Cyclin D1 Induction of Cellular Migration Requires $p27^{KIP1}$

Zhiping Li, Xuanmao Jiao, Chenguang Wang, Xiaoming Ju, Yinan Lu, Liangping Yuan, Michael P. Lisanti, Sanjay Katiyar, and Richard G. Pestell

Kimmel Cancer Center, Departments of Cancer Biology and Medical Oncology, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract

The *cyclin D1* gene is amplified and overexpressed in human breast cancer, functioning as a collaborative oncogene. As the regulatory subunit of a holoenzyme phosphorylating Rb, cyclin D1 promotes cell cycle progression and a noncatalytic function has been described to sequester the cyclin-dependent kinase inhibitor protein p27. Cyclin D1 overexpression correlates with tumor metastasis and cyclin D1–deficient fibroblasts are defective in migration. The genetic mechanism by which cyclin D1 promotes migration and movement is poorly understood. Herein, cyclin D1 promoted cellular migration and cytokinesis of mammary epithelial cells. Cyclin D1 enhanced cellular migratory velocity. The induction of migration by cyclin D1 was abolished by mutation of K112 or deletion of NH_2-terminal residues 46 to 90. These mutations of cyclin D1 abrogated physical interaction with $p27^{KIP1}$. *Cyclin D1*$^{-/-}$ cells were $p27^{KIP1}$ deficient and the defect in migration was rescued by $p27^{KIP1}$ reintroduction. Conversely, the cyclin D1 rescue of *cyclin D1*$^{-/-}$ cellular migration was reversed by $p27^{KIP1}$ small interfering RNA. Cyclin D1 regulated $p27^{KIP1}$ abundance at the posttranslational level, inhibiting the Skp2 promoter, Skp2 abundance, and induced $p27^{KIP1}$ phosphorylation at Ser^{10}. Together, these studies show cyclin D1 promotes mammary epithelial cell migration. $p27^{KIP1}$ is required for cyclin D1–mediated cellular migration. (Cancer Res 2006; 66(20): 9986-94)

Introduction

Several lines of evidence show a key role for cyclin D1 in human tumorigenesis. The *cyclin D1* gene is the *BCL1* oncogene, located at a breakpoint of the characteristic t(11;14)(q13;q32) clonal translocation in mantle cell lymphomas and a subset of multiple myeloma. Initially cloned by three independent groups, structural homology to eukaryotic cyclins led to the understanding that cyclin D1 functions as a regulatory subunit of a holoenzyme phosphorylating several downstream targets, including the retinoblastoma, pRb, protein. The finding that cyclin D1 is overexpressed in human tumors, the identification of cyclin D1 within driver amplicons in human cancer, the common coexpression of cyclin D1 with amplification, together with molecular genetic studies in murine models, have led to an understanding that cyclin D1 is an important collaborative oncogene in human cancer (1, 2).

As a regulatory subunit of the holoenzyme that phosphorylates Rb, cyclin D1 encodes the rate-limiting step in transitions from the G_1 to S phase of the cell cycle in cultured cells. Molecular genetic analysis in transgenic animals has shown an essential role for cyclin D1 in the onset and progression of mammary tumorigenesis induced by Ras or ErbB2 (3) or gastrointestinal tumorigenesis induced by mutation of the *Apc* gene (4). In human tumors, cyclin D1 overexpression correlates with cellular metastases (5, 6), but does not correlate with indices reflecting cellular proliferation (7). Although cyclin D1 phosphorylation of Rb inactivates E2F repressor function in cultured cells, global gene expression of human tumors overexpression cyclin D1 suggested a lack of correlation between cyclin D1 overexpression and regulation of E2F target genes (8). Recent evidence suggests that cyclin D1 physically interacts with several proteins through distinct subdomains, including the p160 coactivator proteins, p300/CBP, and histone deacetylases (HDAC; ref. 1, 9, 10). Furthermore, a noncatalytic function of the cyclin D–cyclin-dependent kinase (CDK) complexes include sequestering the CDK-inhibitor proteins $p21^{Cip1}$ and $p27^{KIP1}$ (10).

The CDK inhibitor family p21 includes the members $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. $p27^{KIP1}$ inhibits most cyclin-CDK complexes, including cyclin E-CDK2, and induces cell cycle arrest in several cell types. p21 and $p27^{KIP1}$ bind to CDK4/CDK6/cyclin D complexes but do not interfere with their kinase activity under stoichiometric conditions. $p27^{KIP1}$ binding to cyclin D/CDK4,6 during the early phase of the cell cycle stabilizes the complex (11, 12). Curiously, CDK2 is dispensable for the cell cycle inhibition during the G_1 phase of the cell cycle and is dispensable for their tumor-suppressor properties (13). Reduction in $p27^{KIP1}$ abundance in tumors, however, correlates with tumor aggressiveness and dedifferentiation (14–16).

Recent evidence showed a role for $p27^{KIP1}$ in cellular migration (ref. 17; reviewed in ref. 18). Intriguingly, although $p27^{KIP1}$ inhibited migration of endothelial cells and vascular smooth muscle cells (19, 20), in a subset of cells, $p27^{KIP1}$ stimulated cellular migration. In hepatocellular carcinoma cells and mouse embryonic fibroblasts (MEFs), $p27^{KIP1}$ induced cellular migration (17, 21). The promigratory function of $p27^{KIP1}$ was distinct from its cell cycle regulatory function. The NH_2-terminal domain of $p27^{KIP1}$ was considered to play an essential role in functional interactions regulating cell cycle progression. In contrast, the COOH-terminal 28 amino acids played a key role in migratory inhibitory activity (22).

In view of the previous studies demonstrating a correlation between cyclin D1 overexpression and cellular migration, analyses were recently conducted of *cyclin D1*$^{-/-}$ MEFs (23). These studies show cyclin D1–induced cellular migration, reducing cellular attachment. The induction of cellular migration was associated with the inhibition of Rho-GTPase activity and inhibition of thrombospondin secretion (23). As cyclin D1 is frequently overexpressed in human breast cancer, the current experiments were

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Richard G. Pestell, Kimmel Cancer Center, Departments of Cancer Biology and Medical Oncology, Thomas Jefferson University, 233 South 10th Street, BLSB, Room 1050, Philadelphia, PA 19107. Phone: 215-503-5649; Fax: 215-503-9334; E-mail: Richard.Pestell@jefferson.edu.

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conducted to determine if cyclin D1 also regulated migration and transwell movement of mammary epithelial cells. Next, studies were conducted to determine the mechanism by which cyclin D1 induces cellular migration. Introduction of cyclin D1 promoted cellular migration and transmigration across a membrane. Mutational analysis identified a role for residues 47 to 90 in the NH_2 terminus of cyclin D1 for cellular migration. These residues of cyclin D1 were required for enhancement of cellular migratory velocity. Molecular dissection of proteins binding to cyclin D1 mutants, defective in regulating cellular migration, identified a key requirement for these domains in physical association with $p27^{KIP1}$ and CDK4, both in immunoprecipitation–Western blotting and mammalian two-hybrid analysis. Cyclin D1 inhibited Rho expression and coassociated with $p27^{KIP1}$ in a complex with active Rho substrates. Depletion of $p27^{KIP1}$, through small interfering RNA (siRNA), abrogated rescue by cyclin D1 of cellular migration. Conversely, $p27^{KIP1}$ reintroduction obviated a defect of transmigration of cyclin D1–deficient cells. Cyclin D1–deficient cells show reduced levels of $p27^{KIP1}$. Cyclin D1 induced $p27^{KIP1}$ protein levels by inhibiting the Skp2 component of the SCF complex that degrades $p27^{KIP1}$ and by inducing phosphorylation of $p27^{KIP1}$ at Ser^{10}. Together, these studies identify a novel molecular mechanism by which cyclin D1 promotes cellular migration through inducing the expression of $p27^{KIP1}$ and by physically associating with $p27^{KIP1}$ to promote cellular migration.

Materials and Methods

Mice. All animal experiments were done in accordance with the guidelines for the care and use of laboratory animals at Georgetown University and Thomas Jefferson University. *Cyclin D1*$^{-/-}$ mice were maintained on a mixed C57Bl/6x129/SvJ background as described previously (4). Genotyping was done on tail DNA by PCR as described before (4).

Plasmids. The MSCV-IRES-GFP retroviral vector and cyclin D1 wild-type (23) or mutant constructs were cloned into the *Eco*RI site of the vector. pCMV5/human $p27^{Kip1}$ full length, NH_2 terminus (1-86 amino acids), and COOH terminus (77-198 amino acids) were provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). The full-length human $p27^{Kip1}$ cDNA (*Kpn*I to *Bam*HI fragment from pCMV5/$p27^{Kip1}$) was inserted into the MSCV-IRES-GFP vector at the *Eco*RI site upstream of the IRES driving expression of green fluorescent protein (GFP).

Cell culture. *Cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ primary MEF cultures were prepared as described previously (24). Human kidney 293, 293T, and MCF-7 cells were maintained in DMEM containing penicillin and streptomycin (100 mg of each/L) and supplemented with 10% fetal bovine serum (FBS).

For culture of murine mammary epithelial cells, cells were resuspended in DMEM/F12 medium supplemented with 10% FBS, 10 μg of insulin/mL, 20 ng cholera toxin/mL, 1 μg of hydrocortisone/mL, and 10 ng epidermal growth factor/mL.

Retroviral production and infection. Retroviral production and infection of *cyclin D1*$^{-/-}$ MEFs and MCF-7 cells were described in detail before (23). Fluorescence-activated cell sorting (FACS; FACStar Plus; BD Biosciences, San Jose, CA) sorted GFP^+ cells were used for subsequent analysis.

Scanning electron microscopy. Cells were plated on fibronectin-coated glass coverslips and grown to ~80% confluence. The cells were prepared as described previously (25) for observation under scanning electron microscopy.

Immunofluorescence. Phalloidin staining was conducted as previously described (25). The samples were visualized on a Zeiss LSM 510 META Confocal Microscope with a 63× objective.

Immunoprecipitations and Western blotting. *Cyclin D1*$^{-/-}$ MEFs infected with GFP vector control or cyclin D1 wild type or a series of mutants were lysed in immunoprecipitation buffer (10 mmol/L Tris-HCL at pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.5% IGEPAL CA-630, 10% glycerol, 1 mmol/L sodium orthovanadate and protease inhibitor cocktail; Boehringer Mannheim, Mannheim, Germany). For each immunoprecipitation, 500 μL lysate (500 μg protein) and 10 μL anti-FLAG M2-agarose affinity gel (Sigma, St. Louis, MO) were incubated overnight at 4°C. Immunoprecipitates were washed five times in immunoprecipitation buffer, and 20 μL of 2× sample buffer was added to the bead pellet. The immunoprecipitates, as well as 50 μg proteins of the corresponding lysates were subjected to Western blotting as previously described (26). The following antibodies were used for Western blotting: mouse M2 anti-FLAG antibody, antivinculin antibody, and anti-α-tubulin antibody (Sigma), mouse DCS-6 anti-cyclin D1 antibody, rabbit polyclonal anti-$p27^{Kip1}$ antibody, anti-$p27^{Kip1}$ Ser^{10} phosphorylated antibody, anti-cdk4 antibody, anti-cdk5, anti-Skp2, anti-$p57^{Kip2}$, anti-β-tubulin antibody, and anti-CRM1 (Santa Cruz Biotechnology), rabbit anti-stathmin (Cell Signaling Technology, Danvers, MA).

Migration assay. Transwell migration assays were done as described before (27). Briefly, GFP^+ cells were seeded on 8-μm-pore transwell filter insert (Corning Incorporated, Corning, NY) coated with 10 μg/mL fibronectin (Sigma). After 16-hour incubation at 37°C and 5% CO_2, cells adherent to the upper surface of the filter were removed using a cotton applicator. Cells were fixed with 3.7% formaldehyde, stained with crystal violet, and the numbers of cells on the bottom were counted. Data are from three experiments done in triplicate (mean ± SE).

Time-lapse video. For time-lapse observation of cell movement, cells on 12-well plates were maintained in DMEM with 10% FCS and HEPES. Cells were placed in a temperature and CO_2 controlled incubator to maintain the temperature at 37°C and CO_2 at 5%. The cell movement videos were taken at 5-minute intervals for 2 hours by using a Nikon Eclipse TE-300 Inverted Microscope System. The cell movement velocity was determined by tracing the single cells at different time points using MetaMorph software.

Mammalian two-hybrid system. Mammalian two-hybrid was done by following the instruction of the manufacturer (Promega, Madison, WI). Human $p27^{Kip1}$ cDNA was cloned into pBIND vector and human cyclin D1 cDNA (wild type or mutants) was cloned into pACT vector to generate fusion proteins with the DNA-binding domain of GAL4 (Gal4-$p27^{Kip1}$) and the activation domain of VP16 (VP16-cyclin D1), respectively. Gal4-$p27^{Kip1}$, VP16-cyclin D1, and pG5*luc*, which contains five Gal4 binding sites upstream of a minimal TATA box and the firefly luciferase gene (Promega) were cotransfected into MCF-7 cells with Superfect reagent. Two days after transfection, the cells were lysed and the amount of luciferase activity was quantitated as previously described (24).

Rho pull down assay. The activated Rho complex present in *cyclin D1*$^{-/-}$ MEFs infected with wild-type cyclin D1 or vector control was determined by using the Rho activation assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY) according to the instruction of the manufacturer. The presence of cyclin D1 and $p27^{Kip1}$ in the activated Rho complex was detected by immunoblotting.

siRNA. For suppression of cellular $p27^{Kip1}$ expression, siRNA that specifically targets mouse $p27^{Kip1}$ mRNA was purchased from Santa Cruz Biotechnology. MEFs were transfected with the $p27^{Kip1}$ siRNA or control siRNA (Santa Cruz Biotechnology) using the Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer.

Results

Previous studies had shown defective migration of cyclin D1–deficient macrophages (25). To identify the molecular mechanisms by which cyclin D1 promoted cellular migration, a series of cyclin D1 expression plasmids were constructed. The CDK-binding defective mutant (K112E), the pRb-binding defective mutant [CC7,8GH(GH)], and the p160(SRC-1) coactivator-binding defective mutant of cyclin D1 [LL254, 255AA, (LLAA)] were previously described (24). Deletion constructs of the cyclin D1 carboxyl terminal 268 to 295 region (N1), deletion of the COOH-terminal region residues 179 to 295 (N4), deletion of the NH_2-terminal 45

amino acids (C7) or NH_2-terminal 90 amino acids (C6) were assessed (Fig. 1*A*). These cDNAs were cloned into the MSCV-IRES-GFP expression vector and analyzed for their abundance in cultured cells through Western blotting to the NH_2-terminal FLAG epitope. These studies showed levels of expression of these mutant proteins were similar to that of the cyclin D1 wild type when normalized to the loading control protein, GDI (data not shown). Scanning electron microscopy of MEF derived from cyclin D1–deficient cells, at passage 3, showed flattened spread morphology of *cyclin D1*$^{-/-}$ cells. Reintroduction of cyclin D1 and either the GH, LLAA, N1 or N4, or the C7 construct rescued the aberrant cellular morphology, returning the *cyclin D1*$^{-/-}$ MEFs to the rounded fibroblastoid morphology of wild-type cells (Fig. 1*B*). In contrast, the C6 mutant failed to rescue the aberrant cellular morphology (Fig. 1*B*).

Filamentous actin (F-actin) staining was conducted using rhodamine-phalloidin staining; reintroduction of cyclin D1 wild type into cyclin D1–deficient cells induced the fibroblastoid distribution of F-actin (Fig. 1*C*). Similarly, the reintroduction of the expression vectors (GH, LLAA, N1, C7) rescued the spread morphology. However, the reintroduction of the expression vector C6 or the CDK binding defective mutant KE revealed the flattened spread morphology of the *cyclin D1*$^{-/-}$ cells, consistent with the failure of these two cDNAs to rescue the cellular morphologic appearance (Fig. 1*C*).

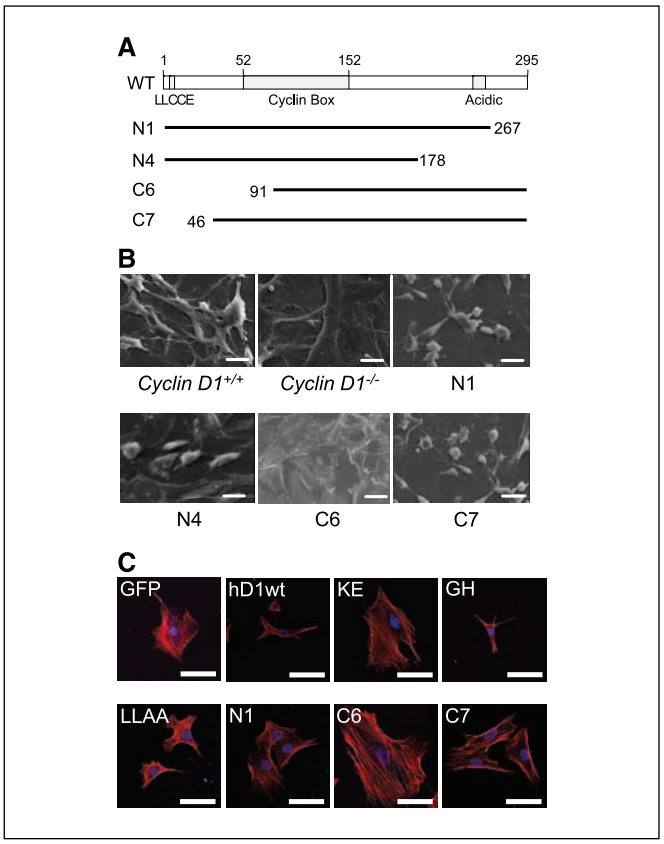

Figure 1. The cyclin D1 NH_2 terminus and K112 regulate polar cellular morphology. *A,* schematic representation of wild-type and mutant human cyclin D1 expression vector structure. *B, cyclin D1*$^{+/+}$, *cyclin D1*$^{-/-}$ MEFs, and *cyclin D1*$^{-/-}$ MEFs infected with mutant human cyclin D1 mutants N1, N4, C6, and C7 were plated on fibronectin (10 μg/mL)–coated coverslips and examined by scanning electron microscopy (×1,000). Bar, 20 μm. *C,* rhodamine-phalloidin staining of *cyclin D1*$^{-/-}$ MEFs transduced with control vector (*GFP*) or human cyclin D1 wild type (*hD1wt*) or KE mutant (*KE*), GH mutant (*GH*), LLAA mutant (*LLAA*), N1 mutant (*N1*), C6 mutant (*C6*), C7 mutant (*C7*). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Bar, 50 μm.

Cyclin D1 is overexpressed in ~50% of human breast cancers and is sufficient for the induction of mammary adenocarcinoma when targeted to the mammary gland in transgenic mice. We, therefore, sought to determine whether cyclin D1 is capable of promoting mammary epithelial cell migration. Primary murine epithelial cells (MEC) were prepared from *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ mice. *Cyclin D1*$^{-/-}$ MEC showed a larger diameter when plated on fibronectin-coated plates. Cellular adhesion of primary murine mammary epithelial cells was assessed through crystal violet staining and measurement of absorbance (Fig. 2*A*). Cellular attachment was increased >20% in *cyclin D1*$^{-/-}$ MECs. To examine the biological significance of the flattened cellular morphology of the *cyclin D1*$^{-/-}$ cells, migration assays were conducted. Transwell migration was done in which cells were plated on the superior aspect of a transwell membrane and the ability to transmigrate was assessed by cell counting of viable cells that have transversed the inferior membrane. The migration from the superior aspect of the membrane to the inferior aspect of the membrane was quantitated after 16 hours (Fig. 2*B*). A reduction in transwell migration was shown in *cyclin D1*$^{-/-}$ mammary epithelial cells (Fig. 2*B*). The ~3-fold reduction of transwell migration ($P < 0.05$) was observed in multiple separate MEC preparations from multiple mice of identical strain.

The *cyclin D1*$^{-/-}$ MEFs were also defective in transwell migration (ref. 23; Supplementary Data 1), with ~4-fold less cells migrating compared with littermate control MEFs. Reintroduction of cyclin D1 restored transwell migration to that of wild-type cells. Deletion of amino acids 46 to 90 or point mutation of lysine 112 abrogated the ability of cyclin D1 to promote cellular migration (Supplementary Data 1). Persistence of migratory directionality was next assessed using videomicroscopy (Fig. 2*D*). Migratory velocity was enhanced by either murine or human cyclin D1, with a 3- or 4-fold increase in cellular movement velocity. Again, mutation of amino acid residues 46 to 90 or mutation of lysine 112 abrogated affects on cellular movement velocity (Fig. 2*D*).

Cyclin D1 and $p27^{KIP1}$ function *in trans* during murine development (28). We therefore examined the physical association of cyclin D1 with $p27^{KIP1}$ by transducing *cyclin D1*$^{-/-}$ MEFs with expression vectors for either wild-type or mutant cyclin D1, to determine whether the domains of cyclin D1 regulating cellular migration were required for physical association with $p27^{KIP1}$. Western blot analysis showed similar levels of mutant cyclin D1 proteins compared with wild-type by FLAG antibody to the NH_2-terminus of each cyclin D1 protein (Fig. 3*A*). The relative abundance of $p27^{KIP1}$ was induced by cyclin D1 transduction. The cells transduced with the cyclin D1 KE and C6 mutant did not induce $p27^{KIP1}$ abundance by Western blotting. Using these cellular extracts, immunoprecipitation-Western blotting was conducted. An antibody to the FLAG epitope of the cyclin D1 proteins was used in immunoprecipitation. Western blot to FLAG showed the immunoprecipitation of cyclin D1 and the coassociation of CDK4 and $p27^{KIP1}$. $p27^{KIP1}$ associated with each of the cyclin D1 proteins except the cyclin D1 KE and cyclin D1 C6 mutant (Fig. 3*B*). Cdk4 was coprecipitated with each of the cyclin D1 mutants except the cyclin D1 KE mutant and the cyclin D1 C6 mutant. Collectively, these studies show a correlation between the ability of cyclin D1 to induce $p27^{KIP1}$ abundance and the ability to bind $p27^{KIP1}$ (Fig. 3*C*).

To determine whether cyclin D1 directly interacted with $p27^{KIP1}$, mammalian 2-hybrid experiments were conducted. The

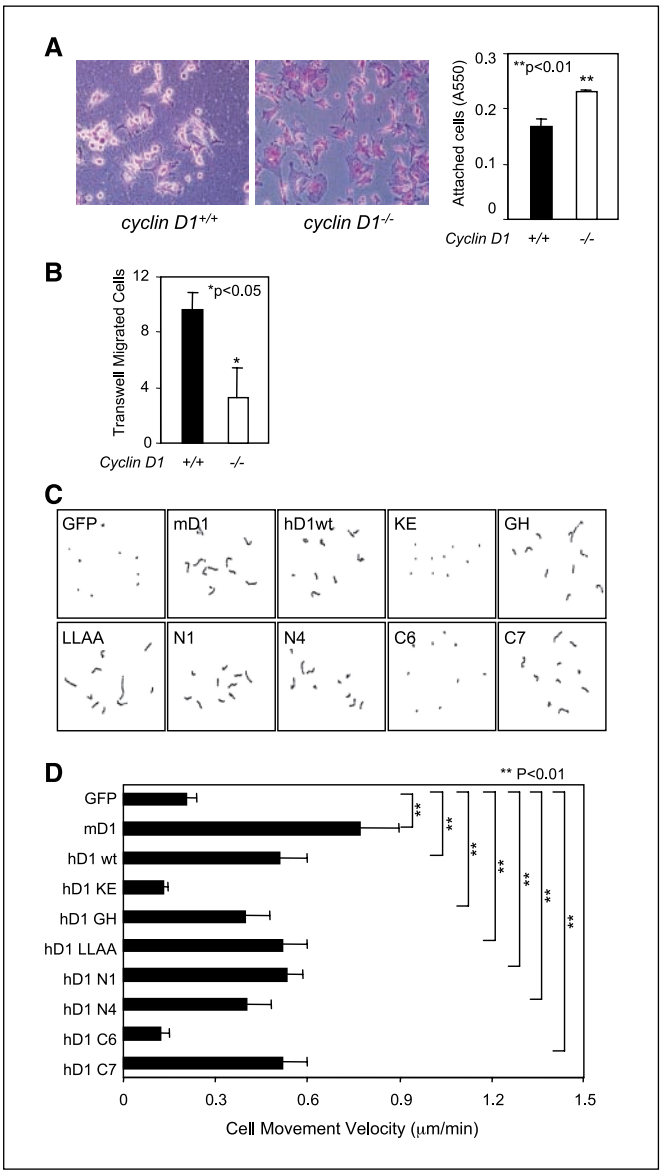

Figure 2. Cyclin D1 promotes mammary epithelial cell migration. *A,* adhesion assay on fibronectin-coated, 96-well plates was conducted in either wild-type or *cyclin D1*$^{-/-}$ MECs. After 30 minutes, plates were washed, fixed, and stained with crystal violet. Adherent cells were observed under phase-contrast microscopy and the attached cells were counted as A550. *B,* transwell migration assay in wild-type and *cyclin D1*$^{-/-}$ murine mammary epithelial cells. * and **, significant difference between wild-type cells and *cyclin D1*$^{-/-}$ cells. *C,* single-cell analysis of cell movement velocity taken at 5-minute time intervals from *cyclin D1*$^{-/-}$ MEFs transduced with control vector (GFP) or mouse cyclin D1 (*mD1*) or human cyclin D1 wild type or KE mutant, GH mutant, LLAA mutant, N1 mutant, N4 mutant, C6 mutant, C7 mutant. *D,* the velocity of cell movement was determined by tracing the movement of single cells with time using MetaMorph software. *Columns,* mean cellular movement velocity for $n \geq 20$ cells assessed at 5-minute intervals for 2 hours; *bars,* SE. **, $P < 0.01$.

Gal4 DNA-binding domain was linked to the p27^{KIP1} protein. Cyclin D1 fragments were linked to the VP16 transactivation domain and the interaction between p27^{KIP1} and cyclin D1 was assessed using a multimeric Gal4 DNA binding site (pG5LUC; Fig. 4*A*). The fusion of cyclin D1 to the VP16 activation domain reduced the transactivity of VP16 by 50%, consistent with the known binding of cyclin D1 to corepressors with HDAC activity (9). The association between p27^{KIP1} and cyclin D1 was shown by the induction of luciferase activity (Fig. 4*A, lane 2* versus *lane 3*). The deletion of amino acid residues 46 to 90 abrogated the interaction between cyclin D1 and p27^{KIP1} in mammalian two-hybrid analysis (*lane 4* versus *lane 3*). Similar analysis showed point mutation of cyclin D1 lysine 112 abrogated interaction between cyclin D1 and p27^{KIP1} (Fig. 4*A*). Cyclin D1 immunoprecipitation Western blot showed cyclin D1 coprecipitated p27^{KIP1} and this association was abolished by deletion of the p27^{KIP1} NH_2 terminus (Fig. 4*B*). Together, these studies show the association with p27^{KIP1}, requires cyclin D1 amino acids 46 to 90 and lysine 112.

Previous studies have shown that p27^{KIP1} promotes fibroblast migration, although the mechanisms by which p27^{KIP1} promotes this activity remains controversial (29). Although the role of p27^{KIP1} in transwell migration had not previously been determined, we considered the possibility that cyclin D1–deficient cells may express less p27^{KIP1} protein, which, in turn, may contribute to the defect in migration of *cyclin D1*$^{-/-}$ MEFs. Western blot analysis revealed reduced p27^{KIP1} abundance in cyclin D1–deficient MEFs (Fig. 5*A*). Recent studies have suggested an important role for

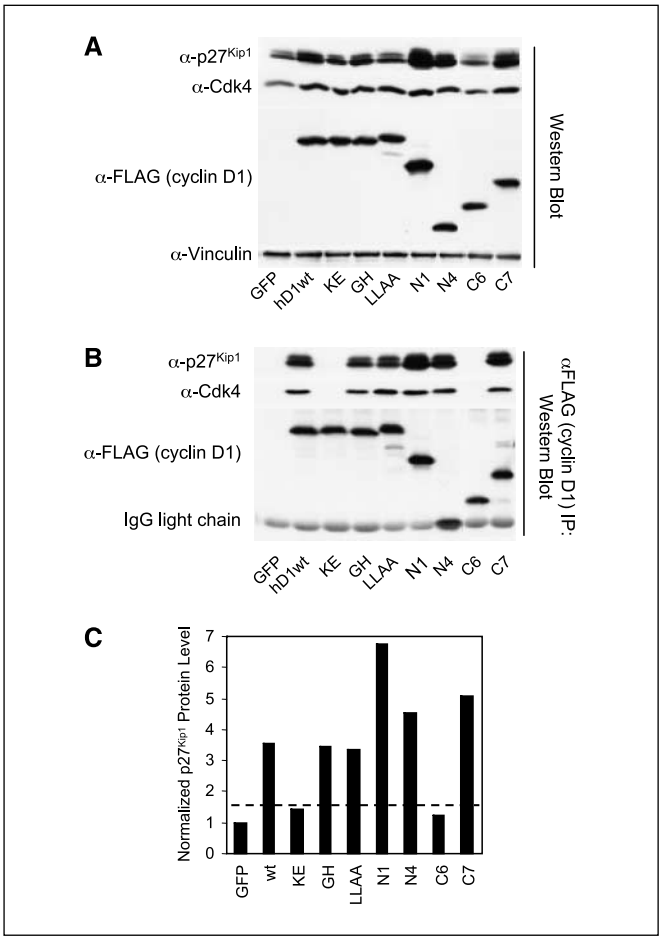

Figure 3. The cyclin D1 NH_2 terminus and K112 are required for p27^{Kip1} binding in cultured cells. *A,* Western blot for the amounts of p27^{Kip1}, Cdk4, FLAG-tagged cyclin D1, and the loading control vinculin in the cell extracts used for immunoprecipitations. *B,* immunoprecipitation (*IP*) with a monoclonal FLAG antibody (M2) in *cyclin D1*$^{-/-}$ MEFs infected with control vector (GFP) or FLAG-tagged human cyclin D1 wild type or KE mutant, GH mutant, LLAA mutant, N1 mutant, N4 mutant, C6 mutant, C7 mutant. Immunoprecipitates were resolved on SDS-PAGE, and membranes were probed with a polyclonal p27^{Kip1} antibody (C19), Cdk4 antibody (C22), and FLAG antibody (M2). *C,* relative abundance of p27^{KIP1} in *cyclin D1*$^{-/-}$ MEFs transduced with cyclin D1 expression vectors.

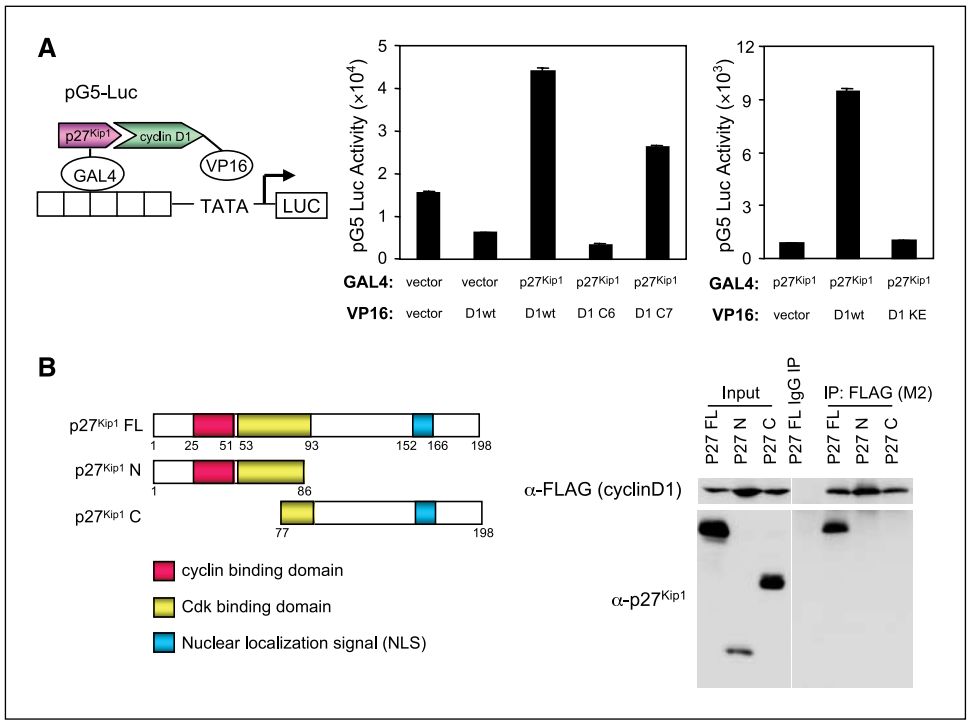

Figure 4. The cyclin D1 NH_2 terminus and K112 are required for $p27^{Kip1}$ binding in mammalian two-hybrid system. *A,* the mammalian two-hybrid system was used to detect the binding of cyclin D1 and $p27^{Kip1}$. *Left,* schematic representation of the system. *Middle* and *right,* pG5*luc* activity in MCF-7 cells cotransfected with 0.3 μg pG5*luc*, Gal4-$p27^{Kip1}$, and VP16-cyclinD1 wild type or mutants. *B, left,* schematic representation of full-length, NH_2 terminus, and COOH terminus of $p27^{Kip1}$; *right,* immunoprecipitation with a monoclonal FLAG antibody (M2) in HEK293T cells cotransfected with FLAG-tagged cyclin D1 and $p27^{Kip1}$ full length, NH_2 terminus, or COOH terminus. Immunoprecipitates were resolved on a SDS-PAGE gel, and membranes were blotted with a polyclonal $p27^{Kip1}$ antibody (M197) and FLAG antibody (M2). The amounts of $p27^{Kip1}$ and FLAG-tagged cyclin D1 in the cell extracts used for immunoprecipitations were also indicated as input. Cyclin D1 binds $p27^{Kip1}$. Neither the $p27^{Kip1}$ NH_2 terminus or COOH terminus are sufficient to bind cyclin D1.

$p27^{KIP1}$ in promoting cellular migration through several distinct hypothesized mechanisms (18, 29). One mechanism by which $p27^{KIP1}$ promotes cellular migration involves inhibition of Rho-GTPase activity. Reintroduction of cyclin D1 into *cyclin D1*$^{-/-}$ MEFs induced cyclin D1 and $p27^{KIP1}$ levels with a commensurate reduction in Rho-GTPase activity (23) assessed by Rho-GTP pull down with $p27^{KIP1}$ and cyclin D1 both detected in the Rho-GTP complex (ref. 23; Fig. 5*B*). We examined further the possible role for $p27^{KIP1}$ in cyclin D1–mediated cell migration. To determine whether $p27^{KIP1}$ rescued the *cyclin D1*$^{-/-}$ cell migration defect, *cyclin D1*$^{-/-}$ cells were transduced with a retroviral vector expression for $p27^{KIP1}$ and transwell migration analyses were conducted. Reintroduction of $p27^{KIP1}$ into *cyclin D1*$^{-/-}$ MEFs induced transwell migration (Fig. 5*C*) with a 2-fold increase in cell migration (Fig. 5*C*). The rescue function in transwell migration assays was similar to that of cyclin D1 in the number of migrating cells induced by $p27^{KIP1}$ transduction (cyclin D1 transduction 72 ± 8 versus $p27^{KIP1}$ transduction 61 ± 4, n = 3). Thus, $p27^{KIP1}$ is sufficient to rescue the defect in migration of cyclin D1–deficient cells. To determine whether $p27^{KIP1}$ functioned as a downstream target of cyclin D1–dependent rescue of cellular migration of cellular movement velocity, $p27^{KIP1}$ siRNA was used (Fig. 5*D-E*). Cyclin D1 transduction of *cyclin D1*$^{-/-}$ cells induced cellular velocity 2.5-fold. Introduction of $p27^{KIP1}$ siRNA abrogated $p27^{KIP1}$ protein levels by Western blot and reduced cell movement velocity induced by cyclin D1 to that of *cyclin D1*$^{-/-}$ cells (Fig. 5*D, E*). Together, these studies show that cyclin D1 induction of cellular migration requires $p27^{KIP1}$.

The finding that the domains of cyclin D1–promoting cellular migration physically associated with $p27^{KIP1}$ suggests that a physical interaction between these two proteins is important in cyclin D1–mediated migration. Thus, cyclin D1 promotes cellular migration through physical association with $p27^{KIP1}$. These findings extend our previous understanding of the role of cyclin D1 in cellular biological activities. These studies also suggest the reduction in cyclin D1 abundance in *cyclin D1*$^{-/-}$ cells may have a secondary effect of reducing $p27^{KIP1}$ levels, which, in turn, may contribute to the defect in cellular migration.

The finding that $p27^{KIP1}$ protein levels were reduced in *cyclin D1*$^{-/-}$ MEFs led us to examine further the mechanism by which cyclin D1 regulated $p27^{KIP1}$ abundance. $p27^{KIP1}$ abundance is affected by transcriptional and translational pathways. However, the major mechanism is posttranslational phosphorylation of $p27^{KIP1}$ on Thr^{187}, which creates a recognition site for the Skp2-SCF-E3 ubiquitin ligase, thereby promoting ubiquitination and degradation by the proteasome (30, 31). An additional degradation pathway involves phosphorylation of $p27^{KIP1}$ at Ser^{10}. To determine the mechanism by which cyclin D1 induced $p27^{KIP1}$ abundance, we examined the effect of cyclin D1 on $p27^{KIP1}$ mRNA levels. *Cyclin D1*$^{-/-}$ MEFs were transduced with a cyclin D1 expression vector and $p27^{KIP1}$ mRNA abundance was assessed by reverse transcription-PCR (RT-PCR). Cyclin D1 expression did not alter $p27^{KIP1}$ mRNA levels (Fig. 6*A*). We next assessed the effect of cyclin D1 on $p27^{KIP1}$ protein levels. *Cyclin D1*$^{-/-}$ MEFs were stably transduced with either an expression vector encoding control (GFP) or cyclin D1/IRES-GFP. Cells transduced with cyclin D1 showed a 6-fold increase in $p27^{KIP1}$ protein. Inhibition of new protein synthesis led to a reduction in both cyclin D1 and $p27^{KIP1}$ levels, further suggesting the abundance of cyclin D1 protein maintains $p27^{KIP1}$ protein levels (Fig. 6*B*). The abundance of the rate-limiting Skp2 component of the Skp2-SCF-E3 ligase that degrades $p27^{KIP1}$ was next assessed. Skp2 abundance was inhibited by cyclin D1 expression (Fig. 6*C*), commensurate with the induction of $p27^{KIP1}$ protein. Skp2 is regulated by transcriptional induction (32). We therefore assessed the regulation of the Skp2 promoter linked to a luciferase reporter gene. Cyclin D1 repressed the Skp2 promoter in a dose-dependent manner (Fig. 6*D*). Phosphorylation of $p27^{KIP1}$ at Ser^{10} regulates $p27^{KIP1}$ degradation by an ubiquitin ligase other

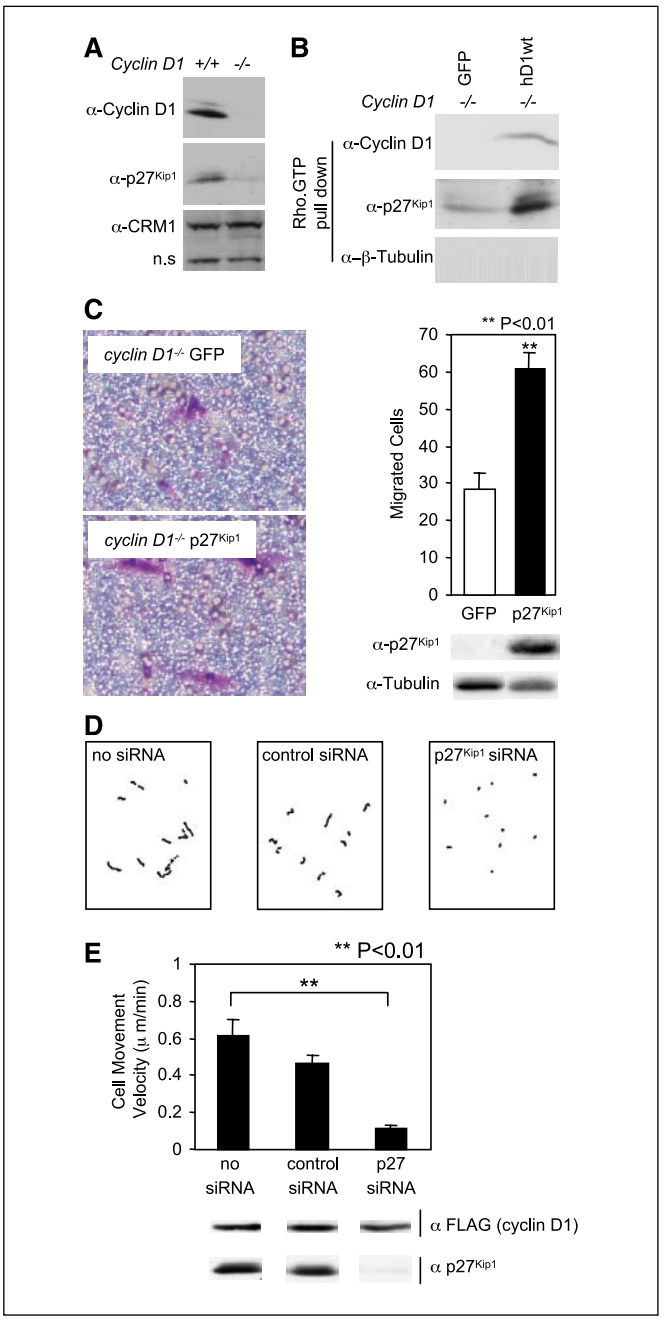

Figure 5. Exogenous $p27^{Kip1}$ rescues the migration defect of *cyclin D1*$^{-/-}$ MEFs. *A,* Western blot for $p27^{Kip1}$, cyclin D1, and CRM1 protein expression in *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ MEFs. *B,* glutathione *S*-transferase pull-down assay for activated Rho (Rho-GTP activity) in *cyclin D1*$^{-/-}$ MEFs infected with wild-type cyclin D1 (hD1wt) or vector control (GFP). The same membrane was blotted with FLAG antibody (mouse M2), $p27^{Kip1}$ antibody (C19), and β-tubulin antibody (H235) for cyclin D1, $p27^{Kip1}$, and β-tubulin expression in the activated Rho complex. $p27^{Kip1}$ and cyclin D1 were detected in the activated Rho-GTP complex. β-Tubulin is a negative control not associated with active Rho. *C,* transwell migration assay was conducted in *cyclin D1*$^{-/-}$ MEFs infected with either MSCV-GFP control vector or wild-type human $p27^{Kip1}$. **, migrated cell number and the significant difference from GFP vector control. *Bottom,* exogenous $p27^{Kip1}$ protein expression in *cyclin D1*$^{-/-}$ MEFs infected with vector control or $p27^{Kip1}$-expressing virus. *D* and *E,* $p27^{Kip1}$ siRNA blocked the promigratory effect of exogenous cyclin D1 in *cyclin D1*$^{-/-}$ MEFs. Phase-contrast, time-lapse video microscopy images of *cyclin D1*$^{-/-}$ MEFs infected with wild-type cyclin D1 without treatment or with control siRNA or $p27^{Kip1}$ siRNA were traced by MetaMorph software (*D*). The cell movement velocity was determined by tracing the single cells at different time points. *Columns,* mean of at least 10 individual cells of the same cell type; *bars,* SE. *Bottom,* $p27^{Kip1}$ protein expression was blocked after siRNA treatment.

than Skp2-SCF that remains to be defined. As $p27^{KIP1}$ phosphorylation at Ser^{10} increases $p27^{KIP1}$ stability, we conducted a Western blot with a phospho-Ser^{10}-specific antibody. *Cyclin D1*$^{-/-}$ cells showed reduced $p27^{KIP1}$ Ser^{10} phosphorylation. Cyclin D1 expression increased the relative abundance of $p27^{KIP1}$ and associated Ser^{10} phosphorylation (Fig. 6*E*; ref. 33). $p27^{KIP1}$ Ser^{10} is phosphorylated by cdk5, and cyclin D1 induced cdk5 abundance (Fig. 6*E*). As cyclin D1 induced p27 and cdk5 abundance, we investigated the possibility that cyclin D1 may induce additional cyclin D1–binding proteins. More than 30 cyclin D1 interactive proteins have been described (1). The well-characterized $p21^{CIP1}$ protein was therefore assessed. Cyclin D1 induced expression of $p21^{CIP1}$ but did not affect expression of $p57^{KIP2}$ or cdk4 (Fig. 6*F*).

Recent studies (23) showed cyclin D1 promoted migration through reducing the expression and transcription of thrombospondin 1. Consistent with previous studies, cyclin D1 repressed TSP-1 (Fig. 6*G*). $p27^{KIP1}$ also repressed TSP-1, consistent with our model in which cyclin D1 and $p27^{KIP1}$ function cooperatively to promote cellular migration. Collectively, these studies suggest that cyclin D1 promotes migration through $p27^{KIP1}$. Cyclin D1 associates with $p27^{KIP1}$ and induces $p27^{KIP1}$ abundance, through reducing $p27^{KIP1}$ degradation by repressing Skp2 and inducing $p27^{KIP1}$ Ser^{10} phosphorylation (Fig. 6*H*).

Discussion

Cyclin D1 is overexpressed in human breast cancer and the current studies identify a novel function for cyclin D1 in mammary epithelial cells. The studies herein show that cyclin D1 reduces attachment and induces mammary epithelial cell migration across a membrane. This new function of cyclin D1 may contribute to the clinical observation that cyclin D1 overexpression correlates with tumor metastasis. The current studies are consistent with and extend recent findings that cyclin D1 promotes motility of fibroblasts (23). These prior studies show that cyclin D1 induces MEF motility by inhibiting Rho GTPase activity. The current studies identify several key molecular mechanisms by which cyclin D1 promotes migration. First, using siRNA to $p27^{KIP1}$, we showed that induction of cellular migration by cyclin D1 requires $p27^{KIP1}$. Second, the domains of cyclin D1 required for the induction of cellular migration are shown to be required for $p27^{KIP1}$ binding and for the induction of $p27^{KIP1}$ abundance. Third, these studies show that cyclin D1 induces $p27^{KIP1}$ abundance through two mechanisms: cyclin D1 represses the Skp2 component of the Skp2-SCF-E3 ligase that degrades $p27^{KIP1}$ and cyclin D1 induces phosphorylation of $p27^{KIP1}$ at Ser^{10}, a modification known to enhance $p27^{KIP1}$ stability (33, 34). Collectively, these studies show a novel function for cyclin D1 in mammary epithelial cells, and a new mechanism by which cyclin D1 regulates cellular migration and a new mechanism by which cyclin D1 regulates $p27^{KIP1}$ abundance.

The domains of cyclin D1 required for the induction of cellular migration included amino acid residues in the NH_2 terminus and Lys^{112}. Both regions of cyclin D1 were shown to be required for association with $p27^{KIP1}$ in mammalian two-hybrid. The requirement for $p27^{KIP1}$ in cyclin D1–mediated migration suggests that the physical association of cyclin D1 with $p27^{KIP1}$ plays an important role in the formation of a promigratory complex. These studies extend previous observations in which cyclin D1 association with $p27^{KIP1}$ functioned to promote cell cycle function and DNA synthesis (35). In prior studies, a noncatalytic function of cyclin D–CDK4 included sequestration of $p27^{KIP1}$ from the cyclin E–CDK2

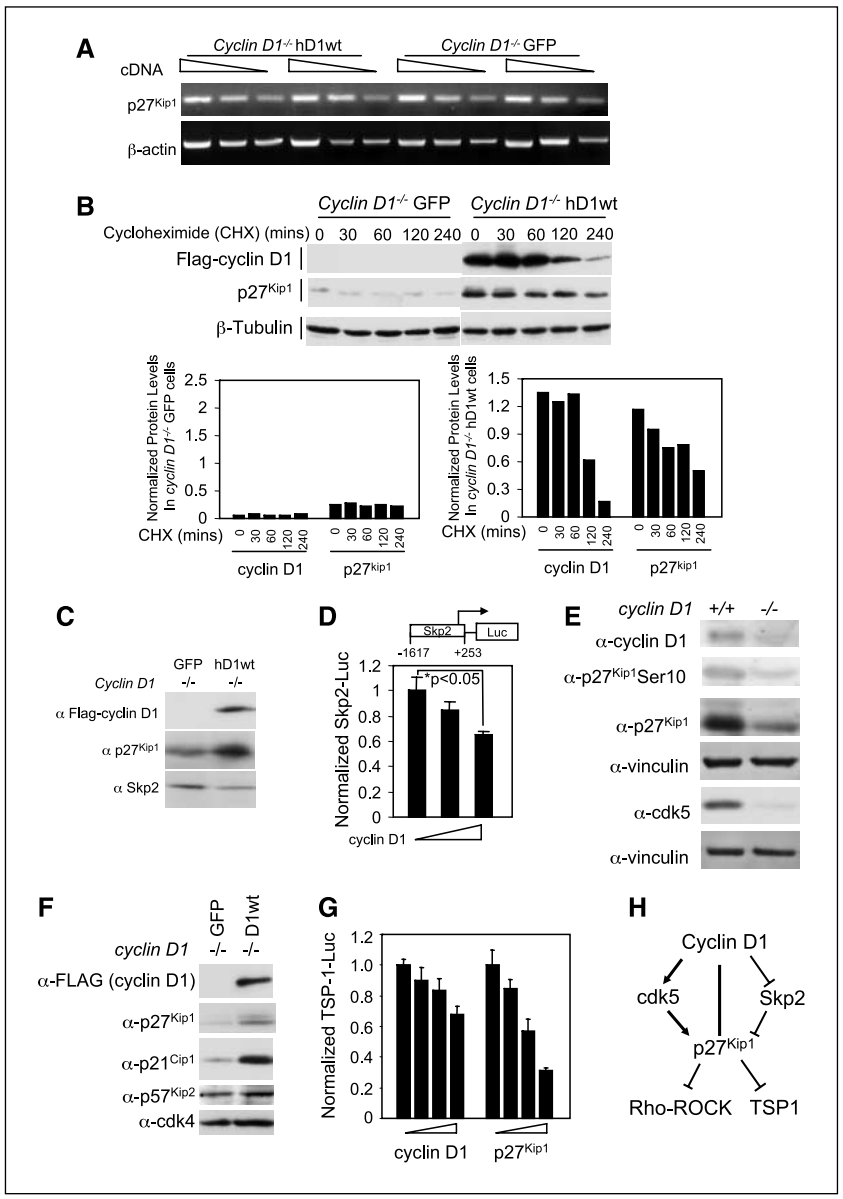

Figure 6. Cyclin D1 induces $p27^{KIP1}$ abundance, reducing the Skp2 component of Skp2-SCF-E3-ligase. *A,* $p27^{KIP1}$ mRNA levels assessed by RT-PCR in *cyclin D1*$^{-/-}$ cells transduced with a cyclin D1 expression vector. Data are shown normalized to β-actin. *B,* Western blot analysis for cyclin D1 and $p27^{KIP1}$. *Cyclin D1*$^{-/-}$ cells, transduced with cyclin D1 expression vector, were subsequently treated with cyclohexamide for the time points indicated. Cyclin D1 and $p27^{KIP1}$ protein abundance is shown graphically. *C,* Western blot for Skp2 and $p27^{KIP1}$ in *cyclin D1*$^{-/-}$ cells, transduced with cyclin D1 expression vector encoding cyclin D1 with an amino terminal FLAG epitope. *D,* activity of the Skp2 promoter in HEK293T cells transfected with a cyclin D1 expression vector. *Columns,* mean of $n > 5$ separate transfections; *bars,* SE. *E* and *F,* Western blot analysis of *cyclin D1*$^{-/-}$ MEFs transduced with a cyclin D1 expression vector was done to assess abundance of $p27^{KIP1}$ and cyclin D1 interacting proteins. *G,* TSP1 promoter activity was repressed by both cyclin D1 and $p27^{KIP1}$ in a dose-dependent manner. *H,* schematic representation of model by which cyclin 1 regulates $p27^{KIP1}$ abundance.

complex (reviewed in ref. 35). These studies extend the prior observations, demonstrating a role for cyclin D1 association with $p27^{KIP1}$ in promoting cellular migration.

These studies also extend prior observations that *cyclin D1*$^{-/-}$ murine bone marrow macrophages show reduced migration in response to CSF1 and increased cellular adhesion (25). This abnormality of cellular migration in cyclin D1–deficient cells was observed herein in fibroblasts and in mammary epithelial cells. Cyclin D1–deficient mammary epithelial cells showed a reduction in migration and reduced cellular velocity. The morphologic appearance of *cyclin D1*$^{-/-}$ MEFs were more spread and flattened as assessed by scanning electron microscopy. This spread morphology was rescued by murine or human cyclin D1 requiring the cyclin D1 NH_2-terminal residues (46-90). The domains of cyclin D1 required for inducing the fibroblastoid polarized morphology were also required for the induction of cellular migratory velocity and transwell migration. The domains of cyclin D1 promoting cellular migration were also required for association with $p27^{KIP1}$. Cyclin D1–deficient cells express reduced levels of $p27^{KIP1}$ and $p27^{KIP1}$ siRNA abrogated cyclin D1–mediated cellular migration. Collectively, these observations show that the cyclin D1–$p27^{KIP1}$ complex is mechanistically involved in promoting cellular migration. Reintroduction of $p27^{KIP1}$ rescued the cellular migratory defect of *cyclin D1*$^{-/-}$, suggesting $p27^{KIP1}$ is a key distal mediator of cyclin D1–mediated migration.

The mechanism by which $p27^{KIP1}$ regulates cellular migration remains controversial. $p27^{KIP1}$ can induce rearrangements of the actin cytoskeleton in either a Rac-dependent manner (21) or RhoA-dependent manner through inhibition of RhoA (17). In endothelial and vascular smooth muscle cells, $p27^{KIP1}$ blocks cellular migration (19, 20). The subcellular distribution of $p27^{KIP1}$ is important in promigratory function and cytoplasmic $p27^{KIP1}$, rather than nuclear $p27^{KIP1}$ promotes cellular migration (36). In addition to inhibiting RhoA activity, $p27^{KIP1}$ binds stathmin, which may counteract the promigratory effects of stathmin, and cyclin D1 may antagonize this effect of $p27^{KIP1}$ (22). Cyclin D1 is known to

regulate E2F activity (37) and E2F transcription factors activate stathmin (38). However, cyclin D1 did not affect either stathmin abundance or stathmin promoter activity (Supplementary Data 2).

The current studies show that cyclin D1 regulates $p27^{KIP1}$ abundance. *Cyclin D1*$^{-/-}$ cells were $p27^{KIP1}$ deficient and reintroduction of cyclin D1 induced $p27^{KIP1}$ abundance. Mutants of cyclin D1 that rescued cellular migration also induced $p27^{KIP1}$ abundance, whereas mutants defective in rescue were also defective in regulating $p27^{KIP1}$ abundance (Fig. 3*C*). Together, these studies suggest an additional mechanism by which cyclin D1 may promote migration indirectly through the induction of $p27^{KIP1}$ abundance. $p27^{KIP1}$ abundance can be regulated at both the transcriptional and posttranslational level. Phosphorylation of $p27^{KIP1}$ at Thr^{187} creates a recognition site for a ubiquitin ligase that degrades $p27^{KIP1}$. The Skp2 SCF ubiquitin ligase (31, 39–41) regulates $p27^{KIP1}$ abundance by degradation. Herein, cyclin D1 repressed the Skp2 promoter and reduced Skp2 abundance, thereby inducing $p27^{KIP1}$ protein abundance. Cyclin D1 is known to regulate the activity of a subset of transcription factors (1) through mechanisms that involve association with HDACs (9), providing a possible mechanism by which cyclin D1 may regulate the Skp2 promoter.

Herein, phosphorylation of $p27^{KIP1}$ at Ser^{10}, known to stabilize $p27^{KIP1}$ (33), was increased by cyclin D1. Several different kinases have been linked to $p27^{KIP1}$ Ser^{10} phosphorylation [Mirk/dyrk1B (42), hKIS (43), AKT (44), cdk5 (45), and extracellular signal-regulated kinase 2 (44)]. Phosphorylation at Ser^{10} is important in the inactivation of $p27^{KIP1}$ tumor-suppressor function (17). In addition, $p27^{KIP1}$ is exported from the nucleus to the cytoplasm at the G_0-G_1 phase of the cell cycle (34, 46, 47). Phosphorylation of Ser^{10} is required for the binding of $p27^{KIP1}$ to CRM1, a carrier protein for nuclear export. KPC1 and KPC2-E3 ubiquitin-promoting complexes ubiquitinate and degrade $p27^{KIP1}$ subsequent to CRM1-dependent nuclear export. Although the mechanism by which cyclin D1 regulates $p27^{KIP1}$ Ser^{10} phosphorylation will require further analysis, AKT kinase that phosphorylates Ser^{10} *in vitro* was unaltered in these cells, suggesting that an alternate kinase, perhaps cdk5, is involved.

The current demonstration of a role for the physical interaction between cyclin D1 and $p27^{KIP1}$ in the induction of cellular motility provides an important new link between the cyclins and the cyclin-dependent kinase inhibitor proteins. The biological significance of these studies is evidenced by the alteration of transwell migration, cellular adhesion, and cellular velocity. The demonstration that a single amino acid residue of cyclin D1 is required for $p27^{KIP1}$ association and the induction of cellular migratory capability lends credence to the possibility that specific inhibitors may be designed to inhibit cyclin D1–dependent cellular migration in tumors.

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Cyclin D1 Induction of Cellular Migration Requires p27^{KIP1}

Zhiping Li, Xuanmao Jiao, Chenguang Wang, et al.

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