

p21^{Cip1} Promotes Cyclin D1 Nuclear Accumulation via Direct Inhibition of Nuclear Export*

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There is increasing evidence that p21^{Cip1} and p27^{Kip1} are requisite positive regulators of cyclin D1-CDK4 assembly and nuclear accumulation. Both Cip and Kip proteins can promote nuclear accumulation of cyclin D1, but the underlying mechanism has not been elucidated. We now provide evidence that p21^{Cip1} promotes the nuclear accumulation of cyclin D1 complexes via inhibition of cyclin D1 nuclear export. *In vivo*, we demonstrate that p21^{Cip1} can inhibit glycogen synthase kinase 3 β -triggered cyclin D1 nuclear export and phosphorylation-dependent nucleocytoplasmic shuttling. Furthermore, we find that cyclin D1 nuclear accumulation in p21/p27 null cells can be restored through inhibition of CRM1-dependent nuclear export. The ability of p21^{Cip1} to inhibit cyclin D1 nuclear export correlates with its ability to bind to Thr-286-phosphorylated cyclin D1 and thereby prevents cyclin D1-CRM1 association.

Cell cycle progression requires the sequential and ordered activation of the cyclin-dependent kinases (CDKs)¹ and inactivation of CDK inhibitors. D-type cyclins (D1, D2, D3), the regulatory subunit of the CDK4/6 kinase, function as critical mitogenic sensors that integrate growth factor-initiated signals with G₁-phase progression (1). Mitogenic stimuli trigger the accumulation of active cyclin D1-CDK4 complexes through both increased cyclin expression and decreased cyclin proteolysis and through the promotion of cyclin D-CDK4 assembly (1). Mitogen-dependent expression of cyclin D1 depends upon growth factor-mediated activation of a signal transduction cascade consisting of Ras, Raf-1, and the extracellular signal-regulated protein kinases (ERK1 and 2) (2–8). Accumulation of cyclin D1 during G₁ also relies upon mitogen-dependent inhibition of glycogen synthase kinase 3 β (GSK-3 β) via activation of phosphatidylinositol 3-kinase and Akt (protein kinase B) (9). The subcellular localization of cyclin D1 complexes also oscillates during the cell cycle, being nuclear throughout G₁-phase and cytoplasmic during the remainder of interphase (9–11). Nuclear export of cyclin D1 is a major determinant of cyclin

D1-CDK4 localization (12). Phosphorylation of cyclin D1 at a single threonine residue, Thr-286, by GSK-3 β facilitates the binding of cyclin D1 with the nuclear exportin, CRM1, and thereby promotes cyclin D1 nuclear export (12). Because neither cyclin D1 nor CDK4 has a recognizable nuclear localization signal, the mechanisms governing cyclin D1 nuclear import remain undefined.

Until recently, members of the Cip/Kip family were considered universal inhibitors of CDK activity. The demonstration that both p21^{Cip1} and p27^{Kip1} are components of active cyclin-CDK complexes (13–15) and that p21^{Cip1} can promote the assembly of cyclin D-CDK4 complexes *in vitro* (14) has stimulated the re-evaluation of this hypothesis. p21^{Cip1} and p27^{Kip1} have also been implicated as nuclear import factors for the cyclin D-CDK4 complex. Unlike cyclin D1, both p21^{Cip1} and p27^{Kip1} contain canonical nuclear localization signal motifs (16, 17) and can promote the nuclear accumulation of cyclin D1-CDK4 complexes in transient transfection experiments (14, 18, 19). However, although p21^{Cip1} can facilitate the nuclear accumulation of cyclin D1, the loss of both p21^{Cip1} and p27^{Kip1} does not abolish cyclin D1 nuclear import (18). Thus, neither p21^{Cip1} nor p27^{Kip1} are strictly required for cyclin D1 nuclear import, and it is possible that they promote the nuclear accumulation of the cyclin D1-CDK4 complex via an alternative mechanism. We now demonstrate that p21^{Cip1} promotes the nuclear accumulation of cyclin D1-CDK4 through its ability to inhibit GSK-3 β -triggered cyclin D1 nuclear export.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions, Transfections, and Virus Production—NIH-3T3 cells and 293T cells were maintained in Dulbecco's modified Eagle's medium containing glutamine supplemented with antibiotics (Cellgro) and 10% fetal calf serum (FCS) (BioWhittaker). MEFs derived from p21/p27^{-/-} mice were maintained on a passage protocol wherein 5 × 10⁶ cells were passaged per 60-mm dish every third day (20). Insect Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FCS. For expression in insect Sf9 cells, HA-tagged p21^{Cip1} or p21 Δ 53–58, constructs (provided by Dr. Guy Adami) were inserted into pVL1393 as *Bam*HI to *Cla*I fragments. Baculovirus encoding HA-CRM1 and FLAG-D1 were previously described. Standard protocols for baculovirus manipulation were followed (21). Derivatives of NIH-3T3 cells engineered to overexpress FLAG-tagged cyclin D1 and were previously described (9). Transient expression of Myc-tagged GSK-3 β , HA-tagged p21 or p21 Δ 53–58, and FLAG-tagged cyclin D1 in NIH-3T3 cells was achieved by calcium phosphate co-precipitation (22). For virus production, Human kidney 293T cells were transfected with 15 μ g of ecotropic helper retrovirus plasmid plus 15 μ g of pSR α -FLAG-D1, FLAG-D1-T286A, or pBabe-p21^{Cip1}118. Supernatants collected every 6 h for 48 h were pooled and filtered. Virus infections were carried out on exponentially growing cells in an 8% CO₂ atmosphere in the presence of 10 μ g/ml Polybrene (Sigma Chemical Co.). Cells were harvested for indicated analysis 48 h post-infection.

Expression and Purification of Proteins—HA-CRM1, HA-p21 p21 Δ 53–58, and FLAG-D1 were cloned into the pVL-1393 baculoviral expression vector (PharMingen), and virus was isolated according to

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¹ The abbreviations used are: CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; LMB, leptomycin B; GSK-3 β , glycogen synthase kinase 3 β ; HA, hemagglutinin; HRP, horseradish peroxidase; FCS, fetal calf serum; Rb, retinoblastoma protein.

established procedures (21). Following infection of insect Sf9 cells at high multiplicity with the indicated viral supernatants, cells were lysed in EBC buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% Nonidet-P40) and cleared by sedimentation in a microcentrifuge for 10 min. FLAG-D1 and FLAG-D1 complexes were purified by affinity chromatography using M2-agarose beads and eluted with an excess of FLAG peptide (5 μ g/ml) solubilized in kinase buffer (50 mM Hepes, pH 8.0, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM dithiothreitol, 20 μ M ATP, 10 mM β -glycerolphosphate, 0.1 mM NaVO₃, and 1 mM NaF).

In Vitro Binding, Immunoblotting, and Immunoprecipitation—Protein complexes were phosphorylated with recombinant GSK-3 β protein (Calbiochem), 1 mM ATP, and 10 μ Ci of [³²P]ATP for 30 min at 30 °C. Phosphorylated complexes normalized to phosphorylated cyclin D1 were mixed with a HA-CRM1 lysate and immunoprecipitated with the 12CA5 antibody. Precipitated complexes were resolved on denaturing polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Millipore), subjected to autoradiography, and then blotted with the cyclin D1 monoclonal antibody (D1-17-13G). Sites of antibody binding were visualized with either protein-conjugated horseradish peroxidase (HRP, EY Laboratories), anti-mouse conjugated HRP, or anti-rabbit conjugated HRP (Amersham Biosciences, Inc.). Pulse-chase experiments were performed as previously described (9).

For detection of both total and P-286 cyclin D1 complexes, NIH-3T3 cells stably overexpressing FLAG-D1 were arrested in G₀ by serum starvation for 36 h and stimulated to re-enter the cell cycle by the re-addition of FCS. Lysates were prepared in EBC buffer (23) during G₁ (9 h)- and S (20 h)-phases, and 1500 μ g of total protein was immunoprecipitated with M2-agarose (Sigma) or the phosphorylation-specific cyclin D1 antibody (P-286). Bound proteins were resolved on denaturing polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Millipore), and blotted with antibodies specific for total cyclin D1 or Thr-286-phosphorylated cyclin D1. Detection of HA-p21 was achieved by immunoblot with either the 12CA5 monoclonal antibody (Fig. 2D) or an antibody raised against a peptide derived from the C terminus of p21^{Cip1} (Santa Cruz Biotechnologies, SC-397). Sites of antibody binding were visualized with protein-conjugated horseradish peroxidase (HRP, EY Laboratories).

Immunofluorescence and Interspecies Heterokaryon Shuttle Assay—NIH-3T3 cells seeded on glass coverslips were transfected with expression vectors encoding the indicated DNAs. Cells were fixed 36–48 h following transfection using 3% paraformaldehyde. For visualization of cyclin D1, coverslips were stained with either a mouse-specific cyclin D1 monoclonal antibody (D1-17-13G) or the FLAG-specific M2 monoclonal (Sigma) in phosphate-buffered saline containing 1% dry milk. Secondary antibody staining was performed for 30 min using fluorescein isothiocyanate-conjugated anti-mouse (Amersham Biosciences, Inc.). For visualization of FLAG-tagged cyclin D1 and HA-tagged p21 in the same cell, fixed cells were incubated with the mouse-specific cyclin D1 monoclonal antibody (D1-17-13G) followed by biotinylated anti-mouse (1:500), and streptavidin Texas Red (1:500). Detection of p21^{Cip1} was achieved by incubation with the p21 C-19 antibody (Santa Cruz Biotechnologies, SC-397) followed by fluorescein isothiocyanate-conjugated anti-rabbit (Amersham Biosciences, Inc.). Detection of Thr-286-phosphorylated cyclin D1 was achieved by incubation with affinity-purified P-286 antibodies (50 μ g/ml). In all cases, DNA was visualized using Hoechst 33258 dye at a 1:500 dilution. Coverslips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories). Heterokaryon shuttle assays were performed as previously described (10).

RESULTS

Constitutive Nuclear Export of Cyclin D1 in p21/p27^{-/-} MEFs—Targeted deletion of both p21^{Cip1} and p27^{Kip1} inhibits efficient nuclear accumulation of wild-type cyclin D1 (18). Because CRM1-dependent nuclear export is a major determinant in cyclin D1 localization, we wondered if an increased rate of cyclin D1 nuclear export might be responsible for its cytoplasmic localization in cells null for both p21^{Cip1} and p27^{Kip1}. Murine embryonic fibroblasts prepared from mice lacking both p21^{Cip1} and p27^{Kip1} (p21/p27^{-/-} MEFs) were infected with retrovirus encoding either wild-type cyclin D1 or D1-T286A. The expression of ectopic cyclin D1 in p21/p27^{-/-} MEFs is necessary, because levels of the endogenous protein is reduced at least 10-fold due to increased proteolysis (18). Localization of cyclin D1 was monitored by indirect immunofluorescence with a monoclonal anti-

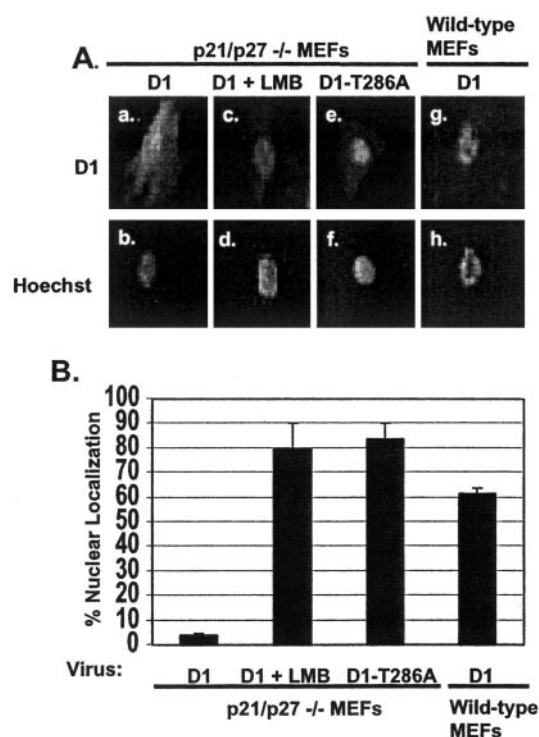


FIG. 1. Inhibition of nuclear export restores cyclin D1 nuclear localization in p21/p27^{-/-} MEFs. A, asynchronously proliferating wild-type (panels g–h) or p21/p27^{-/-} MEFs (panels a–f) were infected with retrovirus encoding either FLAG-D1 or FLAG-D1-T286A. Thirty-six hours post-infection, cells were fixed and processed for immunofluorescence with a cyclin D1-specific antibody (top panels) and Hoechst dye (bottom panels). Where indicated, cells were treated with leptomycin B (LMB) for 2 h prior to fixation. B, nuclear localization of cyclin D1 from part A was quantitated and is shown with bars representing standard deviation from at least three independent experiment.

body specific for murine cyclin D1. In asynchronously proliferating wild-type MEFs, cyclin D1 is predominantly nuclear in greater than 60% of the cells (Fig. 1A, panel g; Fig. 1B, column 4), whereas in p21/p27^{-/-} MEFs wild-type cyclin D1 localized to the cytoplasm in a majority of the cells (Fig. 1A, panel a; Fig. 1B, column 1). In contrast the nuclear export-defective cyclin D1-T286A was exclusively nuclear in p21/p27^{-/-} MEFs (Fig. 1A, panel e; Fig. 1B, column 3). The nuclear localization of cyclin D1-T286A suggests that cyclin nuclear import occurs unimpeded in the absence of p21^{Cip1} and p27^{Kip1} and that the cytoplasmic localization of wild-type cyclin D1 results from an increase in its rate of nuclear export. To test this hypothesis, we determined if cyclin D1 nuclear localization could be restored by inhibition of its nuclear export. Cyclin D1-infected p21/p27^{-/-} MEFs were treated with leptomycin B (LMB), which was previously shown to inhibit cyclin D1 nuclear export via inhibition of CRM1 (12,24). LMB treatment effectively redirected wild-type cyclin D1 to the nucleus of a majority of p21/p27^{-/-} MEFs (Fig. 1A, panel c; Fig. 1B, column 2). These results demonstrate that cyclin D1 nuclear accumulation can be restored in the absence of either p21^{Cip1} or p27^{Kip1} through the specific inhibition of CRM1-dependent nuclear export. Furthermore, these results demonstrate that neither p21^{Cip1} nor p27^{Kip1} is required for cyclin D1 nuclear import.

p21^{Cip1} Overrides GSK-3 β -dependent Nuclear Export of Cyclin D1—Overexpression of GSK-3 β directs the quantitative nuclear export of cyclin D1 via a CRM1-dependent pathway (12). To examine the role of p21^{Cip1} as an inhibitor of cyclin D1 nuclear export, we tested the capacity of p21^{Cip1} to override GSK-3 β -triggered cyclin D1 nuclear export. To characterize the role of p21^{Cip1} in the regulation of cyclin D1 nuclear export, it

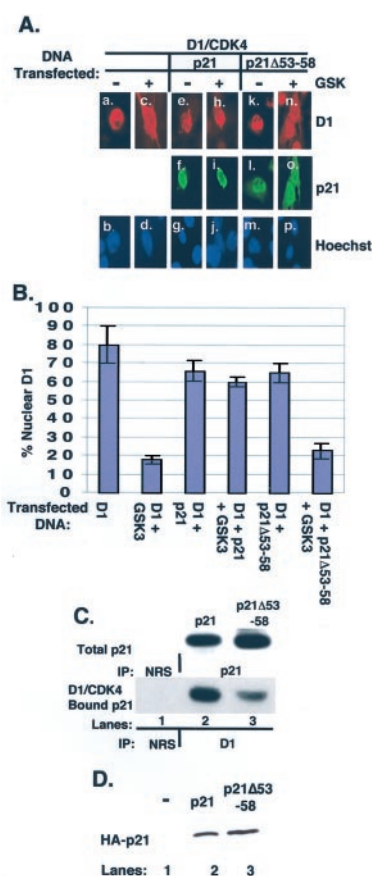


FIG. 2. p21^{Cip1} inhibits GSK-3 β -dependent cyclin D1 nuclear export. **A**, asynchronously proliferating NIH-3T3 cells were transiently transfected with the following expression vector combinations: FLAG-D1 and CDK4 (panels *a–b*); FLAG-D1, CDK4, and GSK-3 β (panels *c–d*); FLAG-D1, CDK4, and p21^{Cip1} (panels *e–g*); FLAG-D1, CDK4, p21^{Cip1}, and GSK-3 β (panels *h–j*); FLAG-D1, CDK4, and p21 Δ 53–58 (panels *k–m*); or FLAG-D1, CDK4, p21 Δ 53–58, and GSK-3 β (panels *n–p*). Twenty-four hours post-transfection, cells were harvested and processed for immunofluorescence with the M2 monoclonal antibody (D1, red), a rabbit antibody raised against the C-terminal peptide of human p21 (p21, green) and cellular DNA was stained with Hoechst dye (blue). **B**, quantitation of three or four independent experiments performed as described for **A**. **C**, Sf9 cell lysates containing cyclin D1 and CDK4, along with either wild-type p21^{Cip1} or p21 Δ 53–58 were precipitated with a p21^{Cip1}-specific antibody (top panel) or the cyclin D1 antibody followed by immunoblot with the p21^{Cip1} antibody. Sites of antibody binding were visualized by enhanced chemiluminescence. **D**, whole cell lysates prepared from NIH-3T3 cells transfected with empty vector (–, lane 1), or vectors encoding either wild-type p21 containing a hemagglutinin antigen (HA) tag (lane 2) or p21 Δ 53–58 containing the HA-tag (lane 3) were subjected to immunoblot analysis with the 12CA5 monoclonal antibody and visualized by enhanced chemiluminescence.

was important to choose a cell type where cyclin D1 localization was properly regulated, dependent upon GSK-3 β , and could be reliably transfected with high efficiency. We therefore utilized NIH-3T3 cells where we have previously documented the dependence of cyclin D1 nuclear export on both CRM1 and GSK-3 β activity. NIH-3T3 cells were transfected with expression vectors encoding FLAG-tagged cyclin D1, CDK4, with or without HA-tagged wild-type p21^{Cip1} and with or without GSK-3 β . Localization of cyclin D1 was determined by indirect immunofluorescence with the cyclin D1-specific monoclonal antibody (Fig. 2A, panels *a, c, e, h, k, n*; Fig. 2B, quantitation). Expression of p21^{Cip1} in the same cell was confirmed with an antibody that recognizes the C terminus of p21^{Cip1} (Fig. 2A, panels *f, i, l, o*). Cyclin D1 localized to the nucleus in the absence of ectopic GSK-3 β (panel *a*) but relocalized to the cytoplasm in its presence (Fig. 2A, panel *c*; Fig. 2B, bar 2). In

contrast, co-expression of p21^{Cip1} along with GSK-3 β restored nuclear accumulation of cyclin D1 (Fig. 2A, panel *h*; Fig. 2B, bar 4). Overexpression of wild-type p21^{Cip1} was also sufficient to override GSK-3 β -dependent cyclin D1 nuclear export (data not shown). p21^{Cip1} binds directly to sequences in both the cyclin and CDK subunits (25). We therefore considered the possibility that inhibition of GSK-3 β -triggered cyclin D1 nuclear export might require direct contact between p21^{Cip1} and both subunits of the cyclin-dependent kinase. Deletion of residues 53–58 of p21^{Cip1} should compromise binding to CDK4 (26). To confirm the effect of this mutation we examined the ability of wild-type p21^{Cip1} or p21 Δ 53–58 to associate with the cyclin D1-CDK4 complex in Sf9 cells. Following infection of Sf9 cells with the appropriate baculovirus, cell lysates were prepared and binding of the respective p21 proteins to cyclin D1-CDK4 complexes was monitored by precipitation with the cyclin D1-specific monoclonal antibody followed by immunoblot analysis with an anti-p21 antibody. Binding of p21 Δ 53–58 to the cyclin D1-CDK4 complex was reduced by greater than 50% (Fig. 2C, lower panel). Because total levels of both mutant and wild-type p21^{Cip1} were comparable, the reduced binding by the mutant was not due to lower protein levels. We next co-transfected NIH-3T3 cells with expression vectors encoding FLAG-tagged cyclin D1 and CDK4, along with HA-tagged p21 Δ 53–58, with or without GSK-3 β . In the presence of overexpressed p21 Δ 53–58 only, cyclin D1 complexes were nuclear (Fig. 2A, panel *k*; Fig. 2B, bar 5). However, upon co-expression of GSK-3 β , cyclin D1 was again cytoplasmic (Fig. 2A, panel *n*; Fig. 2B, bar 6). Ectopic expression of a second p21 mutant, which is defective in cyclin binding (p21 Δ 17–22 (27)), also failed to inhibit GSK-3 β -triggered cyclin D1 nuclear export (data not shown). Because both wild-type and mutant p21 molecules accumulated to the same level in the transfected NIH-3T3 cells (Fig. 2D, lanes 2 and 3), the failure of p21 Δ 53–58 to inhibit cyclin D1 nuclear export was not due to its reduced accumulation in transfected cells. These results demonstrate that p21^{Cip1} does inhibit GSK-3 β -dependent cyclin D1 nuclear export and suggest that p21^{Cip1}-dependent inhibition requires direct binding of p21^{Cip1} with the cyclin D1-CDK4 complex.

Due to the nature of the above experimental approach, we could not rule out the possibility that p21^{Cip1} promotes nuclear accumulation of cyclin D1 by increasing the rate of cyclin D1 nuclear import relative to its rate of nuclear export. To directly assess the capacity of p21^{Cip1} to inhibit cyclin D1 nuclear export, we utilized a heterokaryon shuttling assay. This assay utilizes the fact that fusion of cells of two different species can be induced thereby allowing one to monitor the migration of proteins between heterologous nuclei. For a protein to move from one nuclei to the second, it must undergo one round of nuclear export (from the original nucleus) and one round of nuclear import (into the heterologous nucleus). Proteins that are defective in nuclear export will remain localized to the original nucleus. Nucleoplasmin containing a bi-directional transport signal (M9) and a c-myc epitope tag (Npc-M9) was used as a positive control (12, 28, 29). NIH-3T3 cells seeded on glass coverslips were transfected with cyclin D1 and CDK4 or cyclin D1-CDK4 with either wild-type p21^{Cip1} or p21 Δ 53–58. Thirty-six hours post-transfection, NIH-3T3 cells were co-cultured with HeLa cells for 3 h prior to cell fusion. Thirty minutes following fusion, shuttling between nuclei was monitored by indirect immunofluorescence with the 9E10 monoclonal antibody (Npc-M9) or the mouse-specific monoclonal antibody to cyclin D1. Murine and human cells were distinguished with Hoechst dye, because murine cells display a punctate staining pattern while human cells display a homogeneous staining pattern. The total number of heterokaryons with protein shut-

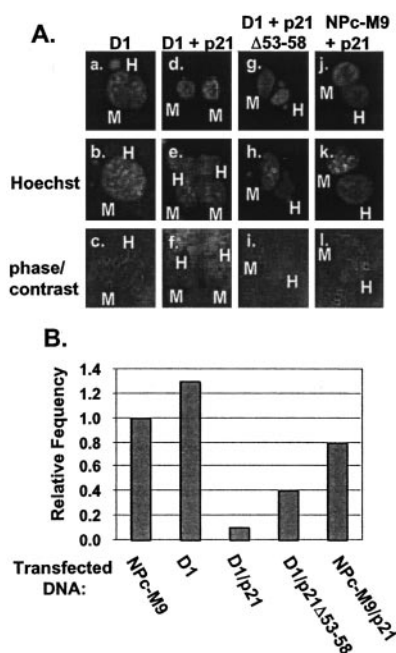


FIG. 3. p21^{Cip1} inhibits cyclin D1 nucleocytoplasmic shuttling. A, NIH-3T3 cells transiently expressing cyclin D1, CDK4 (a–c); cyclin D1, CDK4, p21^{Cip1} (d–f); cyclin D1, CDK4, p21Δ53–58 (g–i); or Npc-M9, p21^{Cip1} (j–l) proliferating on glass coverslips were treated with cycloheximide and fused with HeLa cells. Thirty minutes post-fusion, cells were harvested and processed for immunofluorescence microscopy. Npc-M9 was visualized using the 9E10 monoclonal antibody, and cyclin D1 was visualized with either a monoclonal antibody specific for murine cyclin D1 or the M2 monoclonal antibody, and DNA was visualized with Hoechst dye. The differential staining of human (homogenous) versus murine nuclei (punctate) by Hoechst dye facilitated the identification of fusions composed of murine and human nuclei. B, the number of fusions wherein Npc-M9 and cyclin D1 were observed in HeLa nuclei was quantitated and is expressed.

ting was quantitated (Fig. 3B), and representative fusions are shown (Fig. 3A). The frequency of Npc-M9 shuttling was set at one as a baseline for comparison. As previously demonstrated (12), both Npc-M9 (Fig. 3B, bar 1) and cyclin D1 shuttle between nuclei with a similar frequency (Fig. 3A, panel a; Fig. 3B, bar 2). Although a high frequency of Npc-M9 shuttling was still detected in the presence of ectopic p21^{Cip1} (Fig. 3A, panel j; Fig. 3B, last bar), cyclin D1 shuttling was essentially eliminated in its presence (Fig. 3A, panel d; Fig. 3B, bar 3). The p21Δ53–58 mutant exhibited a reduced capacity to inhibit cyclin D1 shuttling relative to wild-type p21^{Cip1} (Fig. 3A, panel g; Fig. 3B, bar 4). As anticipated, p27^{Kip1} also inhibited cyclin D1 shuttling, but not that of Npc-M9 (data not shown). These results demonstrate that p21^{Cip1} (and p27^{Kip1}) inhibits cyclin D1 nuclear export. Furthermore, these results suggest that p21^{Cip1}-mediated inhibition requires direct contact with both the cyclin D1 and CDK4.

Cyclin D1 nuclear export depends upon functional CRM1 (12). Because the data presented above demonstrate that p21^{Cip1} binding inhibits cyclin D1 nuclear export, we determined if p21^{Cip1} would inhibit CRM1-triggered cyclin D1 nuclear export. NIH-3T3 cells were transiently transfected with FLAG-tagged cyclin D1, CDK4, and CRM1 alone, or along with either p21^{Cip1} or p21Δ53–58. Cyclin D1 localization was monitored by immunohistochemical staining with the M2 monoclonal antibody. Cyclin D1 localized to the nucleus of cell expressing only cyclin D1 and CDK4 (Fig. 4A, panel a; Fig. 4B, bar 1), whereas co-expression of CRM1 promoted the redistribution of cyclin D1 to the cytoplasm (Fig. 4A, panel c; Fig. 4B, bar 2). Co-expression of wild-type p21^{Cip1} but not p21Δ53–58 inhibited CRM1-dependent nuclear

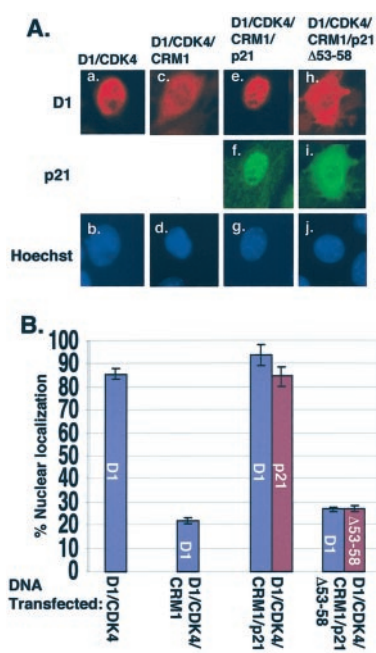


FIG. 4. Inhibition of CRM1-dependent cyclin D1 nuclear export. A, NIH-3T3 cells were transfected with plasmids encoding either wild-type cyclin D1 and CDK4 (panels a–b); cyclin D1, CDK4, and HA-CRM1 (panels c–d); cyclin D1, CDK4, p21^{Cip1}, and HA-CRM1 (panels e–g); or D1, CDK4, p21Δ53–58, and HA-CRM1 (panels h–j). Cyclin D1 was visualized with the M2 antibody (top panels), p21 proteins were visualized with a rabbit anti-p21 antibody, and DNA was visualized with Hoechst dye (bottom panel). B, quantitation of the number of cells expressing exclusively nuclear cyclin D1 from A.

export of cyclin D1 (Fig. 4A; compare panels e and h). These results demonstrate that p21^{Cip1} can promote nuclear accumulation of cyclin D1-CDK4 complexes through inhibition of CRM1-dependent nuclear export.

p21^{Cip1} Inhibits Cyclin D1-CRM1 Association—To determine if inhibition of nuclear export resulted from the capacity of p21^{Cip1} to inhibit GSK-3β-dependent phosphorylation of cyclin D1, whole cell lysates were prepared from Sf9 cells infected with baculoviruses encoding cyclin D1 and CDK4 in the absence or presence of p21^{Cip1}. Total cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blotted for total cyclin D1 or Thr-286-phosphorylated cyclin D1 (P-286). Immunoblot analysis with the P-286-specific antibody (Fig. 5A, bottom panel) revealed the presence of Thr-286-phosphorylated cyclin D1 in the absence (lane 1) and presence (lane 2) of p21^{Cip1}. Parallel immunoblot analysis with the cyclin D1 antibody (top panel) revealed equivalent levels of total cyclin D1. We also examined the ability of p21^{Cip1} to inhibit Thr-286 phosphorylation in NIH-3T3 cells. To facilitate detection of Thr-286-phosphorylated cyclin D1, we utilized FLAG-D1 overexpressing NIH-3T3 cells (D1–3T3). D1–3T3 cells were infected with either control (empty) virus or virus encoding wild-type p21^{Cip1} (Fig. 5B). Thirty-six hours post-infection, cyclin D1 was precipitated with either the phospho-specific antibody (lanes 2 and 5) or the M2 monoclonal antibody (lanes 3 and 6). Levels of cyclin D1 were determined by immunoblot with the cyclin D1 monoclonal antibody. As observed in Sf9 cells, phosphorylated cyclin D1 was detected in the absence or presence of ectopic p21^{Cip1} (compare lanes 2 and 5). We noted a slight increase in both total and phosphorylated cyclin D1 in the presence of ectopic p21^{Cip1}. This increase likely results from decreased turnover of nuclear cyclin D1 (21) (see below). We conclude that p21^{Cip1} does not inhibit phosphorylation of cyclin D1 at threonine 286.

We next determined if p21^{Cip1} could prevent the binding of

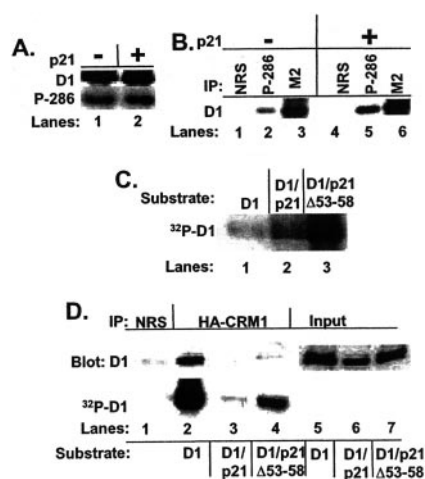


FIG. 5. p21^{Cip1} prevents cyclin D1-CRM1 association. *A*, Sf9 cells expressing either FLAG-D1 and CDK4 (*lane 1*) or FLAG-D1, CDK4, and p21^{Cip1} (*lane 2*) were resolved on a denaturing polyacrylamide gel, transferred to nitrocellulose membrane, and blotted with the phospho-specific P-286 antibody (*bottom panel*) then stripped and re-blotted with the cyclin D1 monoclonal antibody (*top panel*). Sites of antibody binding were visualized by enhanced chemiluminescence. *B*, lysates prepared from D1-3T3 cells infected with empty virus (*lanes 1–3*) or virus encoding p21^{Cip1} (*lanes 4–6*) were precipitated with normal rabbit serum (NRS), the Thr-286 phospho-specific antibody (P-286), or the M2 monoclonal antibody (M2). Precipitated proteins were resolved on a denaturing polyacrylamide gel, transferred to nitrocellulose membrane, and blotted with the cyclin D1 monoclonal antibody. Sites of antibody binding were visualized by enhanced chemiluminescence. *C*, purified FLAG-D1, FLAG-D1-p21^{Cip1}, or FLAG-D1-p21Δ53–58 produced in insect Sf9 cells and phosphorylated with recombinant GSK-3β were resolved on a polyacrylamide gel and visualized by autoradiography. *D*, purified FLAG-D1, FLAG-D1-p21^{Cip1}, or FLAG-D1-p21Δ53–58 complexes normalized from *C* above were mixed with a Sf9 lysate containing HA-CRM1. Complexes were re-purified with the 12CA5 monoclonal antibody, and, following extensive washes, HA-CRM1-cyclin D1 complexes were denatured, resolved on polyacrylamide gels, and transferred to nitrocellulose membrane. Following autoradiography to visualize ³²P-labeled cyclin D1, the membrane was blotted with the cyclin D1 monoclonal antibody (*lanes 1–4*). Equivalent levels of cyclin D1 in the purified complexes were confirmed by direct Western analysis with the cyclin D1 monoclonal antibody (*lanes 5–7*). Sites of antibody binding were visualized by enhanced chemiluminescence.

CRM1 to Thr-286-phosphorylated cyclin D1, D1-p21^{Cip1}, or D1-p21Δ53–58 complexes. Complexes were purified from Sf9 cells by affinity chromatography and phosphorylated with recombinant GSK-3β and [³²P]γATP. The purified complexes were normalized to levels of phosphorylated cyclin D1 (Fig. 5C), mixed with Sf9 lysates containing HA-CRM1 and re-precipitated with the 12CA5 antibody, which is directed against the N-terminal HA-epitope tag encoded by HA-CRM1. Following transfer to nitrocellulose membrane, cyclin D1 was visualized by both autoradiography, to detect ³²P-labeled cyclin D1 (Fig. 5D, *bottom panel*), and by immunoblot with the cyclin D1 antibody (Fig. 5D, *top panel*). Phosphorylated cyclin D1 was readily detectable in the CRM1 precipitates in the absence of p21^{Cip1} (*lane 2*). In contrast, p21^{Cip1}-bound cyclin D1 did not efficiently co-precipitate with CRM1 (*lane 3*). Binding of p21Δ53–58 reduced but did not eliminate D1-CRM1 interactions (*lane 4*) consistent with the reduced capacity of this mutant to bind to and inhibit D1 nuclear export. These results demonstrate that p21^{Cip1} binding prevents association of CRM1 with phosphorylated cyclin D1.

Efficient nuclear export of cyclin D1 requires phosphorylation at Thr-286. The requirement for either p21^{Cip1} or p27^{Kip1} to promote nuclear accumulation of cyclin D1 during G₁-phase implies that cyclin D1 can be phosphorylated on this residue during G₁- and S-phases. D1-3T3 cells were rendered quies-

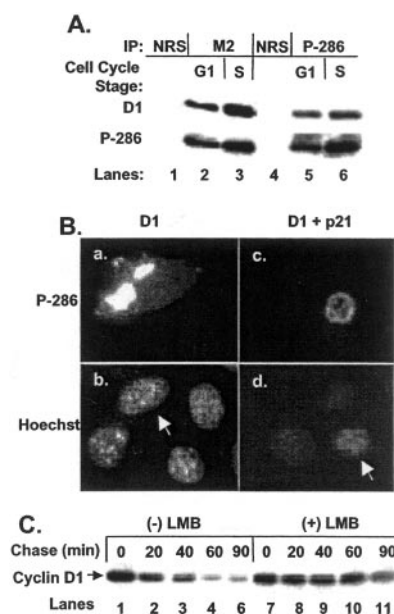


FIG. 6. p21^{Cip1} promotes the nuclear accumulation of Thr-286-phosphorylated cyclin D1. *A*, FLAG-D1-3T3 cells were arrested in G₀ by serum deprivation for 36 h and stimulated to synchronously re-enter the cell cycle by addition of FCS. Cell lysates were prepared at 9 h (G₁-phase; *lanes 1–2, 4–5*) or 20 h (S-phase; *lanes 3 and 6*). Cyclin D1 was precipitated with normal rabbit sera (NRS), the M2 monoclonal antibody (*lanes 2–3*), or the P-286 antibody (*lanes 5–6*). Total cyclin D1 was visualized with the cyclin D1 monoclonal antibody (*top panel*) and the phospho-specific antibody (*bottom panel*). Sites of antibody binding were visualized by enhanced chemiluminescence. *B*, NIH-3T3 cells were transfected with plasmids encoding either wild-type cyclin D1 (*panels a and b*) or cyclin D1 and wild-type p21^{Cip1} (*panels c and d*). Phosphorylated cyclin D1 was visualized with the phospho-286-specific antibody (*top panels*), and DNA was visualized with Hoechst dye (*bottom panel*). *C*, NIH-3T3 cells untreated or treated with 10 ng/ml LMB were pulsed with [³⁵S]methionine for 30 min and “chased” with medium containing excess unlabeled methionine for the indicated intervals. Radiolabeled cyclin D1 was precipitated from lysates and analyzed by SDS-PAGE and visualized by autoradiography.

cent by serum deprivation for 36 h. Cell lysates were prepared following addition of FCS in late G₁-phase boundary (9 h) and late S-phase (20 h). S-phase entry was monitored in parallel by pulse label with bromodeoxyuridine (data not shown). Cyclin D1 complexes were isolated by precipitation with either the M2 monoclonal antibody or the antibody directed against phosphorylated Thr-286. Phosphorylated cyclin D1 could be detected in G₁- and S-phases, although the level of Thr-286-phosphorylated cyclin D1 was increased during S-phase (Fig. 6A, *lanes 5 and 6, top panel*). We noted that less cyclin D1 was detected in the phospho-specific precipitates (compare *lanes 2 with lanes 5 and 6, top panel*) suggesting that only a fraction of the total cyclin D1 is phosphorylated on this residue. Upon blotting with the phospho-specific antibody, equal levels of Thr-286-phosphorylated cyclin D1 were detected in both the M2- and phospho-specific precipitates (compare *lanes 2 and 3 with lanes 5 and 6, second panel*). These data demonstrate that a sub-population of cyclin D1 is phosphorylated on Thr-286 during G₁, with peak levels of phosphorylation occurring during S-phase.

To conclusively demonstrate that p21^{Cip1} promotes the nuclear accumulation of Thr-286-phosphorylated cyclin D1, NIH-3T3 cells were transfected with plasmids encoding either cyclin D1 or cyclin D1 and p21^{Cip1}. The localization of phosphorylated cyclin D1 was determined with the anti-phospho-286 antibody. In the absence of exogenous p21^{Cip1}, phosphorylated cyclin D1 localized to nuclear and cytoplasmic compartments (Fig. 6B, *panel a*). We noted that cytoplasmic phospho-cyclin D1 tended to accumulate as cytoplasmic aggregates (*panel a*). We have

noted similar cyclin D1 localization in cells treated with proteasome inhibitors (data not shown) suggesting that this localization might represent sites of cyclin D1 proteolysis. In contrast, co-transfection of p21^{Cip1} resulted in the exclusive nuclear localization of Thr-286-phosphorylated cyclin D1 (panel c). These results demonstrate that p21^{Cip1} inhibits nuclear export of Thr-286-phosphorylated cyclin D1.

Nuclear localization of cyclin D1 has been hypothesized to potentiate its accumulation via decreased proteolysis (9). Consistent with this hypothesis, overexpression of either p21^{Cip1} or p27^{Kip1}, which enforces the nuclear localization of cyclin D1 complexes (data herein) will decrease the rate of cyclin D1 turnover (18). These results are consistent with the idea that cyclin D1 must be exported from the nucleus for efficient proteolysis. To further test this hypothesis, we compared the half-life of cyclin D1 in untreated *versus* LMB-treated NIH-3T3 cells. We have previously shown that LMB treatment does not inhibit Thr-286 phosphorylation (12); thus, any difference in cyclin D1 turnover can not be attributed to differential cyclin D1 phosphorylation. LMB treatment extended the cyclin D1 half-life greater than 3-fold as determined by pulse-chase analysis (Fig. 6C). These data suggest that Cip/Kip proteins facilitate cyclin D1 accumulation via specific inhibition of cyclin D1 nuclear export thereby resulting in reduced cyclin proteolytic degradation.

DISCUSSION

Regulation of Cyclin D1 Nuclear Export by Cip/Kip—There is increasing evidence that Cip/Kip proteins function as regulators of cyclin D1 nuclear accumulation. Based largely on the ability of p21^{Cip1} and p27^{Kip1} to promote nuclear localization of cyclin D1-CDK4 complexes in transient transfection experiments, it was proposed that Cip/Kip proteins are regulators of cyclin D1 nuclear import. However three pieces of evidence demonstrate that neither p21^{Cip1} nor p27^{Kip1} are strictly required for cyclin D1 nuclear import. First, although inefficient, low levels of cyclin D1 can still enter the nucleus of p21/p27^{-/-} MEFs (Fig. 1) (18). Second, nuclear localization of cyclin D1 in p21/p27-deficient MEFs can be completely restored by inhibition of the nuclear exportin CRM1 with leptomycin B. Third, cyclin D1-T286A, a cyclin D1 mutant that is refractory to CRM1-dependent nuclear export, is exclusively nuclear in the absence of both p21^{Cip1} and p27^{Kip1}. These data demonstrate that cyclin D1 nuclear import remains intact in the absence of either Cip1 or Kip1. In contrast our data suggest that deletion of p21^{Cip1} and p27^{Kip1} results in an increase in the rate of cyclin D1 nuclear export. We noted that both p21^{Cip1} and p27^{Kip1} can abolish cyclin D1 nucleocytoplasmic shuttling, a process that is dependent upon nuclear export (28). In addition, ectopic expression of either p21^{Cip1} or p27^{Kip1} inhibited GSK-3 β triggered cyclin D1 nuclear export. Finally, p21^{Cip1} efficiently inhibited the ability of ectopic CRM1 to shuttle cyclin D1 out of the nucleus and abolished cyclin D1-CRM1 association. These observations in concert with the ability of LMB to restore nuclear accumulation of cyclin D1 in p21/p27^{-/-} cells imply that p21^{Cip1} and p27^{Kip1} are inhibitors of cyclin D1 nuclear export. Accordingly, through inhibition of cyclin D1 nuclear export, p21^{Cip1} and p27^{Kip1} promote cyclin D1-CDK4 nuclear accumulation. In their absence, the rate of cyclin D1 nuclear export is increased such that there is no net cyclin nuclear accumulation. Although neither p21^{Cip1} nor p27^{Kip1} is strictly required for cyclin D1 nuclear import, we cannot eliminate the possibility that they may also facilitate cyclin D1 nuclear import under certain conditions. By increasing nuclear import and inhibiting nuclear export of cyclin D1, Cip/Kip proteins would ensure the efficient nuclear accumulation of cyclin D1 during G₁-phase.

If phosphorylation of Thr-286 is required for cyclin D1 nuclear export, why have cells developed a second mechanism that can override this signal? The answer to this question likely

stems from the fact that the GSK-3 β also shuttles between nuclear and cytoplasmic compartments (9). One could envision leaky regulation of cyclin D1 phosphorylation resulting in Thr-286 phosphorylation during G₁-phase, a point requiring nuclear accumulation of cyclin D1. In point of fact, early experiments demonstrated increasing levels of total cyclin D1 phosphorylation during G₁-phase (23). Our data are consistent with this observation and demonstrate that phosphorylation does occur on Thr-286 during G₁-phase. Phosphorylation of cyclin D1 during G₁-phase will necessitate that an alternative measure be taken to maintain cyclin D1 in the nucleus during G₁-phase. The ability of p21^{Cip1} to block CRM1-cyclin D1 binding and maintain phosphorylated cyclin D1 in the nucleus demonstrates that p21^{Cip1} binding is a dominant event that overrides Thr-286 phosphorylation-triggered cyclin D1 nuclear export. Thus, the binding of Cip/Kip proteins to the cyclin D1 complex will effectively reduce the rate of cyclin D1 nuclear export during G₁-phase, thereby allowing the cell to accumulate a threshold level of cyclin D1-CDK4 kinase necessary to promote restriction point passage. In contrast, the absence of p21^{Cip1} and p27^{Kip1} would result in the rapid export of cyclin D1 and the absence of cyclin D1-CDK4 nuclear accumulation as is observed in p21/p27^{-/-} MEFs.

Given the capacity of Cip/Kip proteins to override phosphorylation-triggered cyclin D1 nuclear export, effective removal of cyclin D1 from the nucleus during S-phase will require two events. The first is the phosphorylation of cyclin D1 at Thr-286. The second will be the liberation of cyclin D1 from p21/p27 proteins. Although we have not established the nature of this latter signal, it is likely that accessibility of p21/p27 to the cyclin D1 complex will be a contributing factor. Accessibility will be sensitive to the rate of Cip/Kip turnover and to Cip/Kip localization within the cell. Cip1 and Kip1 are highly labile proteins, with half-lives of less than 30 min in most cell types (30–33). Accumulation of p21^{Cip1} mirrors that of cyclin D1 subcellular localization. p21^{Cip1} levels peak during late G₁-phase, the point at which cyclin D1 is exclusively nuclear, and its levels dwindle as cells traverse S-phase, the point wherein cyclin D1 becomes cytoplasmic (34).

The subcellular localization of p21^{Cip1} and p27^{Kip1} might also contribute to their capacity to regulate cyclin D1 nuclear export in G₁ *versus* S-phase. Phosphorylation of p21^{Cip1} at Thr-145 is associated with decreased binding to CDK4 and decreased nuclear import (35, 36). Intriguingly, Akt (protein kinase B), the upstream regulator of GSK-3 β , targets p21^{Cip1} at Thr-145 (36). Any shift in Cip/Kip localization resulting in lower levels of nuclear Cip/Kip would result in increased nuclear export of phosphorylated cyclin D1.

Cyclin D1 Nuclear Retention and Cell Cycle Progression—The importance of cyclin D1 nuclear localization is at least 2-fold. First, the critical cell cycle regulatory functions of the cyclin D1 kinase, including phosphorylation of Rb, require its nuclear localization during G₁-phase. As a consequence, the failure of the cyclin D1 kinase to accumulate in the nucleus prohibits G₁-phase progression (19). Second, although cyclin D1 nuclear localization promotes close proximity with Rb, our data also demonstrate that nuclear localization facilitates rapid accumulation of cyclin D1 through inhibition of proteolytic degradation. Cyclin D1 degradation is regulated via the 26 S proteasome, and efficient proteolysis requires phosphorylation of Thr-286. The capacity of phosphorylation to direct cyclin D1 nuclear export lead to the hypothesis that cyclin D1 destruction preferentially occurs in the cytoplasm (9). Consistent with this hypothesis, inhibition of cyclin D1 nuclear export by treatment of cells with LMB reduced the rate of cyclin D1 proteolytic turnover. Because LMB does not inhibit Thr-286

phosphorylation by GSK-3 β (12), we conclude that cyclin D1 must first be shuttled to the cytoplasm where it undergoes ubiquitin-dependent destruction. The capacity of p27^{Kip1} and p21^{Cip1} to reduce the rate of cyclin D1 turnover is also consistent with this notion (18). Thus, Cip/Kip proteins not only directly promote the nuclear accumulation of cyclin D1 during G₁-phase through inhibition of nuclear export, but also facilitate accumulation through inhibition of cyclin D1 degradation.

Duality of Cip/Kip Function—The Cip/Kip inhibitors (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) and INK4 inhibitors (p15, p16, p18, p19) have the functional capacity to limit cell cycle progression via CDK inhibition in response to cellular stresses or through anti-mitogens such as transforming growth factor- β (37). However, it is clear that the Cip/Kip family is required for efficient assembly of the cyclin D-CDK4 kinase (14, 18), and our results now demonstrate that inhibition of cyclin D1 nuclear export by Cip/Kip proteins is required for cyclin D1-CDK4 nuclear accumulation during G₁-phase. The ability of p21^{Cip1} to promote nuclear accumulation of Thr-286-phosphorylated cyclin D1 along with the inability of cyclin D1 to efficiently accumulate in the nucleus in its absence stresses the importance of their capacity to regulate cyclin D1 nuclear export. These data provide direct evidence for the notion that Cip/Kip proteins serve as dual regulators of cell cycle progression: potent inhibitors of the CDK2 kinase and requisite positive regulators of the cyclin D-CDK4 kinase.

Based on our findings, we propose a model wherein cyclin D1 nuclear export is a critical regulatory step necessary to maintain normal mitogen-dependent cell proliferation. In this model the nuclear accumulation of cyclin D1 is regulated by the nuclear exportin CRM1. The binding of CRM1 to cyclin D1-CDK4 complexes requires phosphorylation of cyclin D1 on a single threonine residue by GSK-3 β . Phosphorylation of this residue is in turn determined by the activity of GSK-3 β and access of GSK-3 β to nuclear cyclin D1 complexes. Cip/Kip proteins function as a master switch that can override Thr-286 phosphorylation thereby preventing CRM1-dependent nuclear export and maintaining cyclin D1 complexes within the nucleoplasm.

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