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

Tamara A Potapova, John R Daum, Kendra S Byrd and Gary J Gorbsky
Cell Cycle and Cancer Biology Research Program,
Oklahoma Medical Research Foundation,
825 NE 13th St, Oklahoma City, OK 73104 USA

Running title: Inhibition of Cdk1 in G1 by phosphorylation

Corresponding author:
Gary J Gorbsky
Oklahoma Medical Research Foundation
825 NE 13th St, MS 48
Oklahoma City, OK 73104
Voice 1-405-271-2032
Fax 1-405-271-7312
Gary-Gorbsky@omrf.ouhsc.edu

Running Head: Cdk1 Inhibition in G1 by Phosphorylation

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

Inactivation of Cdk1 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 downregulation 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 re-activation 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 kinase1 (Cdk1) 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 (Glotzer 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 Piwnica-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 induces a robust autoamplification
of Cdk1 activity (O'Farrell, 2001), and are responsible for the switch-like activation of Cdk1 (Pomerening 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% 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) for live imaging experiments, and Lipofectamine L-2000 for biochemical experiments. Both reagents were used according to the manufacturer’s directions. Plasmids encoding both the wild type human cyclin B1-GFP and the non-degradable R42A mutant cyclin B1-GFP were gifts from J. Pines. Plasmids encoding the wild type Cdk1-GFP and phosphorylation mutants (T14A, Y15F and the AF) Cdk1-GFP were gifts from R. Muschel. Experiments were conducted on the day following the transfection.

**Chemical inhibitors.** The Cdk inhibitor, Flavopiridol, provided by the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis) was used at 5-10 µM except for cells overexpressing Cdk1-GFP constructs where it was used at 25µM. The proteasome inhibitor MG132 (Calbiochem) was used at 25µM. The Wee1/Myt1 inhibitor PD0166285 (Pfizer) was used at 0.5 µM. The Aurora kinase inhibitor ZM447439 (AstraZeneca) was used at 25 µM. The Cdc25 phosphatase
inhibitor NSC663284 (Sigma) was used at 25 µM. The general caspase inhibitor Z-VAD-FMK (Calbiochem) was used at 100µM. Nocodazole (Sigma) was used at 100ng/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 non-degradable cyclin B arrest in mitosis spontaneously. The mitotic cells were split into a number of experimental subgroups and treated with specified drugs for indicated periods of time (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 DTT. Protein samples were separated by SDS-PAGE in 4% - 12% Bis-Tris gels (Invitrogen), transferred to PVDF (Millipore) and blocked in 5% non-fat dry milk or 5% BSA. Primary antibodies used were as follows: anti-pNucleolin (gift from P. Davies), anti-pT14Cdk1 (Abcam), anti-pY15Cdk1 (BD Biosciences or Cell Signaling), anti-cyclin B1 (BD Biosciences), anti-Plk1 (Zymed), anti-Wee1 (CellSignaling), anti-
Myt1 (CellSignaling), anti-Cdc25C (CellSignaling) anti-pS10 histone H3 (CellSignaling), anti-nucleolin (Abcam) and anti-Cdk1 (Biomol). Primary antibodies were detected using horseradish-peroxidase conjugated IgG (Jackson ImmunoResearch) and visualized using West Pico Chemiluminescent kit (Pierce).

**Flow cytometry.** For pS10 histone H3 analysis, cells were collected by centrifugation, fixed in 2% formaldehyde in PHEM (60mM PIPES, 25Mm HEPES (pH 6.8), 10mM EDTA, 4mM MgCl₂) for 15 minutes and permeabilized with 90% of -20°C methanol. Later cells were washed three times with 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). For cell cycle analysis, cells were fixed in 90% ethanol for at least 24 hours and stained with 50 μg/ml propidium iodide in PBS in the presence of 100 μg/ml RNAse A (Sigma). Analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences).

**Live imaging.** Cells were grown on 25 mm glass coverslips which were inserted in an Attofluor culture chamber (Molecular Probes) before the experiment. When applicable, cells were pre-incubated 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) and an objective heater (Bioptechs). Time-lapse phase contrast and fluorescent images were collected using Zeiss Axiovert 200M microscopes, either wide-field fluorescent or high-speed Yokogawa spinning disk confocal. Both microscopes were equipped with Hamamatsu ORCA-ERG digital cameras. 63× PlanApochromat oil immersion
objectives were used for all live imaging. Drugs were substituted by exchange of the media after a brief rinse. Time lapse videos were assembled using the Metamorph software (Molecular Devices).

**Immunofluorescence**

Hela cells were blocked in mitosis with nocodazole for 2-3 hours, collected by shake-off, washed in nocodazole-free medium and plated on coverslips in wells containing 0.5 µM PD0166285 or DMSO for 4 hours. Attached cells were fixed in 4% paraformaldehyde/PHEM for 20 min and permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were washed in PBST, blocked in 5%BSA/PBS, incubated with mouse anti-Golgin-97 antibody (Molecular Probes) overnight and secondary anti-mouse antibody conjugated with Alexa-Fluor 546 (Molecular Probes). F-actin was stained with Alexa-Fluor 647 phalloidin (Molecular Probes), DNA was stained with Vybrant®DyeCycle™ Green (Molecular Probes). The images were acquired using Zeiss Axio Observer high-speed Yokogawa spinning disk confocal microscope (63× PlanApochromat oil immersion objective) equipped with a Hamamatsu ORCA-ERG digital camera and processed with SlideBook (Intelligent Imaging Innovations).

**Myt1 kinase assays**

Hela cells were blocked in mitosis with nocodazole for 2-3 hours, collected by shake-off, washed in nocodazole-free medium and incubated without nocodazole for 1,2,3 and 4 hours. Interphase cells were obtained by trypsinizing adherent cells left after shake-off. Equal number of cells (typically between 1.6 to 2 x 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 (50mM TRIS, 50mM NaCl, 5mM MgCl₂, 1% Triton X-100, 1mM DTT supplemented with protease inhibitor cocktail, 1mM NaVO₄ and 400nM 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, was conjugated to Ni-NTA magnetic beads (Qiagen) and incubated in kinase lysates supplemented with 2mM ATP for 30 min at 37ºC. Beads were subsequently washed and the bound proteins released by boiling in NuPAGE protein sample buffer (Invitrogen) for 10 minutes. 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 Comassie 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.

In order to reveal underlying and redundant 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 non-degradable 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) and see 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 a number of 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-alpha tubulin. Cyclin B was preserved by addition of the proteasome inhibitor, MG132, to the medium, or by expressing a non-degradable 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 minutes 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 (Larochelle et al., 2007), by synthesis of small protein kinase inhibitors (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 hours. Mitotic cells were collected by shake-off and treated with Flavopiridol with or without MG132 for 30 or 60 minutes. 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 presence of 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).

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 (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 non-degradable (R42A) cyclin B1 (Figure 1D). For this experiment, cells were transfected with indicated constructs for 24 hours; mitotic cells were collected in nocodazole and treated with Cdk inhibitor for 30 or 60 minutes. Cells expressing the non-degradable (R42A) cyclin B1 lose the ability to re-
phosphorylate nucleolin and histone H3 with time in G1 similarity to MG132-treated cells (lanes 7 vs. 9). The Cdk1 phosphorylation on inhibitory T14 and Y15 also increased (lanes 8 and 9). The wild-type cyclin B was degraded upon Cdk inhibition, and the cells that expressed it did not re-enter 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 minutes 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 non-degradable 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 non-degradable cyclin B for the subsequent experiments, the R42A cyclin B –expressing mitotic cells were collected by shake-off without nocodazole, since 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 1B and 1D 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 (Figures 1C and 1E). 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, though it appeared 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 minutes. 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 minutes 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 minutes of Flavopiridol treatment in the absence of PD0166285.

For HeLa cells expressing non-degradable 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 non-degradable cyclin B1-GFP is shown in Figure 2D and Supplemental video 5. Some cells expressing non-degradable 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 non-degradable cyclin B. We have previously demonstrated 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 non-degradable 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 minutes 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 minutes 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 minutes. Cyclin B was preserved with MG132 (Figure 3A) or by expression of the non-degradable mutant (Figure 3B). PD0166285 prevented Cdk1 phosphorylation on T14 and Y15 and allowed Cdk1 to regain activity when Cdk inhibitor was removed (Figure 3A, lane 4 and Figure 3B, lane 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 hour after the induced mitotic exit, Cdc25 phosphatase(s) have the potential to de-phosphorylate 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 hour 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 minutes 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 hour 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 to T14 in regulation of Cdk1 activity. Together, these results support the hypothesis that blocking Wee1 and Myt1 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 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 minutes. The time-lapse sequence of mitotic exit and reversal of an MG132-treated Xenopus cell subject to 120 minutes 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 minutes in G1 in the presence of the proteasome inhibitor. However, a difference was seen in reversals carried out with HeLa cells expressing non-degradable 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 non-degradable 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 re-entry into mitosis was not induced (Supplemental video 11).
By western blot analysis, Hela cells expressing non-degradable 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 re-phosphorylation of nucleolin and histone H3 reflects the loss of mitotic markers that accompanies cell death in mitosis (Allan and Clarke, 2007).

Since cell death was not observed in cells treated with proteasome inhibitor, we reasoned that in cells expressing non-degradable cyclin B and forced into G1, cell death during reversal might result from re-activation 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 non-degradable 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 to 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 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.

While 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 re-activated 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 hours 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 instance, the antibodies against phospho-Cdk1 T14 and phospho-Cdk1 Y15 show some weak immunoreactivity 3-4 hours after nocodazole release, at the 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 \textit{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 non-degradable \(\Delta 90\) cyclin B. The substrate was incubated in lysates in the presence of ATP and analyzed for phosphorylation on T14 using the phospho-specific antibody. The substrate phosphorylation on T14 begun in lysates of cells released from mitotic nocodazole block for two to three hours. (Figure 6C).
four hours, 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 panel). Together, these results suggest that the Myt1 kinase, and likely the Wee1 kinase, are 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 phosphorylation 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, about 30 minutes 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.

While 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 in by phosphorylation. For example, there exists a poorly explored family of RINGO/Speedy proteins some of which are non-cyclin 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 Cdk’s 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 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. Lastly, virally encoded cyclins, provide another situation
where cell cycle control may be co-opted by non-canonical 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 instance, a recent siRNA study indicated that Myt1 kinase has a specific role in G1, stimulating proper post-mitotic re-assembly 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 cellular catastrophe and cell death. The relevant pathological condition may be illustrated by the apoptosis of post-mitotic neurons in neurodegenerative diseases such as Alzheimer’s. Terminally differentiated neurons permanently exit the cell cycle and stay at resting (G1/G0) phase (reviewed in (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 in (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, while cyclin proteolysis is the dominant control for downregulation 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.

**Acknowledgements.**

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 National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis) 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 OMRF flow cytometry core facility for technical assistance. We thank the members of the Gorbsky, Rankin, and Dresser laboratories for help and advice. This work was supported by a grant from the National Institute of General Medical Sciences (GM50412) and by the McCasland Foundation.
References.


**Figure Legends**

**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 min or 60 min of Flavopiridol treatment assayed by live video microscopy. Prior to 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 non-degradable 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, 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 5µM 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 re-enter 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 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 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 non-degradable R42A (lanes 5-9) cyclin B-GFP. Mitotic cells were collected in nocodazole and induced to exit mitosis by treatment with 5µM Flavopiridol for 30 or 60 min. After removal of the Cdk inhibitor, cells were allowed to re-enter mitosis for an additional 60 min. Cells expressing the wild-type cyclin B1-GFP gradually degrade it upon Flavopiridol treatment and do not re-enter after Flavopiridol washout (lanes 2 and 4). In cells expressing the non-degradable 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 non-degradable cyclin B1 treated as 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).
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. Prior to 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 non-degradable 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 re-enter 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 PD016285 were retained after removal of Flavopiridol. Supplemental video 3 shows the complete timelapse 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 non-degradable 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 non-degradable 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 μm.

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, PD0166285, 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 non-degradable R42A cyclin B1-GFP (in place of incubation with MG132) were treated and analyzed as 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 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 (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).

**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, 12) compared to 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 re-condensed chromosomes but maintained an apparently intact nuclear envelope after Flavopiridol removal (Two expressing T14A-Cdk1-GFP and two 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 show corresponding fluorescent images. Bar, 10 μm. The complete video sequence is shown in Supplemental video 8.

**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 1h 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 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 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 non-degradable cyclin B. (E) The caspase inhibitor Z-VAD-FMK rescues viability during re-entry 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 retained, and Z-VAD-FMK added. Shortly after the Flavopiridol washout, cyclin B1-GFP translocates back into the nuclei, chromosomes re-condense, 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 μm.

**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 μM PD0166285 were fixed, stained with propidium iodide and analyzed by FACS 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 μM
PD0166285 or DMSO after 2-3 hours nocodazole block. Cdk1 activity (phospho-nucleolin), phosphorylated histone H3, inhibitory phosphorylations of Cdk1 on T14 and Y15, protein levels of Wee1, Myt1, Cdc25C and cyclin B1 were analyzed by Western blotting. Dephosphorylation of nucleolin and histoneH3, 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-Δ90cyclin 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 hours after nocodazole release levels of T14 phosphorylation were comparable to that of interphase cells. The absence of ATP or addition of 5µM 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 Comassie 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.
A) Mitotic cells + MG132 → Flavopiridol added 60 min → Flavopiridol washed out 60 min
Lanes: 1,4,7,10
Lanes: 2,5,8,11
Lanes: 3,6,9,12

<table>
<thead>
<tr>
<th>Cdk1-GFP</th>
<th>Flavopiridol washout</th>
<th>n/a</th>
<th>+</th>
<th>n/a</th>
<th>+</th>
<th>n/a</th>
<th>+</th>
<th>n/a</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) % reversed cells

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>T14/A</th>
<th>Y15/F</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/6</td>
<td>3/8</td>
<td>9/11</td>
<td>25/25</td>
</tr>
</tbody>
</table>

C) Flavopiridol and MG132 added → Flavopiridol washed out