Combined Depletion of Cell Cycle and Transcriptional Cyclin-Dependent Kinase Activities Induces Apoptosis in Cancer Cells

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Abstract

Selective cyclin-dependent kinase (cdk) 2 inhibition is readily compensated. However, reduced cdk2 activity may have antiproliferative effects in concert with other family members. Here, inducible RNA interference was used to codeplete cdk2 and cdk1 from NCI-H1299 non–small cell lung cancer and U2OS osteosarcoma cells, and effects were compared with those mediated by depletion of either cdk alone. Depletion of cdk2 slowed G_1 progression of NCI-H1299 cells and depletion of cdk1 slowed G_2-M progression in both cell lines, with associated endoreduplication in U2OS cells. However, compared with the incomplete cell cycle blocks produced by individual depletion, combined depletion had substantial consequences, with G_2-M arrest predominating in NCI-H1299 cells and apoptosis the primary outcome in U2OS cells. In U2OS cells, combined depletion affected RNA polymerase II expression and phosphorylation, causing decreased expression of the antiapoptotic proteins Mcl-1 and X-linked inhibitor of apoptosis (XIAP), effects usually mediated by inhibition of the transcriptional cdk9. These events do not occur after individual depletion of cdk2 and cdk1, suggesting that reduction of cdk2, cdk1, and RNA polymerase II activities all contribute to apoptosis in U2OS cells. The limited cell death induced by combined depletion in NCI-H1299 cells was significantly increased by codepletion of cdk9 or XIAP or by simultaneous treatment with the cdk9 inhibitor flavopiridol. These results show the potency of concomitant compromise of cell cycle and transcriptional cdk activities and may guide the selection of clinical drug candidates. (Cancer Res 2006; 66(18): 9270-80)

Introduction

The cyclin-dependent kinases (cdk) comprise a family divided into two groups based on their roles in cell cycle progression and transcriptional regulation (1, 2). Members of the first group form the core components of the cell cycle machinery, including cyclin D–dependent kinases 4 and 6, cyclin E-cdk2 complexes, cyclin A–dependent kinases 2 and 1, and cyclin B-cdk1 complexes, which facilitate the transition between cell cycle phases. The second group includes cyclin H–dependent kinase 7 and cyclin T–dependent kinase 9 (p-TEFb), which phosphorylate the COOH-terminal domain (CTD) of RNA polymerase II and promote initiation and elongation of nascent mRNA transcripts, respectively. Some of the cdks are involved in both cellular processes. For example, cyclin H-cdk7 is a component of cdk-activating kinase and contributes to cell cycle progression as well as transcription. Cyclin E-cdk2 and cyclin B-cdk1 have been shown to phosphorylate the CTD *in vitro* (3, 4) and therefore may play a role in transcriptional regulation at specific cell cycle phases.

Cdk2 was initially considered a viable antineoplastic drug target, because several approaches inhibiting its activity in cancer cells have induced perturbations during multiple cell cycle phases as well as apoptosis. For example, the inducible expression of a dominant-negative cdk2 mutant in U2OS osteosarcoma cells led to G_2 arrest after low-level induction and arrest during both S and G_2 phases when higher levels were induced (5). Effects on G_1 progression also occurred but were weak and only unmasked when cells were synchronized and released from a nocodazole-induced mitotic block. In exponentially growing transformed cell lines, S and G_2 cell cycle effects have also been described after ectopic expression of $p27^{Kip1}$ (6) or introduction of peptides that inhibit cdk2 activity (7). The latter approaches have also resulted in significant cytotoxicity as has the targeted disruption of cyclin A (8).

These profound effects on S and G_2 cell cycle progression and apoptosis need to be reconciled with the absence of similar cell cycle effects or cell death after introduction of antisense or small interfering RNA (siRNA) targeting cdk2 in cancer cells (9). One possibility is that the former approaches are targeting both cdk1 and cdk2. For example, expression of the dominant-negative cdk2 mutant resulted in reduced cyclin B–associated kinase activity (5). Ectopic $p27^{Kip1}$ would be expected to inhibit both cdks. The reported cdk2-inhibitory peptides were capable of cdk1 inhibition at high concentration, and the proteasomal degradation of cyclin A should affect cyclin A-cdk1 activity as well. In fact, small hairpin RNA (shRNA)–mediated silencing of cdk1 produced greater effects on cellular proliferation in *cdk2*$^{-/-}$ mouse embryo fibroblasts than in wild-type cells (10), suggesting functional redundancy between these cdks. However, whether shRNA-mediated codepletion of cdk2 and cdk1 can similarly affect transformed cells or induce apoptosis has not yet been clarified. Additionally, the definition of the effects of combined depletion in established cancer cells is critical for the design of small-molecule cdk inhibitors. Although compounds highly selective for cdk2 may have limited utility, those targeting both cdks with high potency may have therapeutic value.

Inhibition of the transcriptional cdks has also attracted intense interest because the most sensitive transcripts are those with short half-lives that encode cell cycle regulators, mitotic regulatory kinases, nuclear factor-κB–responsive gene transcripts, and apoptosis regulators, such as Mcl-1 and X-linked inhibitor of apoptosis (XIAP; refs. 11, 12). Diminution of levels of these

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transcripts and their encoded proteins may produce anticancer activity or augment apoptotic responses. Flavopiridol, the most potent known inhibitor of cdk9, has recently shown striking activity in chronic lymphocytic leukemia, which may be in part attributable to a drug-induced decrease in Mcl-1 expression (13, 14). The potential role of cdk2 and cdk1 in RNA polymerase II CTD phosphorylation suggests that their inhibition may similarly deplete transcripts encoding proteins that affect cancer cell survival.

To define the effect of depletion of cdk2 and cdk1 activities in established cancer cells of diverse origin, we have engineered NCI-H1299 non–small cell lung cancer (NSCLC) cells and U2OS osteosarcoma cells to inducibly express shRNA targeting cdk2, cdk1, or both together. This allowed us to probe the effects of relatively acute depletion of these cdk activities, an appropriate model for assessment of these kinases as drug targets. U2OS cells express wild-type p53 and Rb as well as cyclins D1 and D3, and lack $p16^{INK4A}$ expression because of promoter hypermethylation (15, 16). They undergo S and G_2-M arrest and apoptosis in response to cdk2 inhibition by dominant-negative cdk2 mutant expression (5), inhibitory peptides (7), and targeted cyclin A degradation (8) but have not shown cell cycle alterations in response to antisense-mediated specific depletion of cdk2 (9). NCI-H1299 cells are similarly *Rb* wild-type and $p16^{INK4A}$ methylated but carry a *p53* deletion (17, 18). These cell lines show that either G_2-M arrest or apoptosis is the major response to combined cdk2/cdk1 depletion. Apoptosis predominated in U2OS cells and was accompanied by reduced RNA polymerase II expression and phosphorylation. Our results suggest that combined depletion of cell cycle and transcriptional cdk activities may be a reasonable anticancer strategy and that inhibition of cdk2, cdk1, and RNA polymerase II are all important components of the cytotoxic effects observed.

Materials and Methods

Plasmids. Oligonucleotides containing the siRNA sequences targeting cdk1 (GGGGTTCCTAGTACTGCAA) and cdk2 (GGTGGTGGCGCTTAAGAAA) were purchased from Sigma Genosys (The Woodlands, TX), annealed, and ligated into the pSuperior.puro or pSuperior.retro.neo+GFP vectors (Oligoengine, Seattle, WA) precut with *Bgl*II and *Xho*I. Inserts were sequenced before use. The lentiviral shRNA vectors pLKO.1 targeting cdk9 (CTACTACATCCACAGAAACAA) and luciferase (CCTAAGGTTAAGTCGCCCTCG) were obtained from The RNA Interference Consortium (via the laboratory of William Hahn, Dana-Farber Cancer Institute, Boston, MA; ref. 19). Oligonucleotides containing XIAP (GTGGTAGTACCTGTTTCAGC) and p53 shRNA sequences (GACTCCAGTGGTAATCTAC) were cloned into the lentiviral shRNA vector pLVTHM (provided by Didier Trono, University of Geneva, Geneva, Switzerland; ref. 20).

Generation and maintenance of NCI-H1299 and U2OS cells expressing shRNA(s) targeting cdk2, cdk1, and cdk9. NCI-H1299 and U2OS cell lines were obtained from the American Type Culture Collection (Manassas, VA). Tet-resistant starter lines were generated by transfecting cells with the pcDNA6/TR plasmid (Invitrogen, Carlsbad, CA) followed by selection in 10 μg/mL blasticidin. Western blot analysis with an anti-Tet repressor antibody (MoBiTec, Göttingen, Germany) was done to identify clones with the highest Tet repressor expression. Starter lines were transfected with pSuperior.puro/cdk1 or 2 shRNA vectors using Fugene 6 (Roche Applied Science, Indianapolis, IN) and selected with medium supplemented with Tet-free fetal bovine serum (Clontech Laboratories, Inc., Mountain View, CA), 10 μg/mL blasticidin, and 1 μg/mL puromycin. Colonies were screened for cdk1 or cdk2 knockdown after 5 μg/mL doxycycline treatment for 3 days. To generate double-knockdown cell lines, cdk2 or cdk1 shRNA cell lines were cotransfected with pSuperior.puro/cdk1 or cdk2 shRNA vectors as well as a vector encoding a hygromycin selection marker at a 10:1 ratio; individual clones were selected in 200 μg/mL hygromycin with blasticidin and puromycin and tested for knockdown of both cdks after exposure to 5 μg/mL doxycycline for 6 days. For cell growth experiments, cells were plated at 3.75×10^5 per 10-cm dish in the absence or presence of doxycycline. One quarter of the medium was replaced every 3 days.

Lentivirus production and infection. The CalPhos Mammalian Transfection kit (Clontech Laboratories) was used to transfect lentiviral shRNA as well as relevant helper plasmids into 293T cells. A NCI-H1299TR cdk2/cdk1 double shRNA clone was plated for 24 to 48 hours in the absence or presence of doxycycline before application of the appropriate lentivirus at a multiplicity of infection of ~5. Cells were washed in PBS 24 hours after transduction and maintained in medium in the absence or presence of doxycycline for an additional 6 or 7 days for pLKO-cdk9 and pLVTHM-XIAP infection, respectively. One quarter of the medium was replaced every 3 days.

Cell synchronization. Cells were cultured in medium with or without doxycycline for 24 hours and subsequently replated at 6×10^5 per 10-cm dish and grown for an additional 24 hours before synchronization. Cells were treated with 1 mmol/L hydroxyurea for 24 hours or 4 μg/mL nocodazole (both from Sigma-Aldrich Co., St. Louis, MO) for 16 hours to achieve a G_1-S or mitotic block, respectively. Cells were subsequently washed in PBS and maintained in medium in the absence or presence of doxycycline.

Flavopiridol treatment. Flavopiridol was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. A 50 mmol/L stock solution in DMSO was maintained at −20°C. NCI-H1299 cdk2/cdk1 double-knockdown cells were plated at 5×10^5 per 10-cm dish in the absence or presence of doxycycline. Seventy-two hours later, the medium was replaced with or without doxycycline containing DMSO or flavopiridol at the indicated concentrations for an additional 72 hours.

Western blot analysis. Whole-cell and nuclear lysates were prepared as described previously (21) or by sonication in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing protease and phosphatase inhibitors. Protein concentrations were determined by Bradford assay (Bio-Rad, Richmond, CA), and equivalent amounts (10-50 μg) were subjected to SDS-PAGE. Western blotting was done with the following primary antibodies: anti-cdk1, anti-cdk2, anti-cdk9, anti–cyclin B1, anti–cyclin E, and anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Rb and anti-phosphospecific Rb antibodies (Cell Signaling Technology or Biosource International, Camarillo, CA); anti–cyclin D1, anti–cyclin D3, anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-XIAP, and anti-Bcl-XL (Cell Signaling Technology); anti-survivin (Biolegend, San Diego, CA); and anti-tubulin (Sigma-Aldrich). Analysis of RNA polymerase II was done after 6% SDS-PAGE with anti-RNA polymerase II [$pSer^2$] (Abcam, Cambridge, MA), anti-RNA polymerase II [$pSer^5$] (Covance, Berkeley, CA), anti-unphosphorylated RNA polymerase II (Covance), and anti-total RNA polymerase II (Santa Cruz Biotechnology).

Immunoprecipitation and *in vitro* kinase assays. Anti-cdk1, anti-cdk2, anti–cyclin E, and anti–cyclin B1 were used to immunoprecipitate cyclin-cdk complexes from 500 μg lysate extracted from cells grown in the absence or presence of doxycycline for 3 or 6 days. Kinase assays were carried out in the presence of 0.5 μmol/L ATP, 10 μCi [γ-^{33}P]ATP, and 2.5 μg histone H1 (Roche Applied Science) as substrate. After SDS-PAGE and transfer to polyvinylidene difluoride membranes, phosphorylated substrates were visualized by autoradiography and semiquantitiatve analysis of bands was done using ImageJ Software (NIH).

Fluorescence-activated cell sorting analysis and detection of apoptosis by flow cytometry. For cell cycle analysis, fixed cells were stained with propidium iodide and analyzed for DNA content using the ModFit (Verity Software House, Topsham, ME) or CellQuest software (BD Biosciences, Franklin Lakes, NJ). For apoptosis assays, a fluorescein apoptosis detection kit was used (Promega, Madison, WI) as described previously (22).

Statistical analysis. The two-tailed, unpaired Student's *t* test was used. $P < 0.05$ was considered statistically significant.

Results

Generation and characterization of NCI-H1299 and U2OS cells inducibly depleted of cdk2 and/or cdk1 activities. Several NCI-H1299 and U2OS clones were generated, engineered to express shRNA(s) targeting cdk2, cdk1, or both on induction with doxycycline. Representative derivatives are shown in Fig. 1*A* and Supplementary Fig. S1. Overall, cdk expression was depleted and associated kinase activity was reduced after 3 days in doxycycline. The degree of depletion of each cdk was comparable between single- and double-knockdown clones and between NCI-H1299 and U2OS clones engineered in the same way.

Growth curves of subconfluent cells over 6 days in culture in the absence or presence of doxycycline are shown in Fig. 1*B*. In the presence of doxycycline, there is a slight proliferative compromise after cdk2 depletion in NCI-H1299 cells. Compared with cdk2 depletion, the effect of cdk1 depletion on proliferation is greater in both cell lines. For both cell lines, combined cdk2 and cdk1 depletion induces a potent antiproliferative effect, and viable cell numbers suggest cytotoxicity in U2OS cells. These results are largely reflected in 14-day colony formation assays as shown in Fig. 1*C*. In U2OS cells, both cdk1 and combined cdk2/cdk1 depletion prevent the appearance of colonies, although this assay does not distinguish cytostatic and cytotoxic effects.

Depletion of cdk2 activity slows G_1 progression in NCI-H1299 cells. The cell cycle distribution of subconfluent NCI-H1299 cells engineered to inducibly express shRNA targeting cdk2 are shown in the absence and presence of doxycycline in Fig. 2*A*, showing no obvious changes with cdk2 depletion over 6 days. Similarly, cells recruited to the G_1-S boundary with hydroxyurea and then released into S phase traverse S and G_2-M similarly in the absence or presence of doxycycline (Fig. 2*B*). In the presence of doxycycline, cyclin A-cdk2 complexes are largely replaced by cyclin

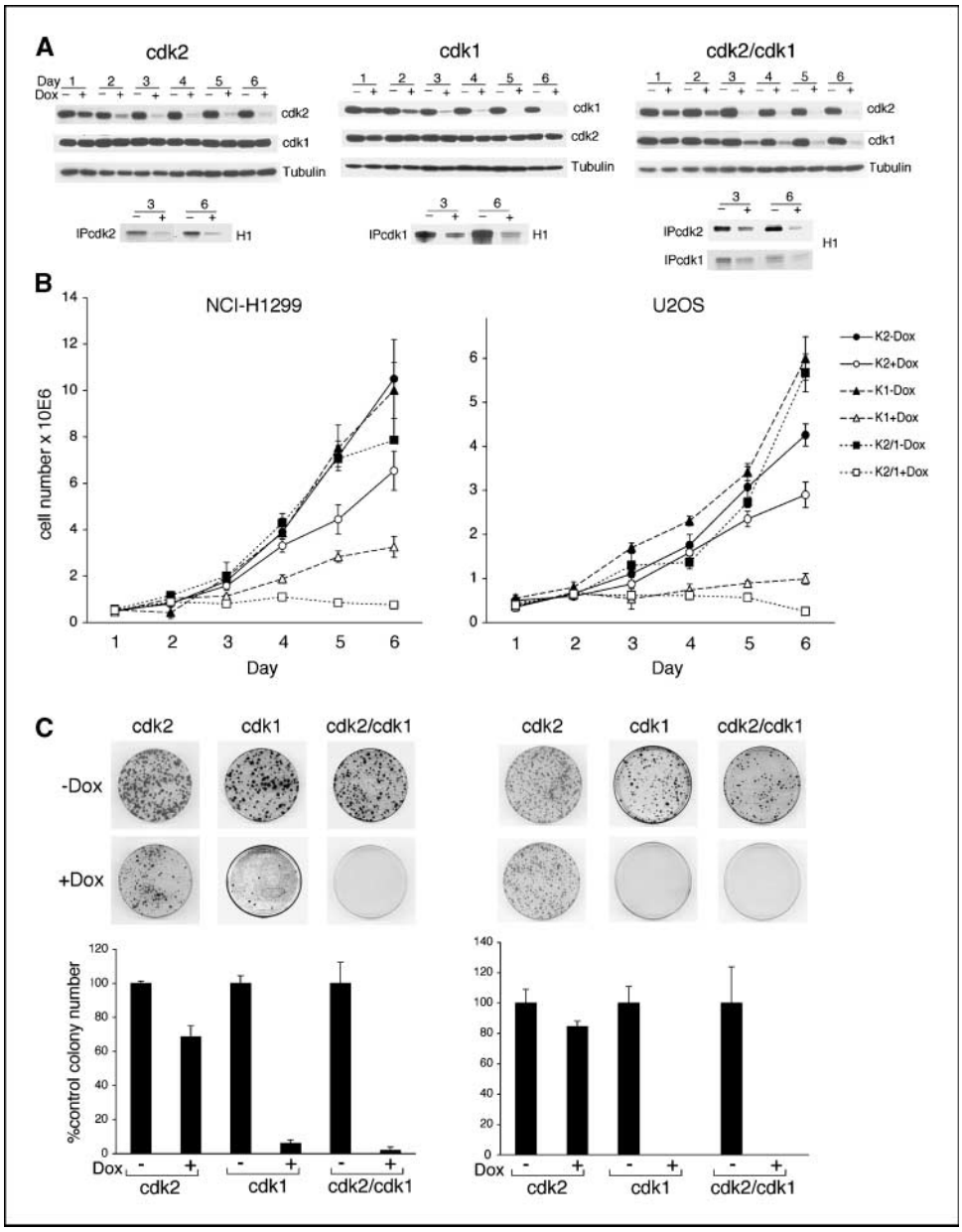

Figure 1. Characterization of cell lines engineered to inducibly express shRNA targeting cdk2, cdk1, or both. *A,* representative NCI-H1299 clones were grown in the absence or presence of doxycycline (*Dox*). Cell lysates were prepared on the indicated days and subjected to Western blotting. On days 3 and 6, cdk2 and cdk1 were recovered by immunoprecipitation and used to direct the phosphorylation of histone H1 *in vitro*. *B,* representative NCI-H1299 and U2OS clones were grown in the absence or presence of doxycycline. On the indicated days, viable cells were counted by trypan blue exclusion. *Points,* average of duplicate cell counts; *bars,* SE. *C,* representative cell lines were plated at 10^3 per 10-cm dish in the absence or presence of doxycycline and allowed to grow for 14 days, after which colonies were stained with crystal violet and counted. *Columns,* average of two experiments; *bars,* SE.

A-cdk1 complexes in these cells; in addition, the depletion of cdk2 does not affect levels of cyclin B found in association with cdk1 (Supplementary Fig. S2).

In contrast, after release from a nocodazole-induced mitotic block, NCI-H1299 cells traverse through the next G_1 more slowly in the presence of doxycycline, showing that cdk2 depletion can affect G_1 progression in these cells (Fig. 2*C*). However, the same delay in G_1 progression was not observed in U2OS cells depleted of cdk2 (Fig. 2*D*). Treatment with doxycycline caused reduced phosphorylation of Rb at Ser^{821} and Thr^{356} cdk2 phosphorylation sites in NCI-H1299 cells but not U2OS cells, reflecting the slowing of G_1 progression in the former (Fig. 2*E*). After depletion of cdk2, both cell lines showed only a slight reduction in cyclin E–dependent kinase activity, attributable to an increase in cyclin E-cdk1 complexes detected (Fig. 2*F*). However, U2OS cells showed an increase in cyclin D3 expression, not detected in NCI-H1299 cells (Fig. 2*G*), suggesting that a modest increase in cyclin D–dependent kinase activity can compensate for cdk2 depletion. Increased cyclin D expression was also found in clones engineered to constitutively express cdk2 shRNA; these clones displayed no apparent alterations in cell cycle progression and routinely overexpressed both cyclins D1 and D3 (Supplementary Fig. S2B and C).

Depletion of cdk1 activity slows G_2-M progression in both NCI-H1299 and U2OS cells and induces endoreduplication in U2OS cells. Cell cycle distributions of cell lines engineered to inducibly express shRNA targeting cdk1 in the absence and presence of doxycycline over 6 days are shown in Fig. 3*A* and *B*. Primary data from which the U2OS bar graphs were derived are shown in Supplementary Fig. S3. In NCI-H1299 cells, there is a slow increase in cells with G_2-M content. The degree of increase in G_2-M content is similar in U2OS cells after cdk1 depletion. However, there was a greater propensity of U2OS cdk1 knockdown derivatives to undergo endoreduplication, with appearance of cells with 8N and 16N DNA content. Despite endoreduplication, cytotoxicity was not observed.

Cell cycle traversal was examined in both cell lines after release from a hydroxyurea-induced block at the G_1-S boundary (Fig. 3*C*

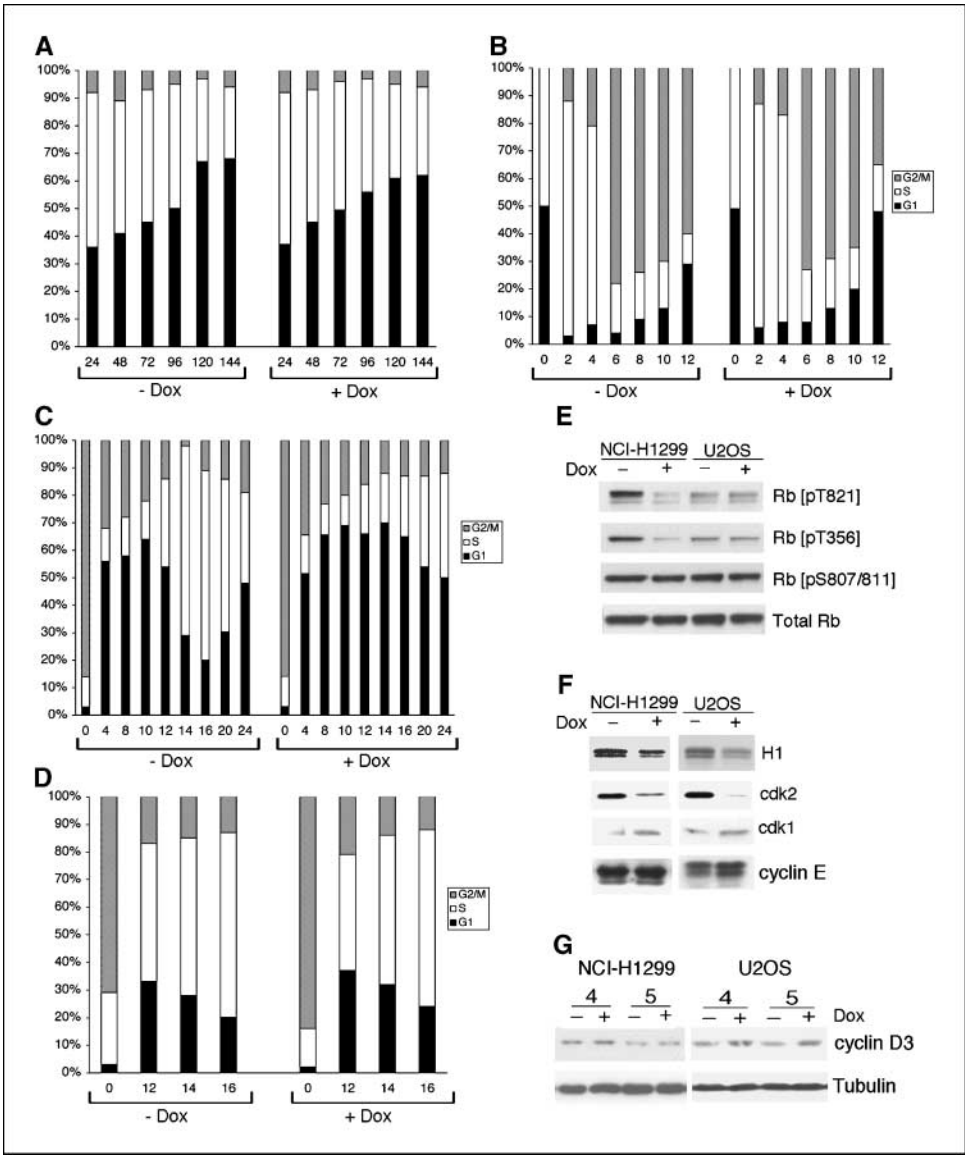

Figure 2. Analysis of cell lines engineered to inducibly express shRNA targeting cdk2. *A,* a representative NCI-H1299 cdk2 shRNA clone was grown in the absence or presence of doxycycline. At the indicated times, cells were collected and analyzed by flow cytometry. *B,* NCI-H1299 cdk2 shRNA cells were synchronized at the G_1-S boundary with hydroxyurea; after release, cells were maintained in the absence or presence of doxycycline for the indicated times and analyzed by flow cytometry. Depletion of cdk2 does not affect S and G_2-M progression. *C,* NCI-H1299 cdk2 shRNA cells were treated with nocodazole to induce a mitotic block; after release, cells were maintained in the absence or presence of doxycycline for the indicated times and analyzed by flow cytometry. Depletion of cdk2 causes slowing of G_1 progression. *D,* U2OS cdk2 shRNA cells were treated as in (*C*), showing that cdk2 depletion does not affect G_1 progression in these cells. *E,* representative NCI-H1299 and U2OS cdk2 shRNA clones were grown in the absence or presence of doxycycline for 6 days; nuclear lysates were subjected to Western blotting with the indicated antibodies, showing the compromise in cdk2-mediated Rb phosphorylation at T821 and T356 (but not the cdk4 S807/S811 phosphosite) in NCI-H1299 cdk2 shRNA cells. *F,* cells were grown in the absence or presence of doxycycline for 6 days; cyclin E–containing complexes were recovered from whole-cell lysates by immunoprecipitation and used to direct phosphorylation of histone H1 *in vitro* (*top blot*) or subjected to Western blotting with the indicated antibodies (*bottom three blots*). Cyclin E–dependent kinase activity is affected slightly; reduced amounts of cyclin E-cdk2 complexes are replaced by increased levels of cyclin E-cdk1 complexes in both cell lines. *G,* cells were grown in the absence or presence of doxycycline for the indicated days and lysates were subjected to Western blotting, showing increased cyclin D3 expression in U2OS cells after cdk2 depletion.

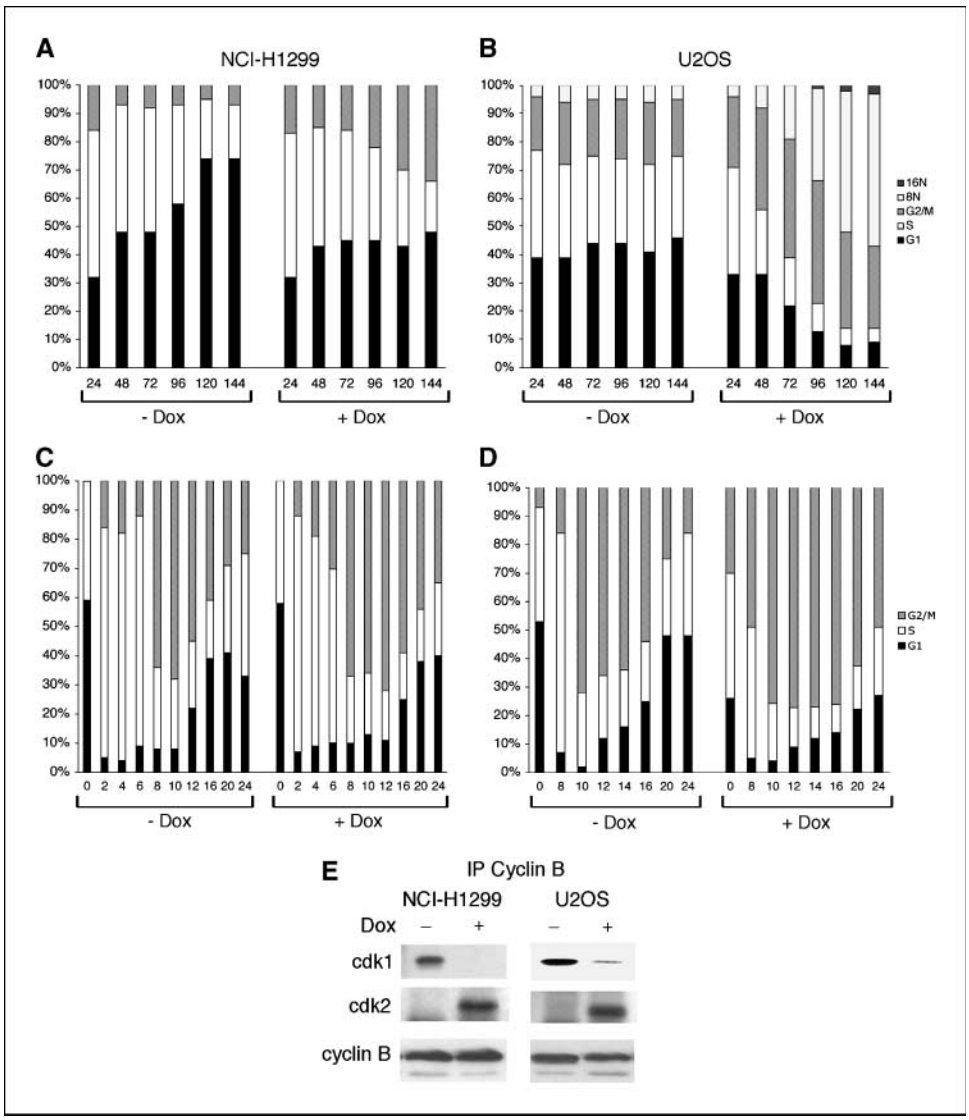

Figure 3. Analysis of cell lines engineered to inducibly express shRNA targeting cdk1. *A,* a representative NCI-H1299 clone was grown in the absence or presence of doxycycline. At the indicated times, cells were analyzed by flow cytometry. *B,* similar analysis for a representative U2OS clone. *C,* NCI-H1299 cdk1 shRNA cells were synchronized at the G_1-S boundary with hydroxyurea; after release, cells were maintained in the absence or presence of doxycycline for the indicated times and analyzed by flow cytometry. *D,* similar analysis for U2OS cdk1 shRNA cells. In both cell lines, movement through G_2-M to the next G_1 is slowed in doxycycline; the effect is more apparent in U2OS cells. *E,* cells were grown in the absence or presence of doxycycline for 6 days; whole-cell lysates were subjected to immunoprecipitation for cyclin B followed by Western blotting with the indicated antibodies. In the presence of doxycycline, cyclin B-cdk1 complexes are depleted and replaced by cyclin B-cdk2 complexes.

and *D*); in U2OS cells, this experiment was done before substantial endoreduplication occurred. Cdk1 depletion did not affect S-phase progression but slowed G_2-M progression to the next G_1; the delay is longer in U2OS cells. Interestingly, after depletion of cdk1 in these cell lines, a cyclin B-cdk2 complex is readily detectable, perhaps providing compensation and permitting traversal of G_2-M (Fig. 3*E*).

Combined depletion of cdk2 and cdk1 induces G_2-M arrest in NCI-H1299 cells. Figure 4*A* shows the cell cycle distribution of subconfluent NCI-H1299 cells engineered to concomitantly express shRNAs targeting cdk2 and cdk1 in the absence or presence of doxycycline over 6 days. Representative flow cytometry patterns are shown in Fig. 4*B*. Compared with either cdk2 or cdk1 depletion alone, combined depletion induces substantial G_2-M arrest after 3 days in doxycycline. This arrest persists, and between 4 and 6 days, there is associated endoreduplication with appearance of a small sub-G_1 peak, suggestive of apoptosis (Fig. 4*A*; Supplementary Fig. S4). Flow cytometry–based terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays confirm ~5% to 10% apoptosis by 6 days in the presence of doxycycline (see Fig. 6*C*). The potency and persistence of G_2-M arrest are shown after release from a hydroxyurea-induced block at the G_1-S boundary; in the presence of doxycycline, there is a substantial delay in G_2-M progression, significantly greater than that seen with cdk1 knockdown alone (Fig. 4*C* and *D*; compare Fig. 4*C* with Fig. 3*C*).

Combined depletion of cdk2 and cdk1 induces apoptosis in U2OS cells. Figure 5*A* shows cell cycle patterns of U2OS cells engineered to concomitantly express shRNAs targeting cdk2 and cdk1 in the absence or presence of doxycycline over 6 days. Initially, in the presence of doxycycline, an increase in G_2-M content occurs along with an increase in S-phase DNA content. A sub-G_1 peak is evident after 3 days and substantially increases by 4 days, with progressive cell death thereafter, so that by 6 days the majority of the culture is dead. TUNEL assays and assessment of PARP cleavage confirm substantial apoptosis after doxycycline-induced cdk2 and cdk1 depletion (Fig. 5*B* and *C*; data not shown). After release from a hydroxyurea-induced block at the G_1-S boundary, U2OS cells traverse S phase more slowly in the presence of doxycycline and die abruptly as they progress through the S and G_2 phases (Fig. 5*D*).

Combined depletion of cdk2 and cdk1 reduces RNA polymerase II expression and CTD phosphorylation in U2OS cells. The CTD of RNA polymerase II contains 52 tandem repeats

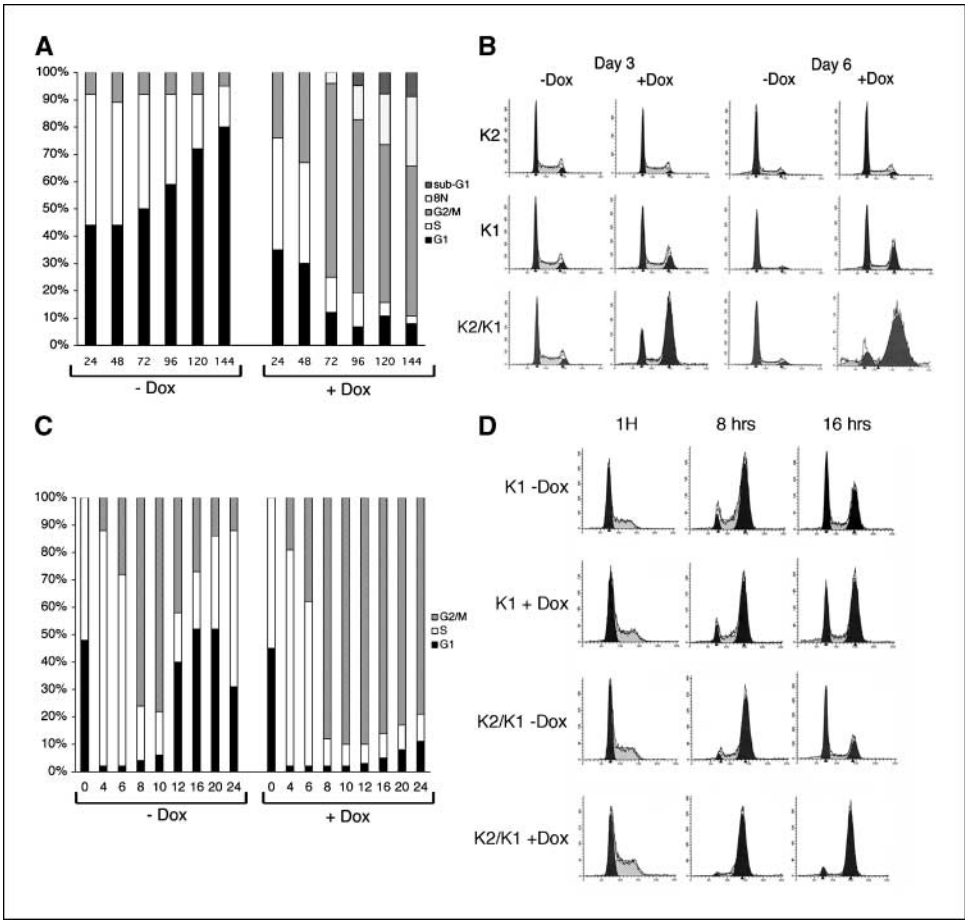

Figure 4. Analysis of NCI-H1299 cells engineered to inducibly express shRNAs targeting both cdk2 and cdk1 together. *A,* a representative NCI-H1299 cdk2/cdk1 shRNA clone was grown in the absence or presence of doxycycline. At the indicated times, cells were analyzed by flow cytometry. *B,* representative flow cytometry patterns of NCI-H1299 cells engineered to express shRNAs targeting cdk2, cdk1, or both after growth for 3 or 6 days in the absence or presence of doxycycline. *C,* NCI-H1299 cdk2/cdk1 shRNA cells were synchronized at the G_1-S boundary with hydroxyurea; after release, cells were maintained in the absence or presence of doxycycline for the indicated times and analyzed by flow cytometry. Depletion of both cdk2 and cdk1 slows G_2-M progression. *D,* NCI-H1299 cdk1 or cdk2/cdk1 shRNA cells were synchronized at the G_1-S boundary (*1H*); after release, cells were maintained in the absence or presence of doxycycline; representative flow cytometry patterns are shown at 8 and 16 hours. After depletion of cdk1 alone, G_2-M progression is slowed; the block is significantly more potent in cells depleted of both cdk2 and cdk1 together.

of the heptapeptide sequence YSPTSPS, phosphorylated at Ser^2 and Ser^5 by cdk9 and cdk7 (23). However, cdk2 and cdk1 have been reported to phosphorylate the CTD of RNA polymerase II *in vitro* (3, 4). In addition, arrested RNA polymerase II elongation complexes are substrates for ubiquitylation, which is dependent on CTD phosphorylation (24). Therefore, we examined the effects of cdk2 and cdk1 depletion on RNA polymerase II expression and phosphorylation in U2OS cells. Figure 5*E* shows that by 84 hours, around the time when cell death is escalating, combined cdk2 and cdk1 depletion causes reduced expression of RNA polymerase II and its associated phosphorylated forms. This is expected to compromise the stability of mRNAs with short half-lives, including those encoding antiapoptotic proteins (12), and depletion of both Mcl-1 and XIAP is observed. These events do not occur in cells individually depleted of cdk2 or cdk1 (Fig. 5*F*). In NCI-H1299 cells depleted of both cdks, effects on CTD phosphorylation occur only to a slight degree at 120 hours and are not associated with compromised Mcl-1 or XIAP expression. These data suggest that combined effects on cell cycle progression and transcription occur in doubly depleted U2OS cells that could contribute to the apoptotic response.

Combined depletion of cdk2, cdk1, and cdk9 or cdk2, cdk1, and XIAP induces substantial apoptosis in NCI-H1299 cells. To determine whether concomitant inhibition of transcriptional cdk activity could affect the response of NCI-H1299 cells to combined cdk2 and cdk1 depletion, double-knockdown cells were infected with a control lentivirus or a lentivirus encoding a shRNA targeting cdk9 in the absence or presence of doxycycline. Figure 6*A* shows that, in the absence of doxycycline, the expression of the cdk9 shRNA reduces expression of cdk9, phosphorylated forms of the CTD of RNA polymerase II and XIAP. Reduction in cdk9 results in a change in cell cycle distribution consistent with cell cycle slowing, with a modest degree of cell death after 6 days of lentiviral infection. In the presence of doxycycline, cdk1 and cdk2 are also depleted, and cell death is significantly increased when the shRNA targeting cdk9 is expressed. These results were confirmed in NCI-H1299 clones engineered to express shRNAs targeting cdk2, cdk1, and cdk9 in the presence of doxycycline (Supplementary Fig. S5).

Double-knockdown cells were also infected with a lentivirus encoding shRNA targeting p53, expected to be inert in p53-deleted NCI-H1299 cells, or one encoding a shRNA targeting XIAP (Fig. 6*B*). XIAP depletion alone does not cause significant cell cycle arrest or cell death, but apoptosis induced by cdk2 and cdk1 depletion is significantly increased when XIAP expression is compromised.

Flavopiridol-mediated cdk9 inhibition can augment apoptosis induced by cdk2 and cdk1 depletion in NCI-H1299 cells. Flavopiridol is a pan-cdk inhibitor but most potently inhibits cyclin T-cdk9 (p-TEFb) with a $K_i \approx 3$ nmol/L compared with K_i values of 40 to 70 nmol/L for cell cycle cdks (25). As shown in Fig. 6*C*, in engineered NCI-H1299 cells, 100 nmol/L flavopiridol compromises CTD phosphorylation, primarily at the cdk9 Ser^2 phosphosite as well as XIAP expression. Flavopiridol treatment for 3 days in the absence of doxycycline induces a small degree of apoptosis, which is

significantly increased when treatment is carried out following cdk2 and cdk1 depletion with doxycycline. Taken together, these data suggest that compromised cdk9 activity and XIAP expression can lower the apoptotic threshold in cells depleted of cdk2 and cdk1.

Discussion

The universal disruption of cell cycle control in human cancer has led to longstanding interest in cdks as possible anticancer drug targets. In the case of cdk2, increased activity in a wide variety of tumors is in part a consequence of cyclin D-cdk4/cdk6-INK4 pathway alterations that cause sequestration of Cip/Kip proteins from cyclin E-cdk2 complexes, where they are inhibitory (26), suggesting that cdk2 inhibition should block G_1 progression. Here, we have shown that depletion of cdk2 can cause slowing of G_1 progression in established cancer cells. This was seen after release from a nocodazole-induced mitotic block in NCI-H1299 cells and is missed if only exponentially growing cells are examined. This result is similar to that described for early-passage synchronized *cdk2*$^{-/-}$ mouse embryo fibroblasts (27, 28). Nonetheless, because synchronization is required to show the slowing of G_1 progression, the effects of cdk2 depletion in NCI-H1299 cells are weak at best at the G_1-S boundary. Neither Rb dephosphorylation nor G_1 arrest is complete, in part related to recently reported compensatory cyclin E-cdk1

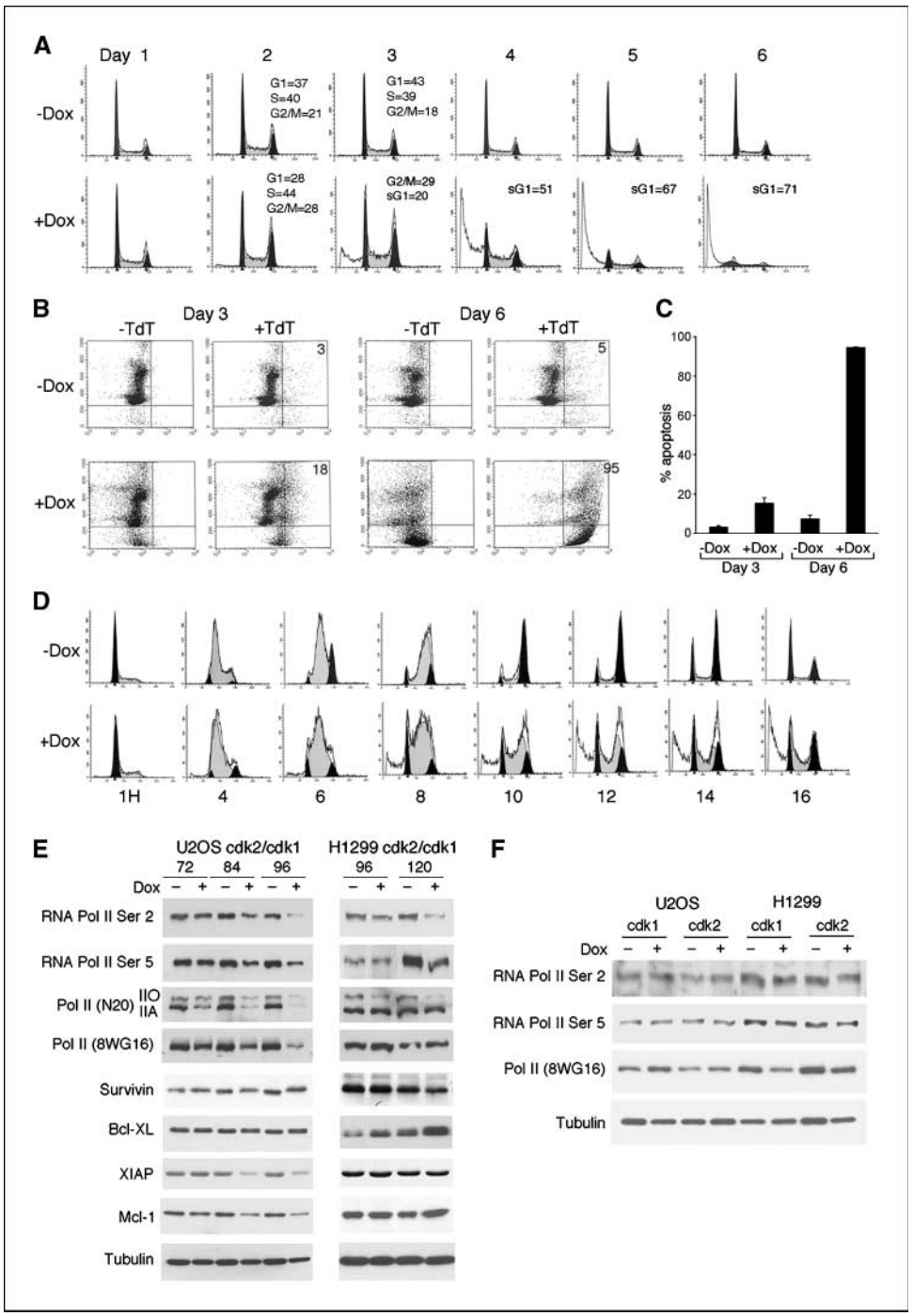

Figure 5. Induction of apoptosis in U2OS cells engineered to inducibly express shRNAs targeting both cdk2 and cdk1 together. *A,* a representative U2OS cdk2/cdk1 shRNA clone was grown in the absence or presence of doxycycline. At the indicated times, cells were analyzed by flow cytometry. In the presence of doxycycline, there is an initial increase in S and G_2-M content followed by the appearance of a sub-G_1 peak, which increases with time. *B,* U2OS cdk2/cdk1 shRNA cells were grown for 3 or 6 days in the absence or presence of doxycycline; fixed cells were subjected to a flow cytometry–based TUNEL assay showing fluorescein shift in cells maintained in doxycycline. *Numbers,* percentages of fluorescein-positive cells. *C,* quantitation of TUNEL assays depicted in (*B*). *Columns,* average of three experiments; *bars,* SD. *D,* U2OS cdk2/cdk1 shRNA cells were synchronized at the S and G_1 boundary with hydroxyurea; after release, cells were maintained in the absence or presence of doxycycline for the indicated times (hours) and analyzed by flow cytometry, showing cell death during S and G_2 progression. *E,* representative U2OS and NCI-H1299 cdk2/cdk1 shRNA clones were grown in the absence or presence of doxycycline. At the indicated times, whole-cell lysates were subjected to Western blot analyses with the indicated antibodies, indicating reduced expression of RNA polymerase II and its phosphorylated forms in U2OS cells depleted of both cdk2 and cdk1, with concomitant depletion of Mcl-1 and XIAP. *IIO,* hyperphosphorylated RNA polymerase II; *IIA,* hypophosphorylated RNA polymerase II. *F,* similar analysis for U2OS and NCI-H1299 cdk1 or cdk2 shRNA clones grown in the absence or presence of doxycycline for 6 days before the preparation of lysates.

complexes (10), which were detected in both cell lines in our study. In addition, U2OS cells also compensate for lower cdk2 activity with increased expression of D-cyclins; cyclin D–dependent complexes likely contribute to the phosphorylation of Rb sites typically phosphorylated by cdk2 (28). Interestingly, in contrast to results seen with siRNA-mediated cdk2 depletion both here and described previously, expression of a dominant-negative cdk2 mutant in U2OS cells induced slowing of G_1 progression after release from synchronization (5). Sequestration of Cip/Kip proteins by the dominant-negative mutant could have affected the assembly of cyclin D–dependent kinase complexes, facilitating G_1 arrest.

The analyses of NCI-H1299 cells and U2OS cells show cell line–specific differences. Compared with NCI-H1299 cells, the effects of cdk2 depletion on G_1 progression are even more potent in melanoma cells, where there is correlation in expression between the mirophthalmia-associated transcription factor (MITF) and cdk2. Melanomas with low MITF expression have low levels of cdk2 and are particularly susceptible to G_1 arrest induced by siRNA-mediated cdk2 depletion (29).

Just as cell type–specific differences occur with the effects of cdk2 depletion on G_1 progression, differences may also occur for S and G_2-M progression. In, NCI-H1299 and U2OS cells, cdk2 depletion did not affect these cell cycle phases. There was neither a decrease in levels of cyclin B in complex with cdk1 nor a change in migration of cdk1 to suggest an accumulation of the inactive phosphorylated form as has been described for dominant-negative cdk2 (5), which may have sequestered anaphase-promoting complex components and cdc25 phosphatases, resulting in cyclin B destabilization and lack of cdk1 activation. Furthermore, cyclin A, which was associated primarily with cdk2 in the absence of doxycycline, was found in complex with cdk1 after cdk2 depletion. In contrast, siRNA-mediated depletion of cdk2 in HT29 colon carcinoma cell caused concomitant depletion of cyclin B, with resultant accumulation of S- and G_2-M-phase cells (19). It is possible that cyclin A-cdk1 complexes do not compensate as well in this cell line. Alternatively, a more complete depletion of cdk2 activity may have been achieved than in our experiments. Nonetheless, substantial cdk2 depletion in NCI-H1299 and U2OS cells did not yield the same results. Taken together with data in other cell lines where cdk2 depletion also had minimal effects (9), it is likely that in a large proportion of cell types cyclin A-cdk1, cyclin E-cdk1 and cyclin D–dependent kinases all conspire to circumvent highly selective cdk2 inhibition. S- and G_2-phase effects may be most easily achieved with the simultaneous direct targeting of cdk2 and cdk1 activities.

Specific cdk1 depletion in NCI-H1299 and U2OS cells showed an approximate doubling of cells with G_2-M DNA content and a slight to moderate slowing of G_2-M progression after release from synchronization. A novel cyclin B-cdk2 complex was readily detected in cells depleted of cdk1 that may facilitate G_2-M progression. The major difference between the two cell lines was the greater propensity of U2OS cells to undergo endoreduplication after depletion of cdk1 activity. Endoreduplication following cdk1 inactivation occurs in a human fibrosarcoma cell line carrying a single conditionally active cdk1 allele (30). Cdk1 activity has been linked to cytokinesis (31); following a cytokinetic defect, endoreduplication may ensue in cells lacking an efficient postmitotic checkpoint, likely the case in U2OS cells.

Although cdk1 depletion had a substantial effect on colony formation over 14 days, significant cell death was not detected in our derivative clones. The ability of cdk1 depletion alone to induce cytotoxicity may be cell line dependent; in a preliminary analysis of similar derivatives of HCT-116 colon cancer cells, increased G_2-M content and endoreduplication are accompanied by a moderate degree of apoptosis.[3]

In all cell lines examined, combined depletion of cdk1 and cdk2 induced more apparent antiproliferative effects than those induced by either cdk individually. In NCI-H1299 cells, combined depletion induced G_2-M arrest that was more pronounced than that induced by cdk1 alone, suggesting that both cdks contribute to G_2-M control. Interestingly, in experiments in which cdk1 shRNA was expressed in *cdk2*$^{-/-}$ mouse embryo fibroblasts, decreased bromodeoxyuridine (BrdUrd) incorporation was observed after release from serum starvation, suggesting effects of combined cdk2/cdk1 depletion at the G_1-S boundary (although decreased BrdUrd incorporation does not rule out arrest at the G_2-M boundary in these cells). Attempts to determine whether G_1 progression was slowed in double-knockdown NCI-H1299 cells were complicated by their inability to be synchronized by starvation and progressive endoreduplication following release from a nocodazole-induced mitotic block (data not shown). However, the propensity for endoreduplication and the absence of evident G_1 effects in asynchronously growing cells suggests that G_1 progression was largely intact. It is possible that reduction in cyclin E-cdk2 and cyclin E-cdk1 activities will promote G_1 arrest in nontransformed cells but may be insufficient to induce G_1 arrest in transformed cells.

In U2OS cells, the increase in G_2-M content observed at early time points (i.e., 48 hours) was similar in cells depleted of cdk1 alone or both cdk2 and cdk1 as has been described after transient siRNA transfection (32). In exponentially growing cdk1-depleted cells, there was reduced S-phase content (Fig. 3*B*), whereas S-phase content was slightly increased in cells subjected to combined depletion (Fig. 5*A*). In addition, with combined depletion, apoptosis occurred, beginning at 72 hours and reaching a substantial degree by 96 hours. Synchronization showed slowing of S-phase progression and cytotoxicity during S- and G_2-phase traversal. Multiple mechanisms may contribute to the death of S- and G_2-phase cells. Both cdk2 and cdk1 contribute to the phosphorylation and appropriately timed down-regulation of E2F-1 during the S and G_2 phases (reviewed in ref. 2). Reduced levels of these cdks may result in inappropriately persistent E2F-1 activity and induce apoptosis in transformed cells that have high baseline levels and may more readily overcome the threshold required to induce apoptosis than nontransformed cells. Consistent with this model, apoptosis induced by several pharmacologic cdk inhibitors during S phase is E2F-1 dependent (21, 33, 34) and selective for transformed cells (22) as is the death induced by cdk-inhibitory peptides that block the interaction of cyclin A-cdk2 with E2F-1 (7).

Recently, it has been shown that cdk inhibition during S phase elicits an intra-S-phase checkpoint that shares components of the pathway activated by dsDNA breaks, with accumulation of activated forms of ATM and chk2, as well as nuclear foci containing phosphorylated substrates of ATM, including histone H2AX, a marker for double-strand breaks (35, 36). Further work will be required to determine whether the combined depletion of cdk2 and cdk1 can induce or predispose to DNA damage. The contribution of wild-type p53 expression to the apoptotic response in U2OS cells also remains to be elucidated.

The depletion of cdk2 and cdk1 in U2OS cells also compromises the phosphorylation and expression of the CTD of RNA polymerase

[3] D. Cai and G.I. Shapiro, unpublished observations.

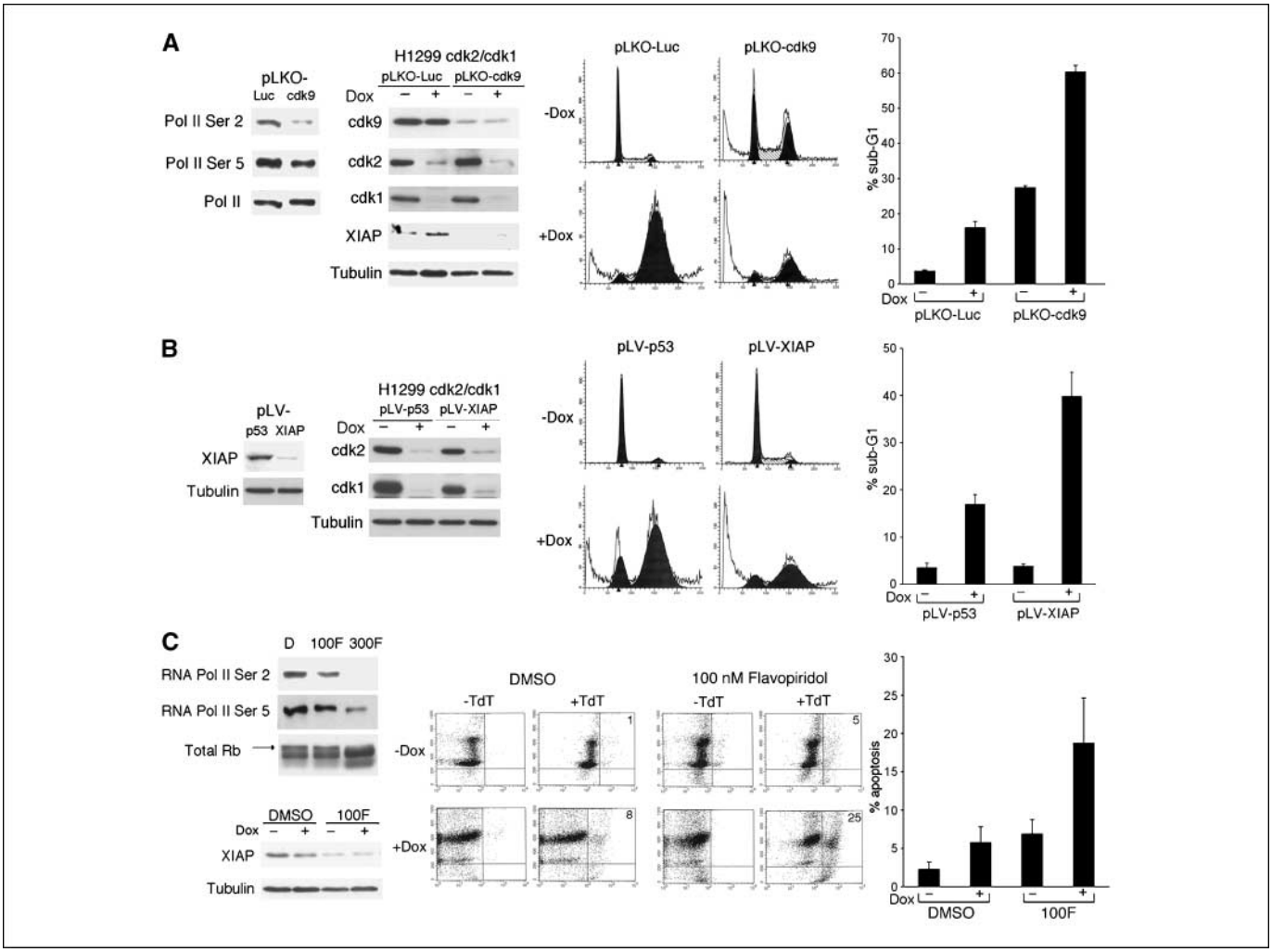

Figure 6. shRNA- or flavopiridol-mediated inhibition of cdk9 or depletion of XIAP along with cdk2 and cdk1 induces apoptosis in NCI-H1299 cells. *A, left,* NCI-H1299 cdk2/cdk1 shRNA cells were infected with lentiviruses expressing shRNA targeting either luciferace (*pLKO-Luc*) or cdk9 (*pLKO-cdk9*) for 6 days in the absence of doxycycline. Lysates were subjected to Western blotting with the indicated antibodies, showing reduced phosphorylation of the CTD of RNA polymerase II at the Ser^2 and Ser^5 sites. In addition, cells were maintained in the absence or presence of doxycycline for 48 hours before infection with either pLKO-Luc or pLKO-cdk9 lentivirus for an additional 6 days in the absence or presence of doxycycline. Lysates were subjected to Western blotting with the indicated antibodies. *Middle,* NCI-H1299 cdk2/cdk1 shRNA cells were infected with the indicated lentiviruses in the absence or presence of doxycycline; at the end of 6 days of infection (and 8 days in doxycycline), cells were collected and analyzed by flow cytometry, showing markedly increased sub-G_1 DNA content following cdk9 depletion in the presence of doxycycline. *Right,* quantification of sub-G_1 peaks over 3 experiments. *Bars,* SD. In the presence of doxycycline, cdk9 depletion results in a statistically significant increase in the fraction of cells with sub-G_1 DNA content ($P = 0.000008$). *B, left,* NCI-H1299 cdk2/cdk1 shRNA cells were infected with lentiviruses expressing shRNA targeting either p53 (*pLV-p53*) or XIAP (*pLV-XIAP*) for 6 days in the absence of doxycycline. Lysates were subjected to Western blotting with the indicated antibodies. In addition, cells were maintained in the absence or presence of doxycycline for 24 hours before infection with either pLVTHM-p53 or pLVTHM-XIAP lentivirus for an additional 8 days in the absence or presence of doxycycline. Lysates were collected and subjected to Western blotting with the indicated antibodies. *Middle,* NCI-H1299 cdk2/cdk1 shRNA cells were infected with the indicated lentiviruses in the absence or presence of doxycycline; at the end of 8 days of infection (and 9 days in doxycycline), cells were collected and analyzed by flow cytometry, showing markedly increased cell death following XIAP depletion in the presence of doxycycline. *Right,* quantification of sub-G_1 peaks over 3 experiments. *Bars,* SD. In the presence of doxycycline, XIAP depletion results in a statistically significant increase in the fraction of cells with sub-G_1 DNA content ($P = 0.0021$). *C, left,* NCI-H1299 cdk2/cdk1 shRNA cells were treated with DMSO (*D*), 100 nmol/L flavopiridol, or 300 nmol/L flavopiridol for 72 hours in the absence of doxycycline. Nuclear lysates were subjected to Western blotting with the indicated antibodies, showing reduced CTD phosphorylation at Ser^2 after exposure to 100 nmol/L flavopiridol. Rb phosphorylation is only compromised after exposure to the higher concentration. *Arrow,* phosphorylated Rb. In addition, cells were maintained in the absence or presence of doxycycline for 3 days, replated, and treated with DMSO or 100 nmol/L flavopiridol for 3 days in the absence or presence of doxycycline, after which lysates were collected and subjected to Western blotting with the indicated antibodies. *Middle,* cells were treated after DMSO or flavopiridol in the absence or presence of doxycycline and subjected to TUNEL assay to quantify apoptosis. *Numbers,* percentage of fluorescein-positive cells. *Right,* quantification of fluorescein-positive cells over three experiments. *Bars,* SD. Compared with DMSO, flavopiridol induces significantly more apoptosis in the presence of doxycycline ($P = 0.023$). Flavopiridol also induces significantly more apoptosis in the presence compared with the absence of doxycycline ($P = 0.029$).

II. Although cdk7 and cdk9 are the primary transcriptional activating kinases that phosphorylate the CTD, both cdk2 and cdk1 have been shown to phosphorylate the CTD *in vitro*. In HIV-infected cells, cyclin E-cdk2 has been found to be a component of the complex required for Tat-mediated transcriptional elongation; an association of Tat with cyclin E-cdk2 stimulates phosphorylation of the CTD by cdk2 (3). Cdk1 may phosphorylate TFIIH subunits, including cdk7, as well as the CTD, events that may serve to repress transcription during mitosis but could conceivably participate in the restoration of transcription during G_1 (37, 38). Both cdk2 and cdk1 phosphorylate the CTD at Ser^2 and Ser^5, and in U2OS cells, reduced phosphorylation at these positions is evident after 3.5 days in doxycycline.

A concomitant decrease in RNA polymerase II expression is also seen at this time point, likely related to the ubiquitylation of polymerases subjected to transcriptional arrest (24). Importantly, *R*-roscovitine, a 2,6,9-trisubstituted purine, has been shown to reduce both expression and phosphorylation of RNA polymerase II (39). Similarly, the imidazo[1,2-*a*]pyridine AZ703, applied to U2OS cells at concentrations that primarily inhibit cdk2 and cdk1, also compromises RNA polymerase II expression,[3] implicating cdk2 and cdk1 in RNA polymerase II homeostasis in these cells. The transcripts most sensitive to reduced CTD phosphorylation are those with short half-lives, including those encoding antiapoptotic proteins. Decreased expression of Mcl-1 and XIAP occurs in response to pharmacologic agents that inhibit transcriptional cdks (12, 40). Depletion of these proteins may be adequate to induce cell death in some instances and may also sensitize cells to other apoptotic stimuli, including those induced by inhibition of the cell cycle cdks as in U2OS cells.

NCI-H1299 cells did not display S-phase retardation and abrupt cell death in response to cdk2 and cdk1 depletion. Similarly, effects on CTD phosphorylation were less marked and not observed until 6 days after depletion, without effects on expression of antiapoptotic proteins. The compensatory mechanisms permitting their escape from apoptosis and ultimate arrest in G_2-M remain to be elucidated. We have previously documented cell line–specific differences in the duration of S phase (22); a shorter time of S-phase traversal as well as slowed G_1 progression after cdk2 depletion may limit the time in S phase when cytotoxic effects are most likely to occur. In addition, differences in the participation of cdk2 and cdk1 in CTD phosphorylation likely contribute to different responses to cdk depletion. Preliminary data in A549 NSCLC and HCT-116 colon cancer double-knockdown lines confirm the dichotomy of response to combined cdk2 and cdk1 depletion, with G_2-S arrest in A549 cells and cell death in HCT-116 cells.[3] Nonetheless, the small amount of apoptosis occurring late after cdk depletion in NCI-H1299 cells (or A549 cells) can be enhanced by concomitant shRNA-mediated depletion of cdk9 or XIAP, indicating that effects on CTD phosphorylation and expression of antiapoptotic proteins contribute to cytotoxicity following inhibition of cdk activities.

The cdk9 inhibitor flavopiridol can augment apoptosis when applied with combined cdk2 and cdk1 depletion, indicating that our results are reflected with small-molecule cdk inhibitors. Similarly, AZ703 potently inhibits cdk2 and cdk1 with IC_{50}s of ~30 nmol/L and moderately inhibits cdk9 with an IC_{50} of ~500 nmol/L. When U2OS and NCI-H1299 cells are treated at micromolar concentrations, G_2-S arrest and cell death are observed, although the degree of cytotoxicity is greater in U2OS cells. However, depletion of cdk9 from NCI-H1299 cells enhances the degree of AZ703-induced apoptosis together with a more marked decline in Mcl-1 and XIAP expression (41). Similarly, apoptosis in response to other compounds targeting cell cycle cdks in tumor cell lines is associated with depletion of IAP proteins (42).

As new cdk inhibitor compounds enter clinical trial, it will be important to assess their relative activities against the cell cycle and transcriptional cdks. To date, pharmacologic cdk inhibitors have produced only modest activity against solid tumors, although the most extensively tested drugs are relatively selective for cdk9 (flavopiridol) or cdk2 (*R*-roscovitine, BMS-387032; reviewed in ref. 2). The inducible cell lines developed here show that acute depletion of a combination of cdk activities can arrest or kill malignant cells and suggest that compounds capable of potent cdk2, cdk1, and cdk9 inhibition may be worthy of clinical development.

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