UBE1L causes lung cancer growth suppression by targeting cyclin D1

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Abstract
UBE1L is the E1-like ubiquitin-activating enzyme for the IFN-stimulated gene, 15-kDa protein (ISG15). The UBE1L-ISG15 pathway was proposed previously to target lung carcinogenesis by inhibiting cyclin D1 expression. This study extends prior work by reporting that UBE1L promotes a complex between ISG15 and cyclin D1 and inhibited cyclin D1 but not other G1 cyclins. Transfection of the UBE1L-ISG15 deconjugase, ubiquitin-specific protein 18 (UBP43), antagonized UBE1L-dependent inhibition of cyclin D1 and ISG15-cyclin D1 conjugation. A lysine-less cyclin D1 species was resistant to these effects. UBE1L transfection reduced cyclin D1 protein but not mRNA expression. Cycloheximide treatment augmented this cyclin D1 protein instability. UBE1L knockdown increased cyclin D1 protein. UBE1L was independently retrovirally transduced into human bronchial epithelial and increased cyclin D1 protein. UBE1L was independently found by UBE1L-ISG15 preferentially inhibiting cyclin D1. Molecular therapeutic implications are discussed. [Mol Cancer Ther 2008;7(12):3780–8]

Introduction
Lung cancer is the leading cause of cancer-related mortality for men and women in the United States (1). Despite advances in chemotherapy, radiation therapy, and surgery, only a minority of lung cancer patients is cured (1). Novel targets for lung cancer therapy and chemoprevention are needed. Prior work with classic and nonclassic retinoid receptor agonists found G1 cyclins were pharmacologic targets for lung carcinogenesis (2–8). Abrupt expression of cyclins D1 and E in human preneoplastic and malignant lung lesions implicated these species as therapeutic or chemopreventive targets (9).

That aberrant cyclin expression caused lung carcinogenesis was found in transgenic mice with the human surfactant C promoter targeting cyclin E expression in the lung. This caused chromosome instability, hedgehog pathway activation, appearance of pulmonary dysplasia, and multiple lung adenocarcinomas along with other changes that recapitulated features of clinical lung carcinogenesis (10). Together, these findings implicated cyclin deregulation as an early step in lung carcinogenesis and as an antineoplastic target. That view was supported by results of clinical proof-of-principle trials with the epidermal growth factor receptor-tyrosine kinase inhibitor erlotinib or the retinoid X receptor (retinoid) agonist bexarotene where intratumoral repression of cyclin D1 was uncovered as a pharmacodynamic marker of antineoplastic response (11,12). Reduced cyclin D1 expression was also detected in post-treatment versus pretreatment buccal swabs following combined erlotinib and bexarotene treatments (13).

One cancer chemoprevention mechanism already identified involved induced proteasomal degradation of cyclins D1 and E by retinoids and retinoids (3–8). This confers G1 arrest and permits repair of genomic DNA damage by carcinogens (2,3). Another mechanism engaged a previously unrecognized retinoid target gene, which inhibited cyclin D1 (14,15). Microarray analyses of all-trans retinoic acid-treated human bronchial epithelial (HBE) and acute promyelocytic leukemia cells revealed UBE1L (ubiquitin-activating enzyme E1-like) induction (14–16). UBE1L conjugates the IFN-stimulated gene, 15-kDa protein (ISG15), a member of the ubiquitin-like protein family...
which is also retinoid induced (15). UBE1L is located near a chromosome 3 region deleted in lung cancers (17). UBE1L mRNA expression is often reduced in lung cancer cells, but its genomic structure is intact (18, 19). Prior work established UBE1L as a retinoid target gene conferring PML-retinoic acid receptor α repression in acute promyelocytic leukemia cells (14) and reduced cyclin D1 expression in HBE cells (16). These and other findings implicated UBE1L as a growth- or tumor-suppressive species.

This study was undertaken to uncover UBE1L-dependent mechanisms for lung cancer growth suppression. Findings reported here identify a complex between ISG15 and cyclin D1, which provides a basis for UBE1L-mediated inhibition of cyclin D1 protein but not mRNA expression. UBE1L transduction suppressed cyclin D1 expression and growth of HBE and lung cancer cells. In contrast, UBE1L knockdown increased cyclin D1 expression. UBE1L confers growth suppression by preferentially targeting cyclin D1. This was confirmed by UBE1L transfection and treatment with cycloheximide, which increased cyclin D1 protein instability.

To ascertain clinical relevance, immunohistochemical expression profiles of UBE1L, ISG15, cyclin D1, and Ki-67 were each examined in a bexarotene proof-of-principle lung cancer trial. These and other findings highlight UBE1L-ISG15 as a distinct growth-suppressive pathway exerting antineoplastic effects by targeting cyclin D1 for repression. Implications for cancer therapy and chemoprevention are discussed.

Materials and Methods

Cell Culture

BEAS-2B HBE cells were cultured in LHC-9 medium (Biofluids; refs. 2, 3). The H358 lung cancer cell line was cultured in RPMI 1640 (Invitrogen) containing l-glutamine, 10% fetal bovine serum, and 1% antibiotic-antimycotic solution (Cellgro; ref. 11). Cells were incubated at 37°C in a humidified incubator with 5% CO₂. Bexarotene treatments of BEAS-2B HBE and lung cancer cell lines were accomplished as described previously (16, 17). Culture conditions for the murine ED-1 monocyte/macrophage cell line (14–16) were identical with the proteasome inhibitor ALLN was used (24). Quantification of signal intensities was scored as before (15, 16, 22, 23). Lysates were size-fractionated by SDS-PAGE assays before transfer to nitrocellulose membranes (Schleicher and Schuell Bioscience). A polyclonal antibody recognizing the UBE1L amino terminus of UBE1L (14–16) was used for immunoblot and immunohistochemical assays. Other primary antibodies for immunoblot assays included a rabbit polyclonal antibody recognizing cyclin D1 (M-20; Santa Cruz Biotechnology), a murine monoclonal antibody against HA-tagged proteins (Babco), and a goat polyclonal antibody recognizing actin (Santa Cruz Biotechnology). Anti-mouse and anti-rabbit antisera were purchased (Amersham Biosciences) as was anti-goat antisera (Santa Cruz Biotechnology) and these were used as respective secondary antibodies. Membranes used for immunoblot analyses were treated with the MemCode reversible stain (Pierce). Treatment with the proteasome inhibitor ALLN was used (24). Quantification of signal intensities was scored as before (22–24). To assess cyclin D1 protein stability following UBE1L transfection, cells were treated with or without cycloheximide (40 μg/mL), as in prior work (23).

Immunoblot Analyses

Cells were lysed with ice-cold radioimmunoprecipitation assay lysis buffer using established techniques (15, 16, 22, 23). Lysates were size-fractionated by SDS-PAGE assays before transfer to nitrocellulose membranes (Schleicher and Schuell Bioscience). A polyclonal antibody recognizing the UBE1L amino terminus of UBE1L (14–16) was used for immunoblot and immunohistochemical assays. Other primary antibodies for immunoblot assays included a rabbit polyclonal antibody recognizing cyclin D1 (M-20; Santa Cruz Biotechnology), a murine monoclonal antibody against HA-tagged proteins (Babco), and a goat polyclonal antibody recognizing actin (Santa Cruz Biotechnology). Anti-mouse and anti-rabbit antisera were purchased (Amersham Biosciences) as was anti-goat antisera (Santa Cruz Biotechnology) and these were used as respective secondary antibodies. Membranes used for immunoblot analyses were treated with the MemCode reversible stain (Pierce). Treatment with the proteasome inhibitor ALLN was used (24). Quantification of signal intensities was scored as before (22–24). To assess cyclin D1 protein stability following UBE1L transfection, cells were treated with or without cycloheximide (40 μg/mL), as in prior work (23).

Immunoprecipitation and Pull-Down Assays

After BEAS-2B cells were transiently transfected with indicated expression vectors, transfectants were lysed with radioimmunoprecipitation assay buffer for immunoprecipitation or for Ni-NTA-agarose (Invitrogen) pull-down using optimized procedures (22). Anti-HA antibody (Santa Cruz Biotechnology) and protein A/G beads (Santa Cruz Biotechnology) were used. Ni-NTA-agarose pull-down assays were done as described (22).

Translational Research Studies

Paraffin-embedded and formalin-fixed tissues were obtained from an institutional review board-approved proof-of-principle bexarotene lung cancer trial (12). Tissues were examined for cyclin D1 (11–13), UBE1L (16), ISG15 (25), and Ki-67 (11–13) immunohistochemical expression profiles.
Clonal Growth Assays

Clonal growth assays (2) were done using 5 × 10^2 BEAS-2B and 1 × 10^3 H358 cells. These cells were independently engineered to overexpress UBE1L or a control vector. Colonies were treated with bexarotene or vehicle (DMSO) to determine dose-responsive effects. Two weeks later, visible colonies were fixed and stained with Diff-Quik solution (Baxtor) and quantified with the Col Count instrument (Oxford Optronix).

Results

Our prior work implicated UBE1L as a molecular pharmacologic target inhibiting cyclin D1 (16). This provided a mechanism for the hypothesized tumor-suppressive role of UBE1L (17–19, 21, 22). To determine whether UBE1L affected cyclin expression, BEAS-2B cells were cotransfected with UBE1L and independently with cyclin D1, D2, D3, or E. Only cyclin D1 was inhibited by UBE1L and actin expression was unaffected, as quantified in Fig. 1A.

Immunoblot experiments were conducted following transfection of UBP43, the enzyme leading to ISG15 deconjugation (26). Figure 1B examined dose-dependent effects in BEAS-2B cells of transient UBP43 transfection on cyclin D1 protein. Cyclin D1 expression increased as UBP43 transfection dosage increased. Quantifications appear in Fig. 1B. UBE1L transfection inhibited cyclin

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**Figure 1.** Effects of UBE1L on individual cotransfected G1 cyclins in HBE cells. A, effects of UBE1L on individual transiently transfected cyclins D1, D2, D3, and E species. UBE1L transfection (+) or insertless control vector transfection (−) was accomplished as was immunoblot analyses as in Materials and Methods. Actin expression served as a loading control. Quantification of each signal is displayed. The percent change in expression of each of these cyclins relative to actin expression is displayed. B, dose-dependent effects of UBP43 on exogenously expressed cyclin D1 are shown by “+” depicting 0.3 μg transfected UBP43 and “++” depicting 0.5 μg transfected UBP43 expression plasmid. Transfected UBP43 increased cyclin D1 relative to actin protein expression. Quantification of signals is provided. C, effects of UBP43 cotransfection on UBE1L-mediated inhibition of cyclin D1. UBP43 antagonized UBE1L-mediated inhibition of cyclin D1 while having no appreciable effect on actin expression. Quantification of signals is provided. D, effects of UBE1L transfection on transfected HA-tagged cyclin D1 immunoblot expression in the presence (+) or absence (−) of cycloheximide (CHX; 40 μg/mL) treatment. UBE1L destabilized (versus actin control) exogenous cyclin D1 protein detected by an anti-HA antibody. This was enhanced by cycloheximide treatment. Quantification of signals is provided.
D1 expression in BEAS-2B cells, but UBP43 cotransfection antagonized this effect in Fig. 1C. Quantifications are displayed. To establish UBE1L affected cyclin D1 protein stability, UBE1L was cotransfected with HA-tagged cyclin D1 into BEAS-2B cells in the presence and absence of cycloheximide. Figure 1D revealed UBE1L reduces exogenous cyclin D1 protein stability following cycloheximide treatment. UBE1L transfection also reduced endogenous cyclin D1 protein but not mRNA expression in BEAS-2B cells (Supplementary Fig. S1A and B).7

It was hypothesized the ubiquitin-like protein ISG15 would complex with cyclin D1. BEAS-2B cells were transiently transfected with or without cyclin D1 and with or without UBE1L and ISG15 expression vectors, as in Fig. 2. Lysates were subjected to immunoprecipitation before immunoblot analyses. Figure 2 revealed two major conjugates of cyclin D1 (arrows) following cotransfection of ISG15 and the slightly smaller molecular weight species in lanes 1, 5, and 6 represent a nonspecific band. Co-transfection of UBE1L promoted ISG15 cyclin D1 complex formation. Treatment with the proteasome inhibitor, ALLN, stabilized these species. B, independent Ni-NTA pull-down of these tagged ISG15 species. The same sized species as in A were independently identified with this pull-down assay. Cotransfection with UBE1L and ISG15 expression vectors led to ISG15-cyclin D1 complexes. Arrows, positions of ISG15-cyclin D1 complexes. Cyclin D1-HA conjugates were identified with an anti-HA antibody. Arrows, positions of ISG15-cyclin D1 conjugates formed in HBE cells and were inhibited by UBP43 cotransfection. Arrows, positions of ISG15-cyclin D1 conjugates. D, results in C were independently confirmed in HBE cells by Ni-NTA pull-down of these tagged ISG15 species. Cyclin D1-HA conjugates were identified with an anti-HA antibody. Arrows, positions of ISG15-cyclin D1 conjugates. To confirm equal amounts of input protein lysates loaded, MemCode-stained membrane filters were shown. Positions of molecular weight size markers are displayed for each experiment.7

Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Figure 2. Complex between ISG15 and cyclin D1 and the role of UBP43 in inhibiting this in BEAS-2B HBE cells. A, immunoprecipitation with an anti-HA antibody followed by immunoblot with a second anti-HA antibody revealed ISG15-tagged cyclin D1 complexes. Arrows, positions of larger molecular weight cyclin D1 complexes with ISG15 and the slightly smaller molecular weight species in lanes 1, 5, and 6 represent a nonspecific band. Co-transfection of UBE1L promoted ISG15 cyclin D1 complex formation. Treatment with the proteasome inhibitor, ALLN, stabilized these species. B, independent Ni-NTA pull-down of these tagged ISG15 species. The same sized species as in A were independently identified with this pull-down assay. Cotransfection with UBE1L and ISG15 expression vectors led to ISG15-cyclin D1 complexes. Arrows, positions of ISG15-cyclin D1 complexes. Cyclin D1-HA conjugates were identified with an anti-HA antibody. C, UBE1L-dependent ISG15 complex formation with cyclin D1 was inhibited by transfection of the deconjugase, UBP43. Immunoprecipitation with an anti-HA antibody followed by immunoblot with a second anti-HA antibody revealed ISG15-tagged cyclin D1 complexes formed in HBE cells and were inhibited by UBP43 cotransfection. Arrows, positions of ISG15-cyclin D1 complexes. D, results in C were independently confirmed in HBE cells by Ni-NTA pull-down of these tagged ISG15 species. Cyclin D1-HA conjugates were identified with an anti-HA antibody. Arrows, positions of ISG15-cyclin D1 conjugates. To confirm equal amounts of input protein lysates loaded, MemCode-stained membrane filters were shown. Positions of molecular weight size markers are displayed for each experiment.

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Specific lysines in cyclin D1 affect cyclin D1 protein stability (23). Whether a lysine-less cyclin D1 species was
resistant to UBE1L-mediated inhibition of cyclin D1 was studied and found in Fig. 3A. This was an expected outcome because absence of lysine residues prevented ISG15ylation. To confirm if this inhibition involved a complex between ISG15 and cyclin D1, transfected lysine-less cyclin D1 was immunoprecipitated with anti-HA or pulled down with Ni-NTA, respectively, before immunoblotting with an anti-HA antibody. Figure 3B and C revealed that UBE1L inhibition of cyclin D1 depended on conjugation to lysine(s) within cyclin D1.

Effects of UBE1L expression on BEAS-2B HBE and H358 lung cancer cell growth were studied. Retroviral UBE1L expression was independently accomplished in BEAS-2B and H358 cells in Fig. 4A. Exogenous UBE1L reduced endogenous cyclin D1 expression in both transduced cell lines (relative to controls). A limiting dilution clonal growth assay confirmed UBE1L overexpression conferred a marked repression of clonal growth. Representative experiments are displayed in Fig. 4B and C. SDs are provided for each transductant. Similar results were obtained in a replicate experiment (data not shown). BEAS-2B and H358 UBE1L transductants exhibited a significant repression ($P < 0.001$) of clonal growth versus insertless controls. To determine whether knockdown of UBE1L affected cyclin D1 expression, two independent siRNAs targeting UBE1L and a control siRNA were independently transfected into ED-1 murine lung cancer cells (20), which exhibit high basal UBE1L protein expression in Fig. 4D. Over 90% of these cells are transiently transfected (data not shown). Knockdown of UBE1L by these siRNAs increased cyclin D1 immunoblot expression in ED-1 cells in Fig. 4D.

Prior work revealed that prolonged retinoic acid treatment augmented UBE1L expression in BEAS-2B cells (16). Studies were undertaken to assess UBE1L expression after bexarotene treatments of BEAS-2B and H358 cells because bexarotene was also used in a proof-of-principle lung

![Figure 3](image-url)

**Figure 3.** Effects of cyclin D1 lysine mutations on ISG15-cyclin D1 complex formation and consequences of UBE1L transfection on wild-type and lysine-less cyclin D1 species. **A,** wild-type and lysine-less cyclin D1 species were independently transfected into BEAS-2B HBE cells in the presence or absence of cotransfected UBE1L. Compared with effects on wild-type cyclin D1, UBE1L did not appreciably inhibit lysine-less cyclin D1 expression. Quantification of signals is provided. **B,** ISG15-cyclin D1 complexes were detected in wild-type cyclin D1 species (arrows). Immunoprecipitation with an anti-HA antibody followed by immunoblot with a second anti-HA antibody revealed prominent ISG15-tagged complexes detected in HBE cells with transfected wild-type but not lysine-less cyclin D1 species. **C,** results in **B** were confirmed in HBE cells by Ni-NTA pull-down of these tagged ISG15 species. **Arrows,** positions of cyclin D1 species complexed with ISG15. ISG15 complex formation occurred with transfected wild-type but not with lysine-less cyclin D1. In **B** and **C,** positions of molecular weight size markers are displayed. To confirm equal amounts of input protein loaded, MemCode-stained membrane filters were provided.
Bexarotene repressed cyclin D1 protein in BEAS-2B and H358 cells (12). Bexarotene (1 μmol/L) prominently increased UBE1L immunoblot expression following 10 days treatment of BEAS-2B cells versus vehicle treatment in Fig. 5A. Similar findings were obtained in H358 cells (data not shown). β-Actin expression served as loading control. Bexarotene treatment caused a dose-dependent decline of BEAS-2B clonal growth in Fig. 5B. Prior work revealed that cyclin D1 immunohistochemical expression declined when high bexarotene levels were measured in lung tumors (12). Whether this repression occurred with an increased UBE1L expression in bexarotene post-treatment versus pretreatment lung cancer biopsies was studied. ISG15 immunohistochemical expression was similar in post- and pre-bexarotene treatment biopsies of the lung cancer cases in Fig. 6. In contrast, cyclin D1 expression declined in post-treatment versus pretreatment biopsies when high bexarotene plasma (1.49 μmol/L) and intratumoral (0.31 μmol/L) levels were measured (12), as in Fig. 6A. In this case, UBE1L immunohistochemical expression increased with bexarotene treatment and proliferation, as assessed by Ki-67 immunostaining, decreased in post-treatment versus pretreatment biopsies.

Another representative case was examined. The case shown in Fig. 6B had low plasma (0.13 μmol/L) and intratumoral (0.09 μmol/L) bexarotene levels (12). UBE1L and cyclin D1 immunohistochemical expression profiles were not appreciably altered by bexarotene treatment. Repression of Ki-67 immunostaining was not observed. A total of 5 cases were examined, with only 1 having high...
intratumoral bexarotene levels and also regulation of UBE1L, cyclin D1, and Ki-67 expression. The response rate for UBE1L is 20% (95% confidence interval, 1-72). The odds ratio assessing association between intratumoral bexarotene concentration and UBE1L increase is 6 (95% asymptotic confidence interval, 0.1-354.9). This odds ratio indicates a high probability of UBE1L induction in tumors with high bexarotene compared with tumors with low bexarotene levels.

**Discussion**

UBE1L is the ubiquitin-activating E1-like enzyme for ISG15. Prior work implicated the UBE1L-ISG15 pathway as a molecular pharmacologic target (16). The current study advances prior work by reporting that UBE1L directly inhibits cyclin D1 by destabilizing cyclin D1 protein (Figs. 1 and 2). This confers antiproliferative effects in Figs. 4 and 5 and clinical antineoplastic activity in lung cancer in Fig. 6. These observations build on previous work implicating UBE1L as exerting tumor-suppressive effects (16–19). These findings provide a mechanism for UBE1L triggering cyclin D1 repression because a complex forms between cyclin D1 and ISG15 in Fig. 2. The dependency on UBE1L for cyclin D1 complex formation with ISG15 was shown by transfection of the UBE1L-ISG15 deconjugase, UBP43, which inhibited this complex that depended on cyclin D1 lysines as in Fig. 3. An inverse relationship between UBE1L and cyclin D1 expression was found after UBE1L retroviral transduction and UBE1L knockdown experiments in cells, as shown in Fig. 4. Bexarotene treatment of HBE and lung cancer cells as well as clinical lung cancer augmented UBE1L expression in Figs. 5 and 6.

Cyclin D1 is a proposed antineoplastic target (29). Both tumor cell differentiation and growth suppression are linked to induced cyclin D1 degradation (2–8). It is important to elucidate involved mechanisms. Prior work using retinoids as pharmacologic tools uncovered specific cyclin D1 residues regulating proteasomal degradation (23). A retinoic acid receptor agonist and a retinoid X receptor agonist each conferred cyclin D1 proteolysis (3–8). UBE1L is a retinoid target gene (14–16). This study directly linked UBE1L expression to destabilization of cyclin D1 by showing reduced cyclin D1 protein expression followed UBE1L transfection and cycloheximide treatment (Fig. 1D). These findings, coupled to presence of ISG15-cyclin D1 complexes, implicate this complex as regulating cyclin D1 protein stability. These findings add to prior work (16, 29–31) by highlighting UBE1L-dependent mechanisms as affecting cyclin D1 protein.

This UBE1L-ISG15-cyclin D1 pathway is affected by expression of the deconjugase, UBP43, as in Figs. 1 and 2. UBP43 is a proposed pharmacologic target (29, 30). Notably, UBP43 transfection inhibited ISG15 complex formation and reduction of cyclin D1 protein, as in Figs. 3 and 4. It is hypothesized that an inhibitor of UBP43 would promote conjugation of ISG15 to cyclin D1 and enhance cyclin D1 inhibition. Clinical relevance of the UBE1L-ISG15 pathway was shown by results of a proof-of-principle clinical trial (12) where increased UBE1L within bexarotene-treated lung cancer was linked to cyclin D1 inhibition, as in Fig. 6.

Combination therapy is a tenet guiding cancer therapy and chemoprevention (32). An optimal combination regimen is one where a critical oncogenic target, such as cyclin

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**Figure 5.** Effects of bexarotene treatment on UBE1L expression and clonal growth of BEAS-2B HBE cells. **A,** in vitro treatment (+) of BEAS-2B cells with bexarotene (1 μmol/L) for 10 d. This increased UBE1L immunoblot expression versus vehicle-treated (−) cells. Actin expression served as a loading control. **B,** dose-dependent inhibition of BEAS-2B cell growth by bexarotene.
D1, is affected by different pharmacologic mechanisms converging on it. Future work should determine whether cross-talk between ubiquitination and ISG15ylation pathways affect cyclin D1. Preliminary studies indicate that UBE1L transfection affects ubiquitination in HBE cells (data not shown). Pharmacologic targeting of the UBE1L-ISG15 pathway through inhibition of the deconjugase, UBP43, is an appealing approach to repress cyclin D1 protein. Perhaps use of agents that induce cyclin D1 proteasomal degradation or those that inhibit activity of cyclin D1 binding partners, cyclin-dependent kinases 4 and 6, would cause cooperative clinical antineoplastic effects (29). Future work should explore this possibility by testing in proof-of-principle trials agents engaging these cyclin-dependent pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Sutisak Kitareewan (Dartmouth Medical School) for helpful consultation, Dr. Ernest C. Borden (Cleveland Clinic Foundation) for the anti-ISG15 antibody, Dr. Robert M. Krug (University of Texas) for the pcDNA3-UbcH8 expression vector, Dr. Steven Dowdy (Howard Hughes Medical Institute, Washington University School of Medicine) for the HA-tagged pRcCMV-cyclin D1 expression plasmid, and Dr. Zhongze Li (Dartmouth’s Norris Cotton Cancer Center) for assistance in biostatistical analyses.

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Mol Cancer Ther 2008;7:3780-3788.

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