Fine Tuning the Cell Cycle: Activation of the Cdk1 Inhibitory Phosphorylation Pathway during Mitotic Exit

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Submitted July 29, 2008; Revised December 10, 2008; Accepted January 12, 2009
Monitoring Editor: Orna Cohen-Fix

Inactivation of cyclin-dependent kinase (Cdk) 1 promotes exit from mitosis and establishes G1. Proteolysis of cyclin B is the major known mechanism that turns off Cdk1 during mitotic exit. Here, we show that mitotic exit also activates pathways that catalyze inhibitory phosphorylation of Cdk1, a mechanism previously known to repress Cdk1 only during S and G2 phases of the cell cycle. We present evidence that down-regulation of Cdk1 activates Wee1 and Myt1 kinases and inhibits Cdc25 phosphatase during the M to G1 transition. If cyclin B/Cdk1 complex is present in G1, the inhibitory sites on Cdk1 become phosphorylated. Exit from mitosis induced by chemical Cdk inhibition can be reversed if cyclin B is preserved. However, this reversibility decreases with time after mitotic exit despite the continued presence of the cyclin. We show that this G1 block is due to phosphorylation of Cdk1 on inhibitory residues T14 and Y15. Chemical inhibition of Wee1 and Myt1 or expression of Cdk1 phosphorylation site mutants allows reversal to M phase even from late G1. This late Cdk1 reactivation often results in caspase-dependent cell death. Thus, in G1, the Cdk inhibitory phosphorylation pathway is functional and can lock Cdk1 in the inactive state.

INTRODUCTION

Cyclin-dependent kinase (Cdk) 1 is the key regulator of mitotic transition. Activation of the kinase drives entry into mitosis (Nurse, 1990), and inactivation drives exit from mitosis (Murray *et al.*, 1989). Cdk1 kinase requires an activating partner—a cyclin. Cyclin B is a pivotal activator of Cdk1 in mitosis. During mitotic exit, cyclin B is degraded by the ubiquitin-proteasome pathway (Glutzer *et al.*, 1991). Cyclin B degradation causes irreversible inactivation of Cdk1 and therefore provides directionality for the cell cycle during the M phase to G1 transition. In addition to the activation by cyclins, Cdk1 activity can be negatively regulated by phosphorylation on two inhibitory residues—T14 and Y15. Wee1 is a tyrosine kinase that phosphorylates Y15 (Parker and Piwnicka-Worms, 1992), and Myt1 is a dual-specificity kinase that can phosphorylate both sites (Kornbluth *et al.*, 1994; Mueller *et al.*, 1995), with a propensity toward the T14 (Liu *et al.*, 1997). These inhibitory phosphorylations are removed by Cdc25 phosphatases. The functions of Wee1, Myt1, and Cdc25, in turn, are regulated by Cdk activity itself. During the G2-to-M transition, Wee1 and Myt1 kinases become phosphorylated and inactive when Cdk1 activity rises (Mueller *et al.*, 1995; Watanabe *et al.*, 2005), and the Cdc25 becomes phosphorylated and active (Hoffmann *et al.*, 1993). Therefore, Cdk activity feeds back on itself by activating its activator Cdc25 and inhibiting its inhibitors Wee1 and Myt1. These two mechanisms serve as positive feedback that in-

duces a robust autoamplification of Cdk1 activity (O'Farrell, 2001), and they are responsible for the switch-like activation of Cdk1 (Pomeroy *et al.*, 2005). Here, we show that after mitotic exit, Wee1 and Myt1 kinases become activated and can lock Cdk1 in the inactive state, thus serving to inhibit Cdk1 in the presence of its activators during G1.

MATERIALS AND METHODS

Cell Culture and Transfection

Xenopus S3 cells were grown at 23°C in 70% L-15 medium supplemented with 15% fetal bovine serum (FBS). HeLa cells were grown in DMEM with 10% FBS in 5% CO₂ at 37°C. HeLa cells were transiently transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) for live imaging experiments, and Lipofectamine L-2000 (Invitrogen, Carlsbad, CA) for biochemical experiments. Both reagents were used according to the manufacturer's directions. Plasmids encoding both the wild-type human cyclin B1-green fluorescent protein (GFP) and the nondegradable R42A mutant cyclin B1-GFP were gifts from J. Pines (University of Cambridge, United Kingdom). Plasmids encoding the wild-type Cdk1-GFP and phosphorylation mutants (T14A, Y15F, and the AF) Cdk1-GFP were gifts from R. Muschel (University of Oxford, United Kingdom). Experiments were conducted on the day after the transfection.

Chemical Inhibitors

The Cdk inhibitor flavopiridol, provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD) was used at 5–10 μ M except for cells overexpressing Cdk1-GFP constructs in which it was used at 25 μ M. The proteasome inhibitor MG132 (Calbiochem, San Diego, CA) was used at 25 μ M. The Wee1/Myt1 inhibitor PD0166285 (Pfizer, New York, NY) was used at 0.5 μ M. The Aurora kinase inhibitor ZM447439 (AstraZeneca Pharmaceuticals LP, Wilmington, DE) was used at 25 μ M. The Cdc25 phosphatase inhibitor NSC663284 (Sigma-Aldrich, St. Louis, MO) was used at 25 μ M. The general caspase inhibitor Z-VAD-FMK (Calbiochem) was used at 100 μ M. Nocodazole (Sigma-Aldrich) was used at 100 ng/ml.

Drug Treatments and Western Blotting

HeLa cells were collected by shake-off after being blocked in mitosis as detailed in figure legends. Nocodazole or MG132 block was used for non-transfected cells. For R42A cyclin B transfection experiments in Figures 2–5, mitotic cells were collected without nocodazole addition because the cells transfected with the nondegradable cyclin B arrest in mitosis spontaneously.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-07-0771>) on January 21, 2009.

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Abbreviations used: Cdk1, cyclin-dependent kinase 1; GFP, green fluorescent protein.

The mitotic cells were split into several experimental subgroups and treated with specified drugs for indicated periods (detailed in figure legends). For the flavopiridol washout, cells were pelleted by centrifugation, the flavopiridol-containing medium was aspirated, and cells were resuspended in the medium without flavopiridol. Cells treated with MG132 were maintained in media containing MG132 for the duration of the experiment. For cells collected in nocodazole, it was also maintained in the media throughout experiments except for the nocodazole release studies. At the time of collection, cells were pelleted by centrifugation and subsequently lysed in NuPAGE protein sample buffer (Invitrogen) containing 50 mM dithiothreitol (DTT). Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 4–12% Bis-Tris gels (Invitrogen), transferred to polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA), and blocked in 5% nonfat dry milk or 5% bovine serum albumin (BSA). Primary antibodies used were as follows: anti-pNucleolin (gift from P. Davies, Albert Einstein College of Medicine, Bronx NY), anti-pT14Cdk1 (Abcam, Cambridge, MA), anti-pY15Cdk1 (BD Biosciences, San Jose, CA or Cell Signaling Technology, Danvers, MA), anti-cyclin B1 (BD Biosciences), anti-Plk1 (Zymed Laboratories, South San Francisco, CA), anti-Wee1 (Cell Signaling Technology), anti-Myt1 (Cell Signaling Technology), anti-Cdc25C (Cell Signaling Technology), anti-pS10 histone H3 (Cell Signaling Technology), anti-nucleolin (Abcam), and anti-Cdk1 (BIOMOL Research Laboratories, Plymouth Meeting, PA). Primary antibodies were detected using horseradish-peroxidase-conjugated immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using West Pico Chemiluminescent kit (Pierce Chemical, Rockford, IL).

Flow Cytometry

For pS10 histone H3 analysis, cells were collected by centrifugation, fixed in 2% formaldehyde in PHEM [60 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 25 mM HEPES, pH 6.8, 10 mM EDTA, and 4 mM MgCl₂] for 15 min and permeabilized with 90%, –20°C methanol. Later cells were washed three times with phosphate-buffered saline (PBS), pH 7.2, blocked with 5% BSA in PBS, and labeled with anti-pS10 histone H3 antibody conjugated to Alexa Fluor 647 (Cell Signaling Technology). For cell cycle analysis, cells were fixed in 90% ethanol for at least 24 h and stained with 50 µg/ml propidium iodide in PBS in the presence of 100 µg/ml RNase A (Sigma-Aldrich). Analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences).

Live Imaging

Cells were grown on 25-mm glass coverslips that were inserted in an Att-ofluor culture chamber (Invitrogen) before the experiment. When applicable, cells were preincubated for 30–60 min with MG132. *Xenopus* S3 cells were imaged at room temperature in their normal growth medium. HeLa cells were imaged in L-15 medium with 10% FBS at 37°C. Temperature was maintained with an air curtain incubator (Nevtek, Burnsville, VA) and an objective heater (Bioptechs, Butler, PA). Time-lapse phase contrast and fluorescent images were collected using Axiovert 200M microscopes (Carl Zeiss, Thornwood, NY), either wide-field fluorescent or high-speed Yokogawa spinning disk confocal. Both microscopes were equipped with ORCA-ERG digital cameras (Hamamatsu, Bridgewater, NJ). PlanApochromat oil immersion objectives (63×) were used for all live imaging. Drugs were substituted by exchange of the media after a brief rinse. Time-lapse videos were assembled using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Myt1 Kinase Assays

HeLa cells were blocked in mitosis with nocodazole for 2–3 h, collected by shake-off, washed in nocodazole-free medium, and incubated without nocodazole for 1, 2, 3, and 4 h. Interphase cells were obtained by trypsinizing adherent cells left after shake-off. Equal numbers of cells (typically between 1.6 and 2 × 10⁶) were collected by centrifugation and flash-frozen in liquid nitrogen. For the assay, cell pellets were thawed in 250–300 µl of kinase lysis buffer (50 mM Tris, 50 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM DTT supplemented with protease inhibitor cocktail, 1 mM NaVO₄, and 400 nM Microcystin LR) and cleared by centrifugation. A portion (~5%) of each lysate was spared for Myt1 immunoblotting.

Recombinant 6His-Cdk1(K33R)-Δ90cyclin B1, a generous gift from P. Todd Stukenberg (University of Virginia, Charlottesville, VA), was conjugated to nickel-nitrilotriacetic acid magnetic beads (QIAGEN, Valencia, CA) and incubated in kinase lysates supplemented with 2 mM ATP for 30 min at 37°C. Beads were subsequently washed, and the bound proteins were released by boiling in NuPAGE protein sample buffer (Invitrogen) for 10 min. Protein samples were run on SDS-PAGE, transferred to PVDF, blocked in 5% BSA, and probed with anti-pT14Cdk1 antibody (Abcam). The membrane was subsequently stained with Coomassie Blue to control for protein loading.

RESULTS

Chemical Cdk Inhibitors Drive Reversible Mitotic Exit, but the Ability of Cells to Reenter Mitosis Declines Progressively with Time in G1 and Correlates with Inhibitory Phosphorylation on Cdk1

To reveal regulatory pathways that may play subtle but important roles in cell cycle regulation, we used a system to artificially block cyclin B proteolysis while still allowing mitotic exit. Here, we use this system to explore auxiliary controls that may participate in regulating Cdk1 activities during G1. In previous work, we demonstrated that in the absence of cyclin B1 proteolysis, mitotic exit can be induced by chemical Cdk inhibitors (Potapova *et al.*, 2006). Cyclin B was preserved by treatment with the proteasome inhibitor MG132 or by expressing a nondegradable mutant of the cyclin B protein. Preserving cyclin B1 causes cells to arrest in mitosis with high Cdk1 activity levels. This mitotic arrest could be overcome by treatments with Cdk inhibitors, such as flavopiridol (Losiewicz *et al.*, 1994), which induces cytokinesis and full mitotic exit in the absence of cyclin proteolysis. This ability to induce mitotic exit by application of chemical Cdk inhibitors to cells arrested in M phase in the presence of proteasome inhibitor was recently disputed (Skoufias *et al.*, 2007). However, in our hands, Cdk inhibitors applied to mitotic cells drives complete mitotic exit in both the absence or presence of proteasome inhibitor by all morphological criteria including cytokinesis, midbody formation, chromosome decondensation, reassembly of the nuclear envelope, and rearrangement of the mitotic spindle microtubules into the interphase array (Potapova *et al.*, 2006; Supplemental Videos 3 and 4). As we reported, chromosome segregation fails in the presence of proteasome inhibitor, presumably due to the necessity for degradation of the securin protein. In cells arrested in M phase with microtubule drugs, chemical Cdk1 inhibition induces loss of mitotic phosphorylations again in both the absence and presence of proteasome inhibitors (see Supplemental Figure 1). In our previous work, we found that mitotic exit in the presence of proteasome inhibitors could be driven by several different Cdk inhibitors in addition to flavopiridol (Potapova *et al.*, 2006).

Flavopiridol is a reversible Cdk inhibitor. Thus, when flavopiridol was removed after the induced mitotic exit, the two daughter cells could reverse their cell cycle progression and reenter mitosis. However, the longer cells were incubated with flavopiridol, the less likely they were to reenter mitosis. Thus, the ability of cells to reverse mitotic exit declined with time spent in G1, even though cyclin B was preserved. To understand the loss of reversibility in later G1, we carried out live imaging studies with human HeLa cells and with an immortalized *Xenopus* cell line S3, which expresses GFP-α tubulin. Cyclin B was preserved by addition of the proteasome inhibitor MG132 to the medium, or by expressing a nondegradable mutant CyclinB1 (R42A) tagged with GFP. Flavopiridol was added to the medium to induce mitotic exit and subsequently washed out. When flavopiridol was removed 30 min after its addition, most cells reentered mitosis (Figure 1A). However, after treatment with flavopiridol for 60 min or more, only a small percentage of cells were capable of reversing mitotic exit.

The fact that cells in later G1 failed to reenter mitosis when cyclin B was preserved indicated the existence of additional mechanisms that block reactivation of the Cdk1. In addition to cyclin availability, Cdk1 activity could be controlled by dephosphorylation of its activation site T161 (Laroche *et al.*, 2007), by synthesis of small protein kinase inhibitors

Figure 1. Cells treated for extended periods with the Cdk inhibitor flavopiridol show progressively decreased ability to reactivate Cdk1 and reenter mitosis. (A) Percentages of cells that reenter mitosis after 30 or 60 min of flavopiridol treatment assayed by live videomicroscopy. Before flavopiridol treatment, *Xenopus* S3 cells were arrested at metaphase by addition of proteasome inhibitor MG132. HeLa cells were arrested with MG132 or by expression of nondegradable R42A cyclinB1-GFP. When used, MG132 was retained in the medium after removal of flavopiridol. Fewer cells reverse after 60-min flavopiridol treatment. (B) Cdk1 kinase activity (phospho-nucleolin); phosphorylation of Cdk1 on T14, Y15, and T161; and protein levels of Wee1, Myt1, Cdc25C, and cyclin B1 were analyzed by Western blotting. Mitotic HeLa cells were collected in nocodazole (lane 0) and induced to exit mitosis by treatment with 5 μ M flavopiridol for 30 or 60 min in the absence (lanes 1–4) or presence (lanes 5–8) of proteasome inhibitor MG132. After removal of the Cdk inhibitor, cells were allowed to reenter mitosis for an additional 60 min (lanes 2, 4, 6, and 8). Total Cdk1 protein levels are shown as loading controls. Flavopiridol induces changes in

(Sherr and Roberts, 2004), or by phosphorylation on inhibitory sites T14 and Y15 (Atherton-Fessler *et al.*, 1994). The loss of reversibility accompanying prolonged flavopiridol treatment was not affected by protein synthesis inhibitors in *Xenopus* S3 cells (data not shown), which suggests the de novo synthesis of small protein kinase inhibitors was not involved in the late G1 block. Dephosphorylation of Cdk1 on the activating site T161 did not occur when cyclin was preserved, indicating that the lack of reversibility was not due to loss of the activating T161 phosphorylation (Figure 1B, lanes 5–8).

We next tested the status of Cdk phosphorylation on its inhibitory sites, T14 and Y15, during chemically induced mitotic exit and reversal. Cells were accumulated in mitosis by treatment with nocodazole for 3 h. Mitotic cells were collected by shake-off and treated with flavopiridol with or without MG132 for 30 or 60 min. Flavopiridol was subsequently washed out, and cells were allowed to reenter mitosis for another 60 min. Cdk1 inhibitory phosphorylation was examined by Western blotting using phospho-specific Cdk1 T14 and Y15 antibodies. Cdk1-specific phosphorylation of nucleolin (Dranovsky *et al.*, 2001) served as a readout for Cdk1 activity. Without the proteasome inhibitor, addition of flavopiridol caused degradation of cyclin B (Figure 1B, lanes 1–4). As expected, removal of flavopiridol did not result in reactivation of Cdk1 when cyclin B was not preserved (Figure 1B, lanes 2 and 4). Consistent with the reports that Cdk1 phosphorylation on the inhibitory residues occurs solely when it is bound to cyclin (Solomon *et al.*, 1990), Cdk1 was not phosphorylated on T14 and Y15 when cyclin B was degraded. When cyclin B was preserved by the proteasome inhibitor, flavopiridol treatment resulted in only weak phosphorylation of the inhibitory sites T14 and Y15 at 30 min but strong phosphorylation at 60 min (Figure 1B, lanes 7 and 8). Thus, Cdk1 activity (phospho-nucleolin) returned to high levels in cells reversed after 30 min (Figure 1B, lane 6) but increased only modestly in cells reversed after 60 min (Figure 1B, lane 8).

Figure 1 (cont). electrophoretic mobility of Wee1/Myt1 kinases and Cdc25C phosphatase that are greater after 60 min of Cdk inhibition. In the presence of proteasome inhibitor that preserves cyclin B, inhibitory phosphorylations on T14 and Y15 are weak at 30 min (lanes 5 and 6) but strong at 60 min (lanes 7 and 8). Cdk1 is robustly reactivated when flavopiridol was washed out after 30 min (lane 6) but only weakly reactivated when flavopiridol was washed out after 60 min (lane 8). (C) Mitotic indices of cell populations induced to exit and reenter mitosis. Mitotic HeLa cells were treated as described in B, fixed, stained with antibody to phospho-histone H3 (mitotic marker) conjugated with Cy5 and processed by flow cytometry. Most cells reenter mitosis when treated with flavopiridol for 30 min before washout (lane 6) but only a minority reenter when treated for 60 min (bar 8). (D) HeLa cells were transfected with wild-type (lanes 0–4) or nondegradable R42A (lanes 5–9) cyclin B-GFP. Mitotic cells were collected in nocodazole and induced to exit mitosis by treatment with 5 μ M flavopiridol for 30 or 60 min. After removal of the Cdk inhibitor, cells were allowed to reenter mitosis for an additional 60 min. Cells expressing the wild-type cyclin B1-GFP gradually degrade it upon flavopiridol treatment and do not reenter after flavopiridol washout (lanes 2 and 4). In cells expressing the nondegradable cyclin B, inhibitory phosphorylations on T14 and Y15 are weaker at 30 min (lanes 6 and 7) than at 60 min (lanes 8 and 9). Cdk1 is reactivated more strongly when flavopiridol is washed out after 30 min (lane 7) than 60 min (lane 9). (E) Mitotic indices of cells expressing wild-type or nondegradable cyclin B1 treated as described in D. Cells were fixed, stained with antibody to phospho-histone H3 (mitotic marker) conjugated with Cy5, and processed by flow cytometry. More cells reenter mitosis after 30-min flavopiridol treatment (bar 7) than after 60 min (bar 9).

The activities of Wee1 and Myt1 kinases as well as Cdc25 phosphatase are reflected in their electrophoretic mobilities caused by phosphorylation changes. Wee1 and Myt1 are hyperphosphorylated and inactive in mitosis (Mueller *et al.*, 1995; Watanabe *et al.*, 2005), whereas Cdc25C, is hyperphosphorylated and active (Hoffmann *et al.*, 1993). Chemical inhibition of Cdk1 resulted in increased mobility of all of three, indicating loss of phosphorylation and consequently activation of the Wee1/Myt1 kinases and inactivation of Cdc25C phosphatase. The changes in mobility increased progressively from 30 to 60 min of treatment with flavopiridol, particularly in the presence of MG132 (Figure 1B, lanes 5 and 7). This suggests that the kinases become progressively dephosphorylated and active as cells exit mitosis, whereas Cdc25 becomes inactive, providing a window of time for Cdk1 reactivation when cyclin B is preserved.

We conducted the same type of analysis in HeLa cells expressing the wild-type or the nondegradable (R42A) cyclin B1 (Figure 1D). For this experiment, cells were transfected with indicated constructs for 24 h; mitotic cells were collected in nocodazole and treated with Cdk inhibitor for 30 or 60 min. Cells expressing the nondegradable (R42A) cyclin B1 lose the ability to rephosphorylate nucleolin and histone H3 with time in G1 similarly to MG132-treated cells (Figure 1B, lanes 7 vs. 9). The Cdk1 phosphorylation on inhibitory T14 and Y15 also increased (Figure 1B, lanes 8 and 9). The wild-type cyclin B was degraded upon Cdk inhibition, and the cells that expressed it did not reenter mitosis after flavopiridol washout (lanes 2 and 4) in spite of similar initial cyclin B1 levels. The differences in phosphorylation shifts of Wee1, Myt1, and Cdc25 at 30 versus 60 min of flavopiridol treatment were less sharp than in the MG132 experiment in Figure 1B. This was likely because not all of the nocodazole-blocked mitotic cells expressed the nondegradable cyclin—the typical transfection efficiency was ~70%, and also because expression levels vary among the transfected cells. To get a higher proportion of cells expressing the nondegradable cyclin B for the subsequent experiments, the R42A cyclin B-expressing mitotic cells were collected by shake-off without nocodazole, because they arrest in mitosis spontaneously.

To assess whether the Cdk1 reactivation indicated by phospho-nucleolin labeling correlated with other markers of cells reentering mitosis, cells from each treatment in Figure 1, B and D, were fixed and stained with antibody against the mitotic marker phospho-S10 on histone H3. The cells were analyzed by flow cytometry to determine mitotic indices (Figure 1, C and E). The samples with high Cdk1 activity as delineated by strong blotting signals to phospho-nucleolin correlated with high mitotic indices in the fixed cells. The decreased ability to reactivate Cdk1 after 60 min of flavopiridol treatment was consistent with the loss of reversibility observed by live imaging. Together, these data indicate that despite presence of cyclin B, cells lose the ability to reenter mitosis with longer time in G1, and this loss correlates with phosphorylation of Cdk1 inhibitory sites T14 and Y15.

Chemical Inhibition of Wee1 and Myt1 Kinases Allows Cdk1 Reactivation and Mitotic Reentry from Late G1

To address the functional significance of Cdk inhibitory phosphorylations in G1, we inhibited the Wee1 and Myt1 kinases with a potent and specific Wee1 and Myt1 inhibitor PD0166285 (Wang *et al.*, 2001; Hashimoto *et al.*, 2003; Hashimoto *et al.*, 2006). Judged against mitotic exit of an untreated cell (Supplemental Video 1), PD0166285 had no obvious effect on normal mitotic exit, although it seemed to inhibit complete extension of the cortex in telophase (Supplemental

Video 2). We used live videomicroscopy to study the consequences of Wee1 and Myt1 inhibition on the reversibility of mitotic exit by adding this inhibitor during flavopiridol-induced exit. Cells were arrested at metaphase by treatment with the proteasome inhibitor MG132. Flavopiridol and PD0166285 were added for 60 min. Then, the flavopiridol was removed, and the cells were maintained in PD0166285. Remarkably, under these conditions, 100% of the MG132-treated cells reentered mitosis (Figure 2A). A time-lapse sequence of mitotic exit and reversal of a *Xenopus* S3 cell after 60 min of Cdk inhibition in the presence of PD0166285 is shown in Figure 2B and Supplemental Video 3. In contrast, in the absence of PD0166285, only a small percentage of MG132-treated *Xenopus* S3 cells and HeLa cells were able to reenter mitosis after 60 min of treatment with flavopiridol (Figure 2A). Shown in Figure 2B and Supplemental Video 4 is a typical example of *Xenopus* S3 cell that remained in G1 after 60 min of flavopiridol treatment in the absence of PD0166285.

For HeLa cells expressing nondegradable cyclin B1-GFP, PD0166285 also promoted mitotic reentry of the majority of cells after prolonged G1 (Figure 2A). A time-lapse sequence of a representative reversed HeLa cell expressing nondegradable cyclin B1-GFP is shown in Figure 2D and Supplemental Video 5. Some cells expressing nondegradable cyclin B1 did not reverse after 60 min even when PD0166285 was used. We attribute this lack of reversibility in some cells to insufficient expression of the nondegradable cyclin B. We have demonstrated previously that the reversibility in R42A cyclin B1-expressing cells correlates with expression levels of the mutant as estimated by GFP fluorescence (Potapova *et al.*, 2006). In the three cells that failed to reverse in these experiments the expression of the nondegradable cyclin was relatively low (data not shown). In the absence of PD0166285, very few HeLa cells expressing R42A cyclin B1 were able to reenter mitosis after 60 min of Cdk inhibition (Figure 2A). An example of R42A cyclin B1-GFP-expressing cell in the absence of PD0166285 that did not reverse from G1 after 60 min of flavopiridol treatment is shown in Figure 2E and Supplemental Video 6. The persistence of the GFP fluorescence in the cell demonstrates that the mutant cyclin B1 protein is stable. However, the cell remained in G1 after flavopiridol washout, and eventually the R42A cyclin B1 was transported out of the newly formed nuclei.

To ensure that the reversibility seen in the presence of the Wee1/Myt1 inhibitor PD0166285 occurs only when cyclin B is preserved, we also expressed wild-type cyclin B1-GFP in HeLa cells. Proteolysis of wild-type cyclin B1 is initiated before anaphase onset and continues during anaphase and mitotic exit (Clute and Pines, 1999). For this experiment, cells were treated with flavopiridol before initiation of spontaneous degradation. The fluorescent intensity of the cyclin B1-GFP dropped rapidly upon Cdk1 inhibition, indicating degradation of the cyclin. None of the 13 observed cells cultured in the presence of PD0166285 and expressing wild-type cyclin B1-GFP were able to reenter mitosis after 60 min of flavopiridol treatment. A representative cell expressing wild-type cyclin B1-GFP blocked in G1 after 60 min of flavopiridol treatment in the presence of the PD0166285 is shown in Supplemental Video 7.

We analyzed directly the effect of Wee1/Myt1 inhibition by blotting Cdk1 inhibitory phosphorylations in extracts of mitotic HeLa cells treated with flavopiridol for 60 min. Cyclin B was preserved with MG132 (Figure 3A) or by expression of the nondegradable mutant (Figure 3B). PD0166285 prevented Cdk1 phosphorylation on T14 and Y15 and allowed Cdk1 to regain activity when Cdk inhibitor was re-

moved (Figure 3, A and B, lanes 4). In contrast to the results obtained with PD0166285, the inhibition of another class of mitotic kinases, the Aurora kinases, with the drug ZM447439 did not affect reversibility of mitotic exit when tested with *Xenopus* S3 cells (data not shown).

The inhibitory phosphorylations catalyzed by the Wee1 and Myt1 kinases are opposed by the activity of the Cdc25 phosphatases (Boutros *et al.*, 2006). We asked whether Cdc25 phosphatases were active during mitotic exit reversal. HeLa cells treated with MG132 were induced to exit mitosis for 60 min in flavopiridol. Flavopiridol treatment induced high levels of phosphorylated T14 and Y15 on Cdk1 (Figure 3C, lane 1). These levels remained high when flavopiridol was removed (Figure 3C, lane 2). When PD0166285 was added to inhibit Wee1 and Myt1 kinases at the time of flavopiridol removal the Cdk1 inhibitory phosphorylations diminished and Cdk1 activity returned, indicating that Cdc25 phosphatases were active (Figure 3C, lane 3). To test whether Cdk1 reactivation was due to the activity of Cdc25 phosphatases, we added a specific Cdc25 inhibitor NSC663284 (Pu *et al.*, 2002) together with PD0166285 after the flavopiridol washout (Figure 3C, lane 4). When Cdc25 activity was inhibited, Cdk1 remained highly phosphorylated on T14 and Y15, and Cdk1 kinase activity was not fully restored. Thus, at least for 1 h after the induced mitotic exit, Cdc25 phosphatase(s) have the potential to dephosphorylate and activate Cdk1, but eventually the Wee1/Myt1 kinases outbalance this potential.

Mutation of Inhibitory Phosphorylation Sites on Cdk1 Allows Cdk1 Reactivation and Mitotic Reentry from Late G1

To confirm the results obtained with the chemical Wee1/Myt1 inhibitor PD0166285, we ectopically expressed the phosphorylation site mutants of human Cdk1 (T14A, Y15F, and the double mutant T14A/Y15F) tagged with GFP in HeLa cells. All populations showed similar transfection efficiencies. Cells expressing each of the phosphorylation mutants, but not the wild-type Cdk1, were able to reactivate Cdk1 to differing degrees after 1 h of flavopiridol treatment in the presence of MG132 (Figure 4A, lanes 6, 9, and 12). The Y15F mutant and the double mutant showed stronger recovery of Cdk1 activity than did the T14A mutant. This is consistent with a report that Y15, but not T14, is essential to prevent mitotic entry after the radiation damage (Fletcher *et al.*, 2002). Live imaging of HeLa cells expressing Cdk1 mutants further supported the idea that inhibitory phosphorylation blocks cells in G1 when cyclin B is preserved. Time-lapse sequences of mitotic exit and reversal after 60 min of Cdk inhibition in HeLa cell expressing Cdk1-Y15F in the presence of MG132 are shown in Figure 4C and Supplemental Video 8. Cells expressing Cdk1 mutants reentered mitosis more readily after 1 h of Cdk inhibition than cells expressing wild-type Cdk1 (Figure 4B). Interestingly, a few of the reversed cells expressing the single T14A or Y15F Cdk1 mutants exhibited a prophase-like morphology with condensed chromosomes surrounded by an intact nuclear envelope, perhaps indicating that such cells had intermediate levels of Cdk1 activity. Notably, many of the mitotic cells expressing the double mutant demonstrated aberrant metaphase morphology and reduced viability, consistent with reports that the expression T14A/Y15F Cdk1 causes cells to enter mitosis prematurely and aberrantly (Krek and Nigg, 1991). Overall, the data obtained with Cdk1 mutants were consistent with our results using the Wee1/Myt1 inhibitor PD0166285 and highlighted a more potent contribution of Y15 in comparison with T14 in regulation of Cdk1 activity. Together, these results support the hypothesis that blocking Wee1 and Myt1

Figure 2. The Wee1 and Myt1 inhibitor PD0166285 allows cells to reenter mitosis from late G1. (A) Percentages of cells that reverse after 60-min flavopiridol treatment in the presence or absence of Wee1/Myt1 inhibitor PD0166285 assayed by live videomicroscopy. Before flavopiridol treatment, *Xenopus* S3 cells were arrested at metaphase by the addition of the proteasome inhibitor MG132. HeLa cells were arrested with MG132 or by expression of nondegradable R42A cyclin B1-GFP. MG132 and PD0166285 were retained in the medium after removal of flavopiridol. Inhibition of Wee1/Myt1 kinases allows most of the cells to reenter mitosis after 60 min of flavopiridol treatment. (B) Mitotic exit is reversible after 60 min of flavopiridol treatment if both proteasome and Wee1/Myt1 activities are inhibited. An MG132-arrested *Xenopus* S3 cell was treated with Cdk inhibitor flavopiridol and Wee1/Myt1 inhibitor PD0166285 for 60 min, and then flavopiridol was removed. MG132 and PD0166285 were retained after removal of flavopiridol. Supplemental Video 3 shows the complete

Figure 3. The Wee1 and Myt1 inhibitor PD0166285 prevents inhibitory phosphorylation of Cdk1 during chemically induced mitotic exit and allows Cdk1 to be reactivated in late G1. (A) MG132-treated mitotic HeLa cells (lane 0) were incubated in the Cdk inhibitor flavopiridol without (lane 1) or with (lane 3) the Wee1/Myt1 inhibitor PD 0166285 for 60 min. After removing flavopiridol, cells were allowed to reenter mitosis for an additional 60 min (lanes 2 and 4). Cdk1 kinase activity (phospho-nucleolin), Cdk1 phosphorylation on T14 and Y15, and cyclin B1 protein levels were analyzed by Western blotting. When Wee1 and Myt1 were inhibited by PD0166285, Cdk1 activity was restored after washout of flavopiridol (lane 4). (B) Mitotic HeLa cells expressing nondegradable R42A cyclin B1-GFP (in place of incubation with MG132) were treated and analyzed as described in A. When Wee1 and Myt1 were inhibited by PD0166285, Cdk1 activity was restored after washout of flavopiridol (lane 4). (C) MG132-treated mitotic HeLa cells (lane 0) were incubated in flavopiridol for 60 min (lane 1). Flavopiridol was removed, and cells were incubated for an additional 60 min in medium containing dimethyl sulfoxide (DMSO) (lane 2), Wee1/Myt1 inhibitor PD0166285 (lane 3), or PD0166285 plus the Cdc25 phosphatase inhibitor NSC63284 (lane 4). Samples were analyzed by Western blotting as in described in A. Inhibitory phosphorylations on Cdk1 induced after 60 min of flavopiridol treatment (lane 1) are maintained when flavopiridol is removed (lane 2). Addition of PD0166285 at the time of flavopiridol washout results in dephosphorylation of T14 and Y15 and robust reactivation of Cdk1 (lane 3). Cdk1 dephosphorylation and reactivation was decreased by the addition of the Cdc25 phosphatase inhibitor NSC662385 (lane 4).

kinase action on Cdk1 extends the period of reversibility into G1 when cyclin B is preserved.

Reactivation of Cdk1 in Late G1 Induces Cell Death

Next, we explored the ability of cells to reenter mitosis from later G1. For these experiments, flavopiridol was applied for

Figure 2 (cont). time-lapse sequence. (C) Mitotic exit is not reversible in most proteasome inhibitor-treated cells after 60 min of flavopiridol treatment if Wee1 and Myt1 are active. An MG132-arrested *Xenopus* S3 cell was treated with Cdk inhibitor flavopiridol for 60 min, and then flavopiridol was removed. MG132 was retained after removal of flavopiridol. The complete video sequence is shown in Supplemental Video 4. (D) Mitotic exit is reversible after 60 min of flavopiridol treatment if Wee1/Myt1 activities are inhibited in the presence of nondegradable cyclin B1. A HeLa cell expressing R42A cyclin B1-GFP was treated with flavopiridol and Wee1/Myt1 inhibitor PD0166285 for 60 min, and then flavopiridol was removed. PD0166285 was retained after removal of flavopiridol. The complete video sequence is shown in Supplemental Video 5. (E) Mitotic exit is not reversible in most cells expressing nondegradable cyclin B1 after 60 min of flavopiridol if Wee1 and Myt1 are active. A HeLa cell expressing R42A cyclin B1-GFP was treated with flavopiridol for 60 min, and then flavopiridol was removed. The complete video sequence is shown in Supplemental Video 6. The top panels in B-E show phase-contrast images, and the bottom panels show corresponding fluorescent images. Bars, 10 μ m.

periods up to 120 min before being removed. PD0166285 was used to alleviate the Wee1/Myt1-mediated G1 block. With proteasome function prevented by MG132, HeLa cells regained Cdk1 activity when Cdk inhibitor was removed (Supplemental Figure 2, lane 4). By live videomicroscopy, 100% of *Xenopus* and HeLa cells treated with MG132 reenter mitosis after being exposed to flavopiridol for 120 min. The time-lapse sequence of mitotic exit and reversal of an MG132-treated *Xenopus* cell subject to 120 min of flavopiridol treatment is shown in Supplemental Video 9. Visually, the process of mitotic reentry was essentially identical to that of cells reversed after 60 min in G1 in the presence of the proteasome inhibitor. A difference was seen in reversals carried out with HeLa cells expressing nondegradable cyclin B in lieu of MG132 treatment. All reversed cells remained viable after being treated with flavopiridol for 60 min (Figure 5A). However, after longer periods in G1, cells expressing nondegradable cyclin B were frequently unable to maintain viability during mitotic reentry. Such cells initially underwent morphological changes associated with reentry into mitosis (translocation of cyclin B1 into the nucleus and chromosome condensation) but then began to bleb violently and subsequently died (Figure 5B and Supplemental Video 10). Cells remained viable if the reentry into mitosis was not induced (Supplemental Video 11).

Figure 4. Mutation of inhibitory phosphorylation sites on Cdk1 allows reactivation of Cdk1 kinase activity in late G1. (A) Mitotic HeLa cells expressing wild-type Cdk1-GFP (lanes 1–3), T14A-Cdk1-GFP (lanes 4–6), Y15F-Cdk1-GFP (lanes 7–9), or T14A/Y15F-Cdk1-GFP (lanes 10–12) were blocked in metaphase with MG132. Cells were induced to exit mitosis by treatment with flavopiridol (lanes 2, 5, 8, and 11) for 60 min. Flavopiridol was removed, and cells were allowed to reenter mitosis for an additional 60 min (lanes 3, 6, 9, and 12). Cdk1 kinase activity (phospho-nucleolin), and the protein levels of endogenous Cdk1 and ectopically expressed Cdk1-GFP's were analyzed by Western blotting. Cells expressing mutant Cdk1 proteins show varying degrees of Cdk1 reactivation (lanes 6, 9, and 12) compared with cells expressing wild-type Cdk1 (lane 3). (B) Percentages of HeLa cells expressing wild-type Cdk1-GFP, T14A-Cdk1-GFP, Y15F-Cdk1-GFP, or T14A/Y15F-Cdk1-GFP that underwent reversal from late G1 as assayed by live videomicroscopy. Cells were arrested at metaphase by the addition of the proteasome inhibitor MG132, treated with flavopiridol for 60 min, and then released from flavopiridol. MG132 was retained in the medium. Two cells expressing Y15F-Cdk1-GFP and 4 cells expressing T14A/Y15F-Cdk1-GFP that died during the manipulations were excluded from the analysis. Four cells recondensed chromosomes but maintained an apparently intact nuclear envelope after flavopiridol removal (2 expressing T14A-Cdk1-GFP and 2 expressing Y15F-Cdk1-GFP), and these were scored as reversed. (C) Mitotic exit is reversible after 60 min of flavopiridol treatment when Cdk1 inhibitory phosphorylation sites are ablated. A HeLa cell expressing Y15F-Cdk1-GFP was treated with flavopiridol for 60 min, followed by flavopiridol washout. Cyclin B1 proteolysis was prevented with MG132. The top panel in C shows phase-contrast images, and the bottom panel shows corresponding fluorescent images. Bar, 10 μ m. The complete video sequence is shown in Supplemental Video 8.

By Western blot analysis, HeLa cells expressing nondegradable cyclin B and treated with flavopiridol for 120 min did not show substantial recovery of phospho-nucleolin or phospho-histone H3 after flavopiridol washout even when Cdk1 phosphorylation on T14 and Y15 was blocked by addition of PD0166285 (Figure 5C, lane 4). We suspect that the lack of rephosphorylation of nucleolin and histone H3 reflects the loss of mitotic markers that accompanies cell death in mitosis (Allan and Clarke, 2007).

Because cell death was not observed in cells treated with proteasome inhibitor, we reasoned that in cells expressing nondegradable cyclin B and forced into G1, cell death during reversal might result from reactivation of Cdk1 in the absence of other mitotic proteins that are normally degraded by late G1. To test this hypothesis, we added the proteasome inhibitor MG132 with the flavopiridol to mitotic cells expressing nondegradable cyclin B1. Incubation with MG132 showed substantial rescue of cells during reversal after 120 min of flavopiridol treatment (Figure 5D). One mitotic protein degraded during G1 is polo-like kinase 1 (Plk1) (Lindon and Pines, 2004). Plk1 was substantially degraded upon treatment of cells with flavopiridol for 120 min in the absence of the proteasome inhibitor MG132 (compare lanes 0

and 1, Figure 5C). The presence of MG132 preserved Plk1 and restored the levels of phosphorylated nucleolin after flavopiridol washout (Figure 5C, lane 5).

To determine whether the loss of viability involved the action of caspases, the general caspase inhibitor Z-VAD-FMK was added to the cells at the time of flavopiridol washout. The caspase inhibitor restored phospho-nucleolin in the reversing cells to levels similar to those of cells incubated with MG132 (Figure 5C, lane 6). Plk1 levels were low in these cells compared with the MG132-treated cells, indicating that the proteasome-dependent protein degradation of Plk1 during G1 was not inhibited. By live imaging, caspase inhibitor also restored the viability of the reversing cells to 90% (Figure 5D). A time-lapse sequence of mitotic exit for 120 min and reentry in the presence of Z-VAD-FMK is shown in Figure 5E and Supplemental Video 12. Together, these results indicate that the Cdk activation in late G1 may lead to cell death in a caspase-dependent manner. Many essential mitotic regulators, such as Plk1, are substrates for the anaphase-promoting complex and are degraded in late G1. The cell death that we observed may stem from a lack of one or more of the proteins that are essential for viability of mitotic cells when Cdk1 becomes reactivated in late G1. This

Figure 5. Reentry into mitosis from late G1 induces cell death that is dependent on proteasome and caspase activities. (A) Percentages of HeLa cells expressing R42A cyclin B1-GFP that retain viability after reentry into mitosis from late G1 observed by live videomicroscopy. Mitotic cells were treated with flavopiridol and PD0166285 for 60, 90, and 120 min. Flavopiridol was removed but PD0166285 was retained. Cells that failed to reenter mitosis (3 in the 1-h incubation and 1 in the 90-min incubation) were excluded from the analysis of viability. For the control, flavopiridol and PD0166285 were left on for the duration of the experiment. Reentry into mitosis from late G1 induces cell death in cells expressing R42A cyclin B1. (B) A HeLa cell expressing R42A cyclin B1-GFP was treated with flavopiridol and Wee1/Myt1 inhibitor PD0166285 for 120 min. Flavopiridol was removed but PD0166285 retained. Shortly after the flavopiridol washout, cyclin B1-GFP translocates back into the nuclei; the nuclear envelope disassembles, and the chromosomes recondense (arrowheads). Subsequently, the cell undergoes violent blebbing followed by shrinkage and loss of viability. The complete video sequence is shown in Supplemental Video 10. (C) Mitotic

mechanism of cell death may be relevant to the aberrant Cdk1 activation and apoptosis occurring in quiescent neurons, a feature of certain neurodegenerative diseases such as Alzheimer's disease (Vincent *et al.*, 1997). Our ability to regulate Cdk1 reactivation in G1 cells may provide a model to study the signaling pathways of unscheduled Cdk1 activity under controlled conditions.

Myt1 and Wee1 Activation in Normal G1

Although cyclin degradation plays a key role in inducing exit from mitosis, we have shown that the inhibitory phosphorylations of Cdk1 can lock the kinase in the low activity state in G1. Therefore, the inhibitory kinases Wee1 and Myt1 must be reactivated in G1 after the Cdk1 activity drops. However, cyclin B1 in our experiments was preserved, providing a detectable substrate for their activity. We next asked the question whether the Wee1 and Myt1 were active during the normal physiological G1. For these experiments, cells were synchronized in G1 by release of mitotic cells from the nocodazole block. Under these conditions, most cells are in G1 by 4 h after nocodazole washout as demonstrated by DNA content (Figure 6A). As cells were exiting mitosis, Wee1, Myt1, and Cdc25C underwent electrophoretic mobility shifts corresponding to the active forms of Wee1 and Myt1 and inactive form of Cdc25C (Figure 6B), suggesting that these changes in enzyme activities are normal aspects of mitotic exit. The presence of the Wee1/Myt1 inhibitor did not severely affect these processes, but there were some detectable differences. For example, the antibodies against phospho-Cdk1 T14 and phospho-Cdk1 Y15 show some weak immunoreactivity 3–4 h after nocodazole release, a time when cyclin B levels are very low (Figure 6B). Importantly, phospho-T14 and phospho-Y15 bands were absent in

the Wee1/Myt1 inhibitor-treated cells. We next directly assayed the Myt1 activity of G1 cells *in vitro*. Technical difficulties precluded us from directly assaying the activity of Wee1. The activity of Myt1 toward a specific substrate was tested in lysates obtained from cells undergoing the M-to-G1 transition. For the substrate, we used a recombinant His-tagged kinase-dead (K33R) Cdk1 protein complexed with nondegradable $\Delta 90$ cyclin B. The substrate was incubated in lysates in the presence of ATP and analyzed for phosphorylation on T14 by using the phospho-specific antibody. The substrate phosphorylation on T14 begun in lysates of cells released from mitotic nocodazole block for 2 to 3 h (Figure 6C). By 4 h, when most of the cells have exited mitosis (Figure 6A), the activity of Myt1 was nearly equal the level detected in interphase lysates from cycling cells. The specific inhibitor PD0166285 blocked the activity of Myt1 nearly as well as the lack of ATP. The activation of the kinase corresponded with its electrophoretic mobility shift in lysates (Figure 6C, bottom). Together, these results suggest that the Myt1 kinase, and likely the Wee1 kinase, is activated shortly after mitotic exit. Specific *in vivo* targets for these kinases in G1 are yet to be established and are currently under investigation.

DISCUSSION

The role of Cdk1 inhibition by phosphorylation in limiting Cdk1 activation during G2 and in preventing mitotic entry in response to DNA damage is well established (Jin *et al.*, 1996). However, previously there was no evidence that the kinases that catalyze these phosphorylations had the capability to negatively regulate Cdk1 activity in G1. Here, we suggest that the reactivation of this pathway is initiated by the drop in Cdk1 activity levels and works to progressively activate the Wee1 and Myt1 kinases and inhibit the Cdc25 phosphatases. At early times, ~30 min after induction of mitotic exit with the reversible Cdk inhibitor flavopiridol, cells could be driven back into mitosis simply by removing the flavopiridol. After that, the Wee1 and Myt1 activities begin to outbalance the Cdc25 activity and flavopiridol removal fails to reactivate Cdk1 even when cyclin B is preserved. By inhibiting the Wee1 or Myt1 kinases, or by expressing mutant Cdk1 protein that was not susceptible to inhibition by phosphorylation, we could greatly extend the time of reversibility.

Although the steps in the activation and inactivation of Cdk1 during the transition into and out of M phase have been the subject of considerable investigation, much less is understood about potential alternative controls that may function in later G1. Our evidence suggests that inhibitory phosphorylation kinases Wee1 and Myt1 are active during this time. The lack of known substrates for them in G1 does not mean the absence of substrates *per se*. We speculate that these kinases may participate in less explored processes involving Cdk inhibition by phosphorylation. For example, there exists a poorly explored family of RINGO/Speedy proteins some of which are noncyclin Cdk1 activators. These proteins are expressed in G1 and can bind and activate Cdk1 and Cdk2 (Cheng *et al.*, 2005). Importantly, at least the Myt1 kinase is able to phosphorylate inhibitory sites leading to inhibition of Cdk1 when Cdk1 is bound to RINGO/Speedy protein (Karaiskou *et al.*, 2001). In mice, the knockout data for Cdks and cyclins reveal that Cdk1 can in principle bind many cyclins *in vivo*, including the G1 cyclins (see review by Sherr and Roberts, 2004). Moreover, mouse embryos expressing only a single cyclin dependent kinase, Cdk1, develop to midgestation and cells from these embryos can

Figure 5 (cont). HeLa cells expressing R42A cyclin B1-GFP (lane 0) were treated with flavopiridol for 120 min. Flavopiridol was removed for an additional 120 min (lanes 2, 4, 5, and 6) in the presence or absence of PD0166285. Cells in lane 5 were treated with MG132 during the induced mitotic exit and reversal to block proteasome-dependent protein degradation. Cells in lane 6 were treated with the general caspase inhibitor Z-VAD-FMK after flavopiridol removal. Samples were analyzed as described in A; Western blotting of Plk1 was added as an example of a protein that is normally degraded in G1. Histone H3 phosphorylated on serine 10 was used as an additional mitotic marker. Proteasome inhibitor added during mitotic exit and reversal or caspase inhibitor added during reversal allow recovery of Cdk1 activity upon reentry into M phase. (D) The percentage of viable HeLa cells expressing R42A cyclin B1-GFP that retain viability after reentry into mitosis from late G1. Mitotic cells were treated with flavopiridol and PD0166285 for 120 min. Flavopiridol was removed but PD0166285 retained. In the middle column, MG132 was present throughout the experiment to block proteasome dependent protein degradation that normally occurs during mitotic exit and in G1. In the rightmost column, the general caspase inhibitor Z-VAD-FMK was added at the time flavopiridol was removed. Proteasome inhibitor added during mitotic exit and reversal or caspase inhibitor added during reversal rescue cell viability upon reentry into M phase for cells expressing nondegradable cyclin B. (E) The caspase inhibitor Z-VAD-FMK rescues viability during reentry into mitosis from late G1. A HeLa cell expressing R42A cyclin B1-GFP was treated with flavopiridol and PD0166285 for 120 min. Flavopiridol was removed, PD0166285 was retained, and Z-VAD-FMK was added. Shortly after the flavopiridol washout, cyclin B1-GFP translocates back into the nuclei, chromosomes recondense, and cytokinesis reverses. The complete time-lapse sequence is shown in Supplemental Video 12. Top panels in B and E show phase-contrast images, and the bottom panels show corresponding fluorescent images. Bars, 10 μ m.

Figure 6. Activation of Wee1 and Myt1 kinases during the M to G1 transition. (A) Asynchronously growing cells, mitotic cells collected by nocodazole shake-off or shake-off cells released from nocodazole block in the presence or absence of 0.5 μ M PD0166285 were fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content. Only single cells were included in the analysis, but the released samples contained increased population of doublets, which were likely telophase cells connected by the midbody. Therefore, G1 peaks in nocodazole released samples may underestimate the actual proportion of cells that have divided. (B) Mitotic HeLa cells were released in the growth medium containing 0.5 μ M PD0166285 or DMSO after 2- to 3-h nocodazole block. Cdk1 activity (phospho-nucleolin); phosphorylated histone H3, inhibitory phosphorylations of Cdk1 on T14 and Y15; and protein levels of Wee1, Myt1, Cdc25C, and cyclin B1 were analyzed by Western blotting. Dephosphorylation of nucleolin and histone H3, cyclin B degradation and the electrophoretic mobility shifts of Wee1, Myt1, and Cdc25 were not affected by Wee1 and Myt1 inhibition. However, after nocodazole release antibodies against Cdk1 pT14 and Cdk1pY15 give faint bands, absent in the Wee1/Myt1 inhibitor-treated samples. (C) Lysates for assaying the Myt1 kinase activity were prepared from mitotic HeLa cells released from nocodazole for indicated time or interphase cells. For a substrate, His-tagged kinase-dead (K33R) Cdk1- Δ 90cyclin B was used. Substrate was conjugated to magnetic beads and incubated in indicated cell lysates supplemented with ATP. Beads were then pulled out, washed, and the bound His-Cdk1 was analyzed for inhibitory phosphorylation on T14 by Western blotting. The substrate gradually became phosphorylated on the T14 as cells exited mitosis. By 4 h after nocodazole release levels of T14 phosphorylation were comparable with that of interphase cells. The absence of ATP or addition of 5 μ M Wee1/Myt1 inhibitor PD0166285 prevented the T14 phosphorylation of the substrate. Lane labeled "mock" contained empty beads (without the substrate) incubated with interphase cell lysates and ATP, lane labeled "beads" contained substrate-conjugated beads not incubated in any lysate. To control for loading, the same PVDF membrane was stained with Coomassie Blue. The kinase-containing lysates were analyzed for the Myt1 electrophoretic mobility shift by Western blotting. The shift to the faster mobility, active form corresponded to the phosphorylation of the substrate on T14.

undergo continuous growth where Cdk1 forms functional complexes with a G1, S and G2-M cyclins (Santamaria *et al.*, 2007). Therefore, the back-up mechanism must exist to dampen the activation of Cdk1 molecules that happened to find themselves an activating partner in G1. Last, virally encoded cyclins, provide another situation where cell cycle control may be co-opted by noncanonical pathways (see review by Nebreda, 2006). The activity of Wee1 and Myt1 kinases in G1 may serve other physiological purposes than simply reinforcing the G1. For example, a recent siRNA study indicated that Myt1 kinase has a specific role in G1, stimulating proper postmitotic reassembly of the ER and Golgi networks (Nakajima *et al.*, 2008). Using the inhibitor PD0166285, we observed similar effects on Golgi reassembly (data not shown).

As we have shown here, reactivation of Cdk1 activity in late G1 leads to cell death. The relevant pathological condition may be illustrated by the apoptosis of postmitotic neurons in neurodegenerative diseases such as Alzheimer's disease. Terminally differentiated neurons permanently exit the cell cycle and stay at resting (G1/G0) phase (reviewed by Yoshikawa, 2000), but in Alzheimer's disease, they display aberrant Cdk1 activity (Vincent *et al.*, 1997). Cdk1 activation has been shown to alter

gene expression and precede the formation of the neurofibrillary tangles and apoptosis in neurons (Pei *et al.*, 2002; reviewed by Becker and Bonni, 2004). Thus, it is not surprising that backup mechanisms to restrain Cdk1 activity during the G1 stage of the cell cycle have evolved.

In summary, although cyclin proteolysis is the dominant control for down-regulation of Cdk1 activity during mitotic exit, additional backup mechanisms may contribute to its fine-tuning and to the maintenance of the G1 state. To fully comprehend cell cycle regulation at the systems level, it is critical that all latent networks be identified and their potential roles be investigated. Elucidation of these accessory controls may also contribute to pathologies that involve aberrations in cell cycle control mechanisms, for example in hyperproliferative diseases such as cancer and in neurodegenerative diseases such as Alzheimer's.

ACKNOWLEDGMENTS

We are grateful to Drs. Jonathan Pines, Ruth Muschel, Peter Davies, Todd Stukenberg, and Osamu Hashimoto for generously providing essential reagents. We thank Sanofi-Aventis and the (Drug Synthesis and Chemistry

Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute for providing flavopiridol and Pfizer for providing PD0166285. We are grateful to Dean Dawson and Todd Stukenberg for insightful discussion and advice. We thank the Oklahoma Medical Research Foundation flow cytometry core facility for technical assistance. We thank the members of the Gorbisky, Rankin, and Dresser laboratories for help and advice. This work was supported by grant GM50412 from the National Institute of General Medical Sciences and by the McCasland Foundation.

REFERENCES

- Allan, L. A., and Clarke, P. R. (2007). Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. *Mol. Cell* 26, 301–310.
- Atherton-Fessler, S., Liu, F., Gabrielli, B., Lee, M. S., Peng, C. Y., and Piwnica-Worms, H. (1994). Cell cycle regulation of the p34cdc2 inhibitory kinases. *Mol. Biol. Cell* 5, 989–1001.
- Becker, E. B., and Bonni, A. (2004). Cell cycle regulation of neuronal apoptosis in development and disease. *Prog. Neurobiol.* 72, 1–25.
- Boutros, R., Dozier, C., and Ducommun, B. (2006). The when and wheres of CDC25 phosphatases. *Curr. Opin. Cell Biol.* 18, 185–191.
- Cheng, A., Xiong, W., Ferrell, J. E., Jr., and Solomon, M. J. (2005). Identification and comparative analysis of multiple mammalian Speedy/Ringo proteins. *Cell Cycle* 4, 155–165.
- Clute, P., and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat. Cell Biol.* 1, 82–87.
- Dranovsky, A., Vincent, I., Gregori, L., Schwarzman, A., Colflesh, D., Enghild, J., Strittmatter, W., Davies, P., and Goldgaber, D. (2001). Cdc2 phosphorylation of nucleolin demarcates mitotic stages and Alzheimer's disease pathology. *Neurobiol. Aging* 22, 517–528.
- Fletcher, L., Cheng, Y., and Muschel, R. J. (2002). Abolishment of the Tyr-15 inhibitory phosphorylation site on cdc2 reduces the radiation-induced G(2) delay, revealing a potential checkpoint in early mitosis. *Cancer Res.* 62, 241–250.
- Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132–138.
- Hashimoto, O., Shinkawa, M., Torimura, T., Nakamura, T., Selvendiran, K., Sakamoto, M., Koga, H., Ueno, T., and Sata, M. (2006). Cell cycle regulation by the Wee1 inhibitor PD0166285, pyrido [2,3-d] pyrimidine, in the B16 mouse melanoma cell line. *BMC Cancer* 6, 292.
- Hashimoto, O., Ueno, T., Kimura, R., Ohtsubo, M., Nakamura, T., Koga, H., Torimura, T., Uchida, S., Yamashita, K., and Sata, M. (2003). Inhibition of proteasome-dependent degradation of Wee1 in G2-arrested Hep3B cells by TGF beta 1. *Mol. Carcinog.* 36, 171–182.
- Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2–cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* 12, 53–63.
- Jin, P., Gu, Y., and Morgan, D. O. (1996). Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J. Cell Biol.* 134, 963–970.
- Karaiskou, A., Perez, L. H., Ferby, I., Ozon, R., Jessus, C., and Nebreda, A. R. (2001). Differential regulation of Cdc2 and Cdk2 by RINGO and cyclins. *J. Biol. Chem.* 276, 36028–36034.
- Kornbluth, S., Sebastian, B., Hunter, T., and Newport, J. (1994). Membrane localization of the kinase which phosphorylates p34cdc2 on threonine 14. *Mol. Biol. Cell* 5, 273–282.
- Krek, W., and Nigg, E. A. (1991). Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. *EMBO J.* 10, 3331–3341.
- Larochelle, S., Merrick, K. A., Terret, M. E., Wohlbold, L., Barboza, N. M., Zhang, C., Shokat, K. M., Jallepalli, P. V., and Fisher, R. P. (2007). Requirements for Cdk7 in the assembly of Cdk1/cyclin B and activation of Cdk2 revealed by chemical genetics in human cells. *Mol. Cell* 25, 839–850.
- Lindon, C., and Pines, J. (2004). Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *J. Cell Biol.* 164, 233–241.
- Liu, F., Stanton, J. J., Wu, Z., and Piwnica-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. *Mol. Cell. Biol.* 17, 571–583.
- Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A., and Worland, P. J. (1994). Potent inhibition of CDC2 kinase activity by the flavonoid L86–8275. *Biochem. Biophys. Res. Commun.* 201, 589–595.
- Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995). Myt 1, a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* 270, 86–90.
- Murray, A. W., Solomon, M. J., and Kirschner, M. W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 339, 280–286.
- Nakajima, H., Yonemura, S., Murata, M., Nakamura, N., Piwnica-Worms, H., and Nishida, E. (2008). Myt1 protein kinase is essential for Golgi and ER assembly during mitotic exit. *J. Cell Biol.* 181, 89–103.
- Nebreda, A. R. (2006). CDK activation by non-cyclin proteins. *Curr. Opin. Cell Biol.* 18, 192–198.
- Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* 344, 503–508.
- O'Farrell, P. H. (2001). Triggering the all-or-nothing switch into mitosis. *Trends Cell Biol.* 11, 512–519.
- Parker, L. L., and Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science* 257, 1955–1957.
- Pei, J. J., Braak, H., Gong, C. X., Grundke-Iqbal, I., Iqbal, K., Winblad, B., and Cowburn, R. F. (2002). Up-regulation of cell division cycle (cdc) 2 kinase in neurons with early stage Alzheimer's disease neurofibrillary degeneration. *Acta Neuropathol.* 104, 369–376.
- Pomeroy, J. R., Kim, S. Y., and Ferrell, J. E., Jr. (2005). Systems-level dissection of the cell-cycle oscillator: bypassing positive feedback produces damped oscillations. *Cell* 122, 565–578.
- Potapova, T. A., Daum, J. R., Pittman, B. D., Hudson, J. R., Jones, T. N., Satinover, D. L., Stukenberg, P. T., and Gorbisky, G. J. (2006). The reversibility of mitotic exit in vertebrate cells. *Nature* 440, 954–958.
- Pu, L., Amoscato, A. A., Bier, M. E., and Lazo, J. S. (2002). Dual G1 and G2 phase inhibition by a novel, selective Cdc25 inhibitor 6-chloro-7-[corrected](2-morpholin-4-ylethylamino)-quinoline-5,8-dione. *J. Biol. Chem.* 277, 46877–46885.
- Santamaria, D., Barriere, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Caceres, J. F., Dubus, P., Malumbres, M., and Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 448, 811–815.
- Sherr, C. J., and Roberts, J. M. (2004). Living with or without cyclins and cyclin-dependent kinases. *Genes Dev.* 18, 2699–2711.
- Skoufias, D. A., Indorato, R. L., Lacroix, F., Panopoulos, A., and Margolis, R. L. (2007). Mitosis persists in the absence of Cdk1 activity when proteolysis or protein phosphatase activity is suppressed. *J. Cell Biol.* 179, 671–685.
- Solomon, M. J., Glotzer, M., Lee, T. H., Philippe, M., and Kirschner, M. W. (1990). Cyclin activation of p34cdc2. *Cell* 63, 1013–1024.
- Vincent, I., Jicha, G., Rosado, M., and Dickson, D. W. (1997). Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.* 17, 3588–3598.
- Wang, Y., Li, J., Booher, R. N., Kraker, A., Lawrence, T., Leopold, W. R., and Sun, Y. (2001). Radiosensitization of p53 mutant cells by PD0166285, a novel G(2) checkpoint abrogator. *Cancer Res.* 61, 8211–8217.
- Watanabe, N., Arai, H., Iwasaki, J., Shiina, M., Ogata, K., Hunter, T., and Osada, H. (2005). Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways. *Proc. Natl. Acad. Sci. USA* 102, 11663–11668.
- Yoshikawa, K. (2000). Cell cycle regulators in neural stem cells and postmitotic neurons. *Neurosci. Res.* 37, 1–14.