E2F-1 Overexpression in U2OS Cells Increases Cyclin B1 Levels and cdc2 Kinase Activity and Sensitizes Cells to Antimitotic Agents

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Abstract

The E2F transcription factors play a critical role in coordinating transcription of specific genes essential for G_1-S transition. In early G_1, the retinoblastoma protein (pRB) becomes phosphorylated by cyclin-dependent kinases, disrupting pRB binding to E2F-1-3, allowing "free" E2F to regulate genes involved in proliferation. In the present study, we used a tetracycline E2F-1 inducible U2OS osteosarcoma cell line to investigate the effect of increasing levels of E2F-1 on the cytotoxicity of various chemotherapeutic drugs. Upon overexpression of E2F-1, there was no detectable change in cytotoxicity to doxorubicin, cisplatin, 5-fluorouracil, or etoposide. In contrast, overexpression of E2F-1 resulted in a marked increase in sensitivity to vinblastine and paclitaxel, drugs that are known to be more effective against cells in M phase. Therefore, we investigated the effect of E2F-1 overexpression on proteins regulating the G_2-M transition and M phase, in particular cyclin B1 and cdc2 kinase. Cyclin B1 mRNA and protein levels increased within 24 hours of E2F1 induction together with an increase in associated cdc2 kinase activity. Overexpression of cyclin B1 also resulted in a specific increase in sensitivity to paclitaxel and an increase in the cellular growth rate. Knockdown of cyclin B1 using an RNA interference oligo resulted in a slower cellular growth rate and an increase in resistance to paclitaxel. These studies add support to recent reports that show E2F regulates genes involved in mitotic entry and exit and allow the suggestion that mitotic inhibitors may have selective effects in tumors that overexpress E2F-1. (Cancer Res 2006; 66(14): 7253-60)

Introduction

E2F-1 overexpression induces genes involved in DNA synthesis and thus G_1-S transition (1, 2). Recent studies have also shown that the E2F family of proteins regulates an array of genes involved in DNA repair, chromosomal stability, and apoptosis. Microarray experiments in cells that overexpress E2F1 show that this protein also regulates several genes involved in the M phase of the cell cycle (3). The relationship of E2F-1 overexpression to toxicity of chemotherapeutic drugs has yielded varying results depending on the cell line studied. Increased sensitivity to camptothecin and etoposide was shown in NIH-3T3 cells with a constitutive overexpression of NH_2-terminal deletion mutant E2F-1d87, whereas no difference in sensitivity was found between cells that expressed full-length E2F-1 and control cells (4). Another study showed increased sensitivity to etoposide but not to doxorubicin or the topoisomerase I poison topotecan in 32D.3 myeloid progenitor cells overexpressing E2F1 or E2F1/DP1 (4–6). HT-1080 cells with forced overexpression of E2F-1 became more sensitive to the camptothecin derivative SN38, etoposide, and doxorubicin and more resistant to 5-fluorouracil (5-FU; ref. 7). The present study was undertaken to gain insight into the role E2F-1 plays in sensitivity of cells to various classes of chemotherapeutic agents, using a U2OS E2F-1 TET-off inducible system to obviate the problem of clonal variation.

E2F1 overexpression in U2OS-E2F1 cells led to a marked increase in sensitivity to the antimitotic agents paclitaxel and vinblastine. We followed this observation by investigating the role of E2F-1 in regulating entry of cells into mitosis. Mitosis is initiated by activation of the cyclin B1/cdc2 kinase complex. For mitosis to occur, cdc2 must be activated by phosphorylation (8–10). Cytotoxicity of paclitaxel and vinblastine has been shown to be associated with a mitotic arrest and activation of cdc2 precedes induction of paclitaxel and vinblastine mediated apoptosis (11, 12). A dominant-negative mutant of cdc2 was shown to block paclitaxel-induced apoptosis showing the necessity for cdc2 activation to occur before cytotoxicity (13). Therefore, we investigated the effect of E2F-1 overexpression on proteins regulating G_2-M transition and M phase, in particular cyclin B1 and cdc2 to obtain an understanding of the sensitivity data. Cyclin B1 protein levels increased within 24 hours of E2F1 induction together with an increase in cyclin B1 mRNA and associated cdc2 kinase activity. Overexpression of cyclin B1 using stable clones also resulted in a specific increase in sensitivity to paclitaxel and an increase in the cellular growth rate. Importantly, down-regulation of cyclin B1 using an RNA interference (RNAi) oligo resulted in an increase in resistance to paclitaxel and a slower cellular growth rate.

Materials and Methods

Materials. Paclitaxel, cisplatin, and etoposide were obtained from Bristol-Myers Squibb (New Brunswick, NJ). Vinblastine was purchased from Bedford Labs (Bedford, OH). [γ-^{32}P]ATP was purchased from ICN Radiochemicals (Irvine, CA). Monoclonal antibodies to E2F-1 (KH-95), Chk1 (G-4), Chk2 (A-11), p53 (DO-1), Bcl-2 (C-2), Bax, Bcl-$_{Xl/Xs}$, cyclin B1 (GNS1), the cyclin B1–conjugated agarose antibody (GNS1AC), and the cdc2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The P73 and Apaf-1 antibodies were obtained from PharMingen (San Diego, CA). The α-tubulin (Clone B-5-1-2) and c-myc antibodies were obtained from Sigma Biochemicals (St. Louis, MO), as was the ATP for the histone H1 assay. The mpm-2 mouse monoclonal antibody was obtained from Upstate Biotechnology (Waltham, MA). The Histone H1 protein was purchased from

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Roche Biochemicals (Indianapolis, IN). The secondary antibodies for anti-mouse, anti-rabbit, and mouse IGg were obtained from Santa Cruz Biotechnology. The β-actin (Ab-1) antibody for the Taqman assay was purchased from Calbiochem (San Diego, CA).

Cell culture and tet inducible system. The human osteosarcoma cell line U2OS-TA, a U2OS tetracycline-off E2F1 inducible system, was a gift from Dr. Maxwell Sehested (14). The U2OS-TA cell line is referred to as U2OS-E2F1 throughout this article. Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% TET-approved FCS (Clontech, Palo Alto, CA) and 100 units/mL penicillin, 0.1 mg/mL streptomycin, 2.0 mmol/L L-glutamine, 1.0 μg/mL puromycin, and 1.0 μg/mL tetracycline. For all experiments, the U2OS-E2F1 cell line was maintained in 1.0 μg/mL tetracycline without puromycin to prevent interference.

Cytotoxicity assays. Cytotoxicity of drugs was determined by the sulforhodamine B (SRB) assay in 96-well microtiter plates as described previously (15) with some modifications. Cells were plated in triplicates (3,000 per well), and E2F1 was induced by removing tetracycline and 24 hours later were incubated with either paclitaxel, vinblastine, 5-FU, methotrexate, etoposide, doxorubicin, or cisplatin for another 24 hours in the presence and absence of tetracycline. After 24 hours, the cells were washed and incubated in drug-free medium for an additional 96 hours, and cell viability was assayed. For long-term experiments, the same experiment was done, but the drugs were kept in the media for all 5 days of the experiment. Cells were then fixed with 50% trichloroacetic acid for 1 hour, and 0.4% SRB (Sigma, St. Louis, MO) was added to each well. After 30 minutes, the cells were washed with 1% acetic acid. Plates were read using a Bio Whittaker microplate reader (Walkersville, MD) at 570 nmol/L. The wells with cells containing no drugs and with medium plus drug but no cells were used as positive and negative controls, respectively. Data were plotted as percentage of controls.

Western blot analysis. Cell extracts were prepared for each condition and time point. Log phase cells were harvested and washed with PBS. After one freeze/thaw cycle, cells were lysed in buffer containing 50 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 0.5% NP40, and 0.5% sodium deoxycholate, which contained a protease inhibitor cocktail: 10 μg/mL phenanthrolene, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride (BD PharMingen, San Diego, CA). The lysates were kept on ice for 15 minutes, vortexed twice, and centrifuged at 4°C for 10 minutes at 10,000 × g in an Eppendorf microcentrifuge. Supernatants were transferred to sterile tubes, and protein concentrations were determined using the Bradford assay. Cell extracts (50 μg) were electrophoresed using a 10% to 12% polyacrylamide gel containing SDS. After electrophoresis, gels were transferred for 1 hour at 100 mV onto nitrocellulose paper (Scleicher & Schuell, Keene, NH). Subsequently, blots were probed by methodology described previously (16) using primary antibodies and the corresponding secondary antibody. The protein bands were visualized on X-ray film using the enhanced chemiluminescence reagent from Amersham Biosciences (Arlington Heights, IL).

For RNAi experiments, cells were collected at 24-hour intervals, lysed, and analyzed as described above. For cyclin B1 overexpression, several clones that were transduced with either PCDNA 3.1 or PCDNA 3.1/cyclin B1 were analyzed by Western blot analysis. Three clones that contained PCDNA 3 and three clones transduced with PCDNA 3.1/cyclin B1, one of which did not overexpress cyclin B1, were selected, and cell lines were generated.

Transfections and luciferase assays. To assay promoter activity of E2F1 using luciferase, 1×10^5 cells were plated in each well of six-well plates, and cells were allowed to attach for 24 hours in the presence of 1 μg/mL of tetracycline. Subsequently, 2 μg of the PGL2-Basic (promoterless luciferase vector), PGL2-E2F (containing two E2F consensus sites), and the PGL2-mutant E2F (with abolishing mutations in the E2F sites) constructs were separately transfected into the cells over 24 hours using Lipofectin reagent as indicated by the manufacturer (Life Technologies, Rockville, MD). Following transduction, cells were washed five times with fresh complete media, and then media containing 1 μg/mL tetracycline was then added, and the cells were allowed to grow for another 24 hours. Cells were washed five times with fresh media void of tetracycline, and then tetracycline was added to the non-induced cells. Cells were harvested for luciferase analysis 24 hours after induction of E2F1. Cell extracts were prepared, and luciferase activity was determined using a standard luminometer. Experiments were done a minimum of three times in triplicate.

Immunoprecipitation and histone H1 kinase assay. Initially, histone H1 kinase activity was measured using the method of Bortner and Rosenberg with some modifications (17). Cells (1×10^7) were lysed with a solution containing 100 mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 2% NP40, 0.5% sodium deoxycholate, and 0.2% SDS and the protease inhibitor cocktail. After centrifugation at 4°C for 15 minutes, the supernatant was collected and precleared with protein A/G agarose beads for 30 minutes. The precleared protein extracts (200 μg) were combined with lysis buffer and 10 μg of anti-cyclin B1 agarose conjugate (Santa Cruz Biotechnology) and incubated for 1 hour at 4°C with rotation. Agarose beads were collected by centrifugation and washed four times in lysis buffer and once in kinase buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 2 mmol/L EGTA, and 1 mmol/L DTT]. The beads were resuspended in 20 μL of kinase buffer containing 20 μmol/L ATP 100 μg/mL of histone H1 protein (Roche Laboratories, Indianapolis, IN), and 200 μCi/mL [γ-^{32}P]ATP. After incubation for 20 minutes at 30°C, the reaction mixture was then subjected to 7.5% SDS-PAGE followed by autoradiography.

The second method used for measurement of kinase activity used the Promega Signatect cdc2 kinase assay kit, in which one unit of cdc2-activated human kinase was used per reaction as a positive control (preclearing methodology was the same as above). Twice the amount of antibodies and lysates were used for this experiment; half of the lysate was used for Western blot analysis, whereas the other half was used for kinase assays. Lysates were immunoprecipitated as above except at the last step; half of the beads were used in the kinase reaction using a mixture that contained 0.5 mmol/L ATP, 10 μCi/μL [γ-^{32}P]ATP (3,000 Ci/mmol), cdc2 kinase assay buffer, immunoprecipitated lysates, or pure kinase, and the biotinylated histone peptide and inhibitors when necessary. The reaction mix was incubated for 10 minutes at 30°C, and the reaction was terminated by adding 7.5 mol/L guanidine hydrochloride; 15 μL of reaction mixture was spotted onto each streptavidin matrix membrane and allowed to dry, and radioactivity measured using a standard scintillation counter. Pmol/min of 10 μCi/μL [γ-^{32}P]ATP (3,000 Ci/mmol) incorporated was calculated as described in the manual.

Taqman mRNA quantitation. Cells were plated and grown for various intervals of time (0, 6, and 24 hours) with and without tetracycline. Subsequently, cells were harvested and lysed; mRNA was recovered and reverse transcribed using an Invitrogen cDNA cycle kit. Cyclin B1 mRNA message was quantitated relative to β-actin levels using primer and probes specific for cyclin B1 and β-actin. PCR was done using a Taqman 1000 RXN Gold with buffer A kit using the ABI prism 7700 thermocycler (Taqman, Applied Biosystems, Foster City, CA). The temperature variables used were 95°C (one cycle) followed by T1 (95°C, 15 seconds) and T2 (60°C, 60 seconds; 42 cycles). The primer and probe sequences are as follows: (*a*) Taqman probe, 5′-TCAACCTCTCCAATCTTAGATGCTCTCCGA-3′; (*b*) forward PCR primer, 5′-GGCCTCTACCTTTGCACTTCC-3′; (*c*) reverse PCR primer, 5′-GGCCAAAGTATGTTGCTCGAC-3′. RNAi transduction and clonogenic assay. Cyclin B1 RNAi was designed using software provided by Dharmacon RNA Technologies (Lafayette, CO), which resulted in two potential RNAi oligos with the following sequences: oligo 1, (*a*) sense sequence UAAGGCGAAGAUCAACAUGdtdt and (*b*) antisense sequence CAUGUUGAUCUUCGCCUUAdtdt; oligo 2, (*a*) sense sequence ACAUGAGAGCCAUCCUAAUdtdt and (*b*) antisense sequence AUUAGGAUGGCUCUCAUGUdtdt. Each oligo was diluted with the dilution buffer provided by Dharmacon to a stock concentration of 20 μmol/L. For mock transductions, no RNAi oligo was added, but an equal amount of oligofectamine (Life Technologies) was added. For oligo 1 and oligo 2, 60 μL of each oligo was added to 1,050 μL of Opti-MEM (Life Technologies), and in a separate reaction, 36 μL of oligofectamine was added to 54 μL of Opti-MEM. After 15 minutes, these reactions were combined and allowed to incubate for another 15 minutes and then added to 6×10^5 U2OS-E2F1 cells washed twice with Opti-MEM void of tetracycline. Four hours later, RPMI containing 1 μg/mL of tetracycline and thrice the normal amount of fetal

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bovine serum was added to the 100 mmol/L plate containing the cells and incubated for 24 hours. Cells were trypsinized, counted, and replated (350 per well) in six-well plates and were allowed to settle overnight.

Generation of cells overexpressing cyclin B1. The full-length cyclin B1 cDNA was excised from Bluescript with *Hin*dIII and *Xba*I and was cloned into PCDNA 3.1 using the same restriction sites. U2OS parental cells were plated in 150 mmol/L dishes and then subsequently transduced with either the PCDNA 3.1 empty vector or PCDNA 3.1/cyclin B1 using *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate (Roche Laboratories, Indianapolis, IN) as suggested by the manufacturer. Twenty-four hours after transduction, cells from each plate were divided into three additional plates and allowed to grow for 24 hours. Next, the medium was changed with the addition of 800 μg/mL of G418 (Life Technologies). After 10 days, clones were selected and analyzed by Western blot analysis as described. For growth studies, 25,000 cells were plated in six-well plates and were counted at varying time points using a Coulter counter. All experiments were repeated a minimum of three times.

Statistical analysis. Statistical analyses were done using the Student's *t* test for unpaired data. $P < 0.05$ was considered significant.

Results

Induction of E2F-1 in a U2OSE2F-1 TET-off inducible cell line results in an increase in sensitivity to paclitaxel and vinblastine. The E2F-1 inducible clone was characterized for expression dynamics, control of expression by tetracycline and E2F-1 transcriptional activity. Removal of tetracycline from the media caused a marked increase in E2F-1 protein expression as measured using Western blot analysis. An increase in E2F-1 expression was detectable as early as 1 hour after induction and peaked at 24 hours and was sustained up to 72 hours (Fig. 1). Concentrations of tetracycline as low as 2 ng/mL inhibited expression (data not shown). To show that the increased levels of E2F-1 correlated with an increase in transactivation activity of E2F-1, three constructs, PGL2-basic, PGL2-E2F [containing two E2F consensus sequences from the dihydrofolate reductase (DHFR) promoter], and PGL2-E2Fmut (containing mutations which abolished the E2F consensus sites), were used. Overexpression of E2F-1 resulted in a 5-fold increase in luciferase activity, whereas the mutant constructs did not show any increase in luciferase activity (data not shown). To examine the effect of overexpression of E2F-1 on drug sensitivity, log phase cells were washed five times with medium void of tetracycline, and 2,000 cells per well were plated in 96-well microtiter plates and incubated with or without antibiotic for 24 hours followed by incubation with either doxorubicin, cisplatin, methotrexate, 5-FU, or etoposide for 5 days. For cytotoxicity experiments using vinblastine and paclitaxel, the drugs were added for 24 hours with or without tetracycline and then washed out with fresh medium and incubated in drug-free media for an additional 96 hours. For all drugs, cell viability was assessed by the SRB assay. Although no difference in sensitivity was noted between cells induced and non-induced for doxorubicin, cisplatin, methotrexate, 5-FU, or etoposide (data not shown), overexpression of E2F-1 was associated with a marked increase in sensitivity to both vinblastine and paclitaxel when compared with non-induced controls (Fig. 2). Parental U2OS cells were used in similar experiments with and without tetracycline using cisplatin and paclitaxel to be certain that the effects observed were specific. The addition or removal of antibiotic did not alter the dose-response curves (data not shown). Thus, overexpression of E2F-1 resulted in increased sensitivity only to the antimitotic drugs vinblastine and paclitaxel.

Induction of E2F-1 expression correlates with increased cyclin B1 expression and associated CDC2 kinase activity. The finding that overexpression of E2F1 specifically sensitized cells to mitotic inhibitors indicated that E2F1 may be involved in mitosis. To examine if the increased sensitivity to paclitaxel and vinblastine upon E2F-1 overexpression correlated with expression of cyclin B1 levels, cells were plated and E2F-1 induction was blocked with 1 μg/mL of tetracycline for 24 hours. After 24 hours, the antibiotic was removed, and cells were collected 12, 24, and 36 hours later; cyclin B1 levels were measured by Western blot analysis (Fig. 3). Twelve hours after induction, E2F-1 levels increased when compared with the corresponding control and reached maximum at 24 to 36 hours. In parallel, cyclin B1 levels increased at every time point in which E2F-1 expression increased and were sustained throughout the time course experiment. CDC2 expression levels did not change throughout the time course.

As CDC2 expression levels did not change after E2F-1 induction, we determined whether the increase in cyclin B1 protein levels correlated with an increase in cyclin B1–associated CDC2 kinase activity. Cells were plated as described earlier, and 24 hours later, E2F1 was induced, and cells were collected 24 hours after induction. Twenty-four hours after induction of E2F-1, the increase in cyclin B1 protein levels was associated with a 6-fold increase in cyclin B1–associated CDC2 kinase activity (Fig. 4).

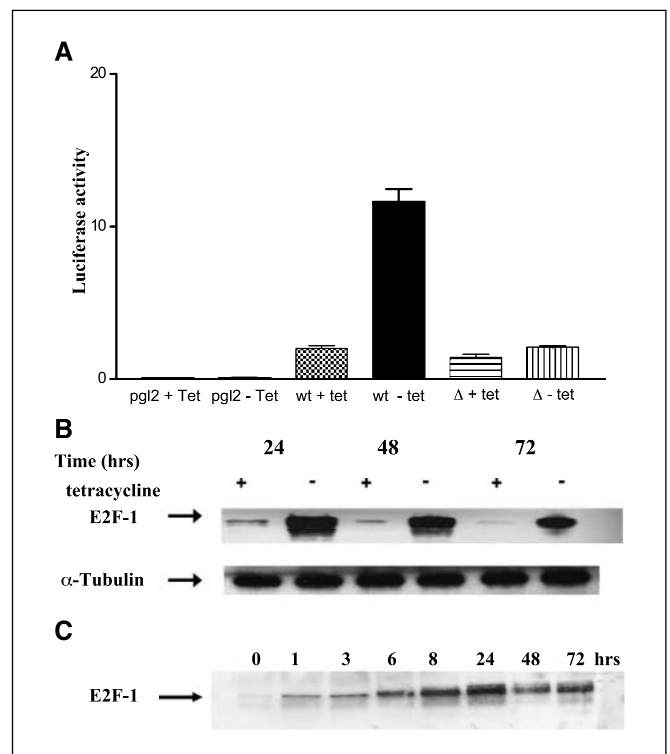

Figure 1. Characterization and dynamics of E2F-1 induction. *A,* U2OS-E2F-1 cells were transfected using Lipofectin reagent (Life Technologies) with PGL2-basic, PGL2-E2F-E2F(wt) (TTTCGCGC, TTTCGCGC), and PGL2-Mutant-Mutant (Δ) (GGATCTCC, GGATCTCC) constructs for 24 hours followed by induction of E2F-1 for 24 hours and subsequent analysis for luciferase activity using a luminometer. *B,* U2OS-E2F-1 cells were plated in 10-cm dishes and allowed to attach for 24 hours in the presence of 1 μg/mL of tetracycline. Subsequently, cells were washed five times with fresh complete medium void of tetracycline; tetracycline (1 μg/mL) was added to the control non-induced cultures. *C,* E2F-1 induction time course using earlier time points.

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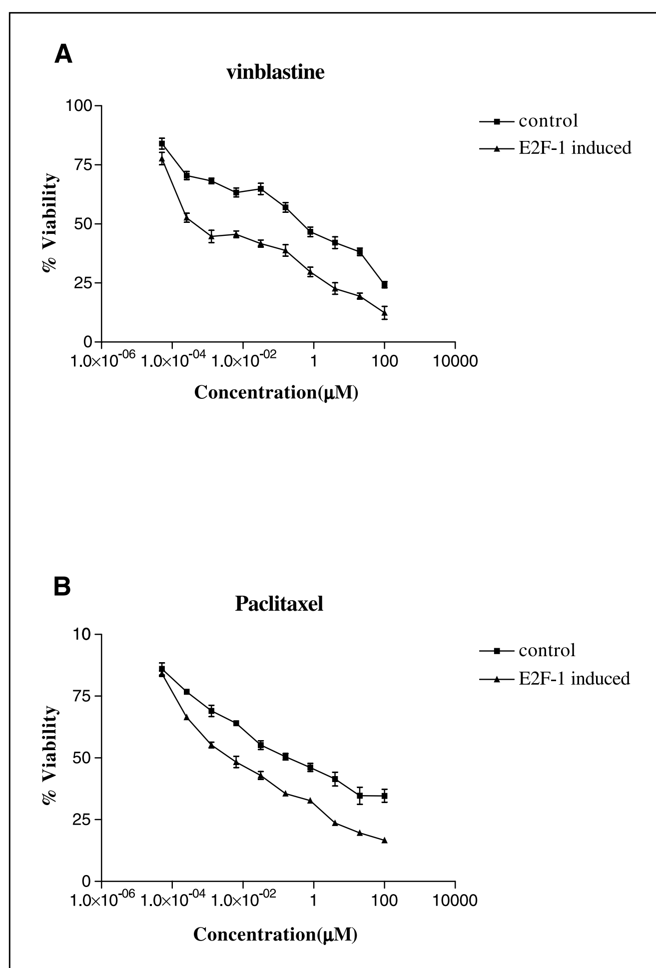

Figure 2. Induction of E2F-1 leads to increased sensitivity to vinblastine and paclitaxel. U2OS-E2F-1 cells were plated in 96-well microtiter plates, and E2F-1 was induced for 24 hours. Subsequently, paclitaxel or vinblastine was added at varying concentrations for 24 hours and then washed out with fresh media, and cells were allowed to grow for 4 days. Cytotoxicity was measured using the SRB assay. *Points,* average of three separate experiments; *bars,* SD. Student's *t* test for unpaired samples for both vinblastine and paclitaxel curves was determined ($P < 0.05$).

Overexpression of cyclin B1 in U2OS cells results in faster growth and an increase in sensitivity to paclitaxel. To determine if the increase in sensitivity to the antimototic agents vinblastine and paclitaxel was mediated by cyclin B1, two cyclin B1–overexpressing cell lines were created. First, the full-length cyclin B1 cDNA was cloned into the PCDNA 3.1 vector as described in Materials and Methods. Next, both the PCDNA 3.1 empty vector and the PCDNA 3.1/cyclin B1 vector were separately transduced into U2OS parental cells. Three clones that were transduced with the PCDNA 3.1 vector alone were selected that had similar levels of cyclin B1. Two clones transfected with PCDNA 3.1/cyclin B1 that overexpressed levels of cyclin B1 and one clone that was transduced with PCDNA 3.1/cyclin B1 but had control levels of cyclin B1 were also selected and cell lines established (Fig. 5*A*). The cyclin B1 overexpressors (clones 7 and 8) showed a faster growth rate (Fig. 5*B*). Clones 1, 2, 3, and 9 showed similar growth dynamics (doubling times = 30, 32, 31, 31 hours, respectively). Whereas clones 7 and 8 showed a marked decrease in doubling time (24 and 22 hours, respectively; $P < 0.05$ for each comparison of clone 7 and 8 with either clones 1, 2, or 3). All six clones used in the above analysis [clones 1, 2, and 3 (PCDNA 3.1) and clones 7, 8, and 9 (PCDNA 3.1/cyclin B1)] were used in a cytotoxicity study to determine if heightened levels of cyclin B1–sensitized cells to the antimitotic agents paclitaxel and vinblastine. Cells were plated in 96-well microtiter plates. Subsequently, 24 hours later, either paclitaxel, vinblastine, or etoposide was added to the plates for each clone separately at varying concentrations; washed out after a 24-hour incubation; and allowed to grow for an additional 4 days. Cytotoxicity was measured using the SRB methodology. Clones 7 and 8 expressing 3- to 4-fold higher levels of cyclin B1 compared with controls showed a marked increase in sensitivity to paclitaxel, whereas the control clones (1, 2, and 3) and the PCDNA 3.1/cyclin B1 clone expressing control levels of cyclin B1 were less sensitive (Fig. 5*C*). Thus, overexpression of cyclin B1 resulted in an increase in sensitivity to paclitaxel and vinblastine but not etoposide, indicating that the increase in sensitivity to paclitaxel is mediated by the level of cyclin B1.

Knockdown of cyclin B1 levels by RNAi results in a decrease in sensitivity to paclitaxel and a decrease in the rate of cell growth of U2OS-E2F1 cells. If cyclin B1 levels are a determinant of sensitivity to antimitotic agents, knockdown of this protein using RNAi would be expected to decrease sensitivity to paclitaxel. U2OS E2F-1 cells (with TET) were plated in 100-mm dishes and either mock transfected with oligofectamine alone or with oligo 1 or oligo 2, two RNAi oligos specifically targeting cyclin B1 RNA. Twenty-four hours after transduction, cells were trypsinized, counted, and replated. One aliquot was counted for the 24-hour time point, and every 24 hours, cells were collected, lysed, and analyzed using Western blot analysis with antibodies specific to cyclin B1 and α-tubulin (loading control). Transduction of oligo 1 did not result in any significant changes in cyclin B1 protein levels, whereas transduction with oligo 2 markedly decreased cyclin B1 protein levels at 24 to 72 hours. At the 96- and 120-hour time points, cyclin B1 protein levels recovered (Fig. 6*A*). Transduction of U2OS-E2F1 cells with oligo 2 significantly inhibited (doubling time = 30 hours for mock, 30 hours for oligo 1, and 25 hours for oligo 2) the growth of the cells compared with the mock- and oligo 1–transduced cells (*P* = not significant for mock versus oligo 1 and $P < 0.05$ for mock versus oligo 2; Fig. 6*B*). To investigate if

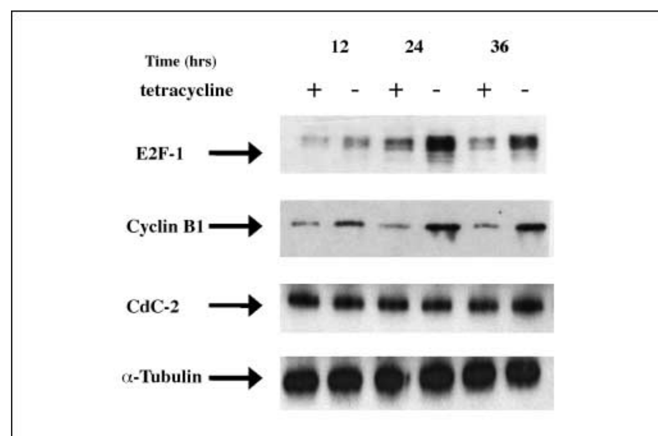

Figure 3. Induction of E2F-1 expression correlates with increased cyclin B1 expression. U2OS-E2F1 cells were plated, and the induction of E2F-1 was blocked with 1 μg/mL of tetracycline for 24 hours. After 24 hours, the tetracycline was removed by washing the cells with fresh complete media as before (Fig. 1); the cells were collected 12, 24, and 36 hours later in the presence or absence of tetracycline; and lysates were prepared for Western blot analysis. Expression of α-tubulin protein served as a loading control.

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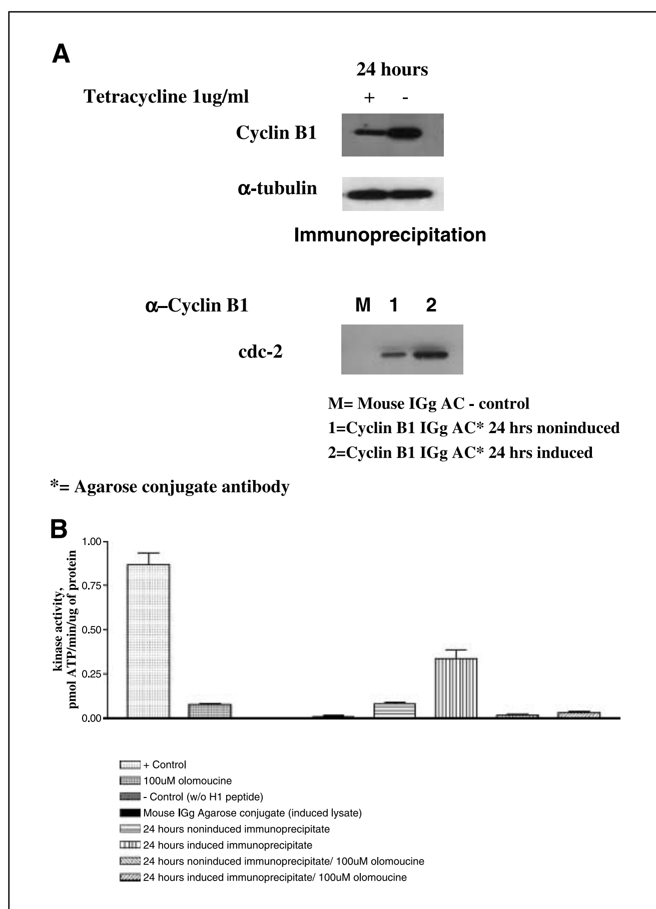

Figure 4. Induction of E2F-1 leads to an increase in cyclin B1/cdc2–associated kinase activity. Lysates from U2OS-E2F-1 cells that were either induced or non-induced for 24 hours were used. Subsequently, cells were collected, lysed, and processed for immunoprecipitation using a control mouse IgG agarose–conjugated antibody, or a cyclin B1 agarose–conjugated antibody. Double the amounts antibodies and lysates were used, and half was used for Western blot analysis, whereas the other half was used for kinase assays. *A,* immunoprecipitation with the mouse control antibody (using induced lysates) did not result in any signal while with the cyclin B1 antibody; cdc-2 was clearly detected, and both were slightly increased in the induced immunoprecipitates. *B,* immunoprecipitates were processed for cdc2-associated kinase activity using a cdc2 kinase assay kit (see Materials and Methods). Immunoprecipitates were combined with reaction mixtures containing biotin-labeled histone H1 that was captured using paper squares containing streptavidin. Subsequently, each reaction was measured for γ-^{32}P activity using a scintillation counter. Induction of E2F-1 resulted in approximately a 6-fold increase in cdc2 kinase activity, which was blocked by the cdc2 kinase inhibitor olomoucine (100 μmol/L).

down-regulation of cyclin B1 also affected the sensitivity of U2OS-E2F1 cells to paclitaxel, cells were mock-transduced, or transduced with either oligo 1 or oligo 2. Twenty-four hours after transduction, 350 cells per well were replated into six-well plates and allowed to attach. Subsequently, cells were exposed to various concentrations of either paclitaxel or etoposide for 24 hours and then washed and allowed to grow for an additional 8 days. Cells were then stained with crystal violet, and colonies were counted. Down-regulation of cyclin B1 levels, using oligo 2, resulted in a significant decrease in sensitivity to paclitaxel but not etoposide, compared with the mock transduction control and oligo 1 (Fig. 6*C*).

E2F-1 expression increases the levels of cyclin B1 mRNA. We next explored the mechanism by which E2F-1 induction caused an increase in cyclin B1 protein levels. U2OS-E2F1 cells were grown in media with or without 1 μg/mL of tetracycline for up to 24 hours. At each time point, cells were harvested and washed with PBS, and cyclin B1 mRNA was quantitated by real-time reverse transcription-PCR. Six hours after induction of E2F1, cyclin B1 mRNA levels slightly increased, but after 24 hours, mRNA levels markedly increased when compared with non-induced controls (Fig. 7).

Discussion

The role of the E2F family members in the cell cycle has been studied extensively(2, 18–20), implicating E2F1-3 in the G_1-S transition via regulation of cell cycle genes (e.g., *TS, DHFR, cyclin A*, and *cyclin E*). In some cells, high levels of E2F-1 have also been shown to induce apoptosis via both p53-dependent and p53-independent mechanisms (2, 18, 19, 21). Most human malignancies express high levels of E2F1, either related to lack of the retinoblastoma protein (pRB), or even in the presence of wild-type pRB. For example, we found that 87% of metastatic colorectal cancers had high levels of E2F1, associated with amplification of the gene coding for this protein (22).

As there have been conflicting reports relating the consequences of overexpression of E2F1 on chemosensitivity, we used a TET-off inducible system to regulate E2F1 levels. A consequence of E2F1 induction in the U2OS-E2F1 cell line was a specific increase in sensitivity to the antimitotic agents vinblastine and paclitaxel without an increase in sensitivity to etoposide, doxorubicin, 5-FU, or cisplatin. In contrast, E2F-1-forced overexpression in the HT-1080 cell line was shown to cause an increase in sensitivity to etoposide and doxorubicin (7). The U2OS cell line contains wild-type p53 and mutated $p14^{ARF}$, whereas the HT 1080 cell line has a mutant p53 and also lacks $p14^{ARF}$. It is likely that drug sensitivity may be cell line specific and thus dependent upon the genetic background of the cell.

In an attempt to understand this increase in sensitivity to antimitotic agents, we studied the relationship between E2F-1 and cyclin B1, because activation of the cyclin B1/cdc2 kinase complex is a key regulatory step for cells to enter the mitotic phase of the cell cycle (10). Overexpression of E2F1 resulted in a sustained and marked increase in cyclin B1 protein levels without an increase in cdc2 protein levels. Increased levels of cyclin B1 protein and associated cdc2 kinase activity correlated with antimitotic agent–induced apoptosis (23); accordingly, the increase in cyclin B1 and associated cdc2 kinase activity observed as a consequence of E2F1 overexpression may explain the sensitivity of these cells to antimitotic agents. Experiments were done to assess the differences in the percentage of cells in M phase in the presence of antimitotic agents. Induction of E2F-1 after pretreatment with 50 nmol/L paclitaxel resulted in sustained expression of cyclin B1 and an increase in the relative amount of cells in the M phase. At 8 hours after induction, a insignificant number of cells were in the M phase. However, 24 and 36 hours after induction, induction of E2F-1 was associated with sustained induction of cyclin B1 and correlated with a >5-fold increase in the relative increase in the relative amount of cells in the M phase as measured by the levels of mpm-2 phosphorylated protein levels (data not shown). In addition, induction of E2F-1 in the presence of paclitaxel resulted in an increase in mitotic cells and sustained mitotic arrest up to 48 hours as measured by the mitotic index (data not shown). This suggests that specific alterations in fidelity of the M phase of the cell cycle may become compromised when E2F-1 is overexpressed for long periods of time, and this warrants further investigation.

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p73 and p53 have been shown to be regulated by E2F1, but neither protein was up-regulated by E2F-1 in this cell line (24–26). Bcl-2 phosphorylation and up-regulation of Bcl-2 family members (e.g., Bax and Bad) have also been associated with antimitotic agent cytotoxicity (27, 28). Induction of E2F1 either in the presence or absence of paclitaxel did not result in an increase in the levels of Bax, or Bcl_{xl}; however, Bcl-2 levels and phosphorylation of Bcl-2 increased (data not shown). Whether or not phosphorylation of Bcl-2 is the cause of the cytotoxicity to antimitotic agents or is a downstream effect is not clear.

As expected, forced overexpression of cyclin B1 resulted in an increase in sensitivity of the cells to paclitaxel and an increase in growth rate. Importantly, knockdown of cyclin B1 caused a decrease in sensitivity to paclitaxel, supporting the premise that the increased sensitivity to antimitotic agents when E2F-1 is induced is mediated through cyclin B1.

Several reports indicated that E2F1 may also regulate genes involved in G_2-M transition and in microtubule dynamics (3) and genes involved in chromatin assembly/condensation, chromosome segregation, and the mitotic spindle checkpoint (29). Of particular

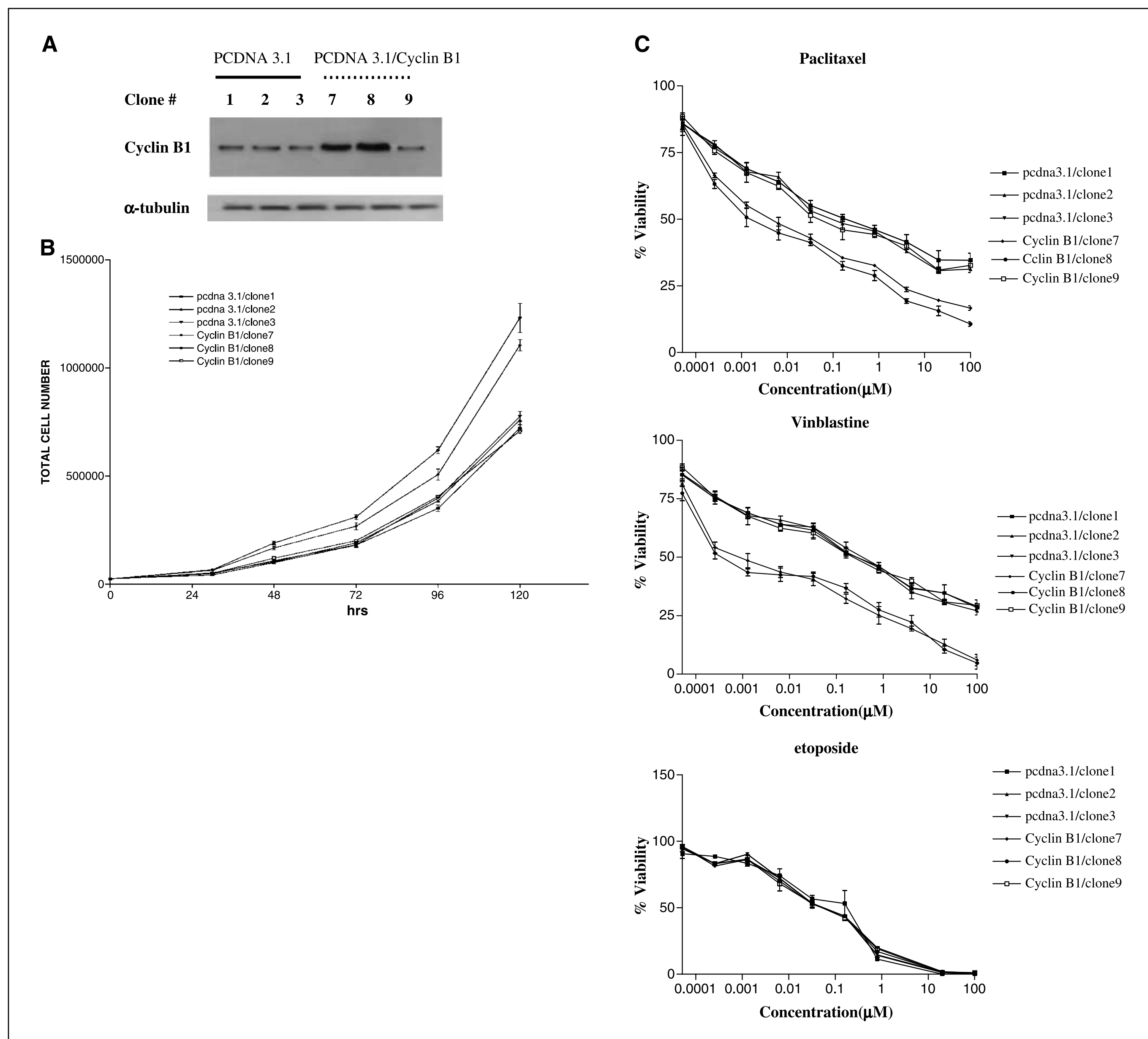

Figure 5. Cells overexpressing cyclin B1 possess a faster growth rate and are more sensitive to paclitaxel in comparison to PCDNA 3.1 control cells. U2OS parental cells were transduced either with PCDNA 3.1 as a control or with cyclin B1 ligated into the PCDNA 3.1 vector. Cell lines were established for each condition (*A*) Western blot analysis showing the levels of cyclin B1 in cell lines from three clones transduced with PCDNA3.1 and 3 clones transduced with PCDNA 3.1/cyclin B1 (clone 9 was transduced with PCDNA 3.1/cyclin B1 but showed similar levels of expression as the PCDNA 3.1 controls). *B,* all six cell lines used in (*A*) were used in a growth assay. Cells were plated in 100-mm plates and followed every 24 hours up to 120 hours, and cell number was counted. *C,* U2OS-cyclin B1 overexpressors (clones 7, 8, and 9) or PCDNA 3.1 control cells were plated in 96-well microtiter plates. Subsequently, 24 hours later, either paclitaxel, vinblastine, or etoposide was added to the plates at varying concentrations and washed out after a 24-hour incubation, and the cells were allowed to grow for an additional 4 days. Cytotoxicity was measured using the SRB assay. Each experiment was done three separate times. *Points,* mean; *bars,* SD. $P < 0.05$ for each comparison of clones 7 and 8 with either clones 1, 2, or 3.

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Figure 6. Knockdown of cyclin B1 hinders growth of U20S-E2F-1 cells and results in decreased sensitivity to paclitaxel. *A,* U2OS-E2F-1 cells were plated in the presence of 1 μg/mL of tetracycline, and 24 hours later, cells were transduced using oligofectamine reagent. Cells were mock transduced (without RNAi) and transduced with either oligo 1 or oligo 2. Twenty four hours later, cells were trypsinized, counted, and replated. Every 24 hours, cells were collected and lysed, and Western blots were done with antibodies specific to cyclin B1 and α-tubulin (loading control). *B,* U2OS-E2F-1 cells were plated in the presence of 1 μg/mL of tetracycline, and 24 hours later, cells were transduced using oligofectamine reagent. Cells were mock transduced (without RNAi) and transduced with either oligo 1 or oligo 2. Twenty-four hours later, cells were trypsinized, counted, and replated. Every 24 hours, cells were collected and counted. *C,* U2OS-E2F-1 cells were plated in the presence of 1 μg/mL of tetracycline, and 24 hours later, cells were transduced using oligofectamine reagent. Cells were mock transduced (without RNAi) and transduced with either oligo 1 or oligo 2. Twenty four hours later, cells were trypsinized, counted, and replated in six-well plates and allowed to attach overnight. Subsequently, either paclitaxel or etoposide was added at varying concentrations and washed out after a 24-hour incubation, and cells were allowed to grow for an additional 8 days. Colonies were stained with crystal violet and then counted. *Points,* mean of three separate experiments; *bars,* SD. For paclitaxel, *P*s were determined: oligo 1 versus mock (*P* = not significant) and oligo 2 versus mock (*P* < 0.05). For etoposide, differences were not significant.

relevance to the present study was the detection of increased expression levels of *cyclin B1* as one of the many genes whose expression levels were increased in cells overexpressing E2F1 as seen in a cDNA microarray analysis (3). This laboratory also reported that both positive and negative regulatory promoter elements were present in the *cyclin B1* gene and in the *cdc2* gene (20). We also found a consensus E2F-1 binding site in the cyclin B1 promoter, and promoter studies showed that the increase in cyclin B1 expression shown after E2F1 induction was due to increased transcription, indicating that the E2F site was functional. In addition, using RNAi to inhibit the expression levels of *B-Myb*, an E2F-regulated gene that is expressed at G_1-S, they found that down-regulation of B-Myb was associated with a decrease in the expression of cyclin B1 and cdc2, providing a link between E2F regulation of G_1-S and G_2-M. Although these studies provide important insights into the control of G_1-S and G_2-M, further studies are necessary to clearly elucidate the key elements involved in the connection between these two phases of the cell cycle.

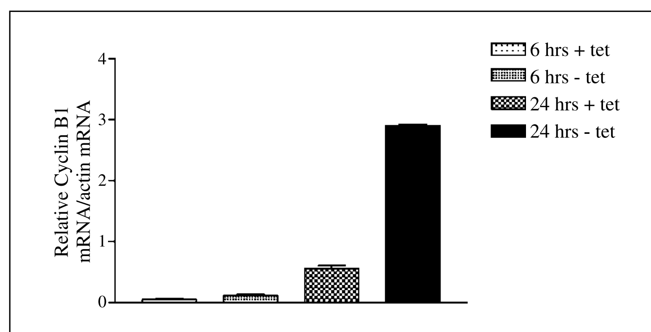

Figure 7. Overexpression of E2F-1 upregulates cyclin B1 mRNA levels. U2OS-E2F1 cells were plated in the presence and absence of tetracycline and allowed to grow for time periods up to 24 hours. Subsequently, mRNA was extracted and reverse transcribed. The relative message quantity was analyzed by Taqman reverse transcription-PCR, using probe and primers specific for cyclin B1 cDNA and normalized to actin message levels. *Columns,* average values of three experiments; *bars,* SD.

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Taxanes and *Vinca* alkaloids are used to treat a variety of human cancers, in particular patients with breast cancer. It is of interest that almost half of breast cancers overexpress E2F1 (30). Although many factors determine drug response, the levels of E2F1 and cyclin B1 expression may be an important determinant of response of cancer patients to taxanes and or *Vinca* alkaloids.

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