fig. 1E, lanes 1 to 4).

To investigate the action of p16ink4a in the same cellular context, A2-4 cells were cultured in the presence of doxycycline (preventing PSM-RB expression) and transduced with adenoviruses encoding either GFP (negative control) or p16ink4a. Under these conditions, p16ink4a was robustly expressed within 24 h (Fig. 1C, left panel). As expected, the effect of p16ink4a was similar to that of PSM-RB, resulting in the specific downregulation of cyclin A but not PCNA protein levels (Fig. 1C, left panel). Additionally, within this same time frame, p16ink4a induced cell cycle arrest with a 2N DNA content (Fig. 1C, right panel). Together, these data are consistent with the model wherein activation of RB and activation of p16ink4a have comparable downstream effects on cell cycle control. However, while p16ink4a expression did not influence total MCM7 or PCNA levels, the chromatin association of both factors was significantly inhibited (Fig. 1D and 1E). These data demonstrate a new function for p16ink4a, which is distinct from the conventional action of activated RB on the replication machinery.

While the replication machinery is highly conserved, the unexpected result that RB and p16ink4a functions had distinct effects on the association of MCM7 with chromatin led us to analyze whether this phenomenon is apparent in other cellular backgrounds. In U2OS (human osteosarcoma) cells, p16ink4a expression resulted in the loss of both PCNA and MCM7 from chromatin-associated fractions (Fig. 1F). In parallel to these studies with p16ink4a, we utilized recombinant adenoviruses to express the RBΔCDK allele (which is resistant to phosphorylation and therefore constitutively active) in U2OS cells (15). While the protein was robustly expressed, downregulation of PCNA (but not MCM) chromatin binding was observed (Fig. 1G, lanes 1 and 2). Thus, the distinct actions of RB and p16ink4a are not restricted to a specific cell line or species.

Since DNA replication proceeds in a highly coordinated manner, we sought to dissect the influence of p16ink4a on other components of the replication machinery, both upstream and downstream of MCM7. Initially, the retention of ORC3 with chromatin was assessed. Before MCM proteins can bind and facilitate DNA replication, the ORC complex must be bound to DNA (8, 67). As shown in Fig. 1F and G, neither RBΔCDK nor p16ink4a influenced ORC3 expression or association with chromatin. Subsequent analyses of MCM2 and MCM5 association with chromatin were performed, as these proteins interact with MCM7 as part of the hexameric MCM complex. Consistent with the results for MCM7, the loss of MCM2 and MCM5 was observed in the chromatin-associated protein in response to p16ink4a expression (Fig. 1F, lane 4). Since formation of a functional preRC is required for the

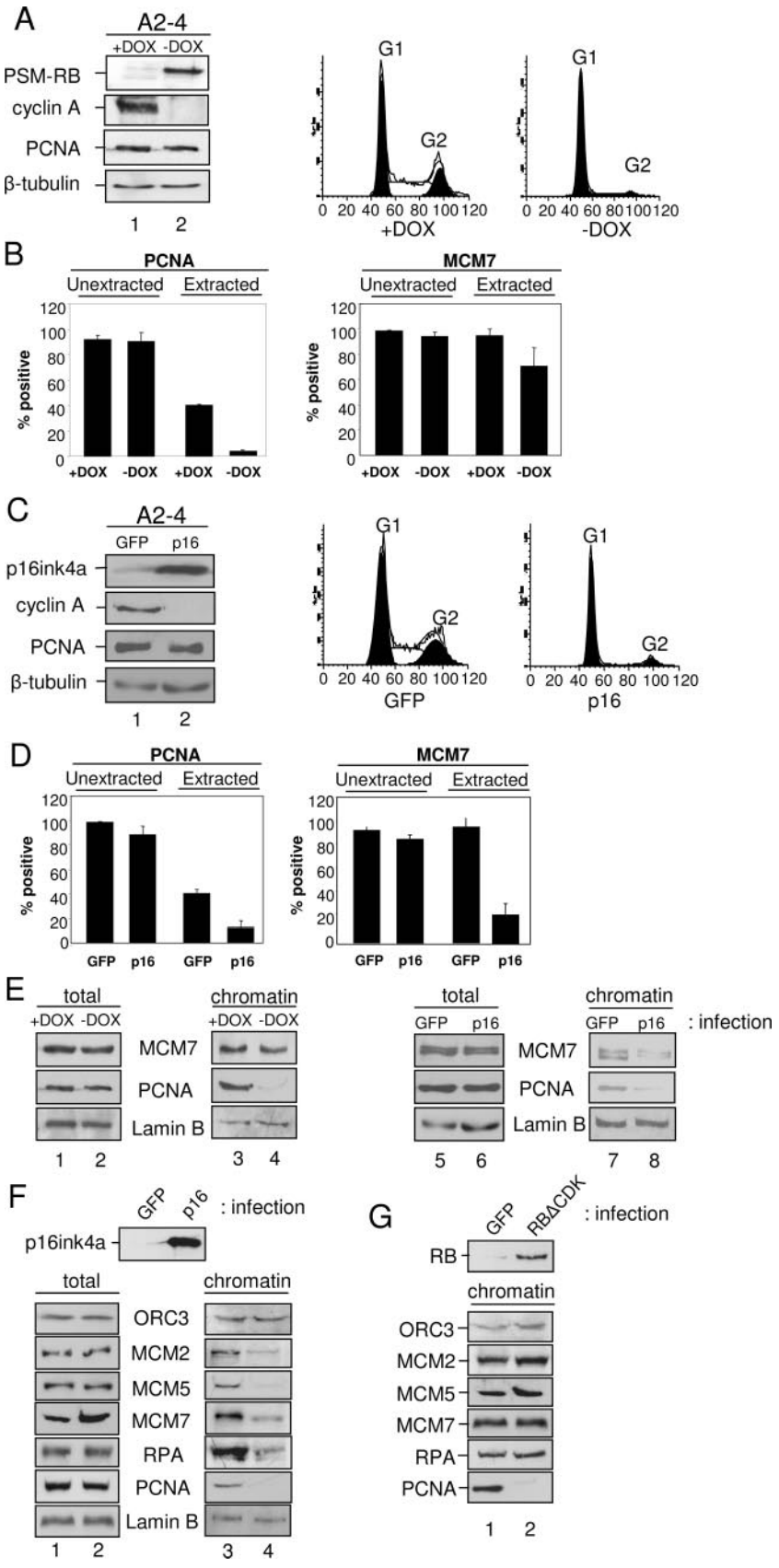


FIG. 1. p16ink4a disrupts PCNA and MCM7 chromatin association, whereas active RB targets only PCNA activity. (A) A2-4 cells were cultured in the presence or absence of DOX (+DOX and −DOX, respectively) for 24 h. Left panel: cells were harvested, equal total protein amounts were

subsequent recruitment of other factors to the replication apparatus, the chromatin association of RPA70 was also assessed, as this event is associated with the initiation of DNA replication. Here, p16ink4a inhibited RPA retention (Fig. 1F, lane 4). Combined, these results indicate that p16ink4a functions to block the assembly of the MCM complex onto chromatin and thus inhibits subsequent downstream events involved in DNA replication (i.e., RPA and PCNA engagement). By contrast, RBΔCDK inhibited only the retention of PCNA with chromatin and did not inhibit RPA chromatin association (Fig. 1G), consistent with previously published data (20, 60). Combined, these data indicate that the function of p16ink4a on the replication machinery is distinct from that of RB and define an earlier point at which the tumor-suppressive pathways intersect to control S phase.

p16ink4a signaling is compromised in a cell cycle phase-specific manner. Given the significant influence of p16ink4a on the replication machinery, the specific action of p16ink4a in S phase was analyzed. While p16ink4a is a well-characterized tumor suppressor, surprisingly little is known regarding its function in controlling S-phase progression. To determine the action of p16ink4a on the replication apparatus in S phase, A2-4 cells were synchronized for 16 h in the presence of aphidicolin. This treatment inhibits S-phase progression and arrests more than 85% of the cells in early S phase (not shown). The cells were maintained in aphidicolin and transduced with either GFP- or p16ink4a-encoding adenoviruses or cultured in the absence of doxycycline. Cells were processed at 0, 4, 8, 16, and 24 h postinfection or following doxycycline withdrawal and assayed for MCM7 and PCNA chromatin association. Under these conditions, PSM-RB induction did not influence MCM7 chromatin association but efficiently displaced PCNA from chromatin (Fig. 2A). In contrast to the significant effect on an asynchronous cell population, p16ink4a expression had minimal influence on MCM7 chromatin association in S phase (Fig. 2B, left panel). Surprisingly, the levels of chromatin-bound PCNA were also unchanged via p16ink4a expression in S-phase cells (Fig. 2B, right panel). These findings were confirmed by immunoblot analyses performed on parallel cell cultures (Fig. 2C). Therefore, although RB efficiently inhibited PCNA activity in S-phase cell populations, p16ink4a had a minimal effect in S-phase cultures. These results additionally indicated that p16ink4a does not directly impact the DNA replication fork or prevent late origin firing and suggested that p16ink4a is incapable of signaling to downstream targets in S phase. To test this idea, cyclin A protein levels were examined. In asynchronous cells transduced with adenovirus encoding p16ink4a or subjected to withdrawal of doxycycline for 24 h, cyclin A levels were diminished dramatically (Fig. 2D, lanes 2 and 3). Induction of PSM-RB in S-phase cells also resulted in the attenuation of cyclin A protein levels (Fig. 2D, lane 5). However, with S-phase-synchronized cells, p16ink4a expression had minimal effect on cyclin A protein levels (Fig. 2D, lane 6). These data were recapitulated with U2OS cells, where expression of p16ink4a in asynchronous cultures dramatically attenuated RB phosphorylation and cyclin A levels (Fig. 2E, lane 2), whereas RB phosphorylation was not completely attenuated and cyclin A expression was retained in aphidicolin-synchronized cells (Fig. 2E, lane 4). To determine the direct influence of p16ink4a on S-phase progression, cells were initially synchronized with aphidicolin and then transduced with p16ink4a- or RBΔCDK-encoding adenoviruses and cultured for 16 h to allow for accumulation of the encoded proteins. Cells were then released from the aphidicolin block to determine the capacity of p16ink4a to mediate the repression of cyclin A or inhibit S-phase progression as monitored by BrdU incorporation. As shown in Fig. 2F, either in the presence of aphidicolin or following a 4-h release, p16ink4a remained incompetent for the downregulation of cyclin A. Under these same conditions, while RBΔCDK expression efficiently inhibited S-phase progression, p16ink4a expression had little influence on DNA synthesis (Fig. 2G).

To further investigate the effect of p16ink4a on the RB pathway, CV-1 cells (either asynchronous proliferating or hydroxyurea synchronized) were transduced with either GFP- or p16ink4a-encoding adenoviruses for the indicated time points (Fig. 2H). While p16ink4a readily inhibited RB phosphorylation in cycling cells and suppressed the expression of cyclin A levels, it was unable to efficiently influence RB phosphorylation in cells blocked in S phase (Fig. 2H, lanes 1 to 4 compared to lanes 5 to 8). To explore the effect on cell cycle control, BrdU incorporation assays were performed in asynchronous and S-phase-synchronized cells. As expected, p16ink4a expression was able to efficiently arrest cycling cells. However,

resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. Right panel: cells were harvested, fixed, stained with propidium iodide, and processed for flow cytometry. Histograms are from 10,000 events. (B) A2-4 cells were cultured in the presence or absence of DOX for 24 h. Cells were subjected to in situ extraction or control extraction (unextracted) and then fixed and immunostained for PCNA (left panel) or MCM7 (right panel). Shown are the percentages of nuclei staining positive for PCNA or MCM7. (C) A2-4 cells cultured in the presence of DOX were transduced with GFP- or p16ink4a (p16)-encoding adenoviruses and harvested at 24 h postinfection. Left panel: cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. Right panel: cells were harvested, fixed, stained with propidium iodide, and processed for flow cytometry. Histograms are from 10,000 events. (D) A2-4 cells cultured in the presence of DOX were transduced with GFP- or p16ink4a-encoding adenoviruses. At 24 h postinfection, cells were subjected to in situ extraction or control extraction (unextracted) and then fixed and immunostained for PCNA (left panel) or MCM7 (right panel). Shown are the percentages of nuclei staining positive for PCNA or MCM7. (E) A2-4 cells were cultured in the presence (lanes 1, 3, 5, 6, 7, and 8) or absence (lanes 2 and 4) of DOX for 24 h or transduced with GFP (lanes 5 and 7)- or p16ink4a (lanes 6 and 8)-encoding adenoviruses and harvested at 24 h postinfection. Equal total protein (lanes 1, 2, 5, and 6) or chromatin-associated protein (lanes 3, 4, 7, and 8) amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (F) U2OS cells were transduced with GFP- or p16ink4a-encoding adenovirus and harvested at 24 h postinfection. Equal total or chromatin-associated protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (G) U2OS cells were infected with GFP- or RBΔCDK-encoding adenoviruses and harvested at 24 h postinfection. Cells were subjected to in situ extraction, equal chromatin-associated protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.

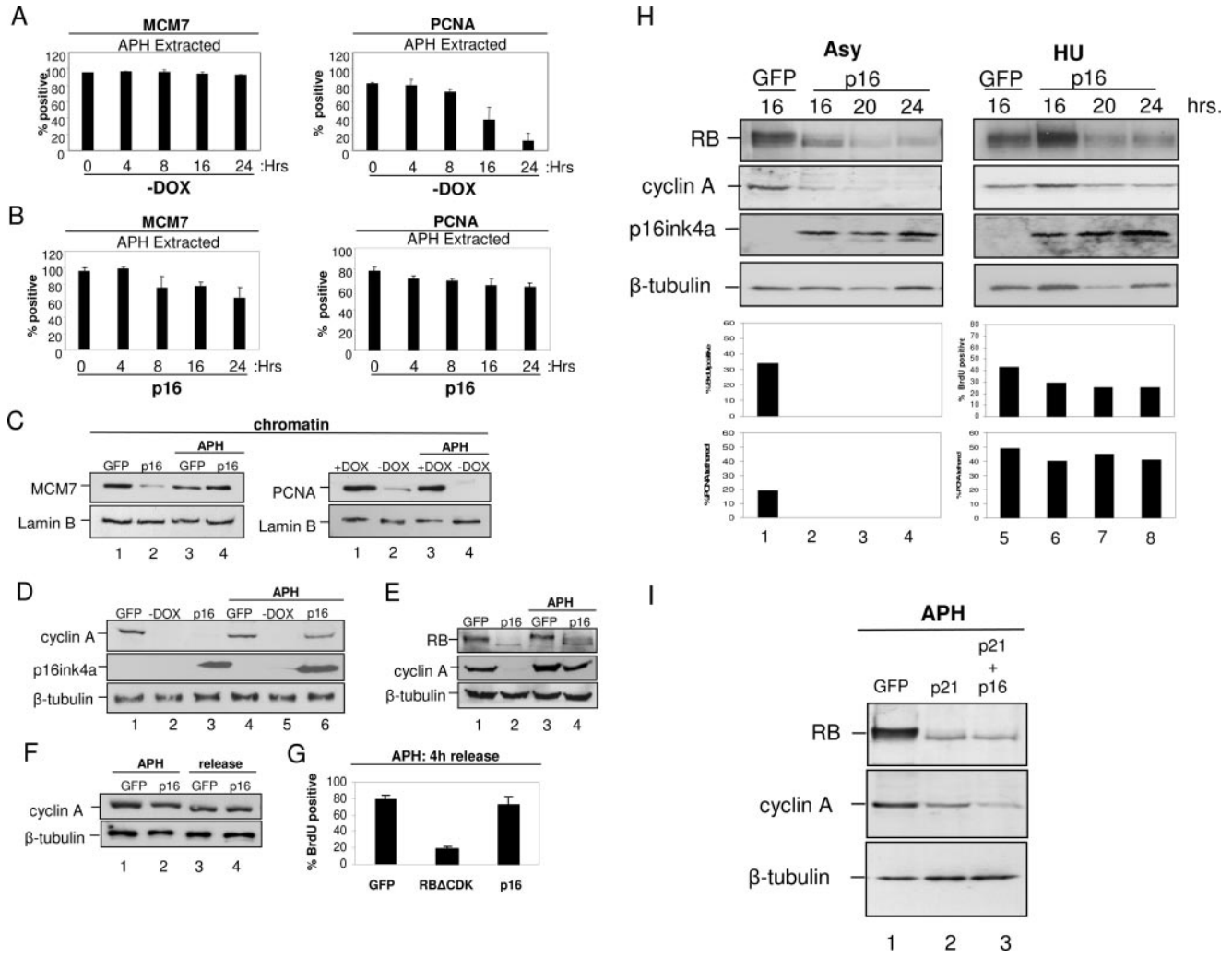


FIG. 2. p16ink4a does not disrupt the maintenance of the preRC in S phase. (A) A2-4 cells cultured in the presence of DOX were synchronized in S phase with aphidicolin for 16 h. Cells were then cultured in the absence of DOX (−DOX) for the indicated periods of time, subjected to in situ extraction or control extraction (unextracted), fixed, and immunostained for MCM7 (left panel) or PCNA (right panel). Shown are the percentages of nuclei staining positive for MCM7 or PCNA. (B) A2-4 cells cultured in the presence of DOX cells were synchronized in S phase with aphidicolin for 16 h. Cells were then transduced with adenoviruses encoding p16ink4a (p16) and subjected to in situ extraction or control extraction (unextracted) at the indicated times postinfection. Cells were then fixed and immunostained for MCM7 (left panel) or PCNA (right panel). Shown are the percentages of nuclei staining positive for MCM7 or PCNA. (C) Left panel: A2-4 cells cultured in the presence of DOX and either asynchronous (lanes 1 and 2) or arrested with aphidicolin (lanes 3 and 4) were transduced with GFP (lanes 1 and 3)- or p16ink4a (lanes 2 and 4)-encoding adenoviruses. At 24 h postinfection, cells were harvested, equal chromatin-associated protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. Right panel: either asynchronous (lanes 1 and 2) or aphidicolin-treated (lanes 3 and 4) A2-4 cells were cultured in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of DOX for 24 h. Cells were harvested, equal amounts of chromatin-associated protein were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (D) Either asynchronous (lanes 1 to 3) or aphidicolin-synchronized (lanes 4 to 6) A2-4 cells were cultured in the absence of DOX (lanes 2 and 5) or transduced with p16ink4a-encoding adenoviruses (lanes 3 and 6). Cells were harvested 24 h later, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (E) U2OS cells asynchronously proliferating (lanes 1 and 2) or arrested with aphidicolin (lanes 3 and 4) were transduced with GFP (lanes 1 and 3)- or p16ink4a (lanes 2 and 4)-encoding adenoviruses. Cells were harvested at 24 h postinfection, equal amounts of total protein were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (F) U2OS cells were treated with aphidicolin for 16 h and then infected with either GFP- or p16ink4a-encoding adenoviruses for 24 h. Cells were then either harvested or released from the aphidicolin block for 4 h and then harvested. Equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (G) U2OS cells were treated with aphidicolin for 16 h and then transduced with either GFP-, RBΔCDK-, or p16ink4a-encoding adenoviruses for 24 h. Cells were then released from the aphidicolin block for 3 h and pulsed with BrdU for 1 h. Coverslips were fixed and immunostained for BrdU incorporation. Data are from three independent experiments, with at least 200 cells scored per experiment. (H) CV-1 cells, asynchronous (Asy) or hydroxyurea arrested (HU), were transduced with either GFP- or p16ink4a-encoding adenoviruses for the indicated time points. Cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting (top panel), immunostained for BrdU incorporation (middle panel), or subjected to in situ extraction and immunostained for anti-PCNA (bottom panel). (I) CV-1 cells either asynchronously proliferating (data not shown) or arrested with APH and transduced with adenoviruses encoding GFP, p16ink4a, or p21Cip1 (p21) for 16 h. Cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.

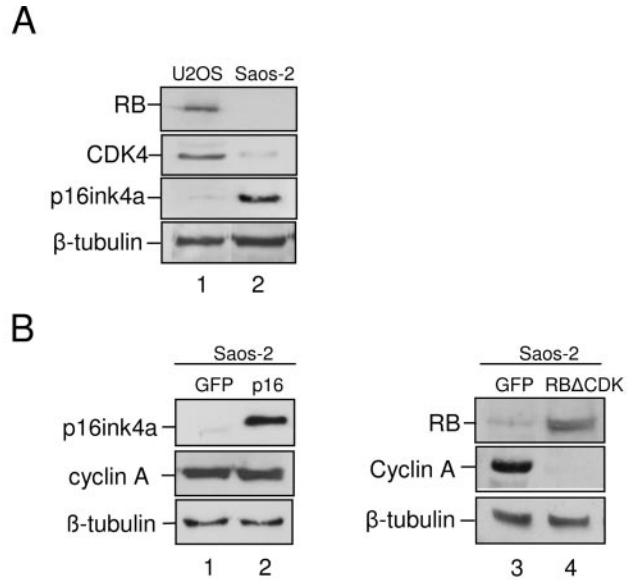
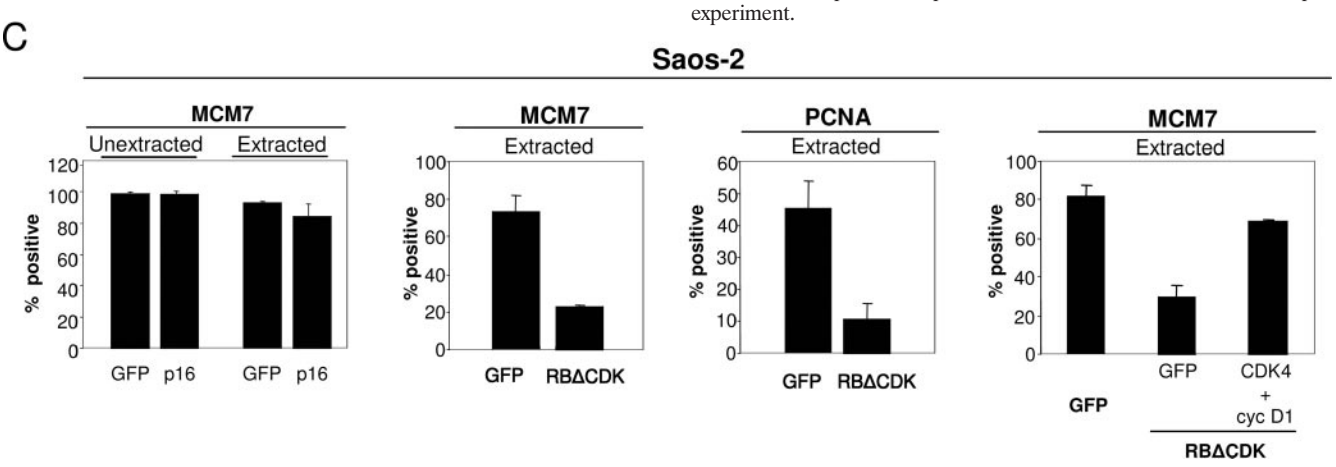


FIG. 3. Requirement of a functional RB pathway in preRC regulation. (A) U2OS and Saos-2 cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) Left panel: Saos-2 cells were transduced with GFP (lane 1)- or p16ink4a (lane 2)-encoding adenoviruses. At 24 h postinfection, cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. Right panel: Saos-2 cells were transduced with GFP (lane 1)- or RBΔCDK (lane 2)-encoding adenoviruses. At 24 h postinfection, cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (C) Left panel: Saos-2 cells were transduced with GFP- or p16ink4a (p16)-encoding adenoviruses. At 24 h postinfection, cells were subjected to in situ extraction or mock extraction (unextracted), fixed, and immunostained for MCM7. Shown are the percentages of nuclei staining positive for MCM7. Middle panels: Saos-2 cells were transduced with adenoviruses encoding GFP or RBΔCDK for 24 h. Cells were subjected to in situ extraction, fixed, and immunostained for MCM7 or PCNA. The percentages of cells staining positive for MCM7 or PCNA are shown. Right panel: Saos-2 cells were transduced with adenovirus encoding GFP or RBΔCDK for 24 h. Cells were then transfected with H2B-GFP or H2B-GFP, CDK4, and cyclin D1 (cyc D1) 24 h postinfection. Cells were subjected to in situ extraction, fixed, and immunostained for MCM7. Percentages of nuclei positive for GFP and MCM7 are shown. Data are from three independent experiments, with at least 200 cells scored per experiment.



p16ink4a had no effect on BrdU incorporation in cells that had been released from the hydroxyurea block (Fig. 2H). This failure of p16ink4a to signal in S phase held true for PCNA chromatin engagement, as indicated in Fig. 2H. Together, these results demonstrate that while active RB can function in S-phase cells, p16ink4a signaling is disrupted both for the activation of the RB pathway and for the disruption of preRC function in this phase of the cell cycle.

Since p16ink4a directly inhibits CDK4, these results likely reflect a minimal role for this kinase in maintaining RB phosphorylation in S phase. To determine the corresponding influence of CDK2 inhibition on RB phosphorylation, we assessed the effects of enforced p21Cip1 expression. For these studies, CV-1 cells were synchronized with aphidicolin and transduced with adenoviruses encoding GFP, p21Cip1, or both p16ink4a and p21Cip1. In contrast with results observed with p16ink4a alone, p21Cip1 provoked RB dephosphorylation in the presence of aphidicolin (Fig. 2I). Thus, CDK inhibitors differentially influence RB phosphorylation, dependent on cell cycle phase.

A functional RB pathway is required for p16ink4a-mediated replicative inhibition. The above-described findings suggested that while RB dephosphorylation/activation is not sufficient to disrupt the preRC, it may be required for p16ink4a to signal to CDK complexes. To determine the role of RB in mediating the effects of p16ink4a on the replication machinery, the RB-deficient osteosarcoma cell line Saos-2 was employed. As expected, Saos-2 cells lack the endogenous RB expression yet express ample levels of endogenous p16ink4a and minimal CDK4 (Fig. 3A). Saos-2 cells efficiently overproduced p16ink4a following infection (Fig. 3B, left panel, lane 2); however, due to the absence of endogenous RB, there was a failure to repress target genes, such as the cyclin A gene (Fig. 3B, left panel, lane 2). In contrast, RBΔCDK expression was able to repress cyclin A (Fig. 3B, right panel, lane 2), indicating that the reconstitution of RB completes a functional RB pathway. In these cells, the ectopic expression of p16ink4a failed to disrupt the association of MCM7 with chromatin (Fig. 3C). Therefore, these data suggest that the inhibition of preRC activity is not due to a direct influence of p16ink4a on the replication machinery but is dependent on RB signaling.

Since Saos-2 cells express high levels of endogenous p16ink4a and relatively low levels of CDK4, reconstitution of

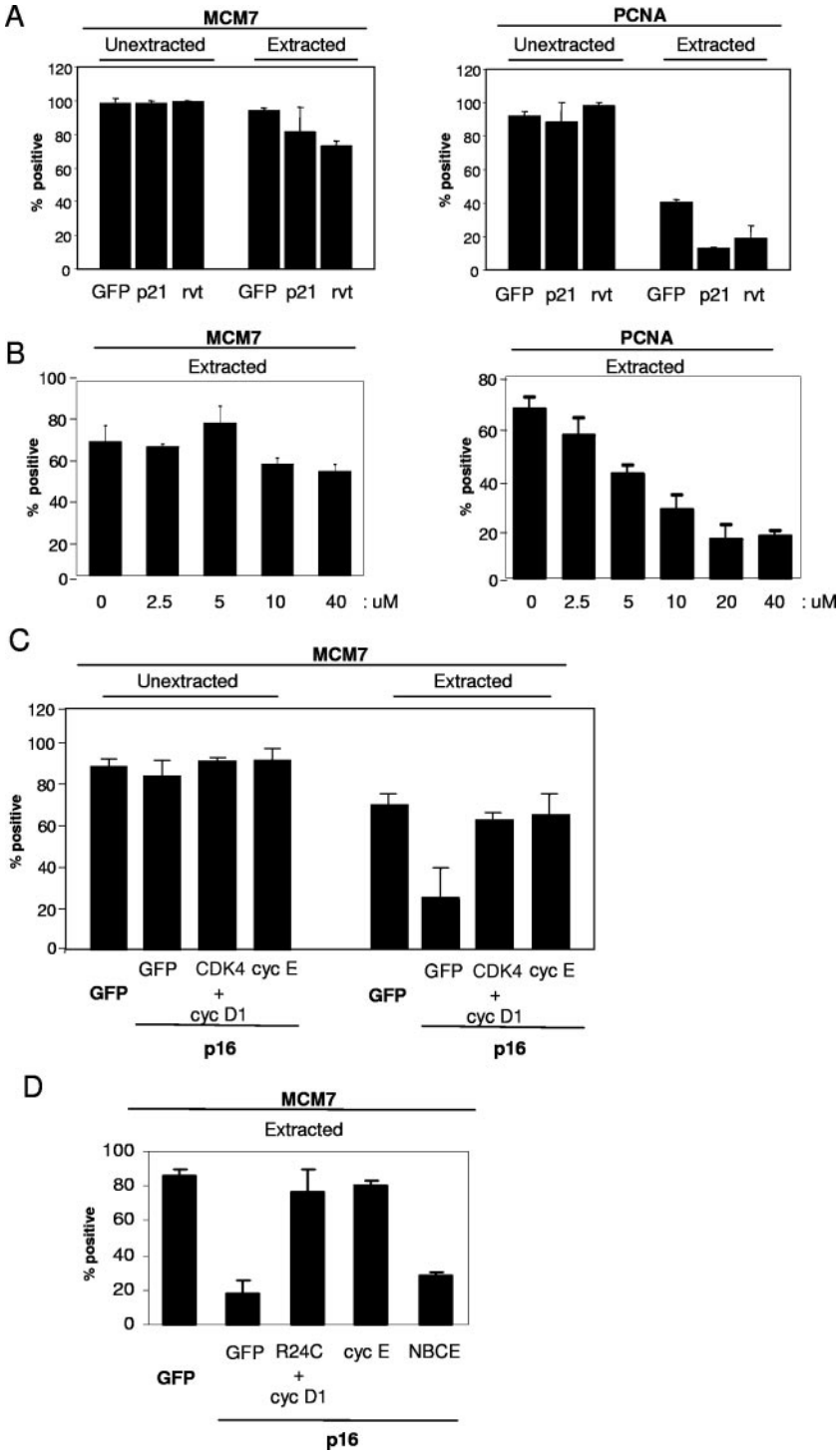


FIG. 4. Coordinate action of p16ink4a inhibits MCM7 chromatin association. (A) A2-4 cells cultured in the presence of DOX were infected with GFP- or p21Cip1 (p21)-encoding adenoviruses or treated with 10 μM roscovitine (rvt). At 24 h posttreatment, cells were subjected to in situ extraction or mock extraction (unextracted), fixed, and immunostained for MCM7 (left panel) or PCNA (right panel). Shown are the percentages of cells staining positive for MCM7 or PCNA. (B) U2OS cells were treated with increasing doses of roscovitine as shown. At 24 h posttreatment, coverslips were subjected to either mock (unextracted; not shown) or in situ extraction, fixed, and immunostained for PCNA or MCM7. (C) U2OS cells were cotransfected with GFP-H2B and the indicated expression plasmids. At 48 h posttransfection, cells were subjected to mock (unextracted) or in situ extraction, fixed, and immunostained for MCM7. The percentages of transfected (H2B-GFP-positive) cells staining positive for MCM7 are shown. Data are from three independent experiments, with at least 200 transfected cells scored per experiment. (D) U2OS cells were cotransfected with H2B-GFP and either p16ink4a (p16); p16ink4a, cyclin D1 (cyc D1), and CDK4-R24C; cyclin E (cyc E); or NBCE for 48 h. Cells were subjected to mock (unextracted) or in situ extraction, fixed, and immunostained for MCM7. The percentages of transfected (H2B-GFP-positive) cells staining positive for MCM7 are shown. Data are from three independent experiments, with at least 200 transfected cells scored per experiment.

RB may be expected to restore the effects of p16ink4a in this cell system. To test this idea, Saos-2 cells were transduced with adenoviruses encoding GFP or RBΔCDK for 24 h. Strikingly, RBΔCDK strongly attenuated chromatin-associated MCM7 and PCNA (Fig. 3C), specifically in Saos-2 cells. To determine whether this specific influence of RBΔCDK on MCM7 loading was due to the low levels of CDK4 activity in Saos-2 cells, the influence of RBΔCDK was challenged with the ectopic expression of CDK4/cyclin D1. As shown in Fig. 3C (right panel), CDK4/cyclin D1 expression efficiently restored MCM7 binding. Thus, these results indicate that Saos-2 cells are sensitive to the effects of RB on the preRC due to the low levels of CDK4 activity and support the idea that high levels of endogenous p16ink4a cooperate with RB.

G_1 CDK inhibition through p16ink4a disrupts MCM loading. The results described above suggest that while there is a dependence on RB function, p16ink4a activity is distinct from that of RB in regulating the preRC. Presumably, such an effect could reflect the ability of p16ink4a to effectively target multiple CDK2 complexes, while RB effectively targets only cyclin A-associated complexes (2, 39). To interrogate this possibility, p21Cip1 was expressed in A2-4 cells. As shown in Fig. 4A (right panel), p21Cip1 expression efficiently disrupted PCNA chromatin association. This result indicates that loss of PCNA tethering is an event common to both CDK inhibitors and RB activation and was also observed with the expression of p27Kip1 (not shown). However, p21Cip1 had minimal influence on MCM7 chromatin association (Fig. 4A, left panel). Since p16ink4a and p21Cip1 both inhibit CDK2 activity, albeit through different mechanisms, the influence of CDK2 activity on MCM7 chromatin association was directly assessed using the pharmacological CDK inhibitor roscovitine. Under conditions of CDK2 inhibition, PCNA chromatin association was maximally inhibited at a 20 μM concentration (Fig. 4A and B). In contrast, the levels of MCM7 were largely unchanged even up to a 40 μM concentration of roscovitine (Fig. 4B, left panel). Together, these results indicate that p16ink4a harbors an activity distinct from those of other CDK inhibitors in the control of MCM7 chromatin association, likely through its ability to coordinately inhibit CDK4 and CDK2 activity.

To test the influence of specific CDK/cyclin complexes in the regulation of preRC function, cells were transiently transfected with p16ink4a expression plasmids and plasmids encoding either cyclin E (to restore CDK2 function) or CDK4 and cyclin D1 (to restore CDK4 activity). In these studies, p16ink4a disrupted the chromatin association of MCM7 in transfected cells (Fig. 4C); however, the ectopic expression of either cyclin E or a combination of both CDK4 and cyclin D1 restored MCM7 retention on chromatin. To determine whether CDK4 activity is directly required for preRC assembly, R24C, a CDK4 mutant that fails to bind p16ink4a, was utilized (70). As shown in Fig. 4D, the effect of p16ink4a on MCM7 chromatin tethering was overcome by CDK4-R24C expression, indicating that CDK4 inhibition failed to block the p16ink4a-mediated inhibition of MCM7 loading onto chromatin. While cyclin E expression can rescue MCM loading in the presence of p16ink4a, we questioned the specific involvement of CDK2 activity in this process. A mutant cyclin E that cannot bind CDK2 (*n*on*b*inding *c*yclin *E* [NBCE]) (13) was incapable of reversing the inhibition of MCM7 chromatin association in the presence of p16ink4a (Fig. 4D). Combined, these data indicate that the coordinate inhibition of multiple G_1 CDK complexes is responsible for the influence of p16ink4a on the preRC.

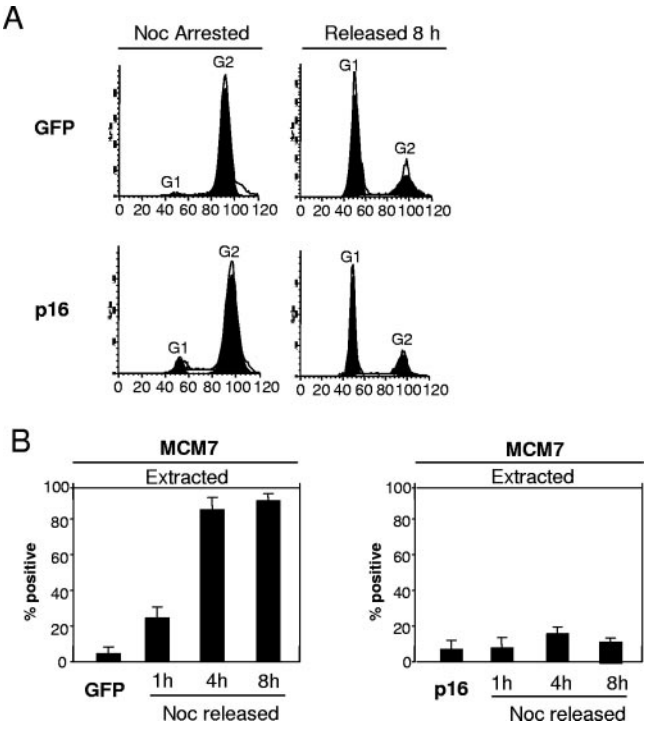


FIG. 5. p16ink4a inhibits preRC formation upon mitotic exit. (A) U2OS cells were treated with 50 ng/ml NOC or transduced with adenovirus encoding p16ink4a and treated with NOC simultaneously for 16 h. Cells were released from the nocodazole block for 0 h and 8 h and harvested for flow cytometric analysis as indicated. (B) Cells were subjected to in situ extraction or control extraction (unextracted) at the indicated times following NOC release, fixed, and immunostained for MCM7.

p16ink4a inhibits preRC assembly as cells progress out of mitosis. The observation that p16ink4a failed to disrupt preformed complexes in early S phase suggested that the influence of p16ink4a is specific to the assembly of the preRC, which occurs in early G_1. To investigate the specific role of p16ink4a in the assembly of the preRC, the maturation of the preRC as cells progressed out of mitosis was investigated. Cells were treated with nocodazole and then infected with GFP- or p16ink4a-encoding adenoviruses. Cells were released from nocodazole at 16 h postinfection and harvested at 1 h, 4 h, or 8 h following release. Under these conditions, both GFP- and p16ink4a-infected cells were efficiently synchronized in G_2/M and progressed into G_1 following release (Fig. 5A). Following treatment, the chromatin association of MCM7 was analyzed kinetically (Fig. 5B). As expected, cells enriched in G_2/M exhibited minimal chromatin association of MCM7. However, as GFP-infected cells entered into G_1, there was a significant increase in the engagement of MCM7 with chromatin at 1, 4, and 8 h following mitotic exit (Fig. 5B, left panel). In contrast, in cells expressing p16ink4a chromatin-associated MCM7, levels did not increase significantly even by 8 h, when the majority of cells were in G_1 (Fig. 5B, right panel). Therefore, these data

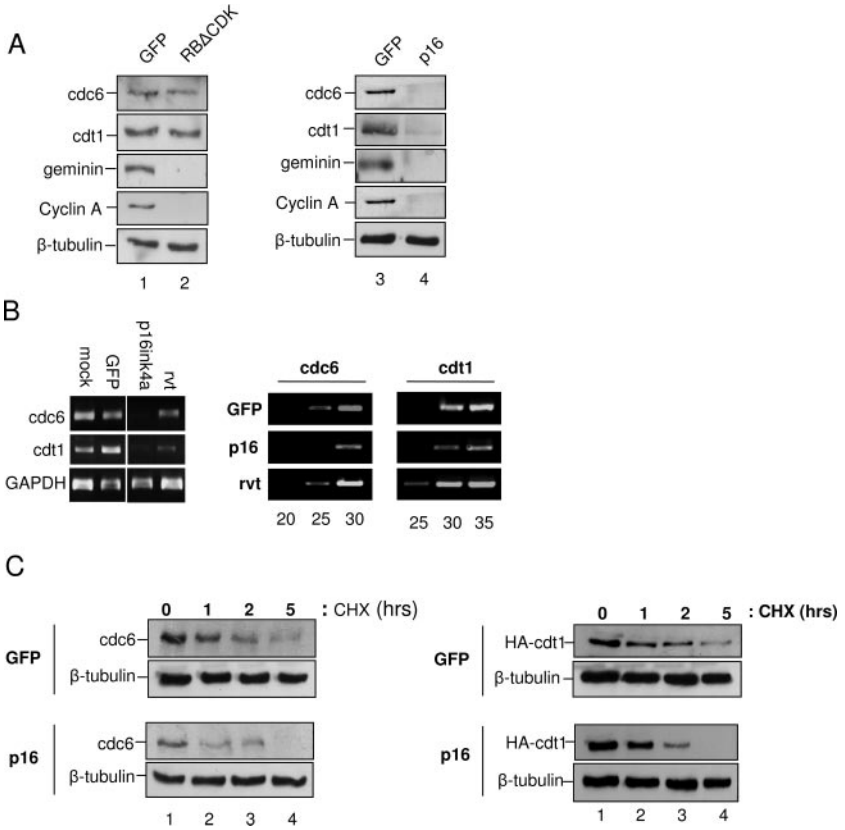


FIG. 6. p16ink4a inhibits preRC at the level of Cdc6 and Cdt1 regulation. (A) U2OS cells were transduced with either GFP (lanes 1 and 3)-, p16ink4a (lane 4)-, or RBΔCDK (lane 2)-encoding adenovirus. At 24 h postinfection, cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) U2OS cells were transduced with the indicated adenoviruses or treated with roscovitine (rvt) for 24 h. RNA was then extracted using TRIzol according to the manufacturer's protocol (Invitrogen), and RT-PCR was performed using primers to amplify Cdc6, Cdt1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). (C) U2OS cells were transduced with adenoviruses encoding GFP or p16ink4a in combination with Cdc6 (left panels) or Cdt1 (right panels) for 24 h. Cells were then treated with cycloheximide (CHX) and harvested at the indicated time points. Lysates were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. Bands were quantitated using densitometry (Metamorph software).

demonstrate that p16ink4a expression specifically prevents MCM loading and thus the formation of the preRC.

Direct inhibition of Cdc6 and Cdt1 by p16ink4a. Loading of MCMs onto chromatin is dependent on the action of Cdc6 and Cdt1. These factors are tightly regulated by multiple mechanisms, including level of expression and the presence of the inhibitory molecule geminin. Therefore, a possible explanation for the observed inhibition of MCM7 chromatin binding is that it was due to the downregulation or inactivation of Cdt1 or Cdc6 or elevated expression of geminin. Initially, the influence of RBΔCDK on Cdt1 and Cdc6 was assessed, and no alteration of protein levels was observed (Fig. 6A, lane 2). These results are consistent with the finding that MCM loading occurs in the presence of dephosphorylated RB. Geminin levels were significantly downregulated by RBΔCDK (Fig. 6A, lane 2). Such a result is consistent with previously published data on the regulation of geminin by the RB pathway (41, 71). By contrast, p16ink4a initiated a significant reduction in the levels of both Cdt1 and Cdc6 proteins (Fig. 6A, lane 4). To determine the corresponding influence on RNA levels, RT-PCR was performed on cells transduced with adenoviruses expressing GFP or p16ink4a or treated with roscovitine. As shown in Fig. 6B, RNA levels of Cdc6 and Cdt1 were inhibited by p16ink4a but not by roscovitine, suggesting one mechanism through which p16ink4a is inhibiting protein levels through transcriptional repression. However, it has recently been demonstrated that Cdc6 and Cdt1 protein levels are modulated as a function of protein stability (4, 22, 37, 40, 55, 58). To determine the influence of p16ink4a on protein stability, we utilized adenovirus-mediated expression of Cdc6 or Cdt1 as a substrate upon which to monitor the effect of p16ink4a. At 24 h postinfection, cells were treated with cycloheximide for 1, 2, and 5 h (Fig. 6C) and protein levels were determined by Western blot analysis. Consistent with a recent study (40), densitometry calculations showed that the half-life of Cdc6 was reduced by approximately 50% with p16ink4a expression. Interestingly, we also found that the Cdt1 protein half-life was reduced by 37% via the expression of p16ink4a. Thus, p16ink4a regulates Cdc6 and Cdt1 at the levels of both RNA level and protein stability.

Loss of Cdt1 and Cdc6 expression correlates with failure in MCM loading. We have shown that p16ink4a signaling is compromised in S phase, resulting in a minimal effect on MCM chromatin association. To determine the corresponding influence of p16ink4a on Cdc6 and Cdt1 in S phase, cells were

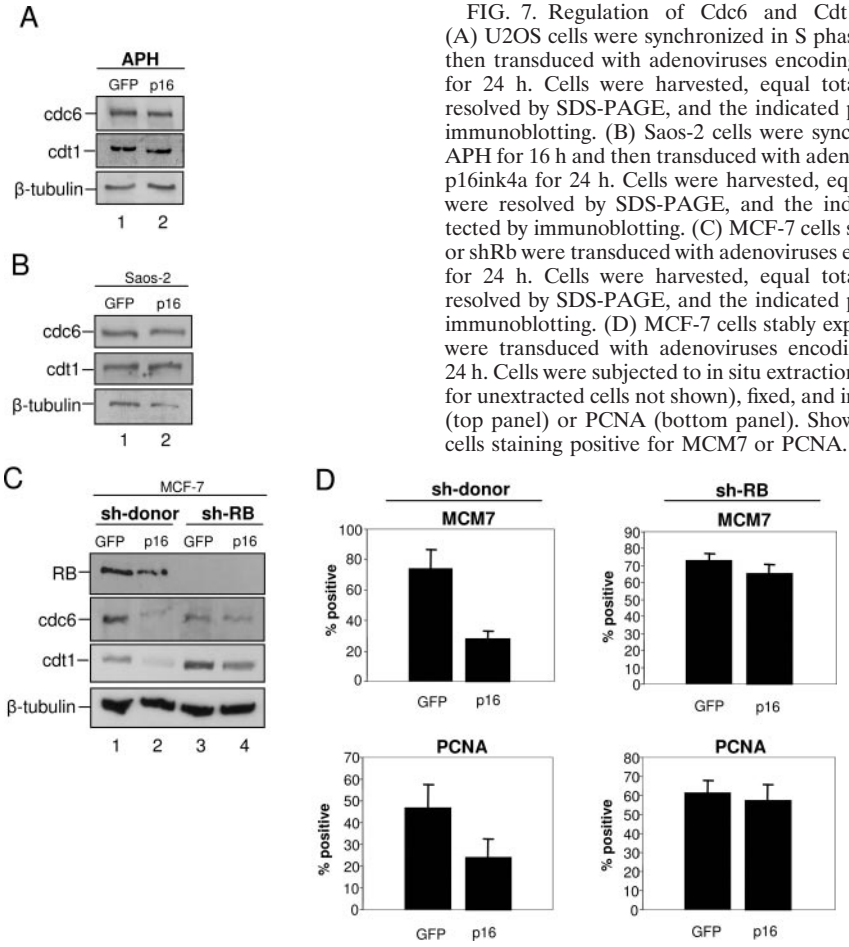


FIG. 7. Regulation of Cdc6 and Cdt1 by the RB pathway. (A) U2OS cells were synchronized in S phase with APH for 16 h and then transduced with adenoviruses encoding GFP or p16ink4a (p16) for 24 h. Cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) Saos-2 cells were synchronized in S phase with APH for 16 h and then transduced with adenoviruses encoding GFP or p16ink4a for 24 h. Cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (C) MCF-7 cells stably expressing shDonor or shRb were transduced with adenoviruses encoding GFP or p16ink4a for 24 h. Cells were harvested, equal total protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (D) MCF-7 cells stably expressing shDonor or shRb were transduced with adenoviruses encoding GFP or p16ink4a for 24 h. Cells were subjected to in situ extraction or mock extraction (data for unextracted cells not shown), fixed, and immunostained for MCM7 (top panel) or PCNA (bottom panel). Shown are the percentages of cells staining positive for MCM7 or PCNA.

synchronized with aphidicolin for 16 h and infected with p16ink4a for 24 h. In this setting, enforced p16ink4a expression had no effect on Cdc6 or Cdt1 in S phase (Fig. 7A), thus correlating the loss of MCM chromatin association with the presence of these loading factors. To determine whether RB was similarly required for this activity, p16ink4a was introduced into Saos-2 cells. Analysis of Cdc6 and Cdt1 revealed no alteration upon p16ink4a expression (Fig. 7B). To further validate this result, stable MCF-7 cell lines containing either an empty short-hairpin RNA vector (shDonor) or short-hairpin RNA directed against Rb (shRb) (Bosco et al., submitted) were transduced with either GFP- or p16ink4a-encoding adenoviruses. While the RB-proficient cells (shDonor) responded to p16ink4a expression by attenuating MCM7 chromatin binding, the RB-negative line (shRB) was refractory to this effect of p16ink4a (Fig. 7D). Under these conditions, p16ink4a similarly failed to influence Cdc6 and Cdt1 protein levels in the absence of RB (Fig. 7C). Thus, RB is required for the action of p16ink4a on both MCM loading and the attenuation of Cdt1 and Cdc6. Combined, these results correlate the influence of p16ink4a on Cdt1 and Cdc6 protein levels and the inhibition of MCM loading.

Combined expression of Cdc6 and Cdt1 restores preRC integrity in the presence of p16ink4a. To determine the action of Cdc6 and Cdt1 in mediating the effects of p16ink4a, adenoviruses encoding either protein were employed. Using these viruses, the expression of either Cdc6 or Cdt1 was efficiently restored in the presence of p16ink4a (Fig. 8A). However, the expression of either factor alone was not sufficient to restore MCM7 chromatin association or trigger the engagement of PCNA (Fig. 8B). In contrast, the coexpression of Cdc6 and Cdt1 leads to the restoration of MCM7 chromatin binding in the presence of p16ink4a (Fig. 8C and D). Under these same conditions, PCNA failed to interact with chromatin (Fig. 8C). Thus, the influence of p16ink4a on the preRC is specifically manifest through Cdt1 and Cdc6 stability and additional facets of CDK inhibition that contribute to the regulation of PCNA.

DISCUSSION

A number of studies have shown the actions of p16ink4a and RB to be equivalent in cell cycle control. In most models, p16ink4a blocks G_1 CDK activity, thus maintaining the antiproliferative function of RB. Given this linear relationship, it was assumed that p16ink4a and RB act in a linear pathway to inhibit DNA replication. Here, we probed the mechanism of p16ink4a-mediated replication control and found that p16ink4a functions distinctly from RB in targeting the preRC. Specifically, p16ink4a targets the assembly of preRCs at the level of MCM loading (Fig. 9). This was observed with a

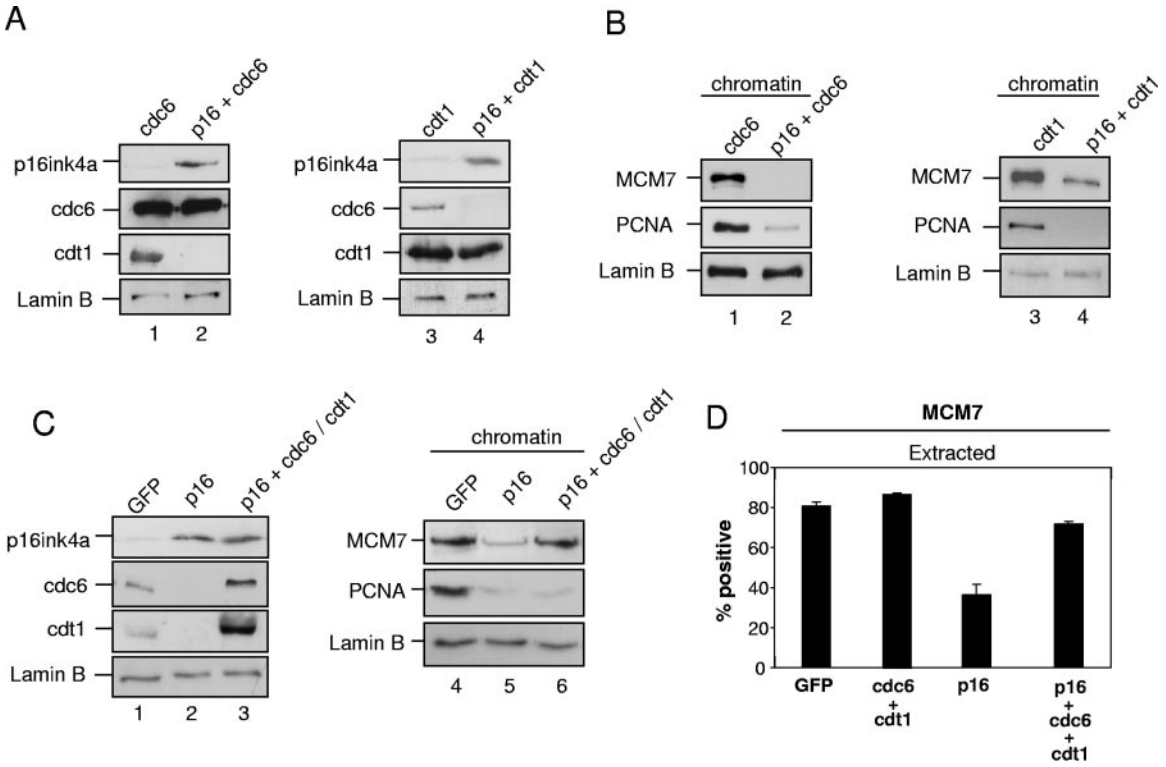


FIG. 8. Cdc6 and Cdt1 expression can rescue MCM7 tethering in the presence of p16ink4a (p16). (A) U2OS cells were transduced with either Cdc6 (lanes 1 and 2)- or Cdt1 (lanes 3 and 4)-encoding adenoviruses in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of p16ink4a-encoding adenovirus. At 24 h postinfection, cells were harvested, equal protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) Cells were transduced with either Cdc6 (lanes 1 and 2)- or Cdt1 (lanes 3 and 4)-encoding adenoviruses in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of p16ink4a-encoding adenovirus. Cells were subjected to in situ extraction 24 h postinfection, equal chromatin-associated protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (C) Cells were transduced with GFP (lanes 1 and 4)-, p16ink4a (lanes 2 and 5)-, or p16ink4a-, Cdc6-, and Cdt1 (lanes 3 and 6)-encoding adenovirus for 24 h. U2OS cells were then harvested or subjected to in situ extraction, equal chromatin-associated protein amounts were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (D) Cells were transduced with adenoviruses encoding the proteins indicated. At 24 h postinfection, cells were subjected to in situ extraction, fixed, and immunostained for MCM7. Shown are the percentages of nuclei staining positive for MCM7.

number of different MCM proteins and did not involve the disruption of the ORC complex. The effect of p16ink4a is not shared with RB, as observed either with activated alleles or through activation/dephosphorylation of endogenous RB (47). p16ink4a function is restricted to G_1 and involves the down-regulation of Cdc6 and Cdt1. These proteins are degraded or become unstable as cells progress from S phase through mitosis (4, 37, 55), and the presented data suggest that p16ink4a specifically compromises RNA and protein accumulation. Interestingly, Cdc6 has been shown to regulate the INK4 locus, suggesting the possibility of a feedback loop through the coordinate actions of these two factors (27). The p16ink4a tumor suppressor is a potent transcriptional repressor functioning through pocket proteins. Surprisingly, p16ink4a regulates the expression of these proteins while RBΔCDK does not, revealing a key role for G_1 CDK complexes in the control of MCM loading. Such a hypothesis is supported by recent findings that CDK activity contributes to the stabilization of Cdc6 (22, 40). Thus, the difference in the actions of p16ink4a and RB on the replication machinery is likely reflective of the manner in which they target CDK/cyclins. p16ink4a targets CDK/cyclin complexes through direct interaction with CDK4, thus mobi-

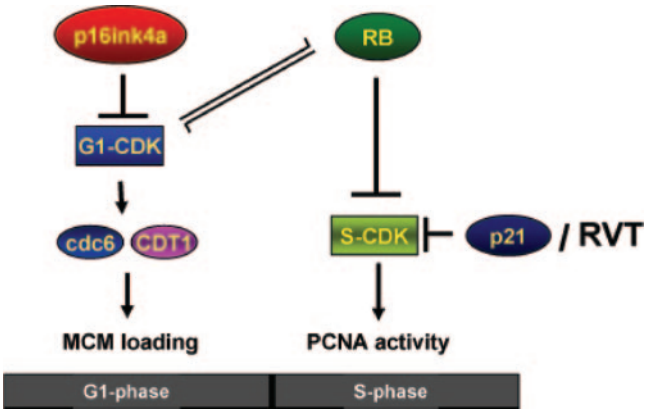


FIG. 9. Model of the RB pathway in coordinating DNA replication. p16ink4a prevents preRC formation by inhibiting the activity of G_1 CDK complexes, leading to a reduction in Cdc6 and Cdt1 protein levels in G_1. RB is negatively regulated by G_1 CDK complexes yet also plays a role in mediating the suppression of G_1 CDK activity. RB acts upon S-phase CDK complexes to inhibit the association of PCNA with chromatin. This activity of RB is shared with other modalities that inhibit CDK2 activity. p21, p21Cip1.

lizing CDK2 inhibitors and preventing RB phosphorylation (which further impinges on CDK2 activity) (63). As such, in cells expressing p16ink4a, G_1 CDK activity is maximally inhibited, and as shown in Fig. 9, there is a subsequent failure to load MCMs. The importance of overall downregulation of CDK activity is demonstrated by the finding that the overproduction of either cyclin E, CDK4/cyclin D1, or CDK4-R24C/cyclin D1 can overcome the effect of p16ink4a on MCM loading. Since CDK4-R24C specifically fails to interact with p16ink4a and thus escapes inhibition, these results indicate that CDK4 activity is critical for MCM loading. Conversely, since a cyclin E mutant which does not mediate activation of CDK2 fails to facilitate MCM loading in the presence of p16ink4a, these data demonstrate that CDK2 kinase activity also contributes to this process. Importantly, the inhibition of CDK2 complexes alone does not recapitulate the effect of p16ink4a on MCM loading. These results suggest that even though CDK2 inhibition destabilizes Cdc6 (22, 40), the relatively low levels of Cdc6 present under conditions of CDK2 inhibition are apparently sufficient to mediate MCM loading. Therefore, we favor the model wherein the combined activity of CDK complexes in early-G_1 cells is required for the assembly of preRC. Such a model takes into account the redundancy of CDK complexes and suggests that the sum activity of the complexes is required for MCM loading (51, 69). Presumably, other INK4 family members would share this effect on DNA replication due to their ability to inhibit both CDK4 and CDK2 activity. A critical aspect arising from this work is the influence of p16ink4a loss on preRC assembly and Cdc6/Cdt1 expression. Using primary fibroblasts deficient for p16ink4a, no significant effects on DNA replication have been observed (35, 61). Additionally, we have found no effect of p16ink4a loss on the expression of MCMs, Cdt1, and Cdc6 (data not shown). Thus, we would speculate that the influence of p16ink4a loss on replication control will be manifest only under specific conditions wherein p16ink4a is induced and required for cell cycle exit (as occurs during prolonged passage or specific cellular stresses). Based on recent data indicating that p16ink4a induction occurs in preneoplastic lesions as a potential barrier to cancer (14, 59), such lesions arising in p16ink4a-proficient and -deficient mice would represent an ideal setting for defining the involvement of p16ink4a loss in replication control in a highly relevant model.

The role of RB in the regulation of preRC, downstream of p16ink4a, could represent distinct effects on CDK2 activity. First, the loss of RB substantially deregulates the expression levels of cyclins E and A (29). Thus, the absence of RB could simply dampen the ability of p16ink4a to sufficiently inhibit CDK2 activity. Second, as we show here, the loss of RB prevents the downregulation of cyclin A, which would further compromise the ability of p16ink4a to inhibit CDK2 activity. Thus, RB indirectly facilitates the action of p16ink4a on MCM loading in G_1 by limiting G_1 CDK activity (Fig. 9). Correspondingly, this G_1 CDK activity targets RB via phosphorylation (Fig. 9). In contrast with the effect of p16ink4a in G_1, RB plays a distinct role in targeting DNA replication in S phase. Cells or tissues deficient in RB exhibit a deregulated S phase and harbor a propensity for overreplication of the genome (5, 42, 43). RB targets replication in S phase via downregulation of PCNA activity, which is dependent on the efficient downregulation of cyclin A protein levels and diminished CDK2 activity (60). This influence of RB is recapitulated by CDK inhibitors and pharmacological inhibitors of CDK2 (Fig. 9). The action of p16ink4a in S phase had heretofore not been explored. However, as shown here, p16ink4a is not able to efficiently lead to the dephosphorylation of RB or the downregulation of cyclin A expression in S-phase-synchronized cells. Thus, while p16ink4a can impinge upon the replication machinery, this effect is restricted to G_1. In contrast, RB functionality is maintained in S phase, and the regulation of RB phosphorylation in S phase must be under controls distinct from those elicited by p16ink4a. As such, it would be predicted that in p16ink4a-deficient cells, RB-dependent control over S phase remains intact. However, signals that act through p16ink4a in G_1 (e.g., response to oncogenic stresses that induce senescence) would be expected to have profound impacts on the replication machinery by targeting the preRC.

Significant emphasis has been placed on determining the action of CDK in coordinating cell cycle progression. Our data indicate that there are two distinct facets of S phase which are regulated by CDK activity. The first occurs in early G_1, wherein the accumulation of Cdt1 and Cdc6 is dependent on G_1 CDK complexes (Fig. 9). The second is in S phase, wherein the activity of PCNA is dependent on the availability of CDK2 complexes (Fig. 9). Strikingly, both of these processes are coordinated through the action of the interrelated p16ink4a and RB tumor suppressors.

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