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ALTERNATE CYCLIN D1 mRNA SPLICING MODULATES P27[KIP1] BINDING AND CELL MIGRATION

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ABSTRACT

Cyclin D1 is an important cell cycle regulator but in cancer its overexpression also increases cellular migration mediated by p27[KIP1] stabilization and RhoA inhibition. Recently, a common polymorphism at the exon 4-intron 4 boundary of the human cyclin D1 gene within a splice donor region was associated with an altered risk of developing cancer. Altered RNA splicing caused by this polymorphism gives rise to a variant cyclin D1 isoform termed cyclin D1b, which has the same N-terminus as the canonical cyclin D1a isoform but a distinct C-terminus. In this study we show that these different isoforms have unique properties with regard to the cellular migration function of cyclin D1. Whereas they displayed little difference in transcriptional co-repression assays on idealized reporter genes, microarray cDNA expression analysis revealed differential regulation of genes including those that influence cellular migration. Additionally, while cyclin D1a stabilized p27[KIP1] and inhibited RhoA-induced ROCK kinase activity, promoting cellular migration, cyclin D1b failed to stabilize p27[KIP1] or inhibit ROCK kinase activity and had no effect on migration. Our findings argue that alternate splicing is an important determinant of the function of cyclin D1 in cellular migration.

The cyclin D1 gene was initially cloned as a breakpoint rearrangement in parathyroid adenoma (1). Subsequent studies confirmed increased cyclin D1 abundance in a variety of tumors including breast and gastrointestinal tumors (2). Molecular analysis demonstrated the cyclin D1 gene encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein, pRb. Immunoneutralizing and antisense experiments revealed the requirement for cyclin D1 in progression through the early G1 phase of the cell cycle (reviewed in (2)). In addition to binding Cdks, cyclin D1 associates with several different intracellular proteins including the estrogen receptor (ERα), PPARγ, P/CAF (p300/CBP associated factor) (3,4) and the cyclin D1 myb-like binding protein (DMP1) (5-8). Cyclin D1 binds to ERα in breast cancer cells and overcomes the BRCA1-mediated repression of ERα activity (9,10). Homozygous deletion of the cyclin D1 gene results in mice with failure of terminal alveolar breast development during pregnancy, increased basal and UV-induced apoptosis, retinal apoptosis, defective angiogenesis, hepatic steatosis, and defective cellular migration (4,11-14).

Previous epidemiological and clinical observations have demonstrated a correlation between cyclin D1 overexpression and cellular metastases (15). Molecular genetic analysis subsequently revealed a key role for cyclin D1 in cellular adhesion and migration (14). In particular, cyclin D1-deficient cells showed enhanced cellular adhesion and reduced migration into a wound, and genetic complementation experiments established a requirement for the CDK-binding but not the pRB-binding domain of cyclin D1 to rescue the cell migratory defect (16,17). Together, these studies demonstrated a key role for cyclin D1 in cell migration, including bone marrow-derived macrophages, fibroblasts, and mammary epithelial cells (14,16,17).

Recently, a common polymorphism identified in the human cyclin D1 gene was found to be associated with an increased risk of cancer development (18-22). This polymorphism (A870G), located at the splice donor region at the exon 4-intron 4 boundary, modulates the efficiency of alternate splicing between exon 4 and 5. As a result of the altered splicing which occurs, the coding region downstream is altered such that the amino acid sequence of the carboxyl-terminus of cyclin D1 is altered (18). Thus, the two isoforms of cyclin D1 produced, the canonical isoform termed cyclin D1a and the alternately spliced isoform termed cyclin D1b, are identical in their N-termini but distinct in their C-termini. Notably, clinical studies have associated this polymorphism with an increased risk of colon and rectal cancer, early-onset squamous cell carcinoma, head and neck cancer, and transitional cell carcinoma of the bladder (23). Elegant in vitro analyses have demonstrated that the cyclin D1a and D1b isoforms have similar half lives in cultured cells, however, the cyclin D1b isoform is primarily nuclear in subcellular localization and relatively more resistant to phosphorylation-dependent nuclear export (24). Cyclin D1a blocks cells at the G1 phase whereas cyclin D1b blocks cells at the G0 phase in some (23), but not all studies (25). Notably, while both isoforms encode regulatory subunits, cyclin D1b has a reduced capacity to phosphorylate pRb (25,26). Yet despite serving as a less effective

component of this pRb kinase, cyclin D1b conveys a greater transforming capacity which enhances colony formation of NIH3T3 cells. Thus, the basis for the enhanced transforming ability of cyclin D1b has been unclear.

The Cdk inhibitor p27^{KIP1} inhibits most cyclin/Cdk complexes, including cyclin E/Cdk2 and cyclin D/Cdk4 (27). In the majority of studies, reduced p27^{KIP1} in tumors correlates with a poorer prognosis (28-31). p27^{KIP1} plays an important role in regulating cellular migration through regulating either Rac (32), Rho GTPase activity (16,33) or Stathmin function (34). Mechanistic analyses presented here reveal that cyclin D1b evades binding to p27^{KIP1} which may explain the increased cell transforming activity of this isoform. Furthermore, cyclin D1b lacks the pro-migratory function of cyclin D1a, implicating the C-terminal region and alternate splicing as important determinants of this function.

Experimental procedures

Cell culture - Cyclin D1$^{+/+}$ and cyclin D1$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) cultures were prepared as described previously (4) and passaged by the original 3T3 protocol (35). Human kidney 293T and MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin and streptomycin (100 mg of each/liter) and supplemented with 10% FBS.

Retroviral production and infection - Retroviral production and infection of cyclin D1$^{-/-}$ 3T3 cells were previously described (16). Fluorescence-activated cell sorting (FACS) (DakoCytomation MoFlo; Dako Denmark) sorted GFP+ cells were used for subsequent analysis.

Immunofluorescence - Phalloidin, paxillin and tyrosine phosphorylated paxillin (pY^{118}) staining were conducted as previously described (14,16). The samples were visualized on a Zeiss LSM 510 META Confocal Microscope with a 63× objective.

Immunoprecipitations and Western blotting - Cyclin D1$^{-/-}$ MEFs or 3T3 cells infected with GFP vector control or cyclin D1 wild type (D1a) or splice variant (D1b) were lysed in immunoprecipitation (IP) buffer (10 mM Tris-HCL at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% IGEPAL CA630, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitor cocktail (Boehringer Mannheim). For each IP, 1 ml lysate (1 mg protein) and 10 µl anti-FLAG M2-agarose affinity gel (Sigma, St. Louis, MO) were incubated overnight at 4°C. Immunoprecipitates were washed 5 times in IP buffer, and 20 µl of 2×sample buffer was added to the bead pellet. The immunoprecipitates, as well as 50 µg of proteins from the corresponding lysates were subjected to Western blotting as previously described (36). The following antibodies were used for Western blotting: mouse M2 anti-FLAG antibody, antivinculin antibody (Sigma, St. Louis, MO), and anti β-tubulin antibody, mouse anti-cyclin D1 antibody

(DCS-6), rabbit polyclonal anti-p27^{KIP1} antibody (C-19), rabbit polyclonal antibody for Ser3 phospho-ADF/cofilin (Santa Cruz Biotechnology, Santa Cruz, CA).

Time-lapse video - For time-lapse observation of cell movement, cells were plated in 12 well plates in DMEM with 10% FCS and HEPES. Cells were placed in a temperature- and CO2-controlled incubator to maintain the temperature at 37°C and CO2 at 5%. The cell movement videos were snapped at 5 minute intervals for 2 hours by using a Zeiss Axiovert 200M Inverted Microscope System. The cell movement velocity was determined by tracing several single cells at different time points using MetaMorph software.

Mammalian two-hybrid and luciferase reporter gene assays - Mammalian two-hybrid was performed by following the manufacturer's instruction (Promega) and was described previously (17). Human p27^{KIP1}, cyclin D1a or D1b cDNAs were cloned into pBIND vector and pACT vector to generate fusion proteins with the DNA binding domain of GAL4 (Gal4-p27^{Kip1}, Gal4-D1a, Gal4-D1b) and the activation domain of VP16 (VP16-p27^{KIP1}, VP16-D1a, VP16-D1b), respectively. Gal4-PPARγ, Gal4-AR, and Gal4-NRF1 were previously described (3,4,37). Gal4p27^{KIP1}, VP16-cyclin D1, and pG5luc, which contains five Gal4 binding sites upstream of a minimal TATA box and the firefly luciferase gene (Promega) or Gal4-cyclin D1, VP16-p27^{KIP1} and pG5luc were cotransfected into MCF-7 cells with Superfect transfection reagent. Two days after transfection, the cells were lysed and luciferase activity quantitated as previously described (4). Luciferase activity was normalized for transfection efficiency using a β-galactosidase reporter (CMVβ-gal) as an internal control.

Northern blot and pulse chase analysis - Total RNA samples were prepared from cyclin D1$^{-/-}$ 3T3 cells infected with cyclin D1a, D1b, or vector control with the Trizol reagent (Invitrogen). For Northern blot, 10 µg of RNA per sample was separated on 0.9% formaldehyde-agarose gel, transferred, and hybridized by using Rapid-hyb Buffer (Amersham/GE Healthcare, Piscataway, NJ). Mouse p27^{KIP1} probe, mouse β-actin probe, and human cyclin D1 probe were labeled with [α^{32}P]dCTP by using the Ready-to-go kit (Amersham/GE Healthcare, Piscataway, NJ). Mouse p27^{KIP1} and mouse β-actin probes were RTPCR products. 1 µg of total RNA from cyclin D1$^{-/-}$ MEFs infected with cyclin D1a was used for cDNA synthesis with the Superscript Reverse Transcriptase (Invitrogen). RT-PCR products were obtained with the following primers: p27^{Kip1} primers: forward: 5'CGTGAGAGTGTCTAACGGG-3' and reverse: 5'-CGAGTCAGGCATTTGGTCC-3'. β-actin primers: forward: 5'-TGTTACCAACTGGGACGACA-3' and reverse: 5'-AAGGAAGGCTGGAAAAGAGC-3'. The human cyclin D1 probe was the fragment between HindIII and NcoI from MSCV-cyclin D1a-IRESGFP retroviral vector. The filters were exposed to X-ray films at –80°C. Pulse chase analysis was conducted as previously described (38).

Real-time RT-PCR - Total RNA samples from cyclin D1$^{-/-}$ cells transfected with MSCV-IRESGFP, MSCV-cyclin D1a-IRES-GFP or MSCVcyclin D1b-IRES-GFP were subjected to DNase I (RQ1 DNase: Promega Corp, Madison, WI) treatment to remove contaminating DNA from RNA preparations followed by their re-purification using Qiagen RNAEasy RNA columns (Qiagen, Valencia, CA). Equal amounts of purified RNA samples were reverse transcribed using Iscript Reverse transcriptase kit (Bio-Rad, Hercules, CA) to form cDNA which was then subjected to SYBR Green based Real-Time PCR relative quantification method for amplification of p27^{KIP1} transcripts using standard hot-start reaction mixes and conditions (39) in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA). The primers used for amplification of p27^{KIP1} were: forward 5' TCTCTTCGGCCCGGTCAAT 3' and reverse 5' GGGGCTTATGATTCTGAAAGTCG 3'. Amplification of 18S rRNA housekeeping gene transcript (primer ref (40)) was performed in every sample and the obtained Ct values for each sample were used for normalization of data for p27^{KIP1} expression in ABI Prism SDS 2.3 software. To calculate the fold change in p27^{KIP1} gene expression between various treatments Ct values obtained from amplification of p27^{KIP1} transcripts in MSCV-IRES-GFP were used for calibration.

Microarray analysis - Total RNA (5µg) was reverse transcribed using Superscript III First-Strand Synthesis System (Invitrogen) using a HPLC purified T7-dT24 primer (Sigma Genosys) which contains the T7 polymerase promoter sequence. The single stranded cDNA was converted to double stranded cDNA using DNA polymerase I (Promega) and purified by cDNA spin column purification using GeneChip Sample Cleanup Module (Affymetrix). The double stranded cDNA was used as a template to generate biotinylated cRNA using Bioarray HighYield RNA Transcription Labeling Kit (Enzo) and the labeled cRNA purified by GeneChip Sample Cleanup Module (Affymetrix). 15µg of cRNA was fractionated to produce fragments between 35–200 bp using 5x Fragmentation buffer provided in the Cleanup Module. The sample was hybridized to mouse 430 2.0 microarray (Affymetrix) representing approximately 39,000 well-characterized mouse transcripts. The hybridization and washing steps were carried out in accordance with Affymetrix protocols for eukaryotic arrays. The arrays were scanned at 570nm with a confocal scanner from Affymetrix.

Array data analysis - Analysis of the arrays was performed using R-statistics package and the limma library of the Bioconductor software package (37). Arrays were normalized using robust multiarray analysis (RMA), and P-value of 0.05 was applied as criteria for statistically significant differentially expressed genes between Cyclin D1a or Cyclin D1b rescued 3T3 compared to GFP control. GO tree analysis of gene function analyzed by "Webgestalt" (http://bioinfo.vanderbilt.edu/webgestalt) (41) and affymetrix gene annotation.

Results

Genes associated with cell migration are preferentially regulated by cyclin D1a but not cyclin D1b. Cyclin D1a functions as a transcriptional coregulator in part by recruiting HDAC and controlling p300 HAT activity (2,42,43). For example, cyclin D1a inhibits PPARγ signaling and transactivation in vitro and in vivo (4,42). To compare the transcriptional repression function of cyclin D1b, we evaluated its effects on PPARγ activity using a heterologous reporter system in which PPARγ was linked to the Gal4-DNA binding domain. Consistent with prior studies, cyclin D1a inhibited PPARγ activity while cyclin D1b also repressed PPARγ activity to a similar extent (Fig. 1A). Cyclin D1 also inhibits mitochondrial biogenesis, in part by inhibiting the transcriptional activity of NRF1 (37). Herein, both cyclin D1a and cyclin D1b inhibited NRF1 activity (Fig. 1A). Consistent with recent studies (44), cyclin D1b repressed androgen receptor activity. To further compare the effects of the cyclin D1 isoforms on transcription, we performed a genome-wide analysis of how they affected gene expression in cyclin D1$^{-/-}$ mouse embryo fibroblast derived 3T3 cells. The cyclin D1$^{-/-}$ cells were transduced with specific retroviral vectors. Previous work has shown that cyclin D1 regulates the expression of genes involved in cellular migration in early passage MEFs (16). 3T3 cells transduced with retroviral vectors expressing each cyclin D1 isoform were processed for mRNA analysis. Similar levels of cyclin D1 isoform were expressed by each vector as determined by Western blotting (Fig. 2C, Fig. 3A). We found that 19 genes were regulated concordantly by both cyclin D1a and cyclin D1b (Fig. 1B). Distinct genes were regulated by cyclin D1a vs. cyclin D1b. Cyclin D1a expression altered the abundance of 661 genes, of which 397 genes were induced and 264 genes repressed >1.3-fold, compared with vector control. Cyclin D1b regulated expression of 60 genes, 1/3 of which (19 genes) were regulated concordantly by cyclin D1a. Cyclin D1a regulated a distinct cluster of genes that function to promote cellular migration and invasion. These genes were not regulated by cyclin D1b (Supplemental Data 1, Table 1, For full details the GEO accession number obtained is GSE9161 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi ?token=zfellwagiuaagfa&acc=GSE9161). Cyclin D1b regulated genes function in DNA-dependent transcription and nucleosome assembly (Supplemental Data 2, Table 2).

Cellular migration is promoted by cyclin D1a but not cyclin D1b. MEFs genetically deficient in cyclin D1 spread with a circumferential distribution of adherent focal contacts (16). Cyclin D1 promotes cellular migration of bone marrow macrophages, fibroblasts, mammary epithelial cells and vascular endothelial cells (14,16,45). To evaluate whether both cyclin D1 isoforms promote focal contact distribution and cellular migration, retroviral transduced cells co-expressing green fluorescence protein (GFP) via an internal ribosomal entry site (IRES) were sorted after transduction for analysis. Consistent with previous studies, cyclin D1$^{-/-}$ MEFs assumed a flattened spread morphology by phase contrast microscopy (Supplemental Data 3). Cell contacts were monitored by confocal microscropy using antibodies specific for tyrosine-phosphorylated paxillin (Y118). Consistent with previous observations, cyclin D1$^{-/-}$ MEFs showed a peripheral distribution of focal contacts as revealed by tyrosine-

phosphorylated paxillin (Y118) (Fig. 2A). Cyclin D1a-expressing cells markedly reduced the peripheral distribution of tyrosine phosphorylated paxillin. In contrast, cyclin D1b failed to reverse this phenotype (Fig. 2A).

To compare effects on cell migration, GFP positive cells were examined by phase contrast video microscopy. Previous studies have demonstrated that cyclin D1 regulates F-actin organization and promotes migration through inhibition of the RhoA-regulated ROCK kinase (16,17). Consistent with these studies, cyclin D1$^{-/-}$ MEFs expressing cyclin D1a appeared polarized (Fig. 2A) and phalloidin staining demonstrated a reduction in F-actin staining. In contrast, although similar protein levels were expressed in cyclin D1b-transduced cells, no alteration in cellular morphology was observed (Fig 2A). Cellular movement velocity was determined at regular 5 min intervals. As expected, velocity was enhanced by expression of cyclin D1a but not by the cyclin D1b isoform (Fig. 2B).

Cyclin D1 enhances cellular migration by inhibiting ROCK kinase activity as evidenced by reduced phosphorylation of the actin-depolymerizing protein cofilin at serine 3 (Ser3) (17). In transduced cyclin D1$^{-/-}$ cells, cyclin D1a reduced ROCK activity as evidenced by a ~2-fold reduction in Ser3 phosphorylation of cofilin relative to vector-only transduced control cells (Fig. 2C). Additionally, cyclin D1a induced p27^{KIP1} abundance ~5-fold (Fig. 2C). In contrast, cyclin D1b did not significantly affect either Ser3cofilin phosphorylation or p27^{KIP1} abundance (Fig. 2C).

p27^{KIP1} is stabilized by cyclin D1a but not cyclin D1b. Because of the strength of the evidence that p27^{KIP1} control is critical for cyclin D1a-mediated cellular migration (17), we further compared how cyclin D1b affected p27^{KIP1} to gain further insight into the basis for its differential effects on cellular migration. Replicate experiments with different cyclin D1$^{-/-}$ MEF preparations indicated that p27^{KIP1} levels were induced ~3-fold on average by cyclin D1a (Fig. 3A). In contrast, cyclin D1b transduction reduced p27^{KIP1} abundance ~40%. This difference was unrelated to levels of isoform expression as similar levels of cyclin D1a and cyclin D1b were produced in cells as confirmed by Western blotting (Fig. 3A). p27^{KIP1} is regulated at multiple levels including transcription (46), translation (47) and post-translational (48). Because cyclin D1a acts as a transcriptional co-regulator we examined whether cyclin D1 regulated p27^{KIP1} mRNA abundance. However, neither isoform affected the steady-state levels of p27^{KIP1} mRNA as determined by Northern blot analysis or QT-PCR (Fig. 3B,C). We next examined whether the increased levels of p27^{KIP1} protein reflected an increase in protein stability. Cyclin D1$^{-/-}$ MEFs were transduced with retroviral vectors encoding cyclin D1a or cyclin D1b and protein abundance determined after cycloheximide treatment. A representative example (Fig. 4A) and mean data of multiplicate experiments (Fig. 4B) are shown. In cells transduced by cyclin D1a, the relative abundance of p27^{KIP1} protein was increased

~2.5 fold at each time point determined after protein synthesis inhibition by cycloheximide, compared to cyclin D1b (Fig. 4). Pulse chase analysis was conducted to determine the effect of the cyclin D1 isoforms on newly synthesized p27^{KIP1} (Fig. 4C). Cyclin D1$^{-/-}$ 3T3s were transduced with retroviral vectors encoding cyclin D1a or cyclin D1b. The rate of newly synthesized p27^{KIP1} was increased approximately 2-fold by cyclin D1a 1 hr after the initiation of the pulse chase, but was similar by 2 hrs. Together these results argue that cyclin D1a increases p27^{KIP1} levels through promoting stabilization of the protein when compared with the effects of cyclin D1b.

Cyclin D1b displays reduced binding to p27^{KIP1}. Previous studies have documented a physical interaction between p27^{KIP1} and cyclin D1a by immunoprecipitation and mammalian two-hybrid assays (17,49). We therefore compared the ability of cyclin D1b to interact with p27^{KIP1} in these assays. In a mammalian two hybrid system, we confirmed that cyclin D1a interacted with p27^{KIP1} and that point mutation of the cdk4-binding domain of cyclin D1a (D1KE) abrogated this interaction (Fig. 5B). By comparison, the cyclin D1b isoform exhibited a relative ~80% reduction in p27^{KIP1} interaction. As the cyclin D1a and cyclin D1b are similar for the amino acids encoded by exons 1-4 but differ in their carboxyl terminal amino acid structure (Fig. 5A), these studies implicated the cyclin D1a carboxyl terminus encoded by exon 5 in the interaction with p27^{Kip1}. A carboxyl terminal deletion mutant of cyclin D1a residues encoding exon 5 (Cyclin D1N2) was therefore generated. This mutant of cyclin D1a was defective in p27^{KIP1} interaction in mammalian 2-hybrid (Fig. 5B). Similar results were obtained by a reciprocal analysis in which the p27^{KIP1} and cyclin D1 polypeptides were swapped in as fusions to the Gal4 DNA-binding domain and VP16 transactivation domain employed in this system (Fig. 5B, right hand panel). We confirmed these findings by an immunoprecipitation analysis conducted in retroviral vector-transduced cyclin D1$^{-/-}$ MEFs that were subjected to GFP-based cell sorting (Fig. 5C). As before, similar levels of cyclin D1a and cyclin D1b were expressed as documented by Western blotting (Fig. 5C; Input). Immunoprecipitation using the amino-terminal FLAG epitope included in the construct confirmed similar amounts of cyclin D1 protein. Western analysis of the cyclin D1 immunoprecipitates identified p27^{KIP1} associated with cyclin D1a. However, only ~1/10 of the amount of p27^{KIP1} present in these immunoprecipitates was present in a similar cyclin D1b immunprecipitate in replicate experiments (Fig. 5C). The role of the cyclin D1a carboxyl terminus in interaction with Cdk4 in mammalian 2-hybrid was next assessed. The cyclin D1b and cyclin D1N2 displayed an approximately 50% reduction in interaction compared with cyclin D1a (Fig. 5D).

Discussion

The present study extends the understanding of cyclin D1 splice variants (26) by

demonstrating that the two distinct isoforms differ in their fundamental biological and biochemical properties. An unbiased genomic approach was used to identify genes regulated by cyclin D1a vs. cyclin D1b. This type of study does not necessarily identify direct transcriptional targets but demonstrated approximately 1/3 or the genes regulated in response to cyclin D1b were also regulated by cyclin D1a. The functions of genes regulated by cyclin D1b only include genes involved in DNA-dependent transcription and nucleosome assembly. Previous studies had characterized transcriptional repression function of cyclin D1a implicating a predicted helix-loophelix domain (4). This domain is conserved with cyclin D1b, and both cyclin D1a and cyclin D1b maintained repression function of the heterologous transcription factor domains for the AR, NRF1 and PPARγ.

Cyclin D1a induces cellular polarity and migration, increasing cellular movement velocity. The molecular mechanism for these effects involves an inhibition of Rho signaling, as evidenced by a specific reduction in the phosphorylation of the actin severing protein cofilin at Ser3. In contrast, cyclin D1b failed to promote cellular polarization and cellular migration, failed to inhibit cofilin phosphorylation, and failed to stabilize p27^{KIP1}. Associated with these defects, cyclin D1b was also defective in its ability to effectively interact with p27^{KIP1}, as measured in mammalian two-hybrid assays and co-immunoprecipitations from mouse cells.

Cyclin D1a increased the abundance of p27^{KIP1} by promoting the stability of this important cell regulator. The crystal structure of the amino-terminal 69 residues of the p27^{KIP1} inhibitory domain bound to phosphorylated cyclin A/Cdk2 contributed to the understanding that p27^{KIP1} contains separate binding sites for the cyclin and the kinase (50). One key mechanism governing p27^{KIP1} stability involves an SCF ubiquitin ligase (E3) complex which induces p27^{KIP1} ubiquitination and degradation by the ubiquitin-proteosome system. An additional ubiquitin ligase, KPC (KIP1 ubiquitination-promoting complex) has been described. The mechanisms by which cyclin D1a induce p27^{KIP1} involves regulation of Skp2 expression, protection of p27^{KIP1} from degradation due to its association with the cyclin, and the induction of p27^{KIP1} phosphorylation at Ser10 (17).

Cyclin D1a regulated expression of genes in 3T3 cells that are known to either inhibit adhesion and/or promote migration. These findings in 3T3 cells are consistent with prior findings that cyclin D1a promotes migration in non-transformed cells including macrophages and mammary epithelial cells (14,16,17). The genes regulated by cyclin D1a include thrombospondin 1 (TSP-1), procollagen, type VIII, alpha 1 (Col8a1), junction plakoglobin (Jup), ras homolog gene family, member B (RhoB), and cadherin 2 (Cdh2). Inhibition of TSP-1 from MEFs reverts abnormal migration (16,51). Cadherin 2 (Cdh2) also known as N-cadherin (NCAD) inhibits Schwann cell migration (52). Cyclin D1a repressed RhoB. Emerging evidence points to a tumor-suppressive role for RhoB (53-55) which antagonizes Ras/PI3K/Akt-dependent transformation, apoptosis and metastasis in vivo (56). Stathmin, which was induced by cyclin D1a, promotes cell

motility (34,57). Cde-12/Elmo1 is required for phagocytosis and cell migration (58). Thus, cyclin D1 downregulates the expression of TSP-1, Cdh2 and RhoB, which inhibit cell migration while upregulating the expression of Stathmin 1 and Elmo1, which are required for cell migration.

The human cyclin D1 gene harbors a common polymorphism that has been associated with increased risk of cancer development. Compared with individuals of the A/G or G/G genotypes, those harboring the A/A genotype have an increased proclivity towards tumorigenesis and worse prognosis (18-20,22). The A allele is associated with synthesis of the cyclin D1b isoform (18). In cells, cyclin D1b has an increased transforming capacity (26). Our finding that $p27^{KIP1}$ levels are reduced in cyclin D1b-expressing cells and that $p27^{KIP1}$ binds cyclin D1b relatively poorly offers one explanation for why production of this isoform might be associated with increased cancer risk, based on the improved ability of cyclin D1b to evade restraint by $p27^{KIP1}$ as compared to cyclin D1a.

The current studies demonstrated the importance of the cyclin D1a carboxyl terminus encoded by exon 5 in binding $p27^{KIP1}$. Cyclin D1b or a carboxyl terminal deletion mutant of cyclin D1 (cyclin D1N2) either reduced or abolished interaction with $p27^{KIP1}$ in mammalian 2-hybrid analysis (Fig. 5A-C). Consistent with our studies of $p27^{KIP1}$, previous studies of $p21^{CIP1}$ assessed in S. Cerevisiae using a LexA-op-lacZ system, had shown that deletion of the C-terminal residues (267-295) reduced association with $p21^{CIP1}$ (59). In addition, these prior studies demonstrated deletion of the cyclin D1a N-terminal 40 residues reduced interaction with $p21^{CIP1}$ indicating the importance of both N and C-terminal interaction made between the cyclin D1 and the cdk inhibitor proteins.

The role of $p27^{KIP1}$ in cellular migration appears to vary with cell and tumor type. Therefore, the effect of cyclin D1a vs. cyclin D1b on migration may also vary by cell type. Reintroducing $p27^{KIP1}$ into $p27^{KIP1-/-}$ cells promotes migration by inhibition of Rho signaling (33). However, $p27^{KIP1}$ inhibits cell migration in fibrosarcoma cells, where $p27^{KIP1}$ binds to and inhibits the function of the microtubule destablizing protein stathmin (34). Binding of $p27^{KIP1}$ to cyclins is important for inhibiting cyclin D/Cdk4 and cyclin E/Cdk2 kinase activity and $p27^{KIP1}$ has an important role as a tumor suppressor. Reduction in $p27^{KIP1}$ levels correlates with tumor aggressiveness. For example, $p27^{KIP1}$ deficiency accelerates cancer formation caused by mutation of the Apc or Neu/ErbB2/HER2 genes (39). Moreover, mice lacking even a single copy of $p27^{KIP1}$ have an increased susceptibility to carcinogenesis (60-62), illustrating the importance of its abundance in tumor suppression. Thus, reduction in $p27^{KIP1}$ binding by cyclin D1b may impact tumor suppressor activity, perhaps in a cell type-specific manner.

We found that cyclin D1a but not cyclin D1b restored defective cellular migration of cyclin D1$^{-/-}$ MEFs. Previous studies have demonstrated a role for p27^{KIP1} in promoting cellular migration through binding to RhoA which regulates cytoskeletal structure, cell adhesion, and cellular migration (33). Cyclin D1a induction of migration is dependent on p27^{KIP1} (17). Our finding of a reduced p27^{KIP1} binding capacity for cyclin D1b suggests that its poor pro-migratory activity reflects a defect in p27^{KIP1}-dependent migration. Thus, by evading restraint by p27^{KIP1}, cyclin D1b may favor cell cycle deregulation at the cost of reduced migratory capability. Further in vivo studies may be useful to distinguish how differential expression of the cyclin D1a and D1b isoforms may impact cancer development and progression at distinct levels.

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Footnotes

Figures

Figure 1. Cyclin D1a and cyclin D1b regulate overlapping and distinct gene expression profiles. (A). Transcriptional activity of PPARγ, NRF1, and AR assayed in HEK 293T cells. Cells were transfected with expression vectors for Gal4-AR, Gal4-PPARγ or Gal4-NRF1 along with either 3xFLAG vector, 3x FLAG cyclin D1a, or 3x FLAG cyclin D1b constructs and the reporter, pG5-LUC. The data represents triplicate experiments and relative luciferase units based on the mean data normalized to basal activity plus standard error of the mean (SEM). (B). Comparison of cyclin D1-regulated genes determined by microarray analysis (For full details the GEO accession number obtained is GSE9161 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zfellwagiuaagfa&acc=GSE9161). Comparison is shown between cyclin D1$^{-/-}$ 3T3 cells transduced with expression vectors encoding cyclin D1a, cyclin D1b or control vector.
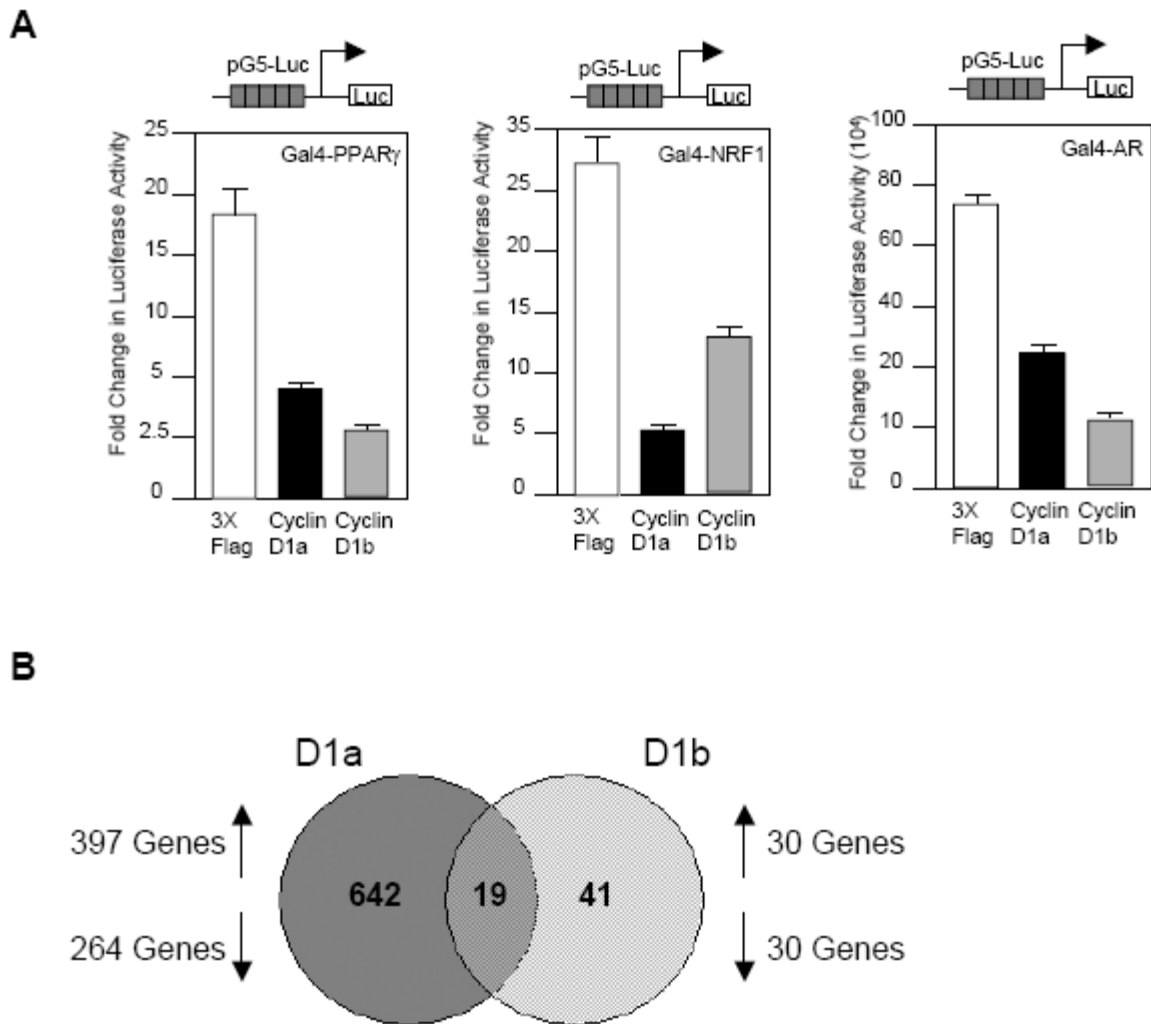
Figure 2. Cyclin D1a but not cyclin D1b enhances cellular migration. (A). Confocal microscopy of cyclin D1$^{-/-}$ MEFs transduced with cyclin D1a or cyclin D1b retrovirus. (left panel) F-actin staining and (right panel) paxillin (green), tyrosine phosphorylated paxillin (Y118) (red) and DAPI (blue). Cyclin D1$^{-/-}$ MEFs were transduced with retroviral expression plasmids encoding either the cyclin D1a or cyclin D1b isoform. The GFP expressing cells were sorted by FACS and were then examined by confocal microscopy. (B). Cellular velocity is shown as mean ± SEM. (C). Western blot analysis of cyclin D1$^{-/-}$ MEFs transduced with retroviral expression vectors cyclin D1a or cyclin D1b. Antibodies were directed

to FLAG (M2), p27^{KIP1}, phospho-cofilin (Ser3) (as a marker of activated Rho signaling), and β-tubulin and vinculin as total protein loading controls.
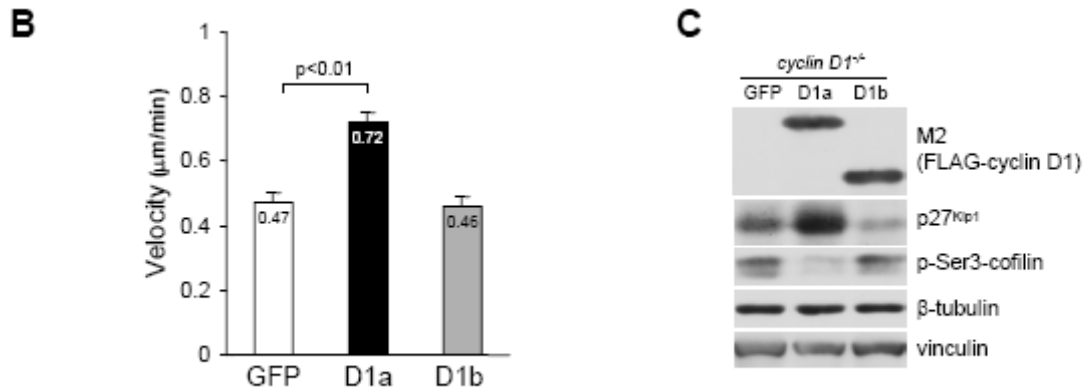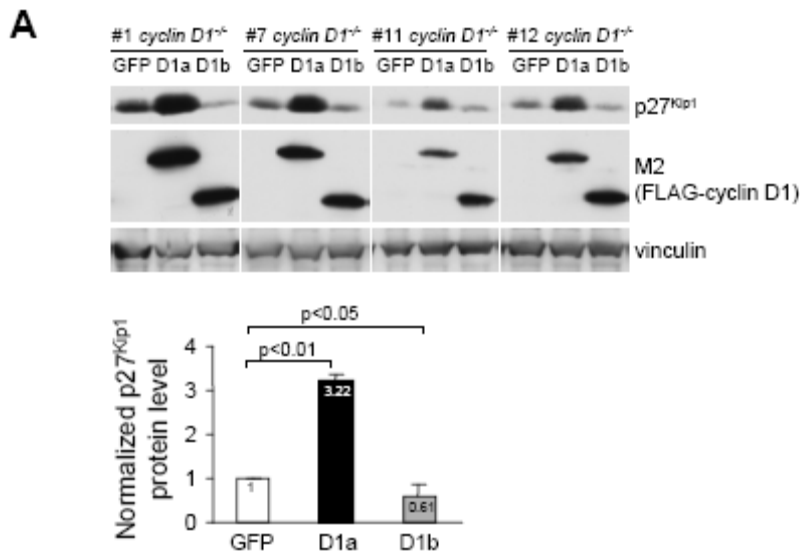

A

Cyclin D1$^{-/-}$ GFP

Cyclin D1$^{-/-}$ D1a

Cyclin D1$^{-/-}$ D1b

Rhodamine-phalloidin (Red)
DAPI (Blue)

Paxillin (Green)
Paxillin (pY^{118}) (Red)
DAPI (Blue)

B

C
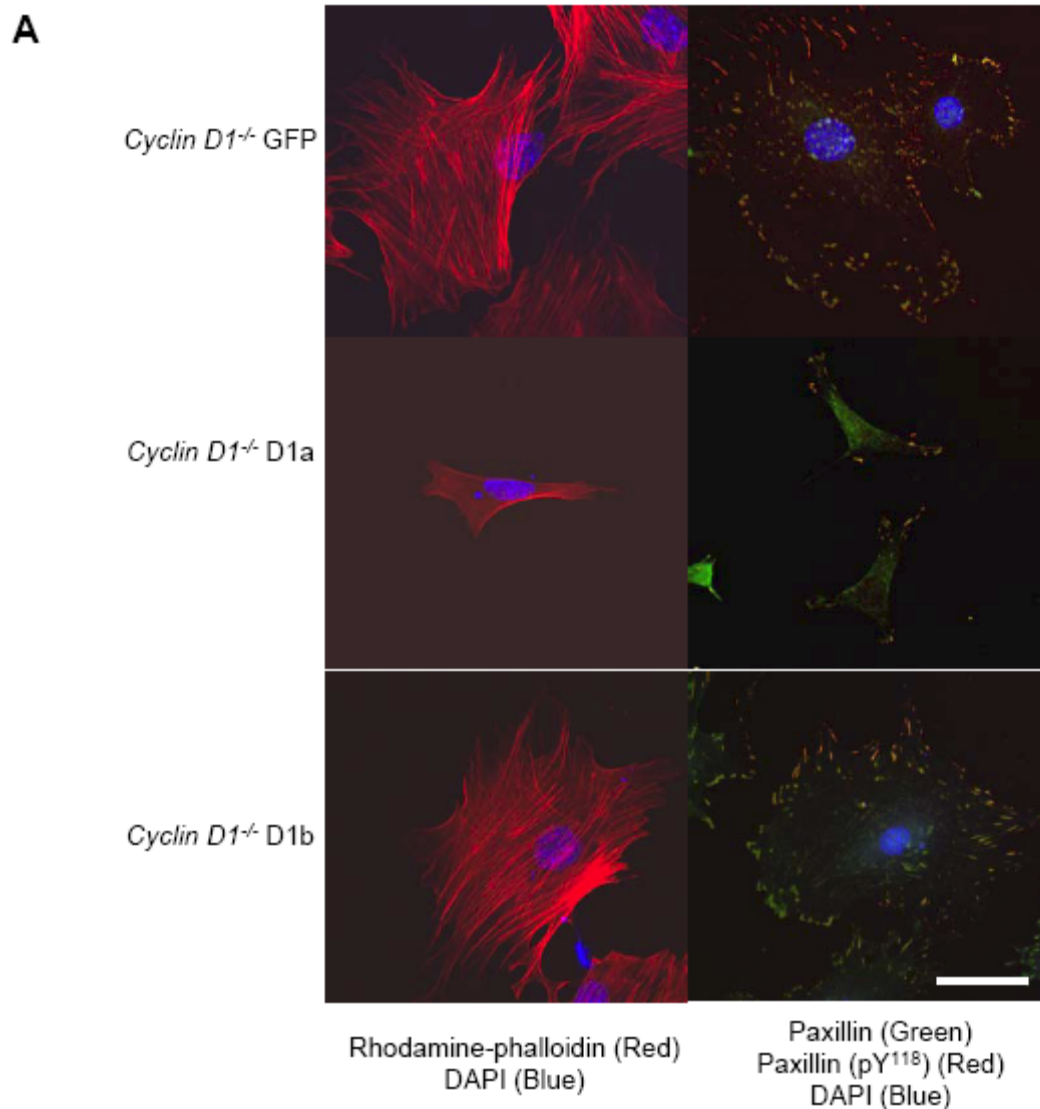
Figure 3. Cyclin D1a but not cyclin D1b increases the abundance of p27^{KIP1} protein. (A). Cyclin D1$^{-/-}$ MEFs were transduced with retroviral vectors for cyclin D1a or cyclin D1b and subjected to Western blot analysis. A number was assigned for each separate batch of MEFs generated and is indicated above each Western blot. (B). Northern analysis for p27^{KIP1} (C). QT-PCR for p27^{KIP1}. Data are shown as mean ± SEM (n=4).
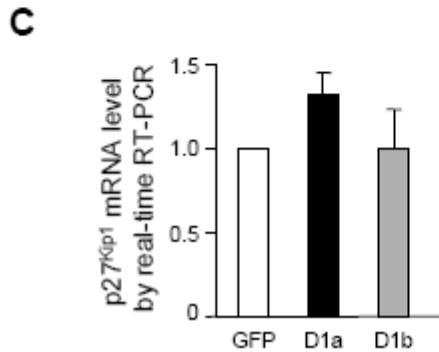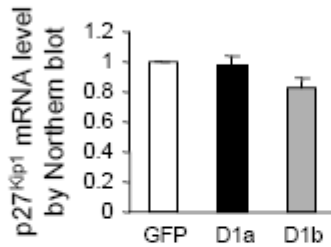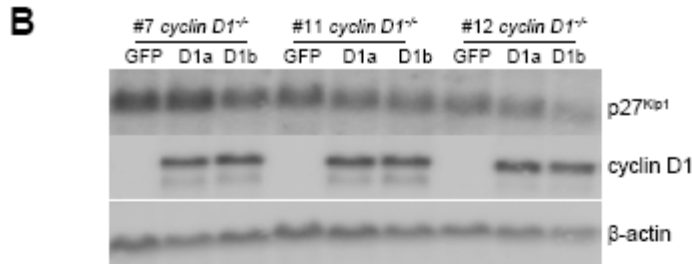
A

B

#7 *cyclin D1⁻ᐟ⁻* #11 *cyclin D1⁻ᐟ⁻* #12 *cyclin D1⁻ᐟ⁻*
GFP D1a D1b GFP D1a D1b GFP D1a D1b

p27^{Kip1}

cyclin D1

β-actin

C

Figure 4. Cyclin D1a increases p27^{KIP1} protein stability. (A,B) Measurement of p27^{KIP1} protein degradation after inhibition of protein synthesis with cycloheximide (CHX). Proliferating MEFs were transduced with the retroviral vectors as indicated and were treated with 10 μg/ml cycloheximide for the indicated times. p27^{KIP1} levels were measured by Western blot shown as representative example. P27^{KIP1} levels were normalized to β-tubulin as a protein loading control and densitometric analysis of representative Western blot studies (B). (C) Pulse chase analysis for p27^{KIP1} abundance. A representative example is shown. The data are mean from densitometry of n=3 separate experiments.
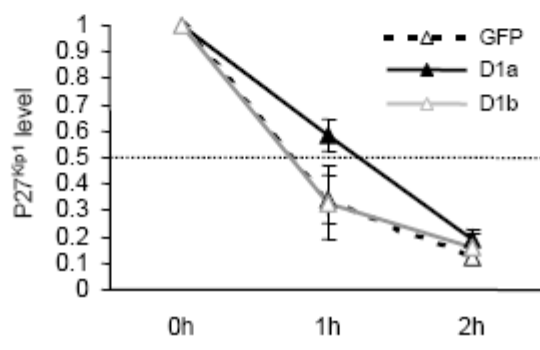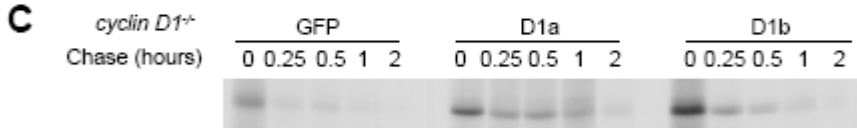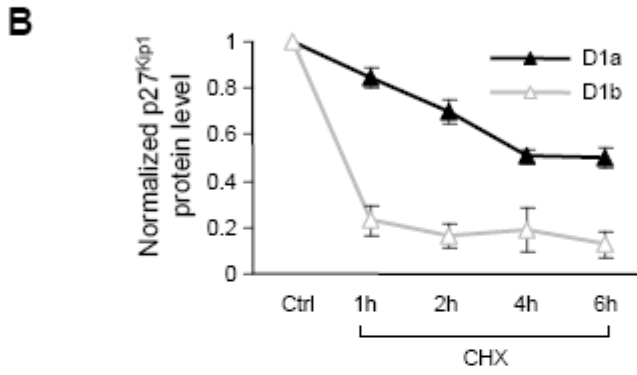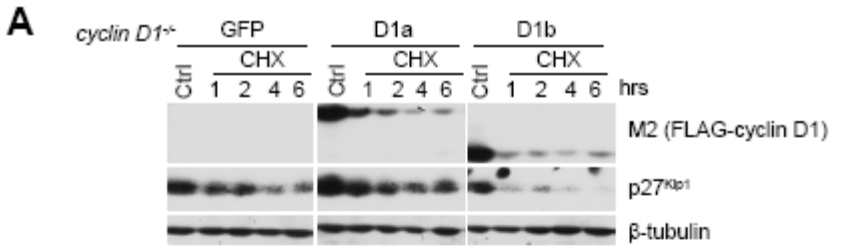
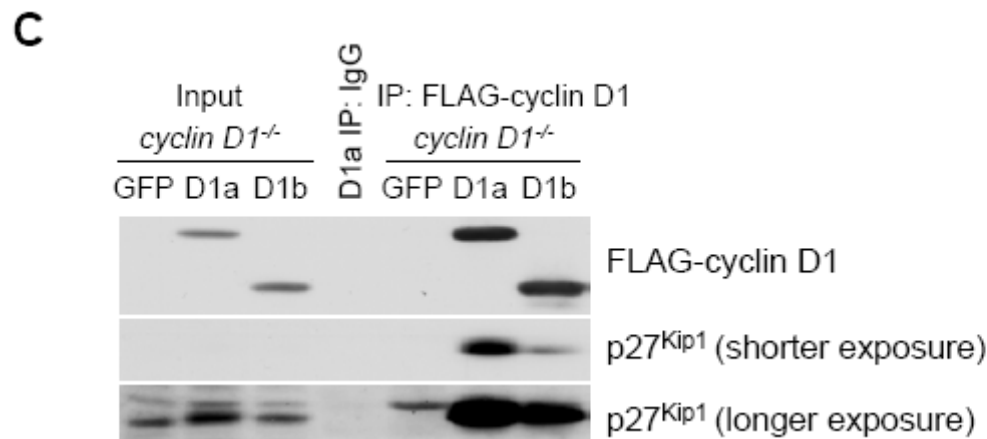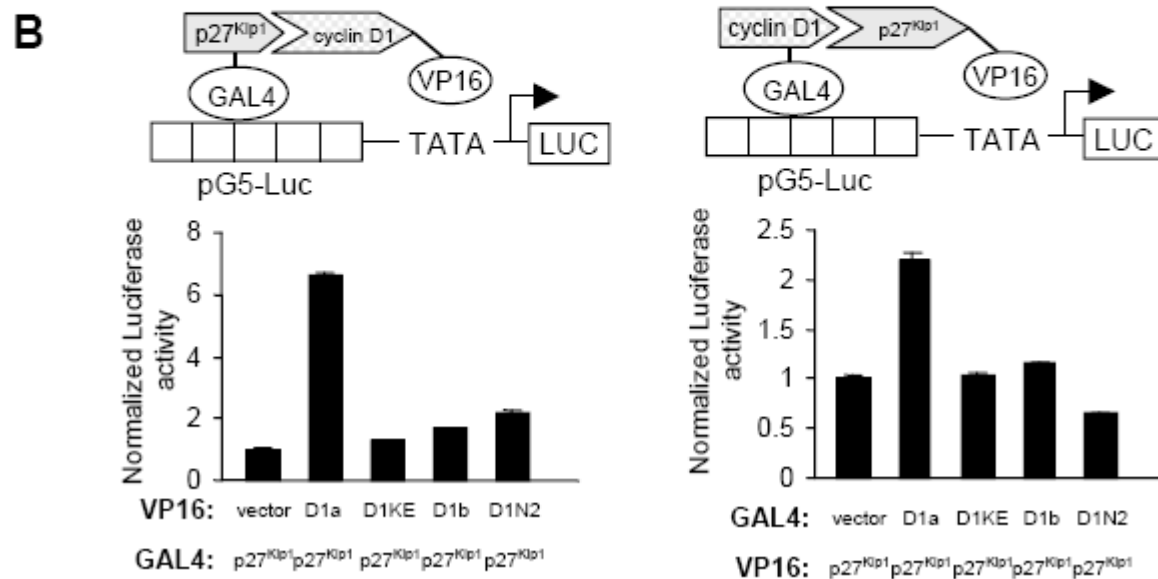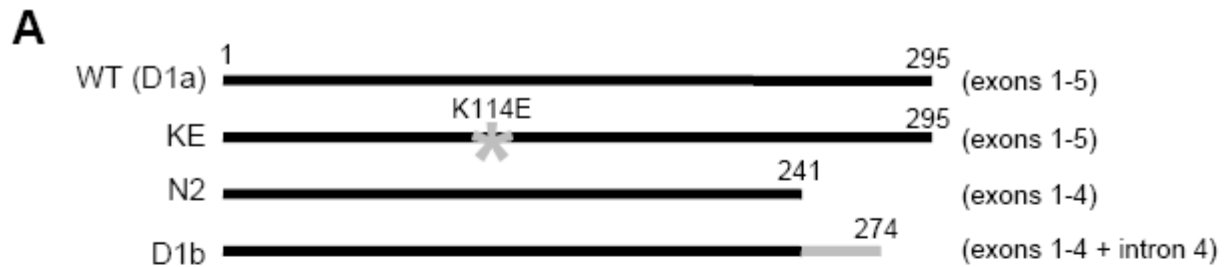

Figure 5. Cyclin D1b is defective in p27[KIP1]-binding. (A). Schematic representation of cyclin D1 constructs used in (B, D) mammalian two hybrid. The cyclin D1 cDNAs shown in (A) were linked to either the VP16 (left panel) transactivation domain or Gal4 (right panel) DNA binding domin. p27[KIP1] was linked to the cognate mammalian 2-hybrid interaction domain. The interaction is shown as relative luciferase activity for N>5 separate transfections as mean ± SEM. (C). Cyclin D1^{-/-} MEFs transduced with retroviral

vectors for cyclin D1a or cyclin D1b were analyzed by Western blot (left hand panel) or immunoprecipitation – Western blot. Cyclin D1 immunoprecipitation was conducted with the FLAG antibody with subsequent Western blot for cyclin D1 (DCS-6) or p27^{KIP1} (C-19). (D). Interaction of cyclin D1 constructs with Cdk4 in mammalian 2-hybrid. The data is shown throughout as mean ± SEM for N> 5 separate experiments.

D

Figure showing bar graph. Y-axis: Luciferase activity (x10^4), ranging 0 to 3.

VP16:	vector	D1a	D1KE	D1b	D1N2
GAL4:	Cdk4	Cdk4	Cdk4	Cdk4	Cdk4