Nucleocytoplasmic Shuttling of the Retinoblastoma Tumor Suppressor Protein via Cdk Phosphorylation-dependent Nuclear Export

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The retinoblastoma (RB) tumor suppressor protein is a negative regulator of cell proliferation that is functionally inactivated in the majority of human tumors. Elevated Cdk activity via RB pathway mutations is observed in virtually every human cancer. Thus, Cdk inhibitors have tremendous promise as anticancer agents although detailed mechanistic knowledge of their effects on RB function is needed to harness their full potential. Here, we illustrate a novel function for Cdks in regulating the subcellular localization of RB. We present evidence of significant cytoplasmic mislocalization of ordinarily nuclear RB in cells harboring Cdk4 mutations. Our findings uncover a novel mechanism to circumvent RB-mediated growth suppression by altered nucleocytoplasmic trafficking via the Exportin1 pathway. Cytoplasmically mislocalized RB could be efficiently confined to the nucleus by inhibiting the Exportin1 pathway, reducing Cdk activity, or mutating the Cdk-dependent phosphorylation sites in RB that result in loss of RB-Exportin1 association. Thus RB-mediated tumor suppression can be subverted by phosphorylation-dependent enhancement of nuclear export. These results support the notion that tumor cells can modulate the protein transport machinery thereby making the protein transport process a viable therapeutic target.

Mutations in cell cycle components that allow cells to bypass quiescence or cellular senescence pathways are important hallmarks of a cancer cell thereby making the cell cycle machinery an important target for anti-cancer therapeutic strategies. Frequent alterations that lead to inactivation of RB tumor suppressor function include overexpression of cyclins and cyclin-dependent kinases (Cdks), inactivation of Cdk inhibitors (CKIs), or loss of RB expression, all of which result in aberrant activation of Cdks. RB is active in its hypo or underphosphorylated state whereas sequential phosphorylation of the sixteen serine/threonine residues of RB by several Cdks leads to inactivation of RB function. Cdk-mediated phosphorylation alters association of RB with its myriad interacting proteins that regulate cell cycle progression and transformation potential. The association of RB with E2F transcription factors is the best characterized and it is believed that phosphorylation of RB on several Cdk phosphorylation sites results in the release of E2Fs and activation or repression of target E2F-dependent promoters.

The normally nuclear localization of RB is facilitated by a bi-partite nuclear localization signal (NLS) in the C terminus. Underphosphorylated RB remains in the nucleus via association of its N terminus with nuclear matrix proteins. In contrast, G1/S phosphorylation results in decreased affinity of hyperphosphorylated RB for the nuclear compartment. The mechanistic details that govern the fate of this hyperphosphorylated RB species and its physiological relevance in relation to RB tumor suppressor function have been obscure and are the subject of this report. We and others have previously shown that inheritance of a p16Ink4a-insensitive Cdk4\(^{Arg24Cys}_{Y188Cys}^{R244Cys} \) allele results in increased Cdk4 kinase activity because of loss of p16\(^{Ink4a} \) inhibition thereby increasing the transformation potential of cells and predisposing mice harboring this mutation to cancer because of loss of RB tumor suppressor function.

Here, we investigated whether the deregulated Cdk activity, often associated with hyperphosphorylation of RB, might alter RB subcellular localization and thereby compromise its tumor suppressor function. We present evidence of cytoplasmic mislocalization of RB generated via enhanced Exportin1-mediated nuclear export. Our results reveal a simple mechanism by which RB-mediated tumor suppression can be subverted during cancer progression by Cdk phosphorylation-dependent enhancement of nuclear export.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments and Plasmids—Mouse embryonic fibroblasts (MEFs) (11, 12) and WI-38 normal human lung fibroblasts (passage 14; ATCC) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS). As described previously (11, 12), cells were synchronized in G0 phase by serum starvation (0.1% FBS) for 72 h, fol-
followed by stimulation to enter the cell cycle in Dulbecco’s modified Eagle’s medium containing 10% FBS, and cell preparations at 0, 8, 12, 18, 20, 24, 30, and 38 h were subjected to immunofluorescence assay.

MEFs were treated with flavopiridol and cells were monitored for viability using the WST-1 assay (Roche Applied Science). Results presented are average of triplicate with standard deviations. Nuclear and cytosolic fractions of cells either untreated or treated with 10 nM or 30 nM LMB (L2913, Sigma) for 5 h were prepared using NE-PER™ reagent (Pierce), and 30-μl aliquots were used for immunoblot analysis. Cells were treated with 10 and 30 nM LMB and incubated for 2–5 h followed by immunofluorescence assay.

GFP-Cdk4R224C was generated by site-directed mutagenesis of the Cdk4 cDNA to convert arginine at position 24 to cysteine followed by cloning into GFP vector (Clontech) and sequence analysis to confirm mutagenesis. GFP and GFP-Cdk4 were used as controls. GFP-Rev, GFP-RB, GFP-RBWTLP, and GFP-RB7LP (14, 15) have been described previously.

Immunoblotting, Immunoprecipitation, and Immunofluorescence—For immunoprecipitation (IP), 300 μg of total protein was incubated with indicated antibodies and protein G beads (Amersham Biosciences) followed by Western blot analysis (WB) using the indicated antibodies. For immunofluorescence (IF), cells were seeded onto collagen I-coated 8-well Biocoat culture slides and were fixed in 1% paraformaldehyde for 10 min and permeabilized with either acetone (2 min) or methanol (2 min). After antibody incubation and mounting in DAPI-containing medium cells were observed on an Olympus IX70 inverted microscope with a Photometric CCD camera. Localization was determined by scoring nuclear/cyttoplasmic and nuclear profiles from at least 100 cells of each sample treatment. Antibody sources are: monoclonal anti-RB antibody (1:200 for IF, 1:500 for WB) from Molecular Probes.

**Semi-quantitative Reverse Transcription-PCR (RT-PCR) Assay**—Total RNA was extracted using the TRI Reagent kit (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR was performed using One Step RNA PCR Kit (AMV) (TAK RR024A, TaKaRa Biomedicals, TaKaRa Shuzo Co., Ltd.) according to the manufacturer’s protocol. Primer sequences were based on cDNA sequences corresponding to each of the genes analyzed, and these primer pairs were selected with the help of PrimerExpress 1.0 software (PerkinElmer Life Sciences). GAPDH (Acc M32599) 5’-ATCACCATCTTCCAGGAC-AGAC-3’ and 5’-ATGCCAGTGAGCTTCCCGTTCAGC-3’; TopoII-alpha (Acc U01915) 5’-GATTCCAGCTTGTGCCTG-3’ and 5’-GGCAATCTACAAATGGTGGAAT-3’; Bcl-2 (Acc M16506) 5’-GAATGAGCGTGACGTACGTACAG-3’ and 5’-CTGGAGCAGCCAGATGATCTCCGTA-3’; Importin-alpha2 (Acc BC003274) 5’-CTTTCTAGAGAGAAACGCCTCC-3’ and 5’-AGCTTCACAAGTTGGGGAACACT-3’. All samples were performed at least in duplicate.

**FRAP**—Fluorescence recovery after photobleaching (FRAP) experiments were performed on Zeiss 510 confocal microscope with a ×100/1.3 N.A. planapochromat oil objective. GFP was excited with the 488 nm line of an Ar laser and GFP emission was monitored above 505 nm. Cells were maintained at 37 °C with a Nvtek ASI 400 Air Stream incubator (Nvtek). In transfected cells a square spot of ∼5 μm inside the nucleoplasm was bleached for ∼600 ms using the 488-nm laser line at 100% laser power. Cells were monitored at 0.5-s intervals for 123 s. For quantitation, the total fluorescent intensities of a region of interest in the bleached area and in the total nuclear area were monitored using Zeiss software. Background fluorescence (BG) was measured in a random field outside the cells. The relative fluorescence intensity double normalized to the pre-bleach value was calculated at each time point as: \[ \text{Intensity}_{(t)} = \frac{\text{Intensity}_{(t)} - \text{BG} \times \text{Intensity}_{(0)} - \text{BG}}{\text{Intensity}_{(0)} - \text{BG}} \] where \( I_{(0)} \) is the average intensity of the entire nucleus during pre-bleach, and \( I_{(t)} \) is the average intensity of the region of interest during pre-bleach. For quantification 15 cells were used.

**Heterokaryon Fusion Assay**—To detect nucleocytoplasmic shuttling, a heterokaryon fusion assay was performed essentially as we have described earlier (15). Cdk4R mutants generated in mammalian cells grown to about 85% confluence, were electroporated with a BTX electro porator ECM 830 using 5 μg of GFP-RB or GFP-Rev and 15 μg sheared salmon sperm carrier DNA in a 2-mm gap cuvette at 200 V, 1-ms pulse, 4 pulses and 0.5-s intervals and transferred on microscopic slides in 6-well plates. 4-h later, RB mutant human SAOS-2 cells were seeded onto the same chambers. SAOS-2 is a human osteogenic sarcoma cell line, which lacks full-length nuclear RB and produces instead a mutant 90kd RB protein that is localized to the cytoplasm (17). Cells were incubated at 37 °C for 24 h. After incubation, the cells were washed thoroughly with pre-warmed PBS and treated with 25 μg/ml of cycloheximide (Sigma) for 30 min to inhibit de novo protein synthesis. After aspirating the medium containing cycloheximide, cells were washed with PBS and immediately overlaid with a solution of 50% polyethylene glycol (PEG; Sigma) and incubated at room temperature for 90 s to induce

**CDK-mediated Nucleocytoplasmic Shuttling of RB**

1. **Cytoplasmic RB**
   - Cells were grown to near confluence, were electroporated with a BTX electroporator ECM 830 using 5 μg of GFP-RB or GFP-Rev and 15 μg sheared salmon sperm carrier DNA in a 2-mm gap cuvette at 200 V, 1-ms pulse, 4 pulses and 0.5-s intervals and transferred on microscopic slides in 6-well plates. 4-h later, RB mutant human SAOS-2 cells were seeded onto the same chambers. SAOS-2 is a human osteogenic sarcoma cell line, which lacks full-length nuclear RB and produces instead a mutant 90kd RB protein that is localized to the cytoplasm (17). Cells were incubated at 37 °C for 24 h. After incubation, the cells were washed thoroughly with pre-warmed PBS and treated with 25 μg/ml of cycloheximide (Sigma) for 30 min to inhibit de novo protein synthesis. After aspirating the medium containing cycloheximide, cells were washed with PBS and immediately overlaid with a solution of 50% polyethylene glycol (PEG; Sigma) and incubated at room temperature for 90 s to induce
cell fusion. The cells were then extensively washed with PBS to completely remove all traces of PEG and transferred back to Dulbecco’s modified Eagle’s medium + 10% FBS containing 25 μg/ml of cycloheximide for 70 min at 37 °C. Cells were then fixed with 3% paraformaldehyde in PBS, washed with PBS and mounted with Vectashield containing DAPI. These cells were then viewed using Zeiss LSM 510 META confocal microscope with 100/1.4 N.A. planapochromat objective. Data were collected via Zeiss/LSM510 software.

**RESULTS**

**Nucleocytoplasmic Localization of RB**—Western blot analyses demonstrate that compared with the low level of RB in Cdk4<sup>WT</sup> cells, Cdk4<sup>R24C/R24C</sup> (Cdk4<sup>R/R</sup>) cells exhibit increased levels of RB with a substantial fraction of RB in its hyperphosphorylated form (Fig. 1A). Western blot analyses of nucleocytoplasmic fractions revealed that in contrast to the exclusively nuclear RB in Cdk4<sup>WT</sup> cells, RB is detected in both nuclear and nucleocytoplasmic compartments of Cdk4<sup>R/R</sup> cells (Fig. 1B). These results were verified using immunofluorescence assays which revealed normal nuclear localization of RB and E2F1 in Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> cells. To evaluate the export process, cells were treated with 30 nM LMB for 3h before fusion and maintained in the presence of LMB after fusion and analyzed as above.

**Live Time-lapse Confocal Microscopy—**Cdk4<sup>R/R</sup> cells were transfected with either GFP-RB or red fluorescent protein (DsRed2) expression vectors followed by mixing the transfected cells as described previously (15). The following day, 30 min before fusion with polyethylene glycol cells were incubated with cycloheximide (25 μg/ml) to prevent de novo protein synthesis. After fusion, cells were maintained in cycloheximide and observed immediately by live time-lapse confocal microscopy. To evaluate the export process, cells were treated with 30 nM LMB for 3h before fusion and maintained in the presence of LMB after fusion and analyzed as above.

**CDK-mediated Nucleocytoplasmic Shuttling of RB**

**FIGURE 1. Nucleocytoplasmic localization of RB.** A, Western blot analysis of total RB, phospho RB<sup>Ser807/811</sup>, underphosphorylated RB, E2F1 and tubulin in Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> MEFs. B, Western blot analysis of total RB, phospho-RB<sup>Ser807/811</sup>, tubulin (cytoplasmic control) and lamin A/C (nuclear control) in nuclear (Nuc) and cytoplasmic (Cyt) fractions of Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> MEFs. C, quantification of RB and phospho-RB in nuclear and nucleocytoplasmic compartments of Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> cells using immunofluorescence assay. Number of cells (%) with nuclear (N; open bars) or nucleocytoplasmic (NC; closed bars) localization of RB and phospho-RB is shown (upper panel). Fluorescence intensity of RB and phospho-RB in the nuclear (N; open bars) or cytoplasmic (C; closed bars) compartments (lower panel). D, RB (red; using clone 4H1 antibody) and E2F1 (green) localization in Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> MEFs with nuclei (DAPI; blue) and overlay images. E, luciferase activities of E2F-dependent Cyclin E promoter (pCE548-Luc) and synthetic E2F-reporter (pE2F-Luc) in Cdk4<sup>WT</sup> (open bars) and Cdk4<sup>R/R</sup> (closed bars) MEFs. F, expression of E2F response genes in Cdk4<sup>WT</sup> and Cdk4<sup>R/R</sup> MEFs. RT-PCR was performed on total RNA to detect mRNA levels of E2F-responsive genes Topoli, Importin-α2 and BCL2. GAPDH levels are shown as control for RNA loading.
data revealed that in comparison to the nearly exclusive nuclear localization of RB and phospho-RB in Cdk4WT cells, greater than 95% Cdk4R/R cells harbored nucleocytoplasmically localized RB and phospho-RB (Fig. 1C, upper panel). Consistent with the Western blot analysis (Fig. 1A), quantification of relative fluorescence intensities over the subcellular compartments revealed that Cdk4R/R cells harbored elevated level of RB and phospho-RB (Fig. 1C, lower panel). The observation of cytoplasmic RB is intriguing since RB is widely believed to be a nuclear protein and nuclear retention is presumed to be important for its tumor suppressor function. To rule out the contribution of fixation and permeabilization conditions in the observed nucleocytoplasmic localization of RB, we performed immunofluorescence assays on Cdk4R/R cells under a variety of fixation/permeabilization conditions. Nucleocytoplasmic RB localization was reproducibly observed in Cdk4R/R cells after fixation in 1% or 4% paraformaldehyde for 10 min followed by permeabilization with either acetone (2 min) or methanol (2 min) (supplemental Fig. S1). Further, the reproducibility of RB nucleocytoplasmic localization was verified under the different fixation/permeabilization conditions using two monoclonal antibodies that recognize distinct epitopes (supplemental Fig. S1). Finally, antibody specificity was verified using RB-deficient (RB−/−) cells where the immunofluorescence signal was comparable to background fluorescence observed in cells treated with only secondary antibodies (supplemental Fig. S1). Together, these studies demonstrate the existence of nucleocytoplasmically localized RB species in Cdk4R/R cells. Consistent with our prior published work demonstrating the increased transformation potential of the Cdk4R24C mutation (12), we observed deregulated E2F activity in Cdk4R/R cells. Enhanced activity of the synthetic E2F-reporter and that of the promoter for the E2F target gene Cyclin E was observed in Cdk4R/R cells (Fig. 1F). Further, elevated levels of E2F target genes (18, 19), TopoII-α, Importin-α2, BCL2 were observed in Cdk4R/R cells (Fig. 1F).

RB phosphorylation occurs at each G1/S transition in normal and cancer cells. Previously, it has been shown using WI-38 normal human lung fibroblasts that the conversion from low salt resistant to low salt extractable hyperphosphorylated RB occurs with transition through the G1/S boundary of the cell cycle (8–10). To monitor whether there was a correlation between RB subcellular localization and position in the cell cycle, we performed immunofluorescence experiments on synchronized cell populations. WI-38 normal human fibroblasts, normal Cdk4WT and Cdk4R/R cells were synchronized by serum starvation and stimulated to enter the cell cycle with 10% serum containing media as described previously (11, 12). Propidium iodide-based fluorescence activated cell sorting assays showed evidence of equivalent numbers of Cdk4WT and Cdk4R/R cells in G1 and S phase of the cell cycle (supplemental Table S1). Immunofluorescence assays were performed using RB antibodies on cell preparations during serum starvation and after serum stimulation. These analyses revealed that RB is confined to the nucleus in normal human (WI-38) and mouse (Cdk4WT) fibroblast cells during all stages of the cell cycle (Fig. 2). Phosphorylation of RB (p-RB) in Cdk4WT cells was evident 24 h after serum stimulation (Fig. 2). In contrast, almost 60% of Cdk4R/R cells harbored nucleocytoplasmic localization of RB between 8–12 h after serum stimulation with majority of RB in a phosphorylated state (Fig. 2). A fraction (33%) of Cdk4R/R cells showed evidence of phosphorylated RB after the 72-h serum starvation (time 0) and throughout the course of the serum stimulation experiment (Fig. 2). It is unclear whether the observed phospho-RB in Cdk4R/R cells at time 0 reflects a pool of cells that are refractory to growth arrest or a property unique to Cdk4R/R cells. Taken together, these observations are indicative of an important role for the Cdk4R24C mutation in mediating changes in subcellular localization of RB.

Cdk-mediated Phosphorylation Regulates RB Subcellular Localization and Function—Predominantly underphosphorylated RB and low levels of phosphorylated RB are observed in Cdk4WT cells (Fig. 1, A and B). In contrast, increased phosphorylation of RB at sites Ser780, Ser807, and Ser811 was observed in Cdk4R/R cells (Fig. 1, A and B; data not shown) with the phosphorylated RB proteins dispersed throughout the nucleus and cytoplasm (Fig. 3A). More interestingly, confinement of majority of underphosphorylated RB in Cdk4R/R nuclei (Fig. 3A) suggested that only phosphorylated species of RB could exit the nucleus. We next investigated the requirement for Cdk activity in determining the nucleocytoplasmic localization of RB. Treatment of Cdk4R/R MEFs with flavopiridol, an inhibitor of phosphokinases with strongest activity against Cdkks (20), was accompanied by nuclear retention of RB (Fig. 3C). Consistent with this, RB was exclusively nuclear in Cdk4KO cells that lack Cdk4 activity (Fig. 3C) further validating the importance of Cdk4 activity in regulating the subcellular localization of RB.

Cdk4R/R cells that harbor nucleocytoplasmically distributed RB exhibit increased cell proliferation rate and enhanced tumorigenesis potential in xenograft-tumor assays (12). Treatment with the Cdk-inhibitor flavopiridol, which caused increased nuclear retention of RB in these cells (Fig. 3C), resulted in reduced viability of Cdk4R/R cells (Fig. 3B). Because there is evidence for both Cdk mediated RB-dependent and RB-independent mechanisms of action involving the anti-tumoral effect of flavopiridol (20), it is plausible that some of the flavopiridol-mediated effects observed here are RB-independent. Together, these results indicate that RB cytoplasmic mislocalization can be effectively reversed by inhibition of Cdk activity.

Phosphorylation of C-terminal Residues of RB Determines Its Subcellular Localization—RB is nuclear in Cdk4KO cells that lack Cdk4 kinase activity (Fig. 3C). Rescue of Cdk4 activity in Cdk4KO cells, by expressing the p16-insensitive activated Cdk4R24C kinase, resulted in efflux of RB from the nucleus into the cytoplasm (Fig. 4A). Similar nucleocytoplasmic RB localization was also seen in Cdk4WT cells transfected with Cdk4R24C expression vectors (Fig. 4A). In contrast, Cdk4KO or Cdk4WT cells transfected with control vectors or vectors expressing wild type Cdk4 showed a typical nuclear RB (Fig. 4A). These observations emphasize the requirement for increased Cdk activity in regulating the cytoplasmic localization of RB.

We next postulated that specific Cdk-phosphorylation sites on RB determine its observed cytoplasmic mislocalization. Sixteen potential Cdk phosphorylatable serine (Ser)/threonine (Thr) residues span the RB protein (3). Six of these sites are in

**CDK-mediated Nucleocytoplasmic Shuttling of RB**
CDK-mediated Nucleocytoplasmic Shuttling of RB

The N terminus that has been previously implicated in mediating interactions of RB with the nuclear lamina (7). Ten remaining phosphorylation sites are located in the large pocket of RB (RB-LP). RB-LP comprises the A/B pocket domain separated by the linker insert domain (I) in addition to the C-terminal domain. The latter itself has seven clustered Cdk sites (3) including Ser780, Ser807, and Ser811, which are all phosphorylated in the cytoplasmic RB observed in Cdk4R/R cells (Figs. 1A and 3A; data not shown). Moreover, RB-LP contains the minimal functional domains required for regulated E2F1 association, transcriptional repression, cell cycle inhibition and tumor suppression (21, 22). To test the role of specific phospho-residues in the subcellular localization of RB, we studied the localization of a phosphomutant of the RB large pocket (GFP-RB7LP).

**FIGURE 2. Subcellular localization of RB during cell cycle progression.** WI-38 normal human lung fibroblasts (upper two panels) and Cdk4WT normal MEFs (middle three panels) and Cdk4R/R cells (lower three panels) were synchronized in G0-phase by 0.1% serum starvation for 72 h. Cells were stimulated to enter the cell cycle upon incubation in media containing 10% fetal bovine serum and cell preparations at indicated time points after serum starvation and after serum stimulation were subjected to immunofluorescence analysis using anti-RB (G3–245 clone) antibody and anti-phospho RB807/811 antibodies (p-RB). RB (red), phospho RB807/811 (green), and DAPI (blue) images are shown. Percentage of cells with nuclear (N) and nucleocytoplasmic (NC) localization of RB and p-RB is indicated in the upper right corner of each panel.
with mutations of all seven C-terminal phosphorylation sites) fused to the GFP (14) in Cdk4−/−R/R cells. As controls we monitored the localization of wild-type RB (GFP-RB) and wild-type RB-LP (GFP-RBWTLP). Equal amounts of GFP-RB, GFP-RBWTLP and GFP-RB7LP were transfected into Cdk4−/−R/R cells (Fig. 4D). Nucleocytoplasmic localization of GFP-RB and GFP-RBWTLP was observed (Fig. 4, B and C). This was consistent with the similar nucleocytoplasmic localization of endogenous RB in Cdk4−/−R/R cells (Figs. 1–3). In contrast, a dramatic nuclear confinement of GFP-RB7LP was seen (Fig. 4, B and C) indicating that these phosphorylation residues in the C-domain of RB are critical in determining its nucleocytoplasmic localization.

Phosphorylation-dependent Association of RB with Exportin1—Nuclear export of the tumor suppressor proteins p27Kip1, APC, Smad4(DPC4), and p53 is known to inactivate their tumor suppressor function (23–30). We reasoned that RB might be subject to similar nucleocytoplasmic shuttling that might influence its tumor suppressor function. Therefore, we next delineated the mechanism of RB nucleocytoplasmic localization by hypothesizing that RB, phosphorylated on its C-domain residues by Cdk2s in the nucleus, is subsequently exported to the cytoplasm. We examined this hypothesis by studying the involvement of nuclear export in the subcellular localization of RB. The karyopherin family of nuclear export receptors (31), notably Exportin1 (CRM1) (32), are involved in trafficking of diverse substrates across the nuclear membrane. Whereas, we detected no major change in the levels of Exportin1 RNA (Fig. 5A), a 2-fold increase in Exportin1 protein level was observed (Fig. 5B), and Western blot analysis of nucleocytoplasmic fractions revealed an increased cytoplasmic Exportin1 expression in Cdk4−/−R/R cells (Fig. 5C). Since RB elicits many of its functions via interaction with its associated proteins (33), we next asked if phosphorylated RB species. To this end, we explored whether the RB-Exportin1 interaction was also dependent on the phosphorylation status of RB. We examined the association of GFP-RB, GFP-RBWTLP and GFP-RB7LP proteins with Exportin1 by co-immunoprecipitation assays. GFP-RB and GFP-RBWTLP proteins efficiently associate with Exportin1 (Fig. 5, F and G), similar to association between endogenous RB and Exportin1 (Fig. 5, D and E). In contrast, we saw no association of Exportin1 with the phosphomutant GFP-RB7LP (Fig. 5G) revealing the importance of Cdk phosphorylation of C-terminal RB in facilitating the RB-Exportin1 interaction. This result indicates that Exportin1 preferentially recognizes and associates with RB phosphorylated on C-terminal residues and thereby selectively mediates export of this phosphorylated RB species. Further detailed analysis will reveal if the RB-Exportin1 association is direct or mediated by interactions with other cellular components.

Hyperphosphorylated RB Exhibits Increased Mobility and Is Subject to Active Nuclear Export—The observed nucleocytoplasmic localization of RB in Cdk4−/−R/R cells is suggestive of a dynamic RB species. To quantify RB in vivo kinetics we examined the association kinetics of GFP-RB in living cells using FRAP microscopy (Fig. 6A). The fluorescence signal of GFP-RB in the nucleoplasm completely recovers in Cdk4−/−R/R cells to pre-bleached value by ~100s. Remarkably, Cdk4−/−R/R cells exhibited a significantly faster recovery of GFP-RB in the nucleoplasm in the range of 30–40s (Fig. 6A). Moreover, similar FRAP assay on the cytoplasmic pool of RB in Cdk4−/−R/R cells revealed a highly mobile RB species (data not shown). These assays thus are evidence of a faster mobility of RB in Cdk4−/−R/R cells, which could facilitate its subcellular transport and shuttling ability.
Association of RB with Exportin1 (Fig. 5, D and E) suggested that RB could be subject to active export. In agreement with this, treatment of Cdk4R/R cells with leptomycin B (LMB), an inhibitor of Exportin1-mediated nuclear export (31, 32), resulted in increased nuclear confinement of RB (Fig. 5H). This is consistent with Western blot analysis of nucleocytoplasmic fractions from LMB-treated cells that exhibited a shift of RB from the cytoplasm to the nucleus (Fig. 5I). These observations are consistent with Exportin1-mediated nuclear export of RB. To confirm active nuclear export of RB, we performed heterokaryon fusion assays (15), where the ability of RB to shuttle from one nucleus to another was assayed. The nuclei in a heterokaryon can be distinguished by DAPI staining where the mouse (Cdk4R/R) nuclei have bright blue chromocenters that are absent in human (SAOS-2) nuclei (Fig. 6B). Cdk4R/R cells were electroporated with GFP-RB and were subsequently fused to SAOS-2 cells. Fluorescence microscopy of the heterokaryons revealed GFP-RB in the SAOS-2 nucleus (Fig. 6B), indicating that it had been exported from the Cdk4R/R nuclei and imported into the SAOS-2 nuclei. The intense nuclear localization of GFP-RB in SAOS-2 cells, that lack sufficient Cdk activity (21, 34, 35) to phosphorylate RB, further corroborates the need for Cdk phosphorylation in facilitating RB nuclear export. In parallel, we used a Rev-GFP construct as a positive control, as the HIV-1 Rev protein shuttles into and out of the nucleus (15). Rev-GFP was observed in human and mouse nuclei of the heterokaryons indicative of its ability to continually shuttle (Fig. 6B).

To further demonstrate the ability of RB to shuttle in and out of the nucleus we performed a modified heterokaryon fusion assay using live time-lapse confocal microscopy (15). We independently transfected Cdk4R/R cells with the DsRed2 fluorescent protein (that is confined to the cytoplasm) or GFP-RB, mixed the two transfected cell pools, and subsequently fused them prior to observing them by live time-lapse confocal microscopy. As expected, we found GFP-RB in DsRed2-positive fused acceptor cells, indicative of its ability to continually shuttle (Fig. 6C). In contrast, when Cdk4R/R cells were treated with LMB for 3 h before fusion and maintained with LMB after fusion, GFP-RB was localized only in the nucleus of GFP-RB trans-
affected cells and did not shuttle to acceptor nuclei of the DsRed2 fused cells (Fig. 6C). These data further support that RB nuclear export is Exportin1-dependent. Collectively, these results illustrate that RB is subject to constant nucleocytoplasmic shuttling.

**DISCUSSION**

The CDK4R24C mutation was originally identified in familial melanoma patients (36, 37) and subsequently we and others demonstrated the enhanced cell transformation and in vivo tumorigenic potential of this point mutation (11–13). However, the plausible mechanism by which this point mutation confers advantage to cancer cells was unclear. Using the Cdk4R24C cell line model, here we uncover a novel mechanism used by cancer cells to circumvent RB-mediated growth suppression by altered CDK-mediated nucleocytoplasmic shuttling of RB. We observe that cells harboring the p16INK4A-insensitive Cdk4R24C kinase display significant cytoplasmic mislocalization of ordinarily nuclear RB. Consistent with our prior published data about the enhanced transformation potential of the Cdk4R24C mutation may be caused by its ability to alter the phosphorylation status and the subcellular distribution of RB. The cytoplasmic RB population is generated by enhanced nuclear export through the Exportin1 pathway. However, based on our observation that expression of the Cdk4R24C kinase also leads to increased levels of RB protein we cannot exclude the possibility of Cdk4R24C enhancing RB protein stability. In such a scenario, although unlikely based on the rest of the data favoring an active nuclear export mechanism, the aberrant subcellular RB localization could be an indirect manifestation of a stabilized RB protein. We demonstrate that the interaction of RB with Exportin1 is dependent on Cdk-mediated phosphorylation of the RB C-terminal domain. Consequently, cytoplasmically mislocalized RB could be efficiently confined to the nucleus by (i) inhibiting the Exportin1 pathway, (ii) reducing Cdk activity by pharmacological intervention, or (iii) mutating the Cdk-dependent phosphorylation sites in RB that resulted in a loss of RB-Exportin1 association.

**Regulation of RB Subcellular Localization: A Novel Cdk Function**—Recent work has documented that the function of tumor suppressor proteins APC, p53, p27kip1, and Smad4 (DPC4) can be inactivated by nuclear export mechanisms (23–30, 38). We illustrate here that RB is similarly targeted by the nuclear transport apparatus and propose that RB-mediated tumor suppression can be effectively subverted during cancer progression by Cdk phosphorylation-dependent enhancement of nuclear export. These observations unravel an additional layer of regulatory control on RB tumor suppressor function that is influenced by Cdk activity. The data presented here reveal that hyperphosphorylated RB is a dynamic species. This is consistent with previous work documenting that hyperphosphorylated RB has reduced affinity for the nucleus and that
Cdks can cause liberation of RB from cell nuclei in vitro (8–10). Cytoplasmic RB is not detected in normal fibroblasts (WI-38 human lung fibroblasts and normal mouse embryonic fibroblasts) at any stage of the cell cycle suggesting that (i) Cdk-mediated phosphorylation may be necessary but not sufficient for nuclear exclusion and (ii) cytoplasmic RB localization may be specific to pathological conditions, such as cancer. It is plausible that cytoplasmic RB performs a, hitherto unknown, tumor-specific function that is facilitated by deregulated Cdk-mediated phosphorylation.

We postulate that the increased mobility of hyperphosphorylated RB facilitates its loss of affinity with the nucleus and allows RB to locate to the nucleoplasm or exit the nuclear compartment and translocate to the cytoplasm. The requirement of Cdk phosphorylation in the observed nuclear exclusion of RB suggests that specific residues need to be phosphorylated (and perhaps other specific residues need to be dephosphorylated) during the nuclear transport process. We infer that distinct Cdks, targeting individual or a combination of similar or different phosphoresidues, may influence subcellular localization of RB in a cell-type and context dependent manner. Although RB features a bi-partite NLS and is normally retained in the nucleus the precise mechanism of nuclear import of RB is not known. We hypothesize that alterations in the nuclear import versus export ratio will have a direct bearing on the observed subcellular localization of RB. Consequently, this might influence the nature and extent of interactions of RB with its many associated proteins and thereby could impact the fidelity of RB tumor suppressor function.

Our observations imply that the C-terminal Cdk phosphorylation sites of RB, in addition to regulating E2F1 association, transcriptional repression, cell cycle inhibition and tumor suppression are also involved in an additional function of regulating RB subcellular localization (Fig. 7A). It is plausible that these functions are interdependent and not mutually exclusive in mediating RB tumor suppressor function. It is intriguing that in contrast to its inability to bind Exportin1, GFP-RB7LP can effi-

![FIGURE 6. Kinetics of RB mobility and active nuclear export of RB. A, cells were electroporated with GFP-RB. At 24-h post-electroporation cells were subjected to nuclear FRAP analysis. Cells were imaged before and after photobleaching of a square area in the nucleoplasm and the recovery of signal was monitored using time lapse microscopy. The bleached area is shown as enlarged pseudo color panels. Fluorescence recovery curves are shown in the bottom panel. Relative fluorescence intensities were determined by comparing the fluorescence intensity of a distal unbleached region of the nucleus to the photobleached area, and the results were plotted over time. B, GFP-RB (RB, top two panels) or GFP-Rev (Rev, bottom panel) was electroporated into Cdk4R/R MEFs and the cells were fused to human SAOS-2 cells to form heterokaryons. Distinctive DAPI fluorescence (blue) identifies the donor mouse (Cdk4R/R) nuclei that have bright blue chromocenters (cells represented by asterisks). These distinctive chromocenters are absent in the recipient human (SAOS-2) nuclei. GFP immunofluorescence was monitored in the nuclei of the heterokaryons to determine the process of nuclear export. Overlay and phase contrast images are provided to allow visualization of component nuclei of the individual heterokaryons. C, Exportin 1-mediated and LMB-sensitive nucleocytoplasmic shuttling of RB. Live fluorescent localization of heterokaryons of Cdk4R/R cells transfected with GFP-RB or DsRed2 expression vectors P-RB or DsRed2 were monitored by fluorescence microscopy after fusion. The presence of GFP-RB in DsRed2-positive cells indicates that GFP-RB shuttles into and out of the cell nucleus (top panels). When Cdk4R/R cell transfected with GFP-RB were not fused with Cdk4R/R cell positive for DsRed2, fluorescence GFP-RB proteins remained in their donor nuclei even though cells were in close proximity and no transport to the DsRed2 cell nucleus is seen (middle panels). GFP-RB does not shuttle into acceptor nuclei of the DsRed2-fused cells 30 min after fusion when Cdk4R/R cells were treated with LMB for 3 h before the fusion and maintained with LMB after fusion (bottom panels).]
CDK-mediated Nucleocytoplasmic Shuttling of RB

gers its association with Exportin1, but we propose two models. In the first model, a CDK phosphorylation-induced conformational change in RB exposes a binding site for Exportin1. In the second model, Exportin1 binds specifically to a phosphopeptide within RB. These two models are not mutually exclusive, because Exportin1 may recognize more than one binding site on RB. Although our observations are consistent with Exportin 1-mediated nuclear export of RB, we cannot exclude the possibility that mislocalization of RB may be caused by stabilization of the hyperphosphorylated form of RB, either directly or indirectly, by the CDK4 \( ^{R24C} \) kinase.

Role of the Nuclear Transport Machinery in Cancer—Cytoplasmic mislocalization and functional inactivation of tumor suppressor proteins has been implicated in cancer progression (23–30, 38). Moreover, alterations in the nuclear transport machinery via truncations or overexpression of the export receptor for karyopherin-\( \alpha \) in colon, breast, and liver neoplasms have been identified (39). Chromosomal rearrangements in the loci coding for nucleoporins in acute myelogenous leukemia, chronic myelogenous leukemia, T-cell acute lymphoblastic leukemia, and myelodysplastic syndrome, further emphasize the relevance of the nuclear transport apparatus in human cancer (39). We show that nuclear export of the tumor suppressor protein RB is governed by its association with Exportin1 that is dependent on CDK-mediated phosphorylation of the C-terminal phosphoresidues of RB. Our observations of nucleocytoplastically localized RB and the evidence of a highly mobile RB species that is subject to LMB-sensitive export is strongly indicative of the ability of hyperphosphorylated RB to continually shuttle between the nucleus and cytoplasm. However, these results do not exclude the possibility that the exported cytoplasmic RB species is retained in the cytoplasm by an “anchor” similar to the one that retains the tumor suppressor p53 (23, 40).

Similar CDK-dependent regulation of nuclear transport has been observed in budding yeast (41–44) where availability of the nutrient phosphate determines subcellular localization of the transcription factor Pho4 and its nuclear export by Msn5. Our work elucidates how mutations in RB loci or alteration in RB expression levels can be dispensable for carcinogenic progression, and changes in protein localization via altered nucleocytoplasmic transport processes that lead to inactivation of RB function suffice. The mechanism of altered nucleocytoplasmic shuttling has implications in RB function that impinge not only on cancer genetics and biology but also on normal development and other disease states that are under the purview of RB proteins. The fact that tumor cells may inherit or acquire a capacity to modulate the protein transport machinery to evade growth regulatory constraints suggests that the protein transport process is a viable therapeutic target.

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CDK-mediated Nucleocytoplasmic Shuttling of RB