The dual specificity phosphatase Cdc25B is capable of inhibiting cellular proliferation, and this occurs in a manner dependent upon its catalytic activity. Here it is shown that this is accompanied by inappropriate cyclin-dependent kinase activation and premature mitotic entry, leading to both p53-dependent and independent checkpoints. Forced expression of Cdc25B inappropriately up-regulated the activity of Cdk1 and Cdk2, by reducing levels of inhibitory phosphorylation. In cells lacking p14ARF, p53 is induced, and components of the ATM and ATR pathways are activated. Cdc25B triggers cell cycle arrest in the G1 and G2 phases that is p53- and p21-dependent and is inhibited by caffeine. Cdc25B also causes cells with an S phase DNA content to enter mitosis prematurely in a p53-independent manner. Synchronization of cells with aphidicolin results in these cells undergoing apoptosis. Thus, inappropriate cell cycle progression and premature mitotic entry via dysregulation of cyclin-dependent kinases results in activation of both p53-dependent and independent responses. Because Cdc25B is known to have oncogenic activity, this provides insight into the multistep nature of cancer development and why there is p53 loss during tumorigenesis.

Cyclin-dependent protein kinases (Cdks) are enzymes that execute progression through the cell cycle (1). Cdk2 is responsible for the onset of DNA replication, and Cdk1 activation ensures entry into mitosis. Given these important roles, the sequence and timing of their activation are strictly regulated at several levels. The final step in activating Cdk1 and Cdk2 is removal of the inhibitory phosphates from threonine 14 and tyrosine 15. In mammalian cells, this dephosphorylation event is brought about by three related dual-specificity phosphatases: Cdc25A, Cdc25B, and Cdc25C (2). Cdc25A has been implicated in the G1/S as well as G2/M transitions. The responsibility for activating pT14pY15Cdk1/cyclin B and triggering mitosis has been delegated to Cdc25B and Cdc25C based on several observations. Microinjection of antibodies into G2 or asynchronous cells blocked entry into mitosis (3, 4). Furthermore, transient transfection of catalytically inactive Cdc25B or Cdc25C led to accumulation cells in the G2 phase (5).

Cdc25B has been classified as an oncogene (2, 6). Cdc25B cooperates with oncogenic Ras or loss of Rb to transform primary rodent cells (7). In a transgenic model, overexpression of Cdc25B in the mouse mammary gland leads to accelerated mammary epithelial proliferation resulting in hyperplasia (8). Cdc25B is up-regulated by the oncogene c-Myc and is phosphorylated and activated by another oncogene, Aurora-A (9). Furthermore, Cdc25B has been found to be overexpressed in a variety of primary human cancers (2, 6).

Recently, it was shown that Cdc25B, as has been shown for other oncogenes, is capable of inhibiting cellular proliferation, and this occurs in a manner dependent upon its catalytic activity (10). Here a molecular basis for this effect is provided. Inappropriate activation of Cdks by forced expression of Cdc25B causes premature entry into mitosis and triggers both p53-dependent and independent checkpoints. The cell cycle arrest is dependent on activation of p53 and its mediator p21 and occurs independently of p14ARF. Furthermore, it is shown that p53 inhibits premature entry into mitosis from the G1, but not the S phase of the cell cycle. Taken together, these results confirm that Cdc25B exerts effects at multiple points of the cell cycle. Its contribution to tumorigenesis is likely due to increased genomic instability in rare cells that survive the lethal consequences of deregulated progression though the cell cycle. The importance of the protective role of the DNA damage checkpoints against inappropriate activity of oncogenes such as Cdc25B is thereby further emphasized.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human U2OS, 293T, and Saos2 cells were grown in monolayer in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 10% penicillin/streptomycin (Invitrogen) at 5% CO₂ and 37 °C.

**Recombinant Adenoviruses**—The FLAG-tagged open reading frames of Cdc25B, mutant Cdc25B, and Cdc25C were cut...
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out of plasmids and subcloned into the pAdTrack-CMV shuttle vectors. These constructs were linearized by digestion with Pmel and were co-transformed together with the adenoviral backbone plasmid, pAdEasy-1, into the homologous recombination-competent Escherichia coli strain B75183. The resultant recombinants were linearized with Pael and transfected into the adenovirus-packaging cell line, 293T. A high titer of recombinant adenoviruses was obtained by several rounds of infection. Cells were infected with 8–12 multiplicity of infection (m.o.i.) of Ad-25B, 5–10 m.o.i. of Ad-25C, and 35–40 m.o.i. of Ad-mt25B.

Flow Cytometry—Cells were fixed in ice-cold 70% ethanol followed by rinsing with PBS (Invitrogen). DNA was stained with propidium iodide (20 μg/ml, Sigma) in the presence of 1 mg/ml Ribonuclease A (Sigma). Cell cycle analysis was performed on FACSCalibur, and data were analyzed using CellQuest. For two-parameter flow cytometry, protocols provided by the manufacturer for p510H3 antibodies (Upstate, Cell Signaling Solutions) were followed.

Histone H1 Kinase Assay—Exponentially growing U2OS cells were infected with Ad-25B or adenovirus alone. The cells were harvested at various time points and were lysed in the buffer described in the Immunoblotting section. Cdk1 and Cdk2 were immunoprecipitated using anti-cyclin B (GNS1, Santa Cruz Biotechnology, Santa Cruz, CA) mixed with protein G-resin (Sigma) and anti-Cdk2 (M2, Santa Cruz Biotechnology) mixed with protein A-resin (Sigma), respectively, according to the manufacturers’ instruction. The immunoprecipitates were first washed three times in lysis buffer followed by 2 × washes with kinase buffer (25 mM HEPES, pH 7.4, 15 mM MgCl2, 10 mM EGTA, 80 mM β-glycerophosphate, 0.1 mM sodium vanadate, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Next the immunoprecipitates were resuspended in a total of 25 μl of kinase buffer that also contained 5 μg of histone H1 (Sigma), 0.1 mM ATP, and 0.5 μl of [γ-32P]ATP (Amersham Biosciences). Samples were incubated at 37 °C for 15 min. The reactions were stopped by adding equal amounts of 2 × SDS-loading buffer, and incubating at 90 °C for 4 min. Samples were centrifuged, and the supernatant containing the proteins was loaded onto SDS-PAGE (12%). The gels were fixed in 7% acetic-acid-10% methanol, dried, and exposed to x-ray films for autoradiography.

Immunoblotting—Cells were lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 mM Na2VO4, 10 mM NaF, 60 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, 5 mg/ml leupeptin, and pepstatin. Lysates were clarified by centrifugation. The supernatant containing the proteins was loaded onto SDS-PAGE and electrophoresed. Proteins were transferred to polyvinylidene difluoride membranes and incubated with the following antibodies: rabbit anti-pS10H3, anti-pS15p53, and mouse anti-pS315 p53; rabbit anti-pT14/pY15Cdk1, anti-Cdk2 (M2), anti-pY15Cdk1, anti-cyclin B (H-433), anti-cyclin A (H-432), anti-pT68Chk2, anti-pS317Chk1, and anti-p21 (C-19) were from Santa Cruz Biotechnology. Rabbit anti-pT160Cdk2, anti-pS15p53, anti-pS315 p53, and mouse anti-pS1981ATM were from Cell Signaling Technology. Rabbit anti-pS10H3 was from Upstate, Cell Signaling Solutions. Mouse anti-β-actin was from Oncogene Research Products. Peroxidase-conjugated goat antibodies against rabbit or mouse IgG (MP, Biomedicals) were used as secondary antibodies. The signals were detected using ECL reagents (Amersham Biosciences) and autoradiography films (Labscientific, Inc.).

Immunostaining—Cells were grown on coverslips (Fisher Scientific). After fixation in 3% paraformaldehyde, cells were washed in PBS and permeabilized in a solution containing 0.5% Triton X-100, 3 mM MgCl2, and 0.2 mM sucrose. After washing in PBS, the cells were blocked in 3% bovine serum albumin for 10 min prior to incubation with a buffer containing 3% bovine serum albumin/PBS and 1:1000 dilution of mouse anti-γ-H2AX (Upstate, Cell Signaling Solutions) for 1 h. When cells were double-stained 1:1000, a dilution of rabbit anti-pS10H3 (Upstate, Cell Signaling Solutions) was included. The cells were then washed in PBS and subsequently incubated with a 1:2000 dilution of Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Invitrogen Detection Technologies) in 3% bovine serum albumin/PBS for 1 h. When cells were double-stained, Alexa Fluor 647 goat anti-rabbit IgG (Molecular Probes, Invitrogen Detection Technologies) was included at a dilution of 1:2000. DNA was stained with DAPI (Sigma) at a concentration of 5 mg/ml PBS. The cells were then washed in PBS. The coverslips were mounted on Microscope slides (Fisher Scientific) covered with Immuno-Mount oil (Thermo, Electron Corp.). The coverslips were sealed with transparent nail polish. The cells were observed using a fluorescence microscope (Nikon Eclipse E800).

Bromodeoxyuridine Incorporation—Bromodeoxyuridine (BrdUrd) incorporation assay was performed according manufacturer’s instructions (BD Biosciences). Mouse anti-BrdUrd antibody was from BD Biosciences. Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Invitrogen Detection Technologies) was used as the secondary antibody. DNA was stained with DAPI (Sigma).

siRNA—p53 siRNA (r(GCAUGAACCGGAGGCCCAU)-dTdT or control siRNA (r(AUGAACGUGAAUUGCUCAUU) were purchased from Qiagen. The p21 siRNA was a pool of four oligonucleotides, (r(GAUGGACUGACUUUGUUU), (r(GCGAUGGAACUCGACUUUUU), (r(CGAUGGACUCUGACUUUGUUU), and (r(CGACUGUGAGCGCAUUGUUU), purchased from Dharmacon. Exponentially growing U2OS cells were transfected with the appropriate siRNA oligonucleotides using DharmaFECT reagent 1 (Dharmacon) following the manufacturer’s instructions.

Lentivirus-expressing shRNA—To construct the lentivirus expressing the p53 shRNA, LV-p53shRNA the following phosphorylated oligonucleotide was used: TGACTCCAGTGGTATCTTACCTAAAGAGGATGATACCACCATGGAGTCCTTTCTC. This was subcloned into the pLL3.7 vector. For the control lentivirus, an empty vector was used. Each of these plasmids was co-transfected into 293T cells together with packaging plasmids, pRE, pPRE, and pVSVG using PolyFect Transfection Reagent (Qiagen) following the manufacturer’s protocol. After 16–18 h, media was replaced. Media containing LV-p53shRNA or the control lentivirus was collected 24, 48, and 72 h later, pooled, and filtered (0.45 mm).
Stable Cells Expressing shRNA—The U2OS cells expressing the p21 shRNA (CAGAACCGGCTGGGGATGTCCGTCAGAAC) was a kind gift of Athena Line (Roswell Park Cancer Institute). U2OS cells were infected with either LV-p53shRNA or the control lentivirus by standard protocols.

FIGURE 1. Ectopic expression of Cdc25B causes inappropriate cell cycle progression and reduces the level of inhibitory tyrosine phosphorylation on the cyclin-dependent kinases Cdk1 and Cdk2. A, U2OS cells were infected with a recombinant adenovirus expressing Cdc25B for the indicated times (0–48 h), a control adenovirus for 48 h (Ad 48 h), treated with 100 ng/ml nocodazole for 24 h, or were untreated during the time course of the experiment (control 72 h). Samples were fixed and double stained with propidium iodide and pS10H3 antibody prior to two-parameter flow cytometry analysis. Representative dot plots are shown. The average of the percentage of cells positive for pS10H3 with either 4N DNA content or with <4N DNA content from three experiments is indicated. B, bar graphs show the percentage of cells positive for pS10H3 with either 4N DNA content (upper panel) or with <4N DNA content (lower panel). The results are again the average of three experiments. C, U2OS cells were infected with adenoviruses expressing the indicated FLAG-tagged proteins or empty vector (LT-Ad: lower titer of adenovirus, same m.o.i. as Ad-25B and Ad-25C and HT-Ad: higher titer of virus, same m.o.i. as Ad-mt25B). 24 h post-infection, cells were lysed and immunoblotted with the indicated antibodies. Extracts from cells treated with 0.05 μg/ml doxorubicin, 100 ng/ml nocodazole, or 1 μg/ml aphidicolin for 24 h were also included as controls to show the status of the indicated cell cycle regulators following treatment with a DNA-damaging agent or arrested in mitosis or S phase, respectively. Actin was used as a loading control. pY15 refers to an antibody that detects phosphorylated tyrosine 15 on either Cdk1 or Cdk2.
RESULTS

Ectopic Expression of Cdc25B Causes Premature Mitotic Entry and Reduces the Level of Inhibitory Tyrosine Phosphorylation on the Cyclin-dependent Kinases Cdk1 and Cdk2—To explore the molecular consequences of untimely cell cycle progression, overexpression of the phosphatase Cdc25B was chosen as an experimental means to inappropriately activate cyclin-dependent kinases. The U2OS cell line (osteosarcoma) was used for two reasons. First, these cells express wild-type p53 and are checkpoint-proficient. Second, they are readily infected with adenoviruses. U2OS cells were infected with a bicistronic recombinant adenovirus expressing human Cdc25B (Ad-25B), and are checkpoint-proficient. Second, they are readily infected with adenoviruses. U2OS cells were infected with a bicistronic recombinant adenovirus expressing human Cdc25B with a FLAG epitope fused to its amino end and green fluorescent protein. Cells that have entered mitosis were detected using immunostaining with an antibody that recognizes histone H3 phosphorylated on serine 10 (pS10H3) with propidium iodide staining used to monitor DNA content. The assay was first validated by examining cells after treatment with nocodazole. As expected for such a mitotically enriched population, the majority (90%) of nocodazole-treated cells were positive for pS10H3 with a DNA content of 4N (Fig. 1A). At various time points following infection with the recombinant adenovirus expressing Cdc25B (Ad-25B), cells were similarly analyzed (Fig. 1A). For quantitation, cells positive for pS10H3 were divided into two groups based on their DNA content, either 4N (R4 box) or <4N (R5 box) (Fig. 1B). Approximately 2% of the exponentially growing culture was in mitosis using the criteria of pS10H3 positivity (Fig. 1A, 0 h). After 6 h of infection with Ad-25B, cells with a 4N DNA content and positive for pS10H3 increased, peaking at 12 h. Thereafter, there was a continuous reduction in the number of these cells. The lowest numbers (at 48 h) were half of that seen with control cells (1.4%). Interestingly, cells positive for pS10H3 with a DNA content <4N increased in Ad-25B-infected cells at 10 h, peaking at 20 h. The number of pS10H3-positive cells with <4N DNA content was undetectable in control or nocodazole-treated cells. This suggests that forced expression of Cdc25B caused cells with <4N DNA content to fail to complete a full round of DNA synthesis. These cells then exited S phase prematurely and inappropriately entered mitosis.

Cdc25B Acts as a Phosphatase That Removes Inhibitory Phosphorylations on the Cyclin-dependent Kinases Cdk1 and Cdk2—To confirm that forced expression of Cdc25B led to a loss of inhibitory phosphorylations on Cdk1 and Cdk2, immunoblotting analysis was performed. As a control, the catalytically inactive mutant of Cdc25B (C488S) (Ad-nt25B) was used. A recombinant adenovirus expressing Cdc25C (Ad-25C) was constructed to compare the effects of this closely related phosphatase. The effects of Cdc25B were also compared with treatments that cause DNA damage (doxorubicin) or cell cycle arrest in mitosis (nocodazole) or S phase (aphidicolin).

Cdk1 is detected as three bands on a SDS gel (12–14). The uppermost band is considered to be phosphorylated on threonine 14 and tyrosine 15 (pT14pY15Cdk1), whereas the middle band is phosphorylated only on tyrosine 15 (pT15Cdk1). The lower band is thought to be fully dephosphorylated on these residues. As expected, treatment with nocodazole led to a single band consistent with the preponderance of the mitotically active, unphosphorylated form of Cdk1, whereas aphidicolin, which arrests cells in early S phase, showed the majority of Cdk1 to be...
fully phosphorylated on these inhibitory residues (Fig. 1C).

Overexpression of Cdc25B gave a similar pattern as treatment with nocodazole, consistent with full dephosphorylation of Cdk1. This was accompanied by reduced levels of cyclin A and cyclin B (Fig. 1C). Cdk2 appears as two bands on a SDS gel. It is thought that pT14pY15Cdk2, which is also phosphorylated on threonine 160, migrates faster (16). Treatment with nocodazole enriched for the slower migrating form of Cdk2, whereas incubation with aphidicolin enhanced the level of the faster migrating form. Again, overexpression of Cdc25B gave a similar pattern as treatment with nocodazole showing loss of phosphorylation on threonine 160. This was directly shown by reduced reactivity with the specific phospho-antibody against threonine 160 (pT160, Fig. 1C). Phosphorylation of this residue has been shown to be necessary for kinase activity (16). Dephosphorylation of tyrosine 15 on both Cdk1 and Cdk2 was further demonstrated by lack of reactivity with the specific phospho-antibody (pY15) against this residue (Fig. 1C). Overexpression of the inactive Cdc25B did not change the overall levels or the phosphorylation status of these cell cycle regulators confirming that the effects caused by Cdc25B overexpression are dependent upon its phosphatase activity. In contrast, overexpression of Cdc25C had no effect nor did infection with empty adenovirus (Fig. 1C). These results demonstrate that overexpression of Cdc25B leads to loss of inhibitory phosphorylations on both Cdk1 and Cdk2, consistent with the notion that inappropriate Cdc25B phosphatase activity leads to premature cell cycle progression.

**Overexpression of Cdc25B Causes Accumulation of Cells in Multiple Phases of the Cell Cycle**—To determine the effects of Cdc25B overexpression on the cell cycle, cells were analyzed by flow cytometry after staining with propidium iodide (Fig. 2A). There was a significant increase in cells with an S-phase DNA content with the majority appearing to accumulate at the start of S phase (Fig. 2A). The number of cells with a 2N DNA content did not change, whereas cells with a 4N DNA content decreased by almost half (Fig. 2A). Similar effects after infection with Ad-25B were seen in two other wild-type p53 tumor cell lines, HT1080 and MCF7 (supplemental Fig. S1A). This suggests that his finding is not peculiar to U2OS cells. By contrast, mutant Cdc25B overexpression at similar levels as the wild-type protein had no effect on the cell cycle at 24 h (data not shown), suggesting the phosphatase activity of Cdc25B was required for this unusual cell cycle profile (Fig. 2A). Previous studies have demonstrated that overexpression of the catalytically inactive Cdc25B leads to accumulation of cells in the G2/M phases (5, 17). Under the conditions here, the inactive mutant Cdc25B had to be overexpressed at considerably higher levels than the wild type to achieve this outcome (Fig. 2A). As controls, cells were infected with either the same amount of empty adenovirus...
as those infected with Ad-25B (lower titer of adenovirus (LT-Ad)) or those infected with Ad-mt25B (higher titer of adenovirus (HT-Ad)). Overexpression of Cdc25C did not affect the cell cycle profile of the cells as compared with the control (Fig. 2A) nor did infection with empty adenovirus (LT-Ad or HT-Ad) (Fig. 2A). Likewise, infection with Ad-25C did not affect the profile of another tumor cell line that expresses wild-type p53, HT1080 (supplemental Fig. S1B).

To determine whether cells overexpressing Cdc25B or mutant Cdc25B are actively synthesizing DNA, bromodeoxyuridine (BrdUrd) incorporation assays were performed. The number of cells positive for BrdUrd was greatly reduced following infection with either Ad-25B or Ad-mt25B as compared with cells infected with empty virus (Fig. 2B). It should be noted that, at the m.o.i. values used here, the levels of mutant Cdc25B are much higher than that of the wild-type protein (see Fig. 1C). Both Cdc25B and its inactive mutant inhibit cellular DNA synthesis and cause cell cycle arrest, but with differing cell cycle profiles and with distinct dose responses.

To confirm that the effects of Cdc25B overexpression are the consequence of hyperactivation of Cdk1 and Cdk2, kinase activity was assessed using histone H1 as a substrate. Following infection with Ad-25B, Cdk1 or Cdk2 were immunoprecipitated and subjected to such assays (Fig. 2C). Enhanced activity of both Cdk1 and Cdk2 was reflected in the increased phosphorylation of histone H1 and was detected at 7 h after infection with Ad-25B (Fig. 2C). Microscopic observation of green fluorescent protein expression confirmed comparable efficiencies of infection. Phosphorylation of histone H1 in both cases increased with time and peaked around 10 h post-infection (Fig. 2C). This was followed by a gradual decline in the phosphorylation of histone H1 such that, at 24 h, little kinase activity was detected. These results further confirm that excess Cdc25B leads to hyperactivation of Cdk1 and Cdk2.

To confirm that hyperactivation of Cdk1 and Cdk2 is a result of dephosphorylation of Cdk1 and Cdk2 on inhibitory residues and not due to increased levels of the relevant cyclins, immunoblot analyses were performed (Fig. 2C). The expression of cyclins A, B, and E was unaffected until 24 h when they decreased. Interestingly, reduction in phosphorylation on threonine 14 and tyrosine 15 was observed at 7 h, which correlates with the increase in Cdk1 and Cdk2 activity (Fig. 2C). The reduction in level of inhibitory phosphorylations continued until 24 h. At this time

![FIGURE 4. The Cdc25B-induced cell cycle arrest in the G1 and G2 phases is p53-dependent. U2OS cells were either untransfected or transfected with either a single siRNA oligonucleotides to p53 or a control siRNA oligonucleotide. 48 h later, cells were then infected with adenoviruses expressing FLAG-tagged Cdc25B proteins or a control virus. A, at the indicated time points, cells were lysed, and the success of siRNA was verified by immunoblotting using antibodies against p53 or p21. Actin was the loading control. B, after similar treatments as in A, cells were fixed and their cell cycle distribution was determined at the indicated time points by flow cytometry. C, at 24 h post-infection, cells treated with p53 siRNA were fixed and immunostained with γ-H2AX antibody. Chromatin was stained with DAPI. The right panel is the differential interference contrast micrograph. D, U2OS cells or a derivatives in which p53 was eliminated by lentivirus-mediated shRNA or its control (transduced with empty lentivirus) were infected with the indicated recombinant adenoviruses. Samples were taken at different time points and used in immunoblotting. E, U2OS cells in which p53 had been eliminated by lentivirus-mediated shRNA were infected with the indicated adenoviruses. At the indicated time points, cells were lysed and immunoblotted for γ-H2AX. F, Saos2 and U2OS cells were infected with Ad-25B and control virus. 48 h after infection cells were lysed and used in immunoblot analyses using specific antibody against Cdc25B. Actin was the loading control. G, infected Saos2 cells were fixed and stained with propidium iodide prior to flow-cytometry analyses.](https://www.jbc.org/content/284/14/9480)
point, however, no Cdk activity was detected, consistent with the reduced levels of the relevant cyclins (Fig. 2C). Thus, these results confirm that excess Cdc25B hyperactivates Cdk1 and Cdk2 by causing dephosphorylation on inhibitory residues. This, in turn, is accompanied by inhibition of cell proliferation (Fig. 2B).

Ectopic Expression of Cdc25B Activates p53 and Components of the ATM and ATR Pathways—The role of p53 in the Cdc25B-dependent cell cycle arrest was then examined. Cells were again infected with recombinant adenoviruses (Fig. 3A). After 24 h, cell lysates were prepared and used in immunoblotting. p53 was induced following overexpression of Cdc25B (Fig. 3A). Mutant Cdc25B, despite being overexpressed at a higher level, caused only a subtle induction of p53 (Fig. 3A), suggesting again that the phosphatase activity of Cdc25B was involved. p21, a known effector of p53, was also induced following infection with Ad-25B, Ad-mt25B, and HT-Ad, but not Ad-25C or LT-Ad (Fig. 3A). Induction of p21 after infection of Ad-mt25B is likely related to the high m.o.i., because this was also detected in cells infected with a similar titer of control virus (Fig. 3A). p53 and p21 were not induced when mutant Cdc25B was expressed at similar level as the wild-type (data not shown) or in response to overexpression of Cdc25C (Fig. 3A).

U2OS cells lack p14ARF. Thus, it became important to ask how elevated levels of Cdc25B might induce p53, if not via a previous characterized pathway that involves p14ARF. Activation of p53 has been shown to occur via a well characterized signaling cascade that includes the ATM and ATR kinases. Thus, the effect on components of the ATM and ATR pathways upon untimely activation of Cdk1 and Cdk2 was examined. As controls, cells were treated with doxorubicin (0.05 μg/ml) or aphidicolin (1 μg/ml) to induce DNA damage and replicative stress, respectively. Overexpression of Cdc25B led to phosphorylation of ATM on serine 1981, Chk2 on threonine 68, Chk1 on serine 317, and p53 on serine 15 (Fig. 3A). These effects are characteristic of activation of these pathways (18). p53 was also acetylated on lysine 382 (Fig. 3A). The phosphorylation levels of proteins were not as high as in cells treated with doxorubicin except with regard to Chk1 (Fig. 3A). Chk1 phosphorylation levels, however, were comparable to that of cells treated with aphidicolin (Fig. 3A). Although Chk1 levels decreased in doxorubicin-treated cells, this did not occur in cells overexpressing Cdc25B or treated with aphidicolin (Fig. 3A). ATM and Chk2 were not phosphorylated in response to aphidicolin treatment with p53 induction and phosphorylation on serine 15 showing only subtle effects (Fig. 3A). No acetylation on lysine 382 was detected in aphidicolin-treated cells (Fig. 3A). These results suggest similarities between the cellular response to DNA damage, replicative stress, and overexpression of Cdc25B. Other hallmarks of activation of these former pathways are degradation of Cdc25A, down-regulation of Cdc25C by p53, and increased phosphorylation on threonine 14 and tyrosine 15 and/or reduction in the levels of Cdk5 or their cyclin partners as observed here in response to doxorubicin or aphidicolin (Figs. 2A and 1C) (19). In response to overexpression of Cdc25B, however, neither levels of Cdc25A nor Cdc25C changed nor were Cdk5 phosphorylated on inhibitory residues (Figs. 2A and 1C). This is not surprising considering the excess amount of Cdc25B and the reduced levels of cyclin B and cyclin A (Fig. 1C). Binding to cyclin partners is considered to be required for phosphorylation of Cdk1 and Cdk2 on threonine 14 and tyrosine 15 (13, 14). Similarly, in doxorubicin-treated cells, Cdk1 was not fully phosphorylated on the inhibitory residues, however, its level and that of cyclins B and A was reduced. Phosphorylation of p53 on serine 315 by Cdk5 has been linked to activation of p53. Phosphorylation of this residue was examined, because Cdc25B is a known activator of the Cdk5. Surprisingly, whereas serine 315 was phosphorylated in response to doxorubicin, no...
Cdk Dysregulation Triggers Checkpoint

A

0 hr

6 hr

8 hr

10 hr

12 hr

14 hr

16 hr

18 hr

20 hr

22 hr

24 hr

48 hr

nocodazole

Ad 48 hr

control 72 hr

B

0 hr

6 hr

8 hr

10 hr

12 hr

14 hr

16 hr

18 hr

20 hr

22 hr

24 hr

48 hr

nocodazole

Ad 48 hr

control 72 hr

C

% of pS\textsuperscript{H3}-positive cells

4N DNA content

% of pS\textsuperscript{H3}-positive cells

<4N DNA content
modification on this residue was detected in cells with excess activity of Cdc25B (Fig. 3A).

To confirm a role for DNA damage, the formation of nuclear foci containing γ-H2AX was detected using immunostaining. Phosphorylation of H2AX on serine 139 (referred to as γ-H2AX) occurs within minutes of DNA damage (20). Interestingly, the nuclei of only 40–45% of cells overexpressing Cdc25B were positive for γ-H2AX (Fig. 3B). The intensity of γ-H2AX staining was so high compared with that caused by doxorubicin or aphidicolin that it was impossible to detect individual foci, suggesting that the DNA damage was extensive (Fig. 3, B and C). Thus, a likely explanation for the activation of the ATM and ATR pathways was in response to damage to genomic DNA. Overexpression of Cdc25C, inactive Cdc25B, or infection with control adenovirus, did not activate these checkpoints (Fig. 3A). These results suggest that the cell cycle arrest induced in response to elevated levels of Cdc25B is, in part, a consequence of activation of DNA damage checkpoints and the phosphatase activity of Cdc25B is required for this.

Morphological examination of the γ-H2AX-positive nuclei revealed that they could be classified into four different groups (Fig. 3, C and D). Group one was round, had uncondensed chromatin, and represented ~20% of the total number. Differential interference contrast microscopy showed that the size or appearance of the cells containing these nuclei did not differ from that of control cells. The second group (15% of the total) showed fragmentation. About half of these nuclei were positive for phosphorylated histone H3 on serine 10 (pS10H3), the mitotic marker, and the chromatin was condensed. Cells harboring these nuclei were large and flattened. The third group represented a small percentage (3–4%) of cells that were micro-nucleated. These cells also were large and flattened. The fourth group (4–5%) had condensed chromatin, were round, and positive for pS10H3. The occurrence of two major morphologically distinct populations of nuclei that were positive for γ-H2AX (i.e. round versus fragmented) and the fact that pS10H3 positivity was found with only the fragmented nuclei suggest that Cdc25B targets two distinct subsets of substrates, each one leading to a distinct outcome. Despite overexpressing Cdc25B, the remaining 55–60% nuclei were negative for γ-H2AX and did not differ from controls in size or shape (Fig. 3C). This is consistent with the idea that Cdc25B-induced DNA damage occurs only in a specific phase of the cell cycle, and some of the cell cycle responses to Cdc25B are triggered in the absence of damage to DNA.

Cdc25B-induced Cell Cycle Arrest in the G1 and G2 Phases Is p53- and p21-dependent—To verify the role of p53 in the Cdc25B-induced cell cycle arrest, p53 was down-regulated by using an siRNA oligonucleotide 48 h prior to infection with Ad-25B or empty adenovirus. Cells were also transfected with a control siRNA before infections. p53 protein level was reduced at the time of infection (0 h) and was significantly eliminated by the time cells were harvested (Fig. 4A). p21 levels were not elevated in the absence of p53 in response to Cdc25B overexpression (Fig. 4A). Both p53 and p21 were induced in response to Cdc25B in cells transfected with control siRNA (Fig. 4A). Flow cytometry analysis revealed an intriguing cell cycle profile. At 24 h after overexpression of Cdc25B in the absence of p53, the number of cells with a 2N or 4N DNA content decreased significantly, whereas that of cells with an S-phase DNA content increased (Fig. 4B). By 48 h the number of cells with an S-phase DNA content started to decline. A concomitant increase in the sub-G1 population suggested that these cells

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**FIGURE 6.** **p53 prevents inappropriate exit from S phase.** U2OS cells were treated as in Fig. 1A after being transduced with a lentivirus expressing p53 siRNA (A) or a corresponding control (transduced with empty lentivirus) (B). Cells were then infected with a recombinant adenovirus expressing Cdc25B for the indicated times (0–48 h), a control adenovirus for 48 h (Ad 48 h), treated with 100 ng/ml nocodazole for 24 h, or were untreated during the time course of the experiment (control 72 h). Samples were fixed and double stained with propidium iodide and pS10H3 antibody prior to two-parameter flow cytometry analysis. Representative dot plots are shown. The average of the percentage of cells positive for pS10H3 with either 4N DNA content or with <4N DNA content from three experiments is indicated. C, bar graphs show the percentage of cells positive for pS10H3 with either 4N DNA content (left panel) or with <4N DNA content (right panel). The results are the average of three experiments. Red bars are the controls, whereas black bars are the p53 shRNA-expressing cells.

**FIGURE 7.** **The effects of Cdc25B are caffeine-sensitive.** A, U2OS cells were treated with 5 mM or 10 mM caffeine for 1 h prior to infection with the indicated adenoviruses or treatment with 0.05 μg/ml doxorubicin or 1 μg/ml aphidicolin. 24 h later, cell lysates were immunoblotted with the indicated antibodies. B, U2OS cells were treated with 5 mM or 10 mM caffeine for 1 h. Cell lysates were immunoblotted for endogenous Cdc25B. C, cells treated as in A were fixed, and DNA was stained with propidium iodide for flow cytometry analysis. D, alternatively, cells were fixed and immunostained with γ-H2AX antibody. DNA was counterstained with DAPI.

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were undergoing apoptosis (Fig. 4B). Similar results were observed in U2OS cells after transduction with a lentivirus expressing an shRNA to p53 (supplemental Fig. S2). Because the shRNA targets a distinct sequence from that of the siRNA oligonucleotide, this suggests that the observed effects may be specific for p53 down-regulation. Indeed, a p53-null tumor cell line, EJ, also shows a similar cell cycle profile after infection with Ad-25B (supplemental Fig. S1C). These results indicate that the cell cycle arrest in the G1 and G2 phases caused by overexpression of Cdc25B are, in fact, p53-dependent.

Immunostaining of p53-depleted cells using γ-H2AX antibody and DAPI revealed that 24 h after overexpression of Cdc25B > 90% of the cells were positive for γ-H2AX (Fig. 4C). This result was further confirmed by immunoblotting using γ-H2AX antibody. For this experiment, p53 was eliminated by means of a lentivirus expressing a shRNA directed against p53, LV-p53shRNA (Fig. 4D). The amount of γ-H2AX increased 24 h after overexpression of Cdc25B in the absence of p53 (Fig. 4E). At this time point, flow cytometric analysis did not reveal a subG1 population (Fig. 4B and data not shown). Thus, the increased detectable DNA damage is unlikely due to apoptosis. These results suggest that p53 prevented intact cells from accumulating DNA damage. Furthermore, these data confirm that not all of the observed cell cycle arrests are associated with DNA damage. Saos2 cells are an osteosarcoma line that lacks functional wild-type p53. Of interest was the observation that overexpression of Cdc25B in Saos2 cells at similar levels as in U2OS cells resulted in cell death. This occurred despite higher levels of endogenous Cdc25B being detected in Saos2 cells (Fig. 4, F and G).

p21 is known to be a major player in the p53-mediated maintenance of G1, and G2 arrests in response to DNA damage (19). To explore the involvement of p21 in the p53-dependent cell cycle arrests accompanying Cdc25B overexpression, U2OS cells in which p21 was eliminated by stable expression of an shRNA or its control were infected with either Ad-25B or empty vector. At the indicated time points, cells were harvested and analyzed. p21 was not detected before (0 h) or after (48 h) infection with Ad-25B or empty vector in p21-depleted cells (Fig. 5A). In control cells, p21 was induced following infection with Ad-25B and not empty vector (Fig. 5A). Flow cytometry analysis revealed a cell cycle profile similar to that of p53-depleted cells overexpressing Cdc25B (Figs. 5B and 4B). At 24 h after overexpression of Cdc25B, the number of cells with 2N or 4N DNA content declined while that of cells with S-phase DNA content increased (Fig. 5B). By 48 h, the number of cells with an S-phase DNA content had started to decline, and cells were undergoing apoptosis as judged by an increase in the sub-G1 population (Fig. 5B). Similar results were obtained when p21 was depleted using transient transfection of a pool p21 siRNA oligonucleotides (supplemental Fig. S3). The sequences targeted by this pool are distinct from that of the shRNA expressed in the stable derivative cell line. This makes it less likely there were off-target effects specific to one oligonucleotide sequence. Thus, p21 is a key mediator of the p53-dependent cell cycle arrest caused by overexpression of Cdc25B.

p53 Inhibits Cdc25B-induced Mitotic Entry of Cells That Have Completed DNA Replication but Not Cells That Prematurely Exit S Phase—To further explore the p53 dependence of these effects, two-parameter flow cytometry analysis using a combination of immunostaining with pS10H3 antibody and propidium-iodide staining for DNA was performed. p53 expression was ablated in U2OS cells by transduction with a recombinant lentivirus expressing shRNA to p53. These were compared with cells that had been transduced with a control lentivirus vector (control shRNA). The efficiency of ablation of p53 expression was verified by immunoblotting (Fig. 4D). The assay was again validated using nocodazole-treated cells that were positive for pS10H3 and had a DNA content of 4N (Fig. 6, A and B). As in Fig. 1 (A and B), cells positive for pS10H3 were divided into two groups based on their DNA content (4N versus <4N). At 6 h following infection with Ad-25B, cells with a DNA content of 4N and positive for pS10H3 were increased in both the control and p53shRNA cells, peaking at 10 h (Fig. 6). In the absence of p53, however, this increase was more pronounced (4% for control cells and 10% for p53 shRNA cells). These results suggest that enforced expression of Cdc25B causes cells in the G2 phase to acquire mitotic characteristics, and p53 can inhibit this. For cells with <4N DNA content, p53 status did not affect outcome. In both control and p53 shRNA cells, there was an increase at 8 h, peaking between 14 and 20 h. These results indicate that overexpression of Cdc25B causes cells in S phase (<4N DNA) to enter mitosis, and p53 expression plays no role. The gradual decrease in the numbers of pS10H3-positive cells is likely due to apoptosis as the sub-G1 population increases with time (Fig. 6). These findings are consistent with the flow cytometric analysis of propidium iodide-stained cells (Fig. 4B).

Although p53 clearly contributes to the cell cycle arrest in the G1 and G2 phases, loss of p53 has no effect on the accumulation of cells with an S-phase DNA content.

The Effects of Cdc25B Are Caffeine-sensitive—To further explore the role of the ATM/ATR pathways in cells with forced expression of Cdc25B, the caffeine sensitivity of the effects were examined (Fig. 7). Caffeine has been shown to act, in part, as an inhibitor of ATM and ATR responses. Interestingly, caffeine treatment caused an increase in the levels of Cdc25B (7A). This effect was not specific to exogenous Cdc25B, because it was also seen with endogenous protein (Fig. 7B). The significance of this observation is unclear. Induction of p53 and p21 and phosphorylation of p53 on serine 15 were reduced after overexpression of Cdc25B in the presence of caffeine (7A).

Consistent with previous published reports, caffeine abrogated the induction of p53 and p21 and phosphorylation of p53 on serine 15 in response to doxorubicin (7A). Only treatment...
with the higher dose of caffeine (10 mM), however, affected the subtle induction of p53 following treatment with aphidicolin (Fig. 7A, compare lanes 1 and 2 with 9 and 10). Flow cytometric analysis revealed that doxorubicin-induced alterations in cell cycle profile were caffeine-sensitive at both the 5 mM and 10 mM doses. Although 5 mM caffeine had little effect on the profiles after overexpression of Cdc25B or treatment with aphidicolin, the higher 10 mM dose blocked any observable effects under both conditions (Fig. 7C). Immunostaining for γ-H2AX showed that caffeine did not affect γ-H2AX positivity upon overexpression of Cdc25B, but did prevent foci formation in response to doxorubicin (Fig. 7D). In the case of aphidicolin, caffeine reduced the DNA damage foci by ~50% (Fig. 7D).

These results confirm that the DNA damage checkpoint pathways that are activated in response to elevated levels of Cdc25B behave similarly to that seen after treatment with doxorubicin.

Cells with an S-phase DNA Content Enter Mitosis and Undergo Apoptosis in Response to Overexpression of Cdc25B—To further explore the fate of the cells that accumulate with a DNA content of <4N following ectopic expression of Cdc25B, U2OS cells were synchronized in S phase by treatment with aphidicolin. Cells were then infected with Ad-25B or empty adenovirus, and at 12 h aphidicolin was removed. Cells infected with control adenovirus exited S phase, traversed through the cell cycle, and by 48 h regained a cell cycle profile consistent with exponential growth (Fig. 8A). The majority of cells overexpressing Cdc25B, however, maintained an S-phase DNA content after aphidicolin removal and by 16 h started to undergo apoptosis as judged by an increase in the population of cells with a hypodiploid DNA content (Fig. 8A). In addition, a small number of cells appeared to achieve a 4N DNA content before dying (Fig. 8A).

At the time of aphidicolin removal, cells had been exposed to Cdc25B for 12 h. At this point, tyrosine 15 phosphorylation on Cdk1 and Cdk2 had decreased compared with the time of infection (when cells had been exposed to aphidicolin for 22 h: *Aph. Tol* (time of infection)), but was not as complete as what is seen in cells overexpressing Cdc25B for 24 h in the absence of aphidicolin (Fig. 8B). This is consistent with the majority of Cdk1 being in the active form (*lower band*) and the bulk of Cdk2 in the slow-migrating form (inactive) (Fig. 8B). The level of cyclin B or A had not changed substantially during this 12-h period (Fig. 8B), however pS10H3 was now detectable. These results suggest that Cdc25B causes cells to enter a mitotic-like state with an S-phase DNA content, thus uncoupling mitosis from DNA replication.

At 4 h post-release, no significant change in the level or phosphorylation of these proteins was detected. At 8 h, the level of cyclin B had decreased, and there was little cyclin A. By 12 h, there were no detectable cyclin B or A. At this and subsequent time points, Cdk1 and Cdk2 had no detectable phosphorylation on tyrosine 15. The level of Cdc25B between 0 and 8 h was similar to that of 24 h in the absence of any treatment with aphidicolin. Throughout the time course, histone H3 phosphorylation levels persisted with the DNA content of the cells remaining less than 4N. Levels of p53 and p21 were initially induced, but a decline in the levels of these proteins from 16 h onward was then detected. This is likely due to ongoing apoptosis as this correlates with the increase in the cells with a hypodiploid DNA content (Fig. 8, A and B).

At the time of aphidicolin release, cells were immunostained for γ-H2AX. Cells infected with control virus had round nuclei and distinct γ-H2AX foci (Fig. 8C). In contrast, the majority of cells overexpressing Cdc25B had nuclei (95%) that displayed fragmented chromatin and the γ-H2AX was much more intense (Fig. 8C). Interestingly, only 10–15% of these cells were positive for pS10H3 (Fig. 8C), suggesting that only a fraction of the cells were forced into mitosis at this time point. Because the level of pS10H3 does not change during the time course of the experiment (Fig. 8B), this suggests that at any given point, there is ongoing entry of a subset of cells into mitosis followed by the beginnings of apoptosis leading to loss of pS10H3 staining.

The two-parameter analysis revealed the presence of apoptotic cells that was not detected when propidium iodide staining alone was performed (Compare Figs. 2A and 6B 24 h for the sub-G₁ population). This is likely due to all cells, including those floating and harvested for the two-parameter analysis, whereas the experiment shown in Fig. 2A assayed only attached cells. The presence of apoptotic cells raises the possibility that the ongoing cell death may be causing the DNA damage that is detected by γ-H2AX. If this were the case, blocking apoptosis should eliminate evidence of DNA damage. To address this, cells were treated with the general caspase inhibitor z-VAD-fmk. As a control, cells were treated with staurosporine, a known apoptosis inducer. Use of z-VAD-fmk resulted in reduction in poly(ADP-ribose) polymerase cleavage in cells overexpressing Cdc25B as well as following treatment with staurosporine (Fig. 8D). This is consistent with the role of z-VAD-fmk as an inhibitor of caspase-mediated cleavage of poly(ADP-ribose) polymerase. In the presence of z-VAD-fmk, the amount of γ-H2AX following overexpression of Cdc25B was only slightly reduced, whereas it was completely eliminated in the staurosporine-treated cells (Fig. 8D). These results argue that the DNA damage induced by overexpression of Cdc25B is not due to ongoing apoptosis (unlike with staurosporine) and confirm that forced expression of Cdc25B in cells in S phase (with <4N DNA content) results in apoptosis.

**DISCUSSION**

Despite the fact that both Cdc25B and Cdc25C are involved in induction of mitosis, only Cdc25B has been characterized as an oncogene. Overexpression of Cdc25B suppressed cell proliferation (Fig. 2B), a quality shared by other oncogenes, including Cdc25A, Aurora-A, Ras, c-Myc, and E1A (9, 21–23). The effect of Cdc25B on the cell cycle profile was not specific to U2OS cells, because it was observed in other cell lines such as HT1080 (fibrosarcoma) and MCF7 (breast carcinoma) (supplemental Fig. S1A). In addition, Cdc25B was previously shown to inhibit cell proliferation in colony formation assays (10). This suggests that the level of Cdc25B needs to be under tight regulation to avoid adverse consequences. In support of this, overexpression of Cdc25B has been shown to cause premature chromosome condensation (29). Consistent with previous studies, overexpression of Cdc25C did not have an effect on cell cycle progression (Fig. 2A). This was again not specific to U2OS cells,
because it was also observed in HT1080 cells (supplemental Fig. S1B). Previous studies have suggested that Cdc25C, to become active, has to be phosphorylated (24, 25). Thus, the activity and/or availability of the activating kinase(s) are important determinants for Cdc25C activity. Cdk1/cyclin B has been proposed to be such a kinase, because overexpression of Cdc25C when accompanied by excess amounts of exogenous cyclin B causes premature chromosome condensation (26, 27).

Given the similarities between Cdc25B and Cdc25C, the question arises as to why only Cdc25B behaves as an oncogene. The role of Cdc25B as the “starter phosphatase” of mitosis may be one answer to this question (3, 5, 9, 28–31). The current model is that Cdc25B is first to activate pT14pY15Cdk1/cyclin B. This, in turn, activates Cdc25C. Cdc25C keeps Cdk1/cyclin B active, and the positive feedback loop between them leads to the execution of mitosis (5, 24, 27–29, 32, 33). Another possibility is the level of Cdc25B is crucial in regulating its activity. In support of that are studies showing Cdc25B is an unstable protein whose overexpression causes premature chromosome condensation (27, 29). Thus, elevated levels of Cdc25B would lead to untimely initiation of mitosis leading to chromosomal abnormalities and genomic instability, hallmarks of cancer (34). Other observations have suggested additional differences between Cdc25B and Cdc25C that can account for the oncogenic activity of Cdc25B. Cdc25B has been shown to activate pT14pY15Cdk2/cyclin A in vitro and be possibly involved in the activation of the G2 pool of this complex in cells (3, 28, 35–37). Cdc25B, distinct from Cdc25C, has been shown to be transcriptionally up-regulated by c-Myc or be targeted for phosphorylation by Raf-1 in vitro and colocalizes with it in cells (38, 39). The preference of Cdc25B for its substrates alternates in a cell cycle-dependent manner. Cdc25B immunoprecipitated from S phase preferentially dephosphorylates pT14pY15Cdk2/cyclin A, whereas it is only active toward pT14pY15Cdk1/cyclin B when immunoprecipitated from G2/M (3, 25). Moreover, Cdc25B activity is also detectable in S phase (3). Thus, it is possible that Cdc25B, in contrast to Cdc25C, might play a role earlier in the cell cycle likely by regulating the S-phase pool of pT14pY15Cdk2/cyclin A. Cdk2/cyclin A activity in S phase ensures proper progression of replication and prevention of re-replication (17, 40, 41). Thus, close regulation of this complex is required for ensuring orderly firing of the origins and duplication of the genome only once, thereby safeguarding genomic integrity.

Oncogene activation is a key step in the development of cancer (42). Thus, elucidating the mechanisms by which abnormal activity of an oncogene contributes to tumorigenesis is of considerable value. Oncogenes that suppress cell proliferation can be transforming depending on cellular context (43, 44). It is hypothesized that the oncogenic and the anti-proliferative activities of Cdc25B are coupled. Thus, clarifying the molecular mechanisms underlying the anti-proliferative activity of Cdc25B could explain how aberrantly elevated Cdc25B protein level can contribute to tumorigenesis. The results presented here suggest that elevated levels of Cdc25B may contribute to genomic instability, a hallmark of cancer, by premature entry into mitosis and deregulated replication. Either of these processes is expected to be lethal due to consequential chromosomal abnormalities. Nevertheless, the selective pressure imposed by the elevated level of an oncogene such as Cdc25B may give rise to cells that have acquired additional abnormalities required for survival. Additional mutations compromise protective mechanisms applied by the cell. Here, the DNA damage checkpoints were shown to be activated in response to increased activity of Cdc25B (Fig. 3). This is consistent with recent studies demonstrating the importance of the DNA damage checkpoints in suppressing oncogenic activity (45–47). Furthermore, p53-dependent pathways are frequently dysfunctional in cancers (48). Two published studies failed to detect a correlation between p53 status and Cdc25B overexpression in human tumors (49, 50). Given the findings presented here, a more careful analysis in patient samples is clearly warranted.

p14ARF has been implicated in signaling oncogene activity to p53 in many instances (43). The results presented here are an example of p53 activation in the absence of p14ARF. The presented data suggest the ATM and ATR pathways are the major transmitters to p53. There is, of course, the additional possibility that there is direct activation of p53 by the hyperactivated Cdks. Lack of phosphorylation of serine 315, however, argues against this (Fig. 3A). Nevertheless, it is possible that the 24-h time point was too late for detecting phosphorylation on this residue, as at this time Cdks are inactive and cells already arrested. It remains possible that serine 315 was phosphorylated when Cdks first encounter elevated levels of Cdc25B at earlier time points.

The survival of mice lacking Cdc25B does not rule out that Cdc25B when present plays an important role in the progression of the cell cycle (51, 52). Studies showing Cdc25B as a target for cell cycle checkpoints support its significance (32, 53). Importantly, Cdc25B is overexpressed in many types of primary human tumors. It is hypothesized that overexpression of Cdc25B in these tumors can contribute to both initiation and continuous propagation of chromosomal abnormalities and genomic instability. These tumors have most likely lost the protective action of the DNA damage checkpoint pathway. Further investigations to provide evidence for these propositions are warranted.

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