Inhibitory Phosphorylation of Cyclin-Dependent Kinase 1 as a Compensatory Mechanism for Mitosis Exit^{▽†}

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The current paradigm states that exit from mitosis is triggered by the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) acting in concert with an activator called CDC20. While this has been well established for a number of systems, the evidence of a critical role of CDC20 in somatic cells is not unequivocal. In this study, we reexamined whether mitotic exit can occur properly after CDC20 is depleted. Using single-cell analysis, we found that CDC20 depletion with small interfering RNAs (siRNAs) significantly impaired the degradation of APC/C substrates and delayed mitotic exit in various cancer cell lines. The recruitment of cyclin B1 to the core APC/C was defective after CDC20 downregulation. Nevertheless, CDC20-depleted cells were still able to complete mitosis, albeit requiring twice the normal time. Intriguingly, a high level of cyclin-dependent kinase 1 (CDK1)-inhibitory phosphorylation was induced during mitotic exit in CDC20-depleted cells. The expression of an siRNA-resistant CDC20 rescued both the mitotic exit delay and the CDK1-inhibitory phosphorylation. Moreover, the expression of a nonphosphorylatable CDK1 mutant or the downregulation of WEE1 and MYT1 abolished mitotic exit in CDC20-depleted cells. These findings indicate that, in the absence of sufficient APC/C activity, an alternative mechanism that utilized the classic inhibitory phosphorylation of CDK1 could mediate mitotic exit.

Cyclin-dependent kinase 1 (CDK1) (also called CDC2) is one of the key protein kinases for promoting mitosis. The activation of CDK1 requires binding to its activating partner (cyclin B1) and the phosphorylation of a residue on the T-loop (Thr161). While CDK1^{Thr161} phosphorylation occurs after cyclin B1 binding, cyclin B1 itself oscillates during the cell cycle, accumulating from S phase, and is destroyed at the end of mitosis (reviewed in reference 10).

Before mitosis, cyclin B1-CDK1 complexes are kept in a CDK1^{Thr14/Tyr15}-phosphorylated and inactive state by two kinases called WEE1 and MYT1. While WEE1 specifically phosphorylates CDK1^{Tyr15} (29), the endoplasmic reticulum/Golgi complex-located MYT1 displays a stronger preference for CDK1^{Thr14} (4, 19). WEE1 itself is regulated by several kinases. WEE1^{Ser123} is phosphorylated by CDK1 at the onset of mitosis, thereby generating a binding motif to allow PLK1 to phosphorylate WEE1^{Ser53} (45, 47). The phosphorylation of WEE1^{Ser123} also independently primes the phosphorylation of WEE1^{Ser121} by CK2 (46). Together, phosphorylated Ser123, Ser121, and Ser53 serve as phosphodegrons that target WEE1 for degradation by the ubiquitin ligase SCF^{B-TrCP} (46), thereby ensuring that WEE1 activity is suppressed during mitosis. Similarly, MYT1 activity decreases during mitosis, coinciding with the phosphorylation by PLK1 and CDK1 (4, 26, 50). At the end of G₂ phase, the stockpile of inactive cyclin B1-CDK1 complexes is activated by members of the CDC25 family.

With the feedback loops that simultaneously activate

CDC25 and inactivate WEE1/MYT1 (reviewed in reference 18), the activation of cyclin B1-CDK1 is essentially a bistable system that becomes autocatalytic once a critical proportion is activated, allowing a rapid entry into mitosis (9). PLK1 is believed to be able to kick-start the bistable system. For example, phosphorylation of CDC25C and CDC25B by PLK1 promotes their nuclear localization and activation of CDK1 (20, 37, 44). Recently, it has been reported that PLK1 itself is activated by Aurora A-dependent phosphorylation, an event that is assisted by Bora (22, 38).

The inactivation of CDK1 at the end of mitosis is mediated by ubiquitin-mediated degradation of cyclin B1. Specifically, this is carried out by the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) loaded with a targeting subunit called CDC20. CDC20 acts both as a substrate-recruiting subunit and a direct activator of APC/C (16). Activated cyclin B1-CDK1 also negatively regulates itself by stimulating the activity of APC/C^{CDC20} through phosphorylation of its subunits, including CDC16, CDC23, CDC27, and CDC20 (reviewed in reference 55).

Activation of APC/C^{CDC20} is initiated only when all the chromosomes have achieved bipolar attachment to the mitotic spindles. Unattached kinetochores or the absence of tension between the paired kinetochores activates a surveillance mechanism termed the spindle assembly checkpoint (reviewed in reference 25). The checkpoint maintains high levels of active cyclin B-CDK1 by inhibiting APC/C^{CDC20}. The underlying mechanism involves the binding of the checkpoint machinery to unattached kinetochores, followed by the formation of a diffusible mitotic checkpoint complex and culminating in the inhibition of APC/C^{CDC20} by MAD2.

APC/C also binds another targeting subunit called CDH1. In marked contrast to CDC20, phosphorylation of CDH1 by cyclin B1-CDK1 prevents its binding to APC/C, thereby keeping

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APC/C^{CDH1} inactive during mitosis (8). The inactivation of CDK1 at the end of mitosis relieves the inhibition of APC/C^{CDH1} (reviewed in reference 30). The phosphatase CDC14 is believed to reverse the phosphorylation carried out by CDK1 on CDH1 (reviewed in reference 43). The activated APC/C^{CDH1} then degrades CDC20 and takes over the task of degrading any remaining cyclin B1, as well as curbing the unscheduled accumulation of cyclin B1 during G₁.

Given the critical roles attributed to CDC20, it is expected that downregulation of CDC20 would lead to a defective mitotic exit. Indeed, mice lacking functional CDC20 display a metaphase arrest at the two-cell stage, with high levels of cyclin B (17). In another study, microinjection of CDC20 antibodies arrested mouse oocytes in the first but not the second meiosis (54). These results support an essential role of CDC20 in the early embryonic cell cycle. However, the roles of CDC20 in the somatic cell cycle are more contentious. It has been shown that the depletion of ~95% of CDC20 by small interfering RNA (siRNA) resulted in mitotic arrest (27, 51). Another study, using a strong inducible short hairpin RNA, revealed neither a mitotic block nor cyclin B stabilization when CDC20 was depleted (3).

In this study, we aimed to see whether somatic cells can exit mitosis after depletion of CDC20 and to elucidate the mechanism involved. We found that depletion of CDC20 with siRNAs led to a delay of anaphase onset. Although CDC20-depleted cells were still able to exit mitosis after the delay, a high level of CDK1 inhibitory phosphorylation was induced. The expression of an siRNA-resistant CDC20 rescued both the mitotic exit delay and the CDK1 inhibitory phosphorylation. Moreover, the expression of a nonphosphorylatable CDK1 mutant (CDK1AF) or depletion of WEE1 and MYT1 abolished mitotic exit in CDC20-depleted cells. Our data indicate an important role of CDC20 in mitotic cells; however, mitotic exit can also occur in the absence of CDC20 due to an alternative mechanism utilizing inhibitory phosphorylation of CDK1.

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

DNA constructs and siRNAs. FLAG-CDK1 and FLAG-CDK1AF in pUHD-P1/PUR were generated as previously described (7). Stealth siRNAs targeting CDH1 (HSS122071), CDC20 (HSS101650 and HSS101651), and WEE1 (HSS111337) and control siRNA were obtained from Invitrogen (Carlsbad, CA). The two siRNAs against CDC20 were CDC20 siRNA#1 (ACGACAUUUGG CAGUGGUGGUAUU) and CDC20 siRNA#2 (GCACCAGUGAUCGACA CAUUCGCAU). When not specified otherwise, CDC20 siRNA#1 was used in the experiment. Two siRNAs against MYT1 (MYT1-siRNA#1, GUGACAUC AACUCAGAGCC, and MYT1-siRNA#2, CUUGGAUUCUCCUCAAGA) were synthesized by Ribobio (GuangZhou, China). CDC20 cDNA was subcloned into pUHD-P3 (21) to generate FLAG-3C-CDC20 in pUHD-P3. To generate a construct resistant to CDC20 siRNA#1, silence mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the oligonucleotides 5'GACATTTAGCTAGCGGTGGTAATGATAA3' and 5'TA CCACCGTAGCTAAATGTCGTCCATC3'.

Cell culture. The HeLa cell line (human cervical carcinoma) used in this study is a clone that expressed the tTA tetracycline repressor chimera (53). To generate APC/C reporter cells, HeLa cells expressing histone H2B-green fluorescent protein (GFP) (6) were transfected with monomeric red fluorescent protein (mRFP)-cyclin B1(CΔ62) in pUHD-P3T and selected with puromycin. Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum (Invitrogen) and 50 U/ml penicillin-streptomycin (Invitrogen) in a humidified incubator at 37°C in 5% CO₂. Unless

stated otherwise, cells were treated with the following reagents at the indicated final concentration: doxycycline (1 μg/ml), MG132 (10 μM), nocodazole (0.1 μg/ml), puromycin (1 μg/ml), RO3306 (Alexis) (10 μM), and thymidine (2 mM). Cells were transfected with plasmids and siRNAs using a calcium phosphate precipitation method (2) and Lipofectamine RNAiMAX (Invitrogen), respectively. Unless stated otherwise, 10 nM siRNA was used for 10-cm plates. Double thymidine synchronization (1) and preparation of cell extracts (33) were performed as described previously. G₂ cells were collected at 8 h upon release from double thymidine block (mitotic cells were first removed by mechanical shake off). For enrichment of prometaphase cells, cells released from double thymidine block were treated with nocodazole for 12 h before the mitotic cells were collected by mechanical shake off. Metaphase cells were collected similarly, except that the cells were pretreated with MG132 (for 1 h) before being released from the nocodazole block and collected after 2 h. G₁ cells were harvested at 14 h after release from double thymidine block.

Antibodies and immunological methods. Monoclonal antibodies against β-actin (6), CDK1 (41), cyclin A2 (52), cyclin B1 (6), and FLAG (11) were obtained from the sources described previously. Mouse monoclonal antibody against CDC20 (for immunostaining) and polyclonal antibodies against CDC20, CDC25C, cyclin B2, cyclin E, geminin, phospho-histone H3^{Ser10}, MYT1, and WEE1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against APC2 (Abcam, Cambridge, United Kingdom), BUBR1 (Bethyl Laboratories, Montgomery, TX), CDH1 (Thermo Fisher Scientific, Waltham, MA), CDC27, MAD2 (BD Transduction Laboratories, Franklin Lakes, NJ), and phospho-CDK1^{Tyr15} (Cell Signaling Technology, Beverly, MA) were obtained from the indicated suppliers. Immunoblotting and immunoprecipitation were performed as described previously (34).

Flow cytometry. Flow cytometry analysis after propidium iodide staining was performed as described previously (40).

Live-cell imaging and immunofluorescent microscopy. Time-lapse microscopy of living cells was performed as previously described (21). For immunofluorescence microscopy, cells grown on poly-L-lysine-treated coverslips were fixed with freshly made 3% formaldehyde and 2% sucrose in PBS at 25°C for 5 min. The cells were then washed three times with PBS for 5 min each, blocked and permeabilized with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 in PBS at 25°C for 30 min, and washed three times with wash buffer (2% BSA and 0.2% Triton X-100 in PBS) for 5 min each time. The cells were incubated with primary antibodies at 4°C for 16 h. After being washed five times with wash buffer, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG secondary antibodies (Invitrogen) for 1 h at 25°C. The cells were further washed five times with wash buffer and stained with Hoechst 33258 (0.5 μg/ml in wash buffer) for 5 min. After being washed three times with wash buffer, the coverslips were mounted with 2% (wt/vol) N-propylgallate in glycerol.

Phosphatase treatment and kinase assays. The expression of recombinant glutathione S-transferase (GST)-CDC25A in bacteria and purification with glutathione-agarose chromatography were as described previously (34). Immunoprecipitates were incubated with 1 μg of GST-fusion proteins in 10 μl of phosphatase buffer (10 mM HEPES, pH 7.2, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol [DTT]) at 25°C for 30 min. After washing in kinase buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM magnesium acetate [MgOAc], 1 mM DTT), the histone H1 kinase activity was assayed as described previously (34). Phosphorylation was quantified with a PhosphorImager (Bio-Rad, Hercules, CA).

RESULTS

Cells undergo a protracted mitosis in the absence of adequate CDC20. Downregulation of CDC20 was carried out with two siRNAs against different regions of CDC20. The results depicted in Fig. 1A show that the siRNAs were effective in downregulating the endogenous CDC20 in HeLa cells (because siRNA#2 was slightly less effective at that concentration, a higher concentration [20 nM per 10-cm plate] was used in later experiments). With serial dilution of the samples, we found that >95% of CDC20 was depleted in G₂ cells (Fig. 1B).

Since it has been reported that CDC20 expression peaks during G₂ metaphase, shortly before it is degraded (49), it is important to confirm the effectiveness of the siRNA in mitotic cells. The results shown in Fig. 1C indicate that CDC20 was

FIG. 1. Downregulation of CDC20 does not affect mitosis. (A) Downregulation of CDC20 with siRNA. HeLa cells were either mock transfected or transfected with two different siRNAs against CDC20. The cells were then harvested at the indicated time points and analyzed by immunoblotting. (B) Efficiency of CDC20 depletion. Cells were harvested 24 h after transfection with either control or CDC20 siRNA. G₂ cell lysates were prepared and diluted by the indicated factors to estimate the relative amounts of CDC20 in the lysates. (C) Depletion of CDC20 during mitosis. HeLa cells transfected with either control or CDC20 siRNA were released from double thymidine synchronization. After 9 h, mitotic cells were collected by mechanical shake off and the remaining cells were designated G₂ cells. Lysates were prepared, and the expression of the indicated proteins was analyzed with immunoblotting. (D) Depletion of CDC20 at kinetochores. HeLa cells transfected with either control or CDC20 siRNA were processed for immunostaining with antibodies against CDC20 and BUBR1. DNA was counterstained with Hoechst 33258. Representative mitotic cells are shown.

reduced to undetectable levels in both G₂ and mitosis after siRNA transfection. The gel mobility shifts of CDC27 and degradation of cyclin A2 indicated that both control and CDC20-depleted cells could successfully enter mitosis.

To further verify the efficiency of CDC20 siRNA, transfected cells were also processed for immunostaining (Fig. 1D). In mock-transfected mitotic cells, CDC20 colocalized with BUBR1 at the kinetochores. In contrast, BUBR1 but not CDC20 was detected in siRNA-transfected mitotic cells, hence excluding the possibility that small amounts of CDC20 could remain at the kinetochores.

We next addressed whether depletion of CDC20 affects cell cycle progression. Cells transfected with CDC20 siRNA were first synchronized with a double thymidine block; they were then released into the cell cycle and harvested at different time points. The results shown in Fig. 2A indicate that the expression of CDC20 in control cells peaked at 9 h after release, coinciding with the peak of histone H3^{Ser10} phosphorylation. The peak of mitosis was further indicated by the expression of other mitotic markers, including cyclin A2 and cyclin B1. As expected, CDC20 was undetectable after siRNA-mediated depletion. In contrast to the results for control cells, the accumulation of histone H3^{Ser10} phosphorylation lasted longer in CDC20-depleted cells (from 9 h to 18 h), suggesting a delay in mitotic exit. Furthermore, the reaccumulation of proteins such as cyclin E during the subsequent cycle was also delayed.

A delay in mitotic exit in CDC20-depleted cells was further confirmed with flow cytometry analysis (Fig. 2B). While the

progression from early S phase to G₂/M was not affected, the appearance of the G₁ population was delayed after CDC20 depletion. A similar delay in mitotic exit was also detected by depleting CDC20 using siRNA#2 (data not shown), confirming that the effects were specific for CDC20.

For a comparison, we also depleted the other targeting subunit of APC/C, CDH1, using siRNA. We found that neither the degradation of the mitotic cyclins nor cell cycle progression was affected by CDH1 downregulation (Fig. 2A and B). Taken together, these data suggest that depletion of CDC20 led to an impairment of the degradation of APC/C targets and a delay in mitotic exit.

Time-lapse microscopy reveals that depletion of CDC20 delays anaphase onset and impairs degradation of APC/C substrates. To monitor the chromosomes and the activity of APC/C in living cells, we generated a HeLa cell line expressing both histone H2B-GFP and an mRFP-APC/C biosensor (containing the D-box fragment from cyclin B1). In control cells, the average duration of prometaphase to anaphase onset was ~44 min (Fig. 3A). As expected, degradation of the APC/C biosensor coincided with anaphase (an example is shown in Fig. 3B; also see Video S1 in the supplemental material). In marked contrast, CDC20-depleted cells took an average of ~90 min to accomplish these mitotic events (Fig. 3A). They stayed in metaphase for a relatively long time before entering anaphase (the metaphase plate actually rotated and flipped, giving the impression that the cell had exited and reentered metaphase) (Fig. 3C; also see Video S2 in the supplemental

FIG. 2. Downregulation of CDC20 delays cell cycle progression. (A) Depletion of CDC20 delays the degradation of mitotic cyclins. HeLa cells were transfected with either control, CDC20 siRNA, or CDH1 siRNA. The cells were synchronized with a double thymidine procedure. At the indicated time points after release, the cells were harvested and subjected to immunoblotting for the indicated proteins. Serial dilutions of control lysates (Extracts) were also loaded as standards. (B) Depletion of CDC20 delays mitotic exit. Cells were treated exactly as described for panel A. At the indicated time points, the cells were harvested and analyzed with flow cytometry.

material). We also observed similar increases in the duration of mitosis after CDC20 knockdown in other human cell lines, including H1299, HCT116, and U2OS (our unpublished observations).

The activation of APC/C in CDC20-depleted cells was also significantly delayed. Quantifying the intensities of the fluorescence signals revealed that the mRFP-APC/C biosensor decreased sharply at the onset of anaphase in control cells (Fig. 3D, at $t = 0$). In contrast, the destruction of the biosensor in CDC20-depleted cells showed a pattern that was both slower and more dispersed. While the half-life of the APC/C biosensor in control cells was ~ 5 min, it was extended to over 20 min in CDC20-depleted cells (Fig. 3E).

It is believed that CDC20 plays a critical role in directing D-box-containing proteins to APC/C for ubiquitination. To

determine whether the stabilization of cyclin B1 in CDC20-depleted cells was due to defective recruitment to APC/C, we examined the direct binding of cyclin B1 to APC/C. In control cells, cyclin B1 was coimmunoprecipitated with CDC27 (one of the components of APC/C) from cells in metaphase but not other phases of the cell cycle (Fig. 3F). In contrast, the interaction between cyclin B1 and CDC27 was significantly diminished in CDC20-depleted cells. The APC/C subunit APC2 was coimmunoprecipitated with CDC27 in both the presence and absence of CDC20, indicating that the core APC/C complex was still intact. As a control, the interaction of spindle assembly checkpoint proteins (MAD2 and BUBR1) with APC/C was determined and was also seen to be disrupted without CDC20.

Taken together, these results show that CDC20 played a major role in the proper timing of anaphase onset and the

FIG. 3. Downregulation of CDC20 delays mitotic exit by suppressing APC/C activity. (A) Mitosis is extended in CDC20-depleted cells. HeLa cells expressing histone H2B-GFP and mRFP-APC/C biosensor were transfected with control or two independent CDC20 siRNAs before being analyzed with time-lapse microscopy. The duration from prometaphase to anaphase onset of individual cells was measured ($n = 200$). The average results and 90% confidence intervals are shown. (B and C) Time-lapse microscopy of HeLa cells expressing histone H2B-GFP and mRFP-APC/C biosensor. The cells were transfected with control (B) or CDC20 siRNA (C). Representative still images from time-lapse microscopy of mitotic cells are shown. Channels of histone H2B-GFP and mRFP-APC/C biosensor are shown. Time unit, h:min. The full videos can be found in the supplemental material (Videos S1 and S2). (D) Degradation of the APC/C biosensor during mitotic exit. HeLa cells expressing histone H2B-GFP and mRFP-APC/C biosensor were transfected with control or CDC20 siRNAs before being analyzed with time-lapse microscopy. The fluorescence intensity of mRFP-APC/C biosensor in individual cells was quantified. The time of anaphase onset is set at $t = 0$. (E) Degradation of the APC/C biosensor during mitotic exit is delayed in CDC20-depleted cells. HeLa cells expressing histone H2B-GFP and mRFP-APC/C biosensor were transfected with control or CDC20 siRNAs before being analyzed with time-lapse microscopy. The fluorescence intensity of mRFP-APC/C biosensor in individual cells was quantified. The mean and standard deviation ($n = 10$) of the time (after anaphase onset) of 50% reduction of the APC/C biosensor were quantified. (F) Attenuation of the recruitment of cyclin B1 to APC/C complexes in CDC20-depleted cells. Cells were transfected with either control or CDC20 siRNA. After enriching cells in G₂ phase, prometaphase (PM), metaphase (M) and G₁ phase, lysates were prepared and subjected to immunoprecipitation (IP) with an antibody against CDC27. Total lysates and the immunoprecipitates were then analyzed with immunoblotting.

FIG. 4. Increase in CDK1 inhibitory phosphorylation during mitotic exit in the absence of cyclin B1 degradation. (A) Mitotic exit in the presence of proteasome inhibitor. HeLa cells were released from double thymidine synchronization and blocked in mitosis with nocodazole (NOC) at 9 h after the release. After 4 h, mitotic cells were collected by mechanical shake off and released either by washing (NOC rel) or RO3306 treatment. The cells were harvested after 2 h and analyzed with immunoblotting. In some samples, the proteasome inhibitor MG132 was included at 1 h prior to the mitotic release. (B) Dose-dependent stimulation of mitotic exit by RO3306. HeLa cells were synchronized with a double thymidine procedure and released into a nocodazole trap. Mitotic cells were isolated by shake off and treated with different doses of RO3306. At the indicated time points, lysates were prepared and the indicated proteins were detected by immunoblotting. (C) RO3306 promotes mitotic exit in both the presence and absence of protein degradation. HeLa cells expressing histone H2B-GFP and mRFP-APC/C biosensor were trapped in mitosis and treated with RO3306 as described for panel A. MG132 was added to the indicated sample at 1 h prior to RO3306 treatment. The mitotic index was then quantified at different time points. (D) Cyclin B1 is protected from degradation by MG132 during RO3306-induced mitotic exit. Cells were trapped in mitosis and treated with MG132 and RO3306 as described for panel C. At the indicated time points, lysates were prepared and the indicated proteins were detected by immunoblotting. (E) Increase in CDK1^{Tyr15} phosphorylation during RO3306/MG132-induced mitotic exit. The CDK1^{Tyr15} phosphorylation signals from the experiment described for panel D were quantified with a densitometer (for 10 μM RO3306 treatment).

recruitment of checkpoint proteins and substrates to APC/C. Without sufficient CDC20, cyclin B1 degradation was impaired and the onset of anaphase was delayed. Importantly, CDC20-depleted cells could still exit mitosis without the complete degradation of cyclin B1, albeit after a delay.

Defective cyclin B1 degradation is compensated by an increase in CDK1 inhibitory phosphorylation. Given that CDC20-depleted cells could exit mitosis with abnormal cyclin B1 degradation, we next investigated whether there are other mechanisms that inactivate CDK1 activity to allow mitotic exit.

To this end, we first examined the importance of cyclin B1 degradation in mitotic exit. Cells were first released from double thymidine synchronization and trapped in mitosis with nocodazole. The cells were then released from mitosis by washing (Fig. 4A). As expected, cells were able to exit mitosis with cyclin B1 degradation. When the cells were also treated with MG132 (a proteasome inhibitor) to stop cyclin B1 degradation, they were unable to exit mitosis even after nocodazole was removed. This was accompanied by the accumulation of phosphorylated histone H3^{Ser10}, CDC27, and CDC25C. These re-

sults indicate the importance of proteolysis in mitotic exit under these conditions.

Alternatively, nocodazole-trapped cells could also be released from mitosis by incubation with a CDK1 inhibitor called RO3306 (Fig. 4A). Interestingly, RO3306 induced cyclin B1 degradation in a dose-dependent manner even in the presence of nocodazole, confirming a tight relationship between the two events (Fig. 4B). These results suggest that inhibition of CDK1 activity could trigger both mitotic exit and cyclin B1 degradation.

Not surprisingly, RO3306-treated cells were able to exit mitosis even when cyclin B1 was stabilized with MG132 (judged by the decrease of phosphorylated histone H3^{Ser10}), suggesting that the inactivation of CDK1 was sufficient to drive mitotic exit without cyclin B1 degradation (6, 36). Significantly, CDK1^{Tyr15} phosphorylation was dramatically increased in these cells (Fig. 4A).

To further verify that RO3306 and MG132 indeed promoted mitotic exit without cyclin B1 degradation, we further examined individual cells using time-lapse microscopy. As expected, cells treated with nocodazole alone were trapped in mitosis (with rounded-up morphology and condensed chromosomes) and the APC/C biosensor was stabilized (see Video S3 in the supplemental material). Similar results were obtained when cells were treated with nocodazole and MG132 (see Video S4 in the supplemental material). In contrast, cells treated with nocodazole followed by RO3306 immediately flattened out and their DNA decondensed; this was accompanied by a rapid degradation of the APC/C biosensor (see Video S5 in the supplemental material). When treated with RO3306 and MG132 together, mitotic exit occurred similarly to mitotic exit without MG132 (Fig. 4C; also see Video S6 in the supplemental material). Notably, both the APC/C biosensor (see Video S6 in the supplemental material) and cyclin B1 (Fig. 4D) were stabilized. Moreover, CDK1^{Tyr15} phosphorylation increased in a time-dependent manner following RO3306 treatment (Fig. 4E). These data confirmed that the mitotic exit stimulated by RO3306 and MG132 was accompanied by an increase in CDK1^{Tyr15} phosphorylation. Based on these premises, we hypothesized that degradation of cyclin B1 may not be the only way to inhibit CDK1 activity at mitotic exit, especially with an insufficiency of APC/C activity.

To see if the increase in CDK1^{Tyr15} phosphorylation was WEE1-dependent, similar experiments were performed after WEE1 was depleted with siRNA (Fig. 5A). As before, the treatment of mitotic cells with RO3306 and MG132 resulted in mitotic exit with high levels of cyclin B1 and CDK1^{Tyr15} phosphorylation (lane 4). Depletion of WEE1 did not affect the level of cyclin B1, but it significantly reduced the level of CDK1^{Tyr15} phosphorylation (lane 8).

To evaluate the effects of the increase in Tyr15 phosphorylation on CDK1, the kinase activities of CDK1 were assayed. Since RO3306 is a reversible inhibitor of CDK1, we were able to assay the intrinsic kinase activity of CDK1 after immunoprecipitation without the direct influence of RO3306. As expected, CDK1 activity was abolished when mitotic cells were treated with RO3306. This was due to the complete degradation of cyclin B1 (Fig. 5B, compare lanes 1 and 3). In contrast, CDK1 activity remained high when cyclin B1 degradation was abolished by MG132 (lane 2). In agreement with the increase

in Tyr15 phosphorylation, treatment of the CDK1 immunoprecipitates with purified CDC25A increased the kinase activity further (lane 6). In contrast, CDC25A did not further increase the kinase activity of CDK1 isolated from mitotic cells (lanes 4 and 8).

The above-described results indicate that cyclin B1 was degraded when cells were forced to exit mitosis with RO3306. The results in Fig. 5C show that the cyclin B1 degradation was CDC20 dependent (lanes 2 and 5). Depletion of CDC20 and CDH1 together completely abolished the degradation of cyclin B1. Interestingly, these cells were still able to exit mitosis after release from a nocodazole block (data not shown), excluding the possibility that CDC20-depleting cells are able to exit mitosis because of the compensatory role of CDH1. In accordance with the above-described results, CDK1^{Tyr15} phosphorylation was stimulated in the presence of MG132 (Fig. 5C, lane 3). Likewise, CDK1^{Tyr15} phosphorylation increased when cyclin B1 degradation was inhibited by CDC20 depletion (compare lanes 2 and 5).

The dependence of cyclin B1 degradation on both CDC20 and CDH1 is somewhat unexpected. During normal mitosis, CDK1 activates APC/C^{CDC20} and inactivates APC/C^{CDH1}, and the situation is reversed after cyclin B1 degradation. However, APC/C^{CDC20} is also under the control of MAD2 when the spindle assembly checkpoint is active. To see if this inhibition was still present when mitotic exit was triggered by CDK1 inhibition, the interaction between MAD2 and CDC20 was analyzed (Fig. 5D). While MAD2-CDC20 complexes could be detected during nocodazole block (lane 6), they were absent after RO3306 treatment (lane 9). The checkpoint was probably not completely inactivated by RO3306 treatment, because small amounts of MAD2-CDC20 complexes could be detected when MG132 was added (lane 10). The partial or inefficient inactivation of the checkpoint may explain why both APC/C^{CDC20} and APC/C^{CDH1} were involved in the degradation of cyclin B1 after RO3306 treatment.

Taken together, these data suggest that when cells were forced to exit mitosis in the absence of cyclin B1 degradation, such as in the presence of MG132 or after CDC20 depletion, CDK1^{Tyr15} became highly phosphorylated. These results implicate a possible role of CDK1-inhibitory phosphorylation in mitotic exit.

An essential protective role of CDK1-inhibitory phosphorylation during mitotic exit. To see if inhibitory phosphorylation of CDK1 plays a major role in mitotic exit when cyclin B1 degradation is compromised, we next examined the kinetics of mitotic exit in CDC20-depleted cells. CDC20 siRNA-transfected cells were trapped in mitosis with nocodazole and released (Fig. 6A). Both the degradation of mitotic cyclins (B1 and B2) and dephosphorylation of CDC27 and CDC25C were delayed. CDC20-depleted cells were able to enter G₁ phase, albeit with a delay in comparison to the time of G₁ entry of control cells (Fig. 6B). Mitotic exit of CDC20-depleted cells was accompanied by an increase in CDK1^{Tyr15} phosphorylation (Fig. 6A).

To ensure that the effects of the CDC20 knockdown were not due to nonspecific effects of the siRNA, an siRNA-resistant CDC20 construct was generated. Expression of the CDC20 construct rescued the delay of mitotic exit induced by CDC20 siRNA (Fig. 6B). Furthermore, the increase in

FIG. 5. WEE1-dependent increase in CDK1^{Tyr15} phosphorylation during RO3306-induced mitotic exit. (A) WEE1-dependent increase in CDK1^{Tyr15} phosphorylation during RO3306-induced mitotic exit. HeLa cells were transfected with either control or WEE1 siRNA. The cells were subjected to double thymidine synchronization and blocked in mitosis with nocodazole (NOC) at 9 h after the release. After 4 h, mitotic cells were collected by mechanical shake off before being treated with RO3306 as indicated. The cells were harvested after 2 h and analyzed with immunoblotting. In some samples, the proteasome inhibitor MG132 was included at 1 h prior to the mitotic release. (B) Inhibition of CDK1 activity by inhibitory phosphorylation during RO3306-induced mitotic exit. Mitotic cells were prepared and treated with MG132 and RO3306 as described for panel A. Lysates were prepared and subjected to either immunoblotting (upper panel) or immunoprecipitation with an antiserum against CDK1. The immunoprecipitates were incubated with either purified GST-CDC25A or GST before being subjected to kinase assays. (C) Attenuation of cyclin degradation leads to an increase in CDK1^{Tyr15} phosphorylation. HeLa cells were transfected with CDC20 siRNA or CDH1 siRNA, synchronized, and treated with MG132/RO3306 as described for panel A. Lysates were then prepared and subjected to immunoblotting. (D) The spindle assembly checkpoint is inactivated during RO3306-induced mitotic exit. Mitotic cells were prepared as described for panel A. The cells were either released from the block by washing (NOC rel) or treated with RO3306. In some samples, the proteasome inhibitor MG132 was added 1 h before release. The cells were harvested after 2 h. Lysates were prepared and subjected to immunoprecipitation with an antiserum against MAD2. Total lysates and the immunoprecipitates were then analyzed with immunoblotting.

CDK1^{Tyr15} phosphorylation was reversed in the presence of the rescue CDC20 (Fig. 6C). These data verify that the delay in mitotic exit and increase in CDK1^{Tyr15} phosphorylation were caused specifically by CDC20 depletion.

The inhibition of CDK1 during mitotic exit was validated by direct measurement of the kinase activity (Fig. 6D). As expected, the kinase activity of CDK1 in control cells declined

immediately after release into G₁ and could not be reactivated by purified CDC25A *in vitro*. In contrast, the loss of CDK1 activity was delayed in CDC20-depleted cells. Moreover, the kinase could be further activated with purified CDC25A, indicating that CDK1 was inhibited by Thr14/Tyr15 phosphorylation in cells that exited mitosis without CDC20.

We next investigated whether the inhibitory phosphorylation

FIG. 6. Inhibitory phosphorylation of CDK1 is increased during CDC20-independent mitotic exit. (A) Mitotic exit in CDC20-depleted cells is accompanied by an increase in CDK1^{Tyr15} phosphorylation. HeLa cells transfected with either control or CDC20 siRNA were released from double thymidine synchronization and treated with nocodazole at 9 h after the release. After 3 h, mitotic cells were collected by shake off and released into G₁ by replating in fresh medium (NOC rel). At the indicated time points, the cells were harvested and the expression of the indicated proteins was detected by immunoblotting. (B) Delay of mitotic exit is specific for CDC20 depletion. HeLa cells were transfected with control vector or plasmids expressing FLAG-CDC20 (a CDC20 siRNA-resistant clone). After enrichment of the transfected cells by with puromycin (48 h), they were transfected with either control or CDC20 siRNA. The cells were then synchronized as described for panel A. At the indicated time points, the cells were harvested and analyzed with flow cytometry. (C) Rescue of CDC20 knockdown effects with siRNA-resistant CDC20. Cells were transfected and synchronized as described for panel B. At the indicated time points, the cells were harvested and analyzed with immunoblotting. (D) CDK1 is inhibited by phosphorylation during mitotic exit in CDC20-depleted cells. Cells were transfected and synchronized as described for panel A. At the indicated time points, lysates were prepared and were subjected to immunoprecipitation with an antiserum against CDK1. The immunoprecipitates were incubated with either purified GST-CDC25A or GST before being subjected to kinase assays.

sites of CDK1 play an essential role in triggering mitotic exit. Wild-type CDK1 and CDK1AF (a nonphosphorylatable mutant with Thr14 and Tyr15 mutated to Ala and Phe, respectively) were expressed in cells, and their effects on mitotic exit were examined by flow cytometry and immunoblotting. Neither CDK1 nor CDK1AF caused a delay in mitotic exit in control cells, as indicated both by the disappearance of histone H3^{Ser10} phosphorylation and cyclin B1 (Fig. 7A) and the appearance of G₁ cells (Fig. 7B). As shown above, mitotic exit

was delayed in CDC20-depleted cells. It was further delayed after the expression of CDK1AF but not CDK1 (Fig. 7B). These data further support the idea that during mitotic exit under conditions in which cyclin B1 degradation is impaired, CDK1 may be inactivated by an alternative mechanism involving inhibitory phosphorylation.

To further substantiate the role of inhibitory phosphorylation in mitotic exit, WEE1 and MYT1 were codepleted together with CDC20. As expected, CDK1^{Tyr15} was phosphory-

FIG. 7. The essential role of inhibitory phosphorylation of CDK1 during mitotic exit in the absence of CDC20. HeLa cells were transfected with constructs that expressed either wild-type CDK1 or CDK1AF. The expression of these proteins was initially suppressed with doxycycline. After enrichment of the transfected cells with puromycin (48 h), they were transfected with either control or CDC20 siRNA. The cells were synchronized with a double thymidine procedure. Nocodazole was added at 9 h after release, and mitotic cells were collected by shake off after 3 h. The cells were released into G₁ by replating in fresh medium (NOC rel); the expression of the recombinant CDK1 was turned on at the same time. At the indicated time points, the cells were harvested and analyzed with immunoblotting (A) or flow cytometry (B).

lated during mitotic exit in CDC20-depleted cells (Fig. 8A). This increase of CDK1^{Tyr15} phosphorylation was abolished after WEE1 and MYT1 were downregulated. While depletion of WEE1 alone was sufficient to mitigate most of the increase of CDK1^{Tyr15} phosphorylation, depletion of MYT1 alone was less efficient (our unpublished data), suggesting that WEE1 was the major contributor of CDK1^{Tyr15} phosphorylation under these conditions. Finally, histone H3^{Ser10} phosphorylation also remained elevated in the absence of WEE1 and MYT1, indicating that mitotic exit was attenuated. This was further verified with flow cytometry analysis (Fig. 8B): while entry into G₁ was delayed after CDC20 knockdown, it was further inhibited when WEE1 and MYT1 were codepleted.

An important prerequisite for this model of mitotic exit without CDC20 is that WEE1 and MYT1 should be present during the mitotic block. It has been reported that human WEE1 is not completely degraded during normal mitosis (48), and the remaining WEE1 is inhibited through a phosphorylation-dependent mechanism (28). MYT1 is also not degraded and is highly phosphorylated during mitosis (26). The results shown in Fig. 8C confirmed that both WEE1 and MYT1 were still present in CDC20-depleted cells during mitosis (albeit WEE1 was at a lower level than in G₂ cells). Collectively, our data indicate that although CDC20-depleted cells could exit mitosis after a delay, cyclin B1 degradation was impaired and CDK1 was inhibited by an alternative

FIG. 8. The role of WEE1 and MYT1 in CDC20-independent mitotic exit. (A) CDC20-independent mitotic exit requires WEE1 and MYT1. HeLa cells were transfected with siRNAs against CDC20, WEE1, and MYT1. The cells were synchronized with a double thymidine procedure and released into a nocodazole trap. Mitotic cells were collected by shake off and released by washing and replating in fresh medium (NOC rel). At the indicated time points, the cells were harvested and analyzed by immunoblotting. (B) Cells were transfected and synchronized as described for panel A. At the indicated time points, the cells were harvested and analyzed with flow cytometry. (C) Both WEE1 and MYT1 are present in CDC20-depleted cells during mitosis. Cells transfected with either control or CDC20 siRNA were released from double thymidine synchronization. After 9 h, mitotic cells were collected by mechanical shake off and the remaining cells were designated G₂ cells. Lysates were prepared and the expression of the indicated proteins was analyzed with immunoblotting. (D) Model of an alternative mechanism of mitotic exit. Unperturbed mitosis involves the sequential phosphorylation and dephosphorylation of CDK1^{Thr14/Tyr15}, followed by the degradation of cyclin B1 by APC/C^{CDC20} (top). Under conditions of insufficient APC/C^{CDC20}, cyclin B1-CDK1 can be inactivated by inhibitory phosphorylation to trigger mitotic exit. See Discussion for details.

mechanism involving WEE1/MYT1-dependent phosphorylation.

DISCUSSION

Mitotic events are required to be highly synchronized to safeguard chromosomal stability. Fundamental to these mechanisms are the abrupt activation and inactivation of cyclin B1-CDK1 during mitosis. These help to ensure that events such as nuclear envelope breakdown and sister chromatid separation are properly executed and minimize a period of potential detrimental conditions during the cell cycle. Inhibitory phosphorylation of CDK1 serves as an elegant mechanism to convert a progressive synthesis of cyclin B1 into an abrupt activation of cyclin B1-CDK1 (see the introduction). Conversely, ubiquitin-mediated proteolysis of cyclin B1 provides an effective mechanism to rapidly inactivate CDK1.

The role of APC/C^{CDC20} in targeting cyclin B1 for ubiquitination has been well established for a number of systems.

For somatic cells, however, the evidence of a critical role of CDC20 is not unequivocal. On one hand, cancer cells (HeLa and U2OS) were found to be arrested in mitosis after the depletion of ~95% of CDC20 using siRNA (27, 51). On the other hand, U2OS cells were found to display neither a mitotic block nor cyclin B stabilization when CDC20 was depleted with an inducible shRNA system (3).

In our study, we were able to deplete >95% of CDC20 with siRNAs (Fig. 1). Downregulation of CDC20 in HeLa cells lengthened mitosis (from ~44 min to ~90 min), mainly through delaying the onset of anaphase (Fig. 3). We also observed similar increases in mitotic length after CDC20 knock-down in other cell lines, including H1299, HCT116, and U2OS. Despite the delay, CDC20-depleted cells were eventually able to exit mitosis without conspicuous morphological defects. The degradation of APC/C substrates, however, was significantly

impeded after CDC20 depletion. This was revealed by the stabilization of endogenous substrates, such as cyclin B1 (Fig. 2A), as well as by an APC/C biosensor in individual cells (Fig. 3D). The impairment of cyclin B1 degradation could be explained by the defective recruitment of substrates to APC/C in the absence of CDC20 (Fig. 3F).

Several other proteins are also degraded by the APC/C during mitotic exit. These include securin, whose degradation by both APC/C^{CDC20} and APC/C^{CDH1} releases separase, thereby allowing the cleavage of cohesin and sister chromatid separation. In the complete absence of CDC20, budding yeast can only proliferate when securin and cyclin B are also absent (39). In mammalian cells, experiments using a nondegradable mutant of securin revealed different extents of chromosomal segregation defects, probably dependent on the level of overexpression (12, 56). But securin is not absolutely required in mammals, as mice lacking securin are viable (13, 23). Securin^{-/-} HCT116 cells are also chromosomally stable (31). We also did not observe any chromosomal segregation defects in CDC20-depleted cells, even though the level of securin remained high at the onset of anaphase (our unpublished data). Nevertheless, it is conceivable that the delay of mitotic exit caused by CDC20 depletion may be due in part to an incomplete degradation of securin. However, we found that codepletion of securin with siRNA did not reverse the CDC20 siRNA-mediated mitotic delay, suggesting that the incomplete degradation of securin may not play a major role in mitotic timing in the absence of CDC20 (our unpublished data).

The current paradigm states that APC/C^{CDC20} is critical for mitotic exit. Why were CDC20-depleted cells still able to complete mitosis? We cannot exclude the possibility that, although >95% of CDC20 was depleted, the residual CDC20 was sufficient to promote mitotic exit, albeit with a delay. Another possibility is that other mechanisms may regulate mitotic exit under conditions of insufficient CDC20. One such mechanism may be the compensation of APC/C^{CDC20} functions by APC/C^{CDH1}. Although downregulation of CDH1 itself had little effect on the timing of mitotic exit (Fig. 2), CDH1 may be responsible for degrading some APC/C substrates in the absence of CDC20 (Fig. 5C). However, cells codepleted of CDC20 and CDH1 could still exit mitosis when released from a nocodazole block (our unpublished data). Therefore, we believe that there must be other compensatory mechanisms leading to the inhibition of cyclin B1-CDK1 activity.

A difficulty in studying mitotic exit is that cells may exit mitosis at slightly different times after mitotic block, thereby masking the molecular changes of CDK1. We addressed this initially by forcing mitotic exit in nocodazole-blocked cells with RO3306. This resulted in the destruction of cyclin B1 (Fig. 4) and exit from mitosis without cell division (see Video S5 in the supplemental material). Although it is still disputable whether the inactivation of CDK1 is sufficient to induce mitotic exit in the presence of a proteasome inhibitor (36, 42), we found that RO3306 could induce rapid mitotic exit in the presence of MG132. This was indicated by the dephosphorylation of histone H3^{Ser10}, CDC25C, and CDC27 and the loss of CDK1 kinase activity (Fig. 4 and 5). Time-lapse microscopy also revealed that cells treated with RO3306 immediately flattened out and their DNA decondensed (see Videos S5 and S6 in the supplemental material).

We found that the RO3306-mediated mitotic exit occurring in the presence of MG132 was accompanied by a dramatic increase in CDK1 inhibitory phosphorylation (Fig. 4 and 5). In support of this result, the CDK1 kinase activity could be reactivated by purified CDC25A *in vitro* (Fig. 5B). A similar conclusion, that inhibitory phosphorylation of CDK1 was activated to lock CDK1 into an inactive state during mitotic exit in the presence of a proteasome inhibitor, was reached by Potapova et al. (35). These results prompted us to consider whether inhibitory phosphorylation of CDK1 plays a role in mitotic exit. As phosphorylation of CDK1^{Thr14/Tyr15} requires the binding of cyclin B1 (4), it is not surprising that there was no observable increase in the inhibitory phosphorylation when cells exited mitosis via the normal degradation of cyclin B1.

We further showed that the inhibitory phosphorylation of CDK1 was upregulated when CDC20 was depleted (Fig. 6A), thereby providing a possible mechanism for CDC20-depleted cells to inhibit CDK1 and exit mitosis. In support of this hypothesis, the expression of CDK1AF inhibited mitotic exit in CDC20-depleted cells (Fig. 7). Likewise, mitotic exit was inhibited after codepletion of WEE1 and MYT1 (Fig. 8). As MYT1 and a portion of WEE1 were still present during the mitotic block in CDC20-depleted cells (Fig. 8C), they should be able to act on cyclin B1-CDK1. The fact that cyclin B1-CDK1 was present after CDC20 depletion provided substrates for WEE1 and MYT1. The disruption of APC/C^{CDC20} should also uncouple the regulation of the cyclin B1-CDK1 bistable switch by WEE1 found in *Xenopus laevis* egg extracts (32). However, the activation of WEE1/MYT1 can only occur once their inhibitory phosphorylation is removed. How this is achieved is currently unclear. It is conceivable that a slight reduction of overall CDK1 activity, either by the slow degradation of cyclin B1 caused by some residual CDC20 or by other proteolysis-independent mechanisms, may be sufficient to turn on some WEE1/MYT1, thus kick-starting the CDK1-WEE1/MYT1 feedback loop. The inactivation of CDK1 may then allow CDH1 to be activated, which may then be responsible for the delayed degradation of APC/C substrates.

These data are consistent with the model that mitotic exit can utilize an alternative mechanism involving the inhibitory phosphorylation of CDK1 (Fig. 8D). During unperturbed mitosis, inhibitory phosphorylation serves an important role in suppressing CDK1 activity during S phase and G₂ phase. Inhibitory phosphorylation is not believed to contribute much to mitotic exit because cyclin B1 destruction normally provides an effective mechanism to inactivate CDK1. However, inhibitory phosphorylation appears to play a critical role in mitotic exit when the activity of APC/C is impaired. With the reasons stated above, whether mitotic exit can occur in the complete absence of CDC20 remained to be deciphered.

One concern of our CDC20-depleted model is the role of the spindle assembly checkpoint. In normal cells, the checkpoint inhibits the activation of APC/C, thereby preventing mitotic exit. It is generally believed that CDC20 is the major target of the checkpoint. How the checkpoint prevents cells from exiting mitosis in our CDC20-depleted model awaits further investigation. One possibility is that the checkpoint acts on other targets to prevent APC/C activation. Another possibility is that the checkpoint somehow also acts on the WEE1/MYT1

network to prevent the precocious inactivation of cyclin B1-CDK1.

Although the biological significance of CDC20 downregulation remains to be defined, the idea that inhibitory phosphorylation of CDK1 plays any role in other conditions is a provocative one. For example, it is conceivable that a transient inhibitory phosphorylation of CDK1 may kick-start the inhibition of CDK1 before the bulk of cyclin B1 is destroyed by the APC/C. The inactivation of CDK1 may turn off the spindle assembly checkpoint and reactivate CDH1, forming an effective feedback loop to promote mitotic exit. However, it will probably be difficult to detect a transient increase in CDK1 phosphorylation during mitotic exit.

It is also possible that, since the expression of the WEE1/MYT1 system is low in the early embryonic cell cycle, inhibitory phosphorylation of CDK1 is unable to compensate for CDC20 deletion in the knockout mouse model (17). CDC20 may also be deregulated in cancer. About half of cancer tissues express high levels of CDC20 (14, 15, 24). Downregulation of CDC20 expression can reduce the growth of cancer cells, suggesting that CDC20 could be a potential target for cancer therapy (14). Hence, it is important to understand how the cell cycle is controlled after CDC20 is depleted.

Another situation in which inhibitory phosphorylation of CDK1 may play a role in mitotic exit is mitotic slippage. Although the presence of unattached kinetochores activates the spindle assembly checkpoint and traps the cell in mitosis, the arrest is not permanent and the cell can exit mitosis by a process known as adaptation or slippage. The underlying mechanism of slippage is not completely understood but may involve a steady degradation of cyclin B1 (5). We speculate that the residual WEE1 and MYT1 may slowly reactivate during prolonged mitotic block. This may contribute to the inactivation of CDK1 and mitotic exit. Consistent with this idea, our preliminary results indicate that mitotic slippage could be delayed by codepletion of WEE1 and MYT1 (our unpublished observation).

In conclusion, the combined results from this study provide evidence that, although depletion of CDC20 led to a delay of mitotic exit, cells were able to exit mitosis. The ability of the cell to exit mitosis without the complete degradation of cyclin B1 was due to an alternative mechanism utilizing the classic inhibitory phosphorylation of CDK1. These observations of a delay in mitotic exit after CDC20 depletion and the existence of an alternative mechanism for mitotic exit may reconcile previous disparate results on this subject.

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