Cyclin D3 Expression in Melanoma Cells Is Regulated by Adhesion-dependent Phosphatidylinositol 3-Kinase Signaling and Contributes to G₁-S Progression

Laurie S. Spofford, Ethan V. Abel, Karen Boisvert-Adamo, and Andrew E. Aplin

From the Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208

D-type cyclins regulate G₁ cell cycle progression by enhancing the activities of cyclin-dependent kinases (CDKs), and their expression is frequently altered in malignant cells. We and others have previously shown that cyclin D1 is up-regulated in melanoma cells through adhesion-independent MEK-ERK1/2 signaling initiated by mutant B-RAF. Here, we describe the regulation and role of cyclin D3 in human melanoma cells. Cyclin D3 expression was enhanced in a cell panel of human melanoma cell lines compared with melanocytes and was regulated by fibronectin-mediated phosphatidylinositol 3-kinase/Akt signaling but not MEK activity. RNA interference experiments demonstrated that cyclin D3 contributed to G₁-S cell cycle progression and proliferation in melanoma cells. Overexpression of cyclin D1 did not recover the effects of cyclin D3 knockdown. Finally, immunoprecipitation studies showed that CDK6 is a major binding partner for cyclin D3, whereas CDK4 preferentially associated with cyclin D1. Together, these findings demonstrate that cyclin D3 is an important regulator of melanoma G₁-S cell cycle progression and that D-type cyclins are differentially regulated in melanoma cells.

G₁ cell cycle progression and entry into S phase are regulated by the activities of cyclin-dependent kinases (CDKs). In early G₁, CDK4 and CDK6 are activated in response to increased expression of D-type cyclins (1). Three D-type cyclins are expressed in mammalian cells: D1, D2, and D3. Activation of CDK4/6 promotes hyperphosphorylation of the retinoblastoma protein, release and derepression of E2F activity, and entry into S phase (2). Genetic deletion studies in mice have illustrated some non-overlapping roles for D-type cyclins. Cyclin D1-deficient mice display defects caused by reduced proliferation of retinal cells and mammary epithelial cells during pregnancy (3, 4), mice lacking cyclin D2 display hypoplasia in the ovaries or testes (5), and cyclin D3-deficient mice display defective thymocyte maturation (6).

A hallmark characteristic of malignant cells is their aberrant G₁-S cell cycle progression and proliferation (7). D-type cyclins are frequently overexpressed in human tumors due either to gene amplification or altered control of signaling pathways, and this overexpression likely contributes to aberrant cell cycle progression in many tumor types (5, 6, 8). Metastatic melanoma is an aggressive skin cancer with a rising incidence rate. Currently, it is only effectively treated by early detection and surgery. Melanoma arises from the transformation of melanocytes, the pigment-producing cells in the skin, and its progression is well characterized (9). Radial growth phase is characterized by cell growth within the epidermis (in situ) or as nests in the papillary dermal layer (microinvasion). The vertical growth phase is associated with growth perpendicular to the skin surface and the gain of tumorigenic properties. Subsequent metastasis of melanoma to distant sites is associated with a poor prognosis.

Expression of cyclin D1 is enhanced in primary and metastatic melanoma (10–12) through constitutive B-RAF-MEK-ERK1/2 signaling (13–16) or, in acral melanoma, due to amplification of the cyclin D1 gene locus (11). In addition to cyclin D1, cyclin D3 expression is readily detected in immunohistochemical studies on melanoma tissue and is associated with a poor prognosis (10). Cyclin D3 is expressed in a wide variety of cell types but compared with other D-type cyclins its regulation is poorly understood (17). Because the role of cyclin D3 in melanoma remains unknown, we analyzed its regulation and contribution to cell cycle progression in human melanoma cells. We find that cyclin D3 is aberrantly expressed in melanoma cells compared with melanocytes, is regulated by a fibronectin adhesion-dependent PI-3 kinase/Akt pathway, and contributes to cell cycle progression. Notably, the regulation and CDK binding partners of cyclin D3 show important differences compared with cyclin D1.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal human epidermal melanocytes (NHEM) were isolated from neonatal foreskins as previously described (14). In brief, foreskins were obtained from The Birth Place at Albany Medical Center in accordance with an Institutional Review Board approved protocol. Foreskin tissue was incubated with dispase to separate the epidermis from the der-
Cells were harvested after a further 68 h. For cyclin D3, two distinct sequences were used: cyclin D3 siRNA #1 was 5'-GGAUCUUUGUUGGCCAAGGAdTdT-3', as previously utilized (6), and cyclin D3 siRNA #2 was 5'-GAUAAUCUCCUGCCAUGUU-3'. For PTEN knockdown, the siRNA sequence was 5'-GUGAAGAUUCCAGACAUUUGU-3'.

**Recombinant Lentiviral Transduction**—Full-length human cyclin D1 cDNA was a gift from Dr. J. Zhao (Albany Medical College). The cyclin D1 sequence was subcloned into pENTR™/D-TOPO vector (Invitrogen) according to the manufacturer’s guidelines. To generate the blunt-ended PCR products for TOPO cloning, we used the KOD Hot Start DNA polymerase kit (Novagen, Darmstadt, Germany) with a reverse primer that omits the stop codon in order to put the open reading frame in-frame with the V5 epitope tag built into pLenti6/UbC/V5-DEST. Primer sequences are available upon request. pENTR/D-TOPO/cyclin D1 was recombinated with pLenti6/UbC/V5-DEST using the LR Clonase II kit and protocol (Invitrogen) to generate pLenti6/UbC/V5-GW/cyclin D1. This construct was verified by DNA sequencing.

Lentiviruses were generated by cotransfecting 293FT with pLenti6/UbC/V5-GW/cyclin D1 or pLenti6/UbC/V5-GW/LacZ (Invitrogen) and three lentiviral packaging plasmids (Invitrogen) according to the manufacturer’s guidelines, with the exception that FuGENE 6 reagent (Roche Applied Science) was used for transfection. Viral supernatants were collected 72 h post-transfection.

For transduction, cells were incubated for 72 h in the presence of lentiviral supernatant. Transduced cell populations were selected with 5 µg/ml blasticidin. Transduction efficiencies were >70%, as judged by transduction with green fluorescent lentivirus and immunostaining for the V5 tag.

**SDS-PAGE and Western Blotting**—Cells lysed in sample buffer were separated by SDS-PAGE, and proteins were transferred electrophoretically onto Immobilon P membranes (Millipore Corp., Bedford, MA). Membranes were blocked with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 for 1 h and subsequently incubated with primary antibody overnight at 4 °C. Primary antibodies used in this study were: Akt (clone 55), CDK4 (clone 97), and cyclin D1 (DCS-6), all from BD Biosciences; phospho-473 Akt, CDK6 (DCS-83), phospho-ERK1/2, phospho-Ser-780 Rb, and PTEN, all from Cell Signaling Technology, Beverley, MA; CDK2 (M2), cyclin A (H-432), and total ERK1/2 (clone K-23), all from Santa Cruz Biotechnology, Santa Cruz, CA; cyclin D3 (DCS-22), from Lab-Vision, Fremont, CA; β-galactosidase, from Promega, Madison WI; tubulin (clone KMX-1), from Chemicon, Temecula, CA. Membranes were washed in phosphate-buffered saline/Tween, and incubated with anti-mouse or anti-rabbit IgG peroxidase conjugates (Calbiochem) for 1 h at room temperature. Western blots were developed using SuperSignal chemiluminescent substrate (Pierce). Immunoreactivity was detected and quantified using a Fluor-S Multi-Imager and Quantity-One software (Bio-Rad).

**Cell Adhesion**—WM793 cells were serum-starved overnight before detachment with trypsin. Cells were pelleted in MCDB 153 medium containing 0.5% bovine serum albumin (Sigma) and 1 mg/ml soybean trypsin inhibitor (Invitrogen). Cells were washed once and then resuspended in MCDB 153, 0.5% bovine serum albumin and re-plated onto fibronectin (BD Biosciences) or poly-lysine-coated dishes for 16 or 24 h. Human fibronectin (BD Biosciences) and poly-lysine (Sigma) were both used at a concentration of 10 µg/ml.

**Immunofluorescence**—For analysis of S-phase entry after cyclin D3 knockdown, WM793 cells were replated onto coverslips for 12 h, and then bromodeoxyuridine (BrdUrd) was added to the medium at a final concentration of 100 µM. After 8 h cells were washed with phosphate-buffered saline and then fixed with 3.7% formaldehyde for 10 min at room temperature. Cells were further washed and permeabilized for 5 min in phosphate-buffered saline containing 0.5% Triton X-100. Slides were then treated with 0.1 units/µl of DNase for 30 min at 37 °C. BrdUrd incorporation was analyzed with a sheep anti-BrdUrd antibody and rabbit anti-sheep IgG conjugated to fluorescein isothiocyanate (BD Biosign, Saco, MA). Nuclei were stained with Hoechst reagent 33342 (Molecular Probes Inc. Eugene, OR) at 1 µg/ml for 10 min at room temperature.

For Ki67 staining cells were fixed in formaldehyde and processed as above but stained with anti-Ki67 (RB-9043, Lab-Vision) and anti-rabbit Alexa-Fluor 488 (Molecular Probes) antibodies. Fluorescent staining was viewed on an Olympus BX60 microscope equipped for epifluorescence. Images were captured using a Spot CCD camera and processed using deconvolution Slide Book software. For cell counting experiments, cells were detached with trypsin and counted using a hemocytometer.

**Immunoprecipitation**—Cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100. Cyclin D3 (Ab-3, Lab-Vision), cyclin D1 (M-20, Santa Cruz), or V5 (Invitrogen) antibodies were incubated with cell lysates overnight at 4 °C. This was followed the next day by the addition of 40 µl of protein A/G agarose and further incubation for 2 h. Protein A/G-agarose complexes were washed three times with cold lysis buffer and boiled with SDS-PAGE sample buffer to dissociate the proteins.
Differential Regulation of D-type Cyclins in Melanoma Cells

RESULTS

Expression of Cyclin D3 Is Enhanced in Human Melanoma Cells—We initially analyzed whether cyclin D3 expression is increased in a panel of human melanoma cell lines that display characteristics of different stages of melanoma progression (18). By Western blot analysis, cyclin D3 was expressed highly in the radial growth-phase cell lines WM35 and SBcl2, the vertical growth-phase WM793 and WM115 cells, and metastatic SK-MEL-28 and SK-MEL-5 cells (Fig. 1). Cyclin D3 was weakly expressed in vertical growth-phase WM278 cells, metastatic SK-MEL-24 cells, and NHEM from distinct donors. These findings show that expression of cyclin D3 is frequently enhanced in human melanoma cell lines. Notably, its expression does not tightly correlate with the expression of B-RAF mutations because SBcl2 cells express wild-type B-RAF (Table 1 and Refs. 13, 19, and 20).

Cyclin D3 Levels and Akt Phosphorylation Are Regulated by Adhesion to Fibronectin—We previously showed that cyclin D1 expression in melanoma cells is independent of integrin-mediated adhesion and spreading (16). To determine whether cyclin D3 was regulated by adhesion, we replated WM793 cells on fibronectin, a matrix that engages integrins, or poly-lysine to which cells attach independently of integrins. Cells attached efficiently to both fibronectin and poly-lysine but only spread on fibronectin. WM793 cells replated on fibronectin showed enhanced levels of cyclin D3 in comparison to cells on poly-lysine (Fig. 2, A and quantitated in B). WM793 cells did not undergo apoptosis in these conditions, as determined by staining for cleaved caspase 3 (21).

Previous reports have shown that PI-3 kinase/Akt signaling regulates cyclin D3 in non-melanoma cell types at the level of translation (22–25). To investigate whether WM793 cell adhesion to fibronectin altered the PI-3 kinase/Akt pathway in melanoma cells, we probed lysates for levels of serine 473-phosphorylated Akt, an indicator of Akt activation. Adhesion of WM793 cells to fibronectin but not attachment to poly-lysine enhanced Akt phosphorylation at serine 473 (Fig. 2, C and D). Similarly, serine 473 phosphorylation of Akt was enhanced after adhesion of SK-MEL-28 cells to fibronectin (Fig. 2E). Thus, PI-3 kinase/Akt signaling and expression of cyclin D3 are adhesion-regulated in melanoma cells.

PI-3 Kinase Signaling Regulates Cyclin D3 Expression—Our findings from Fig. 1 showed that some melanoma cell lines such as WM278 express low levels of cyclin D3 compared with others including WM793 and WM115. All three of these cell lines express mutant B-RAF. We examined the levels of phospho-Akt in these cell lines and observed that Akt phosphorylation was readily detected in WM793 and WM115 but poorly detected in normal melanocytes and WM278 cells (Fig. 3A). Expression of phosphatase and tensin homologue deleted from chromosome 10 (PTEN), a lipid phosphatase that inhibits PI-3 kinase signaling to Akt, is frequently altered in melanoma (26–28). Enhanced Akt phosphorylation in WM793 and WM115 cells correlated with decreased expression of PTEN and the detection of truncated PTEN products. To determine the role of PTEN and subsequent activation of Akt, we utilized RNAi to knock down PTEN in WM278 cells. PTEN-targeting siRNA efficiently down-regulated PTEN in WM278 cells (Fig. 3B).

TABLE 1

| Cell line   | Melanoma stage     | B-RAF status* | Cyclin D3 expression |
|-------------|-------------------|---------------|----------------------|
| NHEM        | Radial growth phase | Wild type    | Low                  |
| SBcl2       | Radial growth phase | V600E        | High                 |
| WM35        | Vertical growth phase | V600E       | High                 |
| WM793       | Vertical growth phase | V600E       | High                 |
| WM115       | Vertical growth phase | V600D       | Low                  |
| WM278       | Vertical growth phase | V600E       | Low                  |
| SK-MEL-5    | Metastatic melanoma | V600E       | High                 |
| SK-MEL-24   | Metastatic melanoma | V600E       | Low                  |
| SK-MEL-28   | Metastatic melanoma | V600E       | High                 |

* The B-RAF statuses of cell lines are from Refs. 13, 19, and 20.

FIGURE 1. Cyclin D3 expression is enhanced in melanoma cells. Serum-starved cultures of NHEM and eight human melanoma cell lines (WM793, WM35, WM115, WM278, SK-MEL-28, SK-MEL-24, SK-MEL-5, and SBcl2) were analyzed by Western blotting (WB) for cyclin D3 and tubulin as a loading control.

FIGURE 2. Adhesion regulation of cyclin D3 and Akt in human melanoma cells. Serum-starved WM793 cells were replated onto either fibronectin (Fn) or poly-lysine (PL) for 16 and 24 h. A, Cells were harvested and lysates analyzed by Western blotting (WB) for cyclin D3 and tubulin. B, graphed is the average cyclin D3 levels relative to tubulin ± S.D. from three independent experiments. C, cell lysates were analyzed for phospho-Ser-473 (pS473) Akt and total Akt. D, graphed is the average phospho-Ser-473 Akt levels relative to total Akt ± S.D. from three independent experiments. E, serum-starved SK-MEL-28 were replated onto either fibronectin or poly-lysine for 16 and 24 h and analyzed by Western blotting for phospho-Ser-473 and total Akt.
Differential Regulation of D-type Cyclins in Melanoma Cells

Concomitant with PTEN down-regulation was an increase in phosphorylation of Akt and cyclin D3 expression.

To further examine the role of PI-3 kinase-Akt signaling in the regulation of cyclin D3 expression, we treated WM793 cells with LY294002, a PI-3 kinase inhibitor. LY294002 treatment was associated with a decrease in Akt phosphorylation and cyclin D3 levels (Fig. 3C). Comparable effects on Akt phosphorylation and cyclin D3 expression were observed after treatment of SK-MEL-28 cells with LY294002 (Fig. 3, D and E). In contrast, treatment of SK-MEL-28 cells with U0126, a MEK inhibitor, had no effect on cyclin D3 levels but reduced phosphorylation of ERK1/2 and cyclin D1 expression (Fig. 3, D and E); these effects of U0126 on cyclin D1 are consistent with our previous report (16). Similar results utilizing these pharmacological inhibitors were observed in SK-MEL-5 cells (data not shown). Thus, PI-3 kinase but not MEK signaling regulates cyclin D3 levels in melanoma cells.

Cyclin D3 Expression Is Required for G1 Cell Cycle Progression—We have previously reported that cyclin D1 is highly expressed and required for G1 cell cycle progression and S phase entry in WM793 cells (16). The requirement of cyclin D3 in melanoma is not known. We utilized RNAi to knock down levels of cyclin D3 in mutant B-RAF-expressing melanoma cells and determined the effects on two markers of progression through G1: phosphorylation of retinoblastoma at serine 780 and cyclin A expression. Cyclin D3 expression was reduced by an average of 84% with targeting siRNA in WM793 cells compared with cells transfected with a non-targeting siRNA (Fig. 4, A and quantitated in B). Importantly, cyclin D3 knockdown did not affect cyclin D1 levels, but phosphorylation of retinoblastoma at serine 780 and levels of cyclin A were reduced by 56 and 74%, respectively. Knockdown of cyclin D3 with a second distinct siRNA elicited similar effects on hyperphosphorylation of Rb and expression of cyclin A (Fig. 4C), indicating that the effects of cyclin D3 knockdown on G1 cell cycle events are not due to off-target effects. To ensure that our results were not exclusive to the WM793 cell line, we reduced cyclin D3 expression by RNAi in a second melanoma cell line, SK-MEL-28. Cyclin D3 knockdown again reduced cyclin A expression (Fig. 4D). The overall efficiency of knockdown was lower in SK-MEL-28 cells compared with WM793; cyclin D3 and cyclin A were reduced by 54 and 39%, respectively, in comparison to controls (Fig. 4E). Together, these data indicate that increased cyclin D3 expression in human melanoma cells contributes to G1 cell cycle progression.

Cyclin D3 Contributes to S-phase Entry and Proliferation in Melanoma Cells—To determine whether cyclin D3 contributes to S phase entry, we measured incorporation of the thymidine analogue, BrdUrd, in control and cyclin D3 knockdown cells by immunofluorescence. BrdUrd incorporation was dramatically reduced after cyclin D3 knockdown (Fig. 5A). Quantitation showed that 34% of the cyclin D3 siRNA-transfected WM793 cells incorporated BrdUrd compared with 58% of control cells. Consistent with these effects, knockdown of cyclin D3 reduced the number of cells staining positively for the proliferation marker Ki67 by more than 50% (Fig. 5B) and decreased cell numbers (Fig. 5C). These results demonstrate that cyclin D3 contributes to melanoma cell S phase entry and proliferation. This requirement for cyclin D3 is in addition to a role of cyclin D1 in these cells (16).

Cyclin D1 Overexpression Does Not Rescue Cyclin D3 Knockdown Effects on Cyclin A—We previously demonstrated a requirement for cyclin D1 in melanoma cell G1 cell cycle progression (16). One possibility is that the effects of cyclin D3 knockdown reflect a requirement for a certain level of total D-type cyclins. To test this, we coordinately overexpressed cyclin D1 and knocked down cyclin D3. We utilized a lentiviral transduction approach to efficiently transduce melanoma cells with V5 epitope-tagged human cyclin D1. As controls, non-transduced cells and lacZ-transduced cells were utilized. Western blotting of cell lysates with the V5 antibody identified tagged cyclin D1 and β-galactosidase in their respective cell lines but not in non-transduced cells (Fig. 6A). The exogenous cyclin D1 migrated slower than the endogenous due to the addition of the V5 epitope tag and was expressed at comparable levels to endogenous cyclin D1.
Immunoprecipitation experiments showed that the exogenous cyclin D1 was functional in that it formed complexes with CDK4 (Fig. 6B). No CDK4 binding was detected in V5-H9252-galactosidase immunoprecipitates. Importantly, knockdown of cyclin D3 led to an efficient and comparable decrease in cyclin A expression in the non-transduced, lacZ-transduced, and cyclin D1-transduced cells (Fig. 6C). These results show that 2-fold enhanced expression of cyclin D1 is not able to rescue the effects of cyclin D3 knockdown on cyclin A expression.

Cyclin D1 and Cyclin D3 Differentially Bind CDKs—To understand how cyclin D3 and cyclin D1 both contribute to G1 progression in melanoma cells, we analyzed their binding to CDKs, 2, 4, and 6 by co-immunoprecipitation. Western blot analysis of whole cell lysates before and after immunoprecipitation demonstrated that the majority of cyclin D3 and cyclin D1 were removed by immunoprecipitation with their respective antibodies (Fig. 7A). Immunoprecipitations were selective since cyclin D1 but not cyclin D3 was detected in D1 immunoprecipitates and vice versa in D3 immunoprecipitates (Fig. 7B). Analysis of CDK binding partners showed that CDK4 preferentially associated with cyclin D1, whereas CDK6 levels were enhanced in cyclin D3 immunoprecipitates (Fig. 7C). CDK2 was associated with cyclin D1 and to a lesser extent with cyclin D3. These findings suggest that cyclin D3 and cyclin D1 exhibit different associations with CDKs in melanoma cells.

Because cyclin D3 but not cyclin D1 bound to CDK6, we utilized RNAi to knock down CDK6. In WM793 cells siRNA targeting CDK6 effectively knocked down expression of CDK6 without reducing expression of CDK4 (supplemental Fig. 1A). However, depletion of CDK6 did not dramatically affect cyclin A expression (supplemental Fig. 1A) or the cell cycle profile as determined by propidium iodide staining (supplemental Fig. 1B). Notably, in CDK6 knockdown cells, cyclin D3 showed enhanced association with CDK2 (supplemental Fig. 1C). This finding may explain, at least partially, the different effects on cell cycle progression upon cyclin D3 and CDK6 knockdown.

**DISCUSSION**

In this study we show that cyclin D3 expression is enhanced in human melanoma cells and is regulated by adhesion-dependent PI-3 kinase/Akt signaling. Importantly, cyclin D3 expression is required for efficient G1-S cell cycle progression and proliferation in melanoma cells even though cyclin D1 is also highly expressed in these cells. Additionally, our data dem-
onstrate that cyclins D3 and D1 are differentially regulated and have preferred CDK binding partners.

Enhanced cyclin D3 expression in human melanoma cell lines supports earlier studies performed on tissue sections (10, 29) and adds to the growing evidence that cyclin D3 levels are increased in many types of cancer (6, 29–31). Importantly, we show that cyclin D3 is highly expressed in several melanoma lines including cells that display characteristics of radial growth phase (Sbcl2 and WM35) and do not express B-RAF mutations (Sbcl2). Hence, up-regulation of cyclin D3 may be an early event in melanoma progression. In melanoma cell lines that do not display enhanced cyclin D3 expression, other cell cycle alterations such as expression of low molecular weight forms of cyclin E may play a key role (32).

Cyclin D3 expression was regulated by adhesion and PI-3 kinase but did not require MEK activity. This regulation contrasts with our previous findings on cyclin D1 in melanoma cells showing that MEK-ERK1/2 signaling controls cyclin D1 levels in an adhesion-independent manner (16). Hence, D-type cyclins are differentially regulated in melanoma cells. Recent studies have provided evidence for the PI-3 kinase/Akt pathway positively regulating melanoma tumor development (26, 33–37). Our data indicate that cyclin D3 is likely to be a major target for PI-3 kinase/Akt signaling in melanoma. PI-3 kinase signaling has been shown to regulate cyclin D3 in other cell types (22–24) at the level of translation (25). In our initial experiments, treatment of melanoma cells with the mTOR inhibitor rapamycin also reduced cyclin D3 levels in SK-MEL-28 cells (data not shown). Evidence for an adhesion-PI-3 kinase-cyclin D3 pathway was primarily obtained from WM793 cells but was supported by experiments in additional melanoma cells including SK-MEL-28 and WM278.

The extent to which D-type cyclins have non-redundant functions is an important issue. Deficiency of individual D-type cyclins reduces susceptibility to transformation mediated by different oncopgenes. Loss of cyclin D1 alone is protective against breast tumors induced by oncopgenic forms of Ras and Neu (38); cyclin D3 deficiency in hematopoietic progenitor cells protects against transformation induced by active Notch1 (6). We show that cyclin D3

**FIGURE 5.** Cyclin D3 knockdown reduces melanoma cell S phase entry and proliferation. A, control (Ctrl) or cyclin D3 knockdown WM793 cells were analyzed for incorporation of BrdUrd as determined by immunofluorescence. Total cell number was determined by nuclear staining with Hoechst reagent. Shown are representative images from one of three independent experiments. Insets are the average percentage of positive cells. Scale bars represent 20 μm. B, control and cyclin D3 knockdown WM793 cells were stained with anti-Ki67 antibodies and Hoechst staining. Graphed are the averages and individual data points for percent of Ki67-positive cells from two independent experiments. C, numbers of control and cyclin D3 knockdown cells were counted after 7 and 10 days after transfections. Graphed are the averages and S.D. from three independent experiments.

**FIGURE 6.** Overexpression of cyclin D1 does not recover effects of cyclin D3 knockdown. A, lysates from non-transduced (−), cyclin D1-transduced, and lacZ-transduced WM793 cells were analyzed by Western blotting (WB) for the V5 epitope tag, cyclin D1, and tubulin (as a loading control). The arrow indicates the exogenous cyclin D1. B, V5 antibody immunoprecipitations (IP) were analyzed by Western blotting for CDK4, cyclin D1, and β-galactosidase (β-Gal). C, non-transduced (−), cyclin D1-transduced, and lacZ-transduced WM793 cells were transfected with either non-targeting (Ctrl) or cyclin D3 duplex #1 siRNA for 72 h. Cell lysates were analyzed by Western blotting for levels of cyclin A, cyclin D3, cyclin D1, and tubulin. Shown are the representative results from one of two experiments. The arrow indicates the migration of exogenous cyclin D1.
expression is necessary for efficient entry into S phase and proliferation of melanoma cells; notably this is in addition to a similar requirement for cyclin D1 (16). Also, our data show that overexpression of cyclin D1 does not rescue cyclin D3 knockdown effects. Combined, our findings indicate that cyclin D3 and cyclin D1 serve at least some non-redundant roles in melanoma cells.

Cyclin D3 preferentially partners CDK6, whereas cyclin D1 associates with CDK4. The former finding is consistent with the similar phenotypes observed between CDK6−/− and cyclin D3−/− mice (6, 39). Interestingly, CDK6-cyclin D3 complexes show enhanced resistance to inhibition by p16INK4a (40). Knockdown of CDK6 did not inhibit cell cycle progression possibly due to a redistribution of cyclin D3 to other CDK partners. The finding that D-type cyclins partner with CDK2 was not surprising since others have described the presence of cyclin D1-CDK2 complexes in breast cancer cells (41) and senescent fibroblasts (42). Furthermore, mouse embryonic fibroblasts deficient in both CDK4 and CDK6 are able to proliferate, albeit slower than wild-type counterparts (39); hence, it is possible that cyclin D-CDK2 complexes play an important role in proliferation. In summary, our data show that cyclin D3 is an important regulator of melanoma cell proliferation and together with other studies (11, 43) highlight therapeutic targeting that more than one D-type cyclin may be necessary to efficiently block melanoma growth.

Acknowledgments—We are indebted to the staff of The Birth Place at Albany Medical Center for providing discarded tissue samples for melanocyte isolation. We are very grateful to Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) for the WM35, BCl2, WM115, WM278, and WM793 melanoma cell lines, Dr. Jihe Zhao for cyclin D1 cDNA, and Dr. Michael DiPersio for critically reading this manuscript.

REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
2. Harbour, J. W., and Dean, D. C. (2000) Genes Dev. 14, 2393–2409
3. Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazelii, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1995) Cell 82, 621–630
4. Fantl, V., Stamp, G., Andrews, A., Rosewell, L., and Dickson, C. (1995) Genes Dev. 9, 2364–2372
5. Sicinski, P., Donaher, J. L., Geng, Y., Parker, S. B., Gardner, H., Park, M. Y., Robker, R. L., Richards, J. S., McGinnis, L. K., Biggers, J. D., Eppig, J. J., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1996) Nature 384, 470–474
6. Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowsky, C., Yu, Q., Ferrando, A. A., Levin, S. D., Geng, Y., von Boehmer, H., and Sicinski, P. (2003) Cancer Cell 4, 451–461
7. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
8. Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. (1995) Oncogene 10, 775–778
9. Clark, W. H., Jr., Elder, D. E., Guerry, D. t., Braimian, L. E., Trock, B. J., Schultz, D., Synnestvedt, M., and Halperrn, A. C. (1989) J. Natl. Cancer Inst. 81, 1893–1904
10. Florens, V. A., Faye, R. S., Maelandsmo, G. M., Nesland, J. M., and Holm, R. (2000) Clin. Cancer Res. 6, 3614–3620
11. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, I., Woofendi, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Har-grave, D., Pritchard-Jones, K., Maitland, N., Chenex-Trench, G., Giggins, G., J., Bigner, D. D., Palmieri, G., Gossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Seiler, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) Nature 417, 949–954
12. Aplin, A. E. (2003) J. Biol. Chem. 278, 34548–34554
13. Calipiel, A., Lefevre, G., Pouponnot, C., Mouriaux, F., Eychene, A., Le Cam, L., and Mascarelle, F. (2003) J. Biol. Chem. 278, 42409–42418
14. Bhattacharya, J., You, R., A., Zhu, L., Lin, W., Tichansky, S., A., Misawa-Furihata, A., Kawakami, Y., and Inazawa, J. (2004) Oncogene 23, 8796–8804
15. Boisvert-Adamo, K., and Aplin, A. E. (2006) Oncogene Epub ahead of print PMID: 16547945
22. Feng, L. X., Ravindranath, N., and Dym, M. (2000) *J. Biol. Chem.* **275**, 25572–25576
23. De Vita, G., Berlingieri, M. T., Visconti, R., Castellone, M. D., Viglietto, G., Baldassarre, G., Zannini, M., Bellacosa, A., Tsichlis, P. N., Fusco, A., and Santoro, M. (2000) *Cancer Res.* **60**, 3916–3920
24. Zhu, X., Kwon, C. H., Schlosshauer, P. W., Ellenson, L. H., and Baker, S. J. (2001) *Cancer Res.* **61**, 4569–4575
25. Naderi, S., Gutzkow, K. B., Christoffersen, J., Smeland, E. B., and Blomhoff, H. K. (2000) *Eur. J. Immunol.* **30**, 1757–1768
26. Stahl, J. M., Cheung, M., Sharma, A., Trivedi, N. R., Shanmugam, S., and Robertson, G. P. (2003) *Cancer Res.* **63**, 2881–2890
27. Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A. F., and Zeuthen, J. (1997) *Cancer Res.* **57**, 3660–3665
28. Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K. L., Vinson, V. L., Gumper, K. L., Ellis, L., El-Naggar, A., Frazier, M., Jasser, S., Langford, L. A., Lee, J., Mills, G. B., Pershouse, M. A., Pollack, R. E., Tornos, C., Troncoso, P., Yung, W. K., Fujiib, G., Berson, A., Stock, P., A., and et al. (1997) *Cancer Res.* **57**, 5221–5225
29. Bartkova, J., Zemanova, M., and Bartek, J. (1996) *Int. J. Cancer* **65**, 323–327
30. Russell, A., Thompson, M. A., Hendley, J., Trute, L., Armes, J., and Germain, D. (1999) *Oncogene* **18**, 1983–1991
31. Zhang, J., Hu, S., Schofield, D. E., Sorensen, P. H., and Triche, T. J. (2004) *Cancer Res.* **64**, 6026–6034
32. Bales, E., Mills, L., Milam, N., McGahren-Murray, M., Bandyopadhyay, D., Chen, D., Reed, J. A., Timchenko, N., van den Oord, J. I., Bar-Eli, M., Keyomarsi, K., and Medrano, E. E. (2005) *Cancer Res.* **65**, 692–697
33. Dhawan, P., Singh, A. B., Ellis, D. L., and Richmond, A. (2002) *Cancer Res.* **62**, 7335–7342
34. Li, G., Kalabis, J., Xu, X., Meier, F., Oka, M., Bogenrieder, T., and Herlyn, M. (2003) *Oncogene* **22**, 6891–6899
35. Dai, D. L., Martinka, M., and Li, G. (2005) *J. Clin. Oncol.* **23**, 1473–1482
36. Jetz, A., Howe, J. A., Horn, M. T., Maxwell, E., Yin, Z., Johnson, D., and Kumar, C. C. (2003) *Cancer Res.* **63**, 6697–6706
37. Stahl, J. M., Sharma, A., Cheung, M., Zimmerman, M., Cheng, J. Q., Bosenberg, M. W., Kester, M., Sandirasegarane, L., and Robertson, G. P. (2004) *Cancer Res.* **64**, 7002–7010
38. Yu, Q., Geng, Y., and Sicinski, P. (2001) *Nature* **411**, 1017–1021
39. Malumbres, M., Sotillo, R., Santamaria, D., Galan, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. (2004) *Cell* **118**, 493–504
40. Faast, R., White, J., Cartwright, P., Crocke, L., Sarcevic, B., and Dalton, S. (2004) *Oncogene* **23**, 491–502
41. Sweeney, K. J., Swarbrick, A., Sutherland, R. L., and Musgrove, E. A. (1998) *Oncogene* **16**, 2865–2878
42. Dulic, V., Drullinger, L. F., Lees, E., Reed, S. I., and Stein, G. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11034–11038
43. Rieber, M., and Rieber, M. S. (1999) *Cell Death Differ.* **6**, 1209–1215