Rapid Cycling and Precocious Termination of G1 Phase in Cells Expressing CDK1AF

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In *Xenopus* embryos, the cell cycle is driven by an autonomous biochemical oscillator that controls the periodic activation and inactivation of cyclin B1-CDK1. The oscillator circuit includes a system of three interlinked positive and double-negative feedback loops (CDK1 $\rightarrow$ Cdc25 $\rightarrow$ CDK1; CDK1 $\vdash$ Wee1 $\vdash$ CDK1; and CDK1 $\vdash$ Myt1 $\vdash$ CDK1) that collectively function as a bistable trigger. Previous work established that this bistable trigger is essential for CDK1 oscillations in the early embryonic cell cycle. Here, we assess the importance of the trigger in the somatic cell cycle, where checkpoints and additional regulatory mechanisms could render it dispensable. Our approach was to express the phosphorylation site mutant CDK1AF, which short-circuits the feedback loops, in HeLa cells, and to monitor cell cycle progression by live cell fluorescence microscopy. We found that CDK1AF-expressing cells carry out a relatively normal first mitosis, but then undergo rapid cycles of cyclin B1 accumulation and destruction at intervals of 3–6 h. During these cycles, the cells enter and exit M phase-like states without carrying out cytokinesis or karyokinesis. Phenotypically similar rapid cycles were seen in Wee1 knockdown cells. These findings show that the interplay between CDK1, Wee1/Myt1, and Cdc25 is required for the establishment of G1 phase, for the normal $\sim$ 20-h cell cycle period, and for the switch-like oscillations in cyclin B1 abundance characteristic of the somatic cell cycle. We propose that the HeLa cell cycle is built upon an unreliable negative feedback oscillator and that the normal high reliability, slow pace and switch-like character of the cycle is imposed by a bistable CDK1/Wee1/Myt1/Cdc25 system.

INTRODUCTION

In the early embryo, the cell cycle is controlled by an autonomous biochemical oscillator that can continue to operate in the face of enucleation (Wasserman and Smith, 1978), spindle poisons (Gerhart *et al.*, 1984), or UV irradiation (Beal and Dixon, 1975). The protein components of this oscillator are well characterized and include (at least) three cyclin–cyclin-dependent kinase (CDK) complexes, the best characterized of which is CDK1–cyclin B1. Once active, the CDK–cyclin complexes phosphorylate hundreds of substrate proteins (Ubersax *et al.*, 2003; Ptacek *et al.*, 2005), culminating in the dramatic transition into mitosis. Active CDK1 also brings about the activation of one form of the anaphase-promoting complex (APC), APC-Cdc20 (Hershko *et al.*, 1994; King *et al.*,

1995, 1996; Sudakin *et al.*, 1995), which catalyzes the polyubiquitylation of the mitotic cyclins. This results in the proteasome-mediated degradation of the cyclins and hence the inactivation of CDK1. CDK1 inactivation allows the embryo to exit mitosis and enter interphase.

The CDK1/APC-Cdc20 system constitutes a negative feedback loop: CDK1 $\rightarrow$ APC-Cdc20 $\vdash$ CDK1. This loop is required for oscillations, as indicated by the fact that non-degradable cyclin proteins cause the cell cycle to arrest in M phase (Murray *et al.*, 1989). In principle, a simple negative feedback loop of this sort can be sufficient to generate sustained oscillations (Goodwin, 1965), and in fact Elowitz and coworkers successfully engineered a synthetic oscillator (the “repressilator”) in *Escherichia coli* based on this design (Elowitz and Leibler, 2000).

However, in addition to the negative feedback loop, there are several interlinked positive and double-negative feedback loops in the CDK1–cyclin B system. Active CDK1–cyclin B brings about the activation of Cdc25, which in turn is an activator of CDK1–cyclin B (CDK1 $\rightarrow$ Cdc25 $\rightarrow$ CDK1), and active CDK1–cyclin B brings about the inactivation of Wee1 and Myt1, two inhibitors of CDK1 (CDK1 $\vdash$ Wee1, Myt1 $\vdash$ CDK1). By themselves, these loops could function as a bistable switch that toggles between a stable interphase state and a stable M-phase state (Novak and Tyson, 1993; Thron, 1996; Tyson and Novak, 2001; Cross *et al.*, 2002), and recent experimental work in *Xenopus* egg extracts has shown that the CDK1/Cdc25/Wee1/Myt1 system is, in fact, bistable (Pomerening *et al.*, 2003; Sha *et al.*, 2003). Moreover, this bistable system, like the negative feedback loop, is essential for normal oscillations in *Xenopus* extracts. When the positive feedback loops are compromised by the introduction of CDK1AF, a mutant CDK1 protein whose sites of Wee1/

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Abbreviations used: ADB, antibody-diluting buffer; APC, anaphase-promoting complex; CDK1, cyclin-dependent kinase 1; CFP, cyan fluorescent protein; d-siRNA, dicer small interfering RNA; GFP, green fluorescent protein; MBS, mitotic biosensor; NEB, nuclear envelope breakdown; NER, nuclear envelope reformation; PCNA, proliferating cell nuclear antigen; pHH3, phospho-histone H3; RFP, red fluorescent protein; RT, room temperature; YFP, yellow fluorescent protein.

Myt1 phosphorylation (Thr 14 and Tyr 15) have been replaced with nonphosphorylatable residues, the activation of CDK1 occurs earlier but less abruptly, and the oscillations of CDK1 activity damp out over several cycles (Pomerening *et al.*, 2005). Thus, both the negative feedback loop (CDK1/APC-Cdc20) and the bistable CDK1/Cdc25/Wee1/Myt1 system are required for sustained, switch-like cell cycle oscillations in *Xenopus* egg extracts.

One way to rationalize how the bistable switch helps the whole circuit to produce oscillations is to consider the hand-off of cell cycle control from CDK1 to APC-Cdc20. Positive feedback makes the activation of CDK1 occur abruptly, which allows CDK1 to become fully activated, or nearly fully activated, before any significant activation of APC-Cdc20 has occurred. Full activation of CDK1 in turn allows APC-Cdc20 to become fully activated, ensuring that CDK1 becomes fully (or nearly fully) inactivated at the end of mitosis. Positive feedback thus helps keep the peaks of CDK1 and APC-Cdc20 activity high in amplitude, spike-like in shape, and separated in time. However, when CDK1AF is introduced, CDK1 activation occurs more gradually. This allows APC-Cdc20 to begin becoming activated before full mitotic levels of CDK1 activity are achieved, blunting the peak activation of CDK1. CDK1 activity then falls, but not as quickly or completely as normal because full APC-Cdc20 activation has not been attained. Ultimately, the amplitudes of the oscillations in CDK1 and APC-Cdc20 become smaller and smaller, until the two activities come into balance in a state that is intermediate between mitosis and interphase (Pomerening *et al.*, 2005).

This rationale explains the damped oscillations in CDK1AF-treated *Xenopus* egg extracts. However, it seemed plausible that in somatic cells, extra control mechanisms like the spindle assembly checkpoint (which does not operate in normal *Xenopus* extracts) could render positive feedback dispensable. With the spindle assembly checkpoint functioning, a gradual increase in CDK1 activity might not necessarily allow APC-Cdc20 to begin becoming activated before maximal mitotic levels of CDK1 are achieved, because the checkpoint would prevent APC-Cdc20 from becoming activated until CDK1 had succeeded in aligning the chromosomes at the metaphase plate. The checkpoint could insulate APC-Cdc20 activation from perturbations in the dynamics of CDK1 activation.

The somatic cell cycle includes other controls that could further diminish the importance of the CDK1/Cdc25/Wee1 system. For example, a second form of the APC, APC-Cdh1, is present in somatic cells but not in *Xenopus* embryos. APC-Cdh1 becomes activated during late mitosis and remains active in G1 phase, until it ultimately brings about its own inactivation and destruction in late G1 phase (Listovsky *et al.*, 2004; Rape and Kirschner, 2004). In yeast, and possibly in other organisms as well, the CDK1-cyclin B/APC-Cdh1 system constitutes a double-negative feedback loop: Cdh1 is inhibited by CDK1-cyclin B and in turn can cause the degradation of cyclin B and the inactivation of CDK1 (Visintin *et al.*, 1997; Jaspersen *et al.*, 1999). This loop may help lock the cell cycle into a stable interphase state, perhaps by functioning as a toggle switch just as the CDK1/Cdc25/Wee1 system does (Nasmyth, 1996; Chen *et al.*, 2004; Ciliberto *et al.*, 2005). If so it could provide a back-up to the latter system.

One straightforward way to test whether the CDK1/Cdc25/Wee1 system is as important in somatic cells as it is in embryos is to ectopically express CDK1AF and observe the consequences. Several groups have reported such studies. The earliest of these indicated that overexpression of CDK1AF in HeLa cells results in premature chromatin con-

densation and nuclear envelope breakdown (Krek and Nigg, 1991). This was the expected result given that similar phenotypes had been seen in *Schizosaccharomyces pombe* (Gould and Nurse, 1989) and *Xenopus* egg extracts (Norbury *et al.*, 1991; Pomerening *et al.*, 2005). However, several subsequent studies showed that although the introduction of CDK1AF does result in premature activation of CDK1 in HeLa cells, it has little or no effect on the timing of chromatin condensation and nuclear envelope breakdown (NEB) (Jin *et al.*, 1996, 1998; Blasina *et al.*, 1997). Moreover, one recent study has shown that human HT2-19 cells can be obtained that both tolerate and require CDK1AF for growth and division (Gupta *et al.*, 2007). These results indicate that the inhibitory phosphorylation of CDK1, and the positive feedback loops that depend upon this inhibitory phosphorylation, are not essential for normal mitotic timing in HeLa cells and not essential for viability in HT2-19 cells.

The surprisingly normal timing of M phase entry in CDK1AF-expressing cells seems to be due to a second level of control over the function of CDK1-cyclin B1. Even though the expression of CDK1AF brings about premature activation of CDK1-cyclin B1, the complexes remain in the cytoplasm and thus presumably do not have access to the substrates required for chromatin condensation and NEB. These events do not occur until the CDK1-cyclin B1 is triggered to accumulate in the nucleus. The nuclear accumulation of CDK1-cyclin B1 requires both Plk1 and CDK-cyclin A2 (Toyoshima-Morimoto *et al.*, 2001; Jackman *et al.*, 2003; Walsh *et al.*, 2003). Thus, the nuclear aspects of mitosis, and presumably APC-Cdc20 activation as well, are insulated from the premature activation of CDK1AF by the requirement for nuclear accumulation.

Nevertheless, the tyrosine phosphorylation of CDK1 and the CDK1/Cdc25/Wee1 feedback loops occur in all species examined, and they seem likely to be of general importance. Therefore, we have looked further into the question of the role of this system in HeLa cells, focusing not just on the entry into mitosis but also on mitotic exit and subsequent cycling. Our approach was to transiently transfect either unsynchronized cells or synchronized cells with wild-type CDK1 (CDK1WT) or CDK1AF together with one or more fluorescent biosensors, and then follow the cells by live-cell epifluorescence microscopy. In agreement with Jin *et al.* (1996, 1998), we found that neither form of CDK1 affected the timing of the first mitosis after thymidine block and release. However, CDK1AF was found to dramatically alter the subsequent dynamics of the somatic cell cycle. Within a few hours of mitotic exit, the CDK1AF-expressing cells began to undergo rapid cycles of chromatin condensation and decondensation, cell rounding and flattening, NEB, and nuclear envelope reformation (NER), and cyclin accumulation and destruction. The period of these cycles was ~3–6 h, compared with ~20 h for normal cell cycles in HeLa cells. The oscillations in cyclin abundance in the CDK1AF-expressing cells were more smoothly sinusoidal and less switch-like than they are in normal HeLa cells or CDK1WT-expressing cells. Similarly rapid cycles were also seen in cells transfected with Wee1 diced small interfering RNAs (dsRNAs), another way of compromising the CDK1/Cdc25/Wee1 system.

We conclude that the phosphorylation of CDK1 at Thr 14 and/or Tyr 15 is required for the production of normal switch-like cell cycle oscillations in HeLa cells, and for the proper activation of the APC. We hypothesize that the somatic cell cycle, like the embryonic cell cycle, relies critically upon a bistable CDK1/Cdc25/Wee1/Myt1 toggle switch.

MATERIALS AND METHODS

Cell Culture, Transfections, and Imaging

HeLa cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin-glutamine (Invitrogen, Carlsbad, CA) under a humidified 37°C environment with 5% CO₂. For imaging experiments, 4000 asynchronous cells were seeded into each well of a Costar 96-well plate, and they were transfected 12 h later with nucleic acids by using the Genesilencer transfection reagent (Genlantis, San Diego, CA) as described previously (Myers and Ferrell, 2005). Transfected DNA never exceeded 60 ng/well and included combinations of the mitotic biosensor (MBS) (red fluorescent protein [RFP]-tagged) (10 ng) and/or CDK1WT (cyan fluorescent protein [CFP]- or green fluorescent protein [GFP]-tagged), CDK1KD (CFP- or GFP-tagged), CDK1AF (CFP- or GFP-tagged), cyclin B1 (yellow fluorescent protein [YFP]-tagged), lamin A1 (YFP-tagged), proliferating cell nuclear antigen (PCNA) (YFP-tagged), and wild type (WT) or R61A-securins (RFP-tagged) (20 ng each) as indicated. At 24 h after transfection, cells were imaged for 14 h (except for PCNA experiments where cells were imaged beginning 6 h after transfection for a duration of 16 h) using an ImageXpress 5000A imaging system (Molecular Devices, Sunnyvale, CA). Images were acquired using a 10× or 20× plan fluor ELWD objective. Image stacks were compiled using IXon software (Molecular Devices), exported as movies in Windows Media format, imported as uncompressed TIFFs into Quicktime, and then subsequently analyzed using ImageJ 1.34m (National Institutes of Health, Bethesda, MD). Montages were either produced via MatLab image analysis scripts (Jin *et al.*, 1998; Gong *et al.*, 2007), or they were manually constructed using Photoshop CS (Adobe Systems, Mountain View, CA). Supplemental videos were produced using Final Cut Pro 4 (Apple Computer, Cupertino, CA).

For experiments involving kinase assays and flow cytometry, 6×10^5 cells were plated in 10-cm dishes and transfected 12 h later, with either 0.67 µg of CDK1WT-CFP or CDK1AF-CFP DNA. These large-scale transfections were performed according to a six-well protocol with volumes scaled up threefold (Myers and Ferrell, 2005).

For the DNA checkpoint experiments, cells were transfected with CDK1WT-CFP or CDK1AF-CFP as described above. Twenty-four hours after transfection, cells were rinsed with phosphate-buffered saline (PBS) and either treated with 15 µJ/m² UV or left untreated. Medium with or without 4 mM caffeine [in 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)-KOH, pH 7.5] was then added back to the cells, which were then either imaged immediately for a duration of 14 h or incubated for an additional 4 h, after which the medium was aspirated and cell lysates were prepared (see below).

For double thymidine block-and-release experiments, cells were treated with 2 mM thymidine for 18 h, released by rinsing with PBS, and then transfected with fluorescent sensor constructs as described. After 9-h release, cells were blocked again for 17 h, released, and then transfected with either 20 ng CDK1WT-CFP or CDK1AF-CFP, or with 20 nM Wee1 diced siRNAs (Liou *et al.*, 2005; Myers and Ferrell, 2005). In Figure 6, cells were imaged for 16 h beginning 8 h after the second release. In Figure 7, cells were treated with nocodazole 10 h after release; at this time point, some of cells had divided and some would divide soon. Cells were then imaged for the next 16 h.

Wee1 Knockdown

For the Wee1 knockdown experiments, siRNA pools were prepared by *in vitro* dicing (Myers and Ferrell, 2005) of double-stranded RNA prepared by *in vitro* transcription of both strands of the last 601 nt of the human Wee1B cDNA sequence, and they were provided by Won Do Heo, Josh Jones, Man Lyang Kim, Jen Liou, Tobias Meyer, and Jason Myers (Stanford University School of Medicine; Liou *et al.*, 2005). Diced GL3 luciferase siRNAs were prepared as controls.

Lysate Preparation, Kinase Assays, Western Blots, and Flow Cytometry

HeLa cell lysates were prepared in a two step process. First, we aspirated the medium and nonadherent cells, pelleted the cells at $600 \times g$, and lysed them in ice-cold 0.6 ml M-PER lysis reagent (Pierce Chemical, Rockford, IL) containing 3 µg/ml aprotinin, 9 µg/ml pepstatin, 10 µg/ml chymostatin, 9 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. Lysates from the pelleted cells were added back to the adherent cells on ice, and these cells were lysed by scraping. Membranes and unlysed cells were pelleted for 1 min at $16,000 \times g$. A portion of each lysate (20 µl) was saved for protein assays, and the remainder was snap-frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical).

For Chk1 and phospho-Chk1 immunoblotting, 50 µg of protein was loaded per lane. The anti-phospho (S345)-Chk1 (Cell Signaling Technology, Danvers, MA) was used at a 1:1000 dilution according to the manufacturer's directions. The Chk1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 0.2 µg/ml in 1% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T) for 2 h at room temperature (RT), rinsed with TBS-T, probed with Zymax anti-rabbit horseradish peroxidase (HRP)-conjugate (Zymed Laboratories,

South San Francisco, CA) at 0.6 µg/ml for 2 h at RT, rinsed with TBS-T, and then exposed to SuperSignal HRP substrate (Pierce Chemical). Chemiluminescent imaging and analysis were done using a ChemiDoc XRS (Bio-Rad, Hercules, CA).

HeLa cells were fixed for flow cytometry by aspirating the medium and nonadherent cells, trypsinizing the adherent cells with 3 ml of trypsin (Invitrogen) for 5 min at 37°C, quenching the trypsin with the original media, pelleting the cells at $600 \times g$ for 5 min, resuspending the pellet in 1 ml of PBS, and then transferring each cell suspension to 10 ml of ice-cold 70% ethanol in 15-ml conical tubes. Cells were fixed overnight at -20°C, split into two 15-ml conical tubes (approx. 3×10^5 cells each), pelleted at $600 \times g$ for 5 min, rinsed in 1 ml of PBS, pelleted again, and then resuspended in 100 µl of antibody-diluting buffer (ADB; 1% bovine serum albumin and 0.1% Triton X-100 in PBS) containing 4.2 µg/ml phospho-histone H3 antibody (clone 3H10; Millipore, Billerica, MA). Samples were incubated for 2 h at RT with occasional shaking, rinsed with 5 ml of ADB, and pelleted at $600 \times g$ for 5 min. The buffer was aspirated and ADB (100 µl) containing 4 µg/ml anti-rabbit (with an Alexa Fluor 488 conjugate) and 20 µg/ml anti-mouse (with an Alexa Fluor 647 conjugate) secondary antibodies (Invitrogen) was added to the cells. Cells and antibodies were incubated in the dark for 2 h at RT with occasional shaking. Cells were rinsed with 5 ml of ADB, pelleted, and resuspended in 500 µl of ADB containing 50 µg/ml propidium iodide (Invitrogen) and 100 µg/ml RNase A (QIAGEN, Valencia, CA) for 1 h. Flow cytometry was performed using a FACSCalibur cytometer (BD Biosciences, San Jose, CA) with a CellQuest software interface, and analysis was done using FlowJo 6.4.7 (Tree-Star, Ashland, OR).

Curve Fitting

In Figure 8, G and H, we estimated the activities of APC-Cdc20 and APC-Cdh1 by plotting the amount of cyclin B1-YFP or R61A-securin remaining as a function of time, beginning at the time point when the rate of protein destruction was highest; the hope was that after this point, the remaining destruction would be well approximated by simple exponential decay. The data were fitted to the simple one-parameter equation percent remaining = $100 \cdot 0.5^{t/\tau}$, where τ is the half-time. Half-times were expressed as the fitted values $\pm$ the asymptotic SEs from nonlinear least squares curve fitting (using the command NonlinearRegress in Mathematica 6.0, Wolfram Research, Champaign, IL).

RESULTS

CDK1AF-GFP Expression Causes Rapid Cycling and Abortive Mitosis

Previous work had shown that overexpressing either CDK1 or CDK1AF has little effect on the timing of chromatin condensation and NEB in HeLa cells released from a double thymidine block (Jin *et al.*, 1996, 1998; Blasina *et al.*, 1997). This leaves open the possibility that there might be abnormalities in subsequent phases of the cell cycle. To test this possibility, we transfected unsynchronized HeLa cells with a fluorescent MBS (Jones *et al.*, 2004), plus either CDK1WT-GFP or CDK1AF-GFP, and we monitored cell cycle progression beginning 24 h later by automated epifluorescence time-lapse microscopy. The MBS consists of a strong nuclear localization signal and weak plasma membrane localization signal fused to a fluorescent protein (RFP in the present case) (Jones *et al.*, 2004). The biosensor accumulates in the nucleus during interphase, disperses to the plasma membrane and cytoplasm when the nuclear envelope breaks down, and then begins to reaccumulate in the nucleus after the nuclear envelope reforms. It provides a convenient readout of nuclear envelope breakdown and reformation without affecting the timing of these processes (Jones *et al.*, 2004).

As shown by immunoblotting, expression of the transfected CDK1WT-GFP and CDK1AF-GFP proteins plateaued within 24 h, the time at which the microscopy began (Figure 1, A and B). The plateau levels of the transfected proteins were 20–40% that of endogenous CDK1, with CDK1WT-GFP typically being expressed at higher levels than CDK1AF-GFP (Figure 1, A–C). Based on the fraction of cells expressing the fluorescent proteins, we estimated the transfection efficiency to be ~30–40%, implying that the amount of transfected protein per transfected cell was similar to or smaller than that of the endogenous CDK1 protein. Thus, on

Figure 1. Rapid cycles in unsynchronized HeLa cells expressing CDK1AF-GFP. Unsynchronized HeLa cells were transfected with various CDK1-GFP expression constructs and an RFP-tDimer2-labeled MBS. CDK1 expression was assessed 12–30 h after transfection by immunoblotting (A and B) and mitotic progression was assessed by time-lapse fluorescence microscopy from 24 to 38 h after transfection (C–E). (A and B) Expression of transfected CDK1WT-GFP and CDK1AF-GFP. CDK1 immunoblots of p13 Suc1 precipitates from lysates of CDK1WT-GFP-transfected (A) and CDK1AF-GFP-transfected (B) HeLa cells. The lower band is the endogenous CDK1 protein. The higher band is the transfected CDK1 chimera. (C) Distribution of CDK1WT-GFP and CDK1AF-GFP expression levels. The same arbitrary fluorescence units were used for both CDK1-GFP proteins. Data are from 50 CDK1WT-GFP- and 50 CDK1AF-GFP-expressing cells. (D) Table of phenotypes observed in cells transfected with different GFP-fused versions of CDK1, or GFP alone, and the MBS. Asterisks indicate treatments where some cells exhibited multiple phenotypes; in these cases the totals across categories add up to >100%. (E) Montage of cells transfected with CDK1WT-CFP and the MBS, showing normal mitotic progression. The images show the MBS channel only. Time stamps are in minutes, with NEB taken to be t = 0. The criterion for metaphase was a maximally compact line of chromosomes at the midline. The criterion for anaphase was a visible separation of the chromosomes into two masses. (F) Montage of a cell transfected with CDK1AF-CFP and the MBS, showing rapid cycling. The images show the MBS channel only. Time stamps are in minutes, with NEB taken to be t = 0.

Figure 2. Rapid cycles of lamin A dispersal and reorganization in unsynchronized HeLa cells expressing CDK1AF-CFP. (A and B) Mitotic entry and exit as assessed by MBS (red) or lamin (green) redistribution in cells cotransfected with CDK1WT-CFP (A) or CDK1AF-CFP (B). Time stamps are in minutes, with $t = 0$ taken to be the first frame of the montage. (C) Mitotic phenotypes in cells transfected with no CDK1 (95 cells), CDK1WT-CFP (210 cells), or CDK1AF-CFP (328 cells). (D) Number of cycles of NEB and NER without karyokinesis or cytokinesis in the CDK1AF-CFP-transfected cells that exhibited at least one abortive mitosis. Cells were followed for 14 h beginning 24 h after transfection.

average, the expression levels of the CDK1WT-GFP and CDK1AF-GFP proteins were modest.

Expression of the MBS plus either CDK1WT-GFP, a kinase-dead version of CDK1 (CDK1KD), or GFP had no obvious effect on mitotic progression compared with cells transfected with the MBS alone (Figure 1D). In all cases, >95% of the cells successfully divided. A typical CDK1WT-GFP-transfected cell is shown in Figure 1E. The cell undergoes NEB, completes nuclear division and cytokinesis, and it has flattened back within 77 min of NEB, and then remains in interphase for the duration of the imaging. In contrast, mitosis was greatly perturbed in most of the cells transfected with CDK1AF-GFP; only 10.9% of the cells carried out mitosis normally. Typical CDK1AF-GFP-transfected cells rounded up and underwent NEB, but then flattened back down and reformed a nucleus (NER) without completing either karyokinesis or cytokinesis (Figure 1, D and F). One such cell is shown in Figure 1F. This cell went into an M phase-like state at $t = 0$, came out 119 min later, and then went back into another M phase at ~266 min and back out by 399 min. Typically, the period between these abortive M phases was 3–6 h, compared with 20 h for the period between mitoses in normal HeLa cells (Hahn, personal communication).

Because there was substantial cell-to-cell variability in the levels of expression of the CDK1 transgenes—from quantitation of the single cell fluorescence data, the variances were 0.95 and 0.91 for CDK1WT-GFP and CDK1AF-GFP, respectively (Figure 1C)—we examined whether there was any connection between the CDK1AF-GFP expression level and the phenotype seen. As shown in Supplemental Figure 1, cells expressing the highest levels of CDK1AF-GFP were the most likely to cease cycling, and cells expressing intermediate levels of CDK1AF-GFP had the most rapid and persistent cycles.

To more directly assess the status of the nuclear envelope during these rapid cycles, we made use of a YFP-lamin A1 biosensor (Moir *et al.*, 2000). Typically, the YFP-lamin A1 dispersed within one frame (7 min) of the exit of the MBS from the nucleus, and it reorganized into recognizable daughter nuclei 14–28 min before the first sign that the MBS was reaccumulating in the nucleus (Figure 2A and Supplemental Movie 1). Of the cells transfected with MBS, YFP-lamin A1, and CDK1, >95% of the cells that entered mitosis completed it successfully (Figure 2, A, C, and D and Supplemental Movie 1). However, more than half of the CDK1AF-CFP-transfected mitotic cells underwent one or more cycles of lamin dispersal and reorganization without

Figure 3. Smooth, nearly-sinusoidal cyclin B1 “flashes” in unsynchronized CDK1AF-CFP-expressing cells. Unsynchronized HeLa cells were transfected with CDK1WT-CFP or CDK1AF-CFP plus cyclin B1-YFP. Cycles of cyclin accumulation and destruction were assessed by time-lapse microscopy from 24 to 38 h after transfection. (A) Montages of one CDK1WT-CFP-transfected cell and two CDK1AF-CFP-transfected cells, showing cyclin B1-YFP fluorescence. (B) Tracings of cyclin B1-YFP fluorescence as a function of time for four CDK1WT-CFP-transfected cells and eight CDK1AF-CFP-transfected cells. The same arbitrary fluorescence units are used for each plot. (C) Mitotic phenotypes in cells transfected with no CDK1 (132 cells), CDK1WT-CFP (176 cells), or CDK1AF-CFP (334 cells). (D) Numbers of cyclin flashes in the course of the 14 h video for cells coexpressing cyclin B1-YFP and CDK1WT-CFP or CDK1AF-CFP.

undergoing cytokinesis (Figure 2, B–D, and Supplemental Movie 2). Thus, both the lamin sensor and the MBS indicated that CDK1AF-CFP-transfected cells underwent rapid cycles between interphase and an M phase-like state.

Cyclin B1 Synthesis and Degradation

YFP-tagged cyclin B1 can be used to measure both the timing of APC activation and qualitative aspects of cyclin B1 dynamics. We coexpressed cyclin B1-YFP plus CDK1WT-CFP or CDK1AF-CFP in unsynchronized cells and monitored cyclin B1-YFP fluorescence beginning 24 h later. In unsynchronized CDK1WT-CFP-transfected cells, cyclin B1-YFP accumulated to a roughly constant level that was maintained for several hours. (Figure 3B, blue plots). Immunoblotting experiments showed that the average level of cyclin B1-YFP expression was typically about twice the endoge-

nous cyclin B1 level. Cyclin B1-YFP was abruptly destroyed at midmitosis (Figure 3A, top, and B, blue plots; and Supplemental Movie 3). Once destroyed, the cyclin B1-YFP remained at low levels for many hours.

Cyclin B1-YFP exhibited strikingly different behavior in the CDK1AF-CFP-transfected cells (Figure 3A, bottom, and B, red plots; and Supplemental Movie 4). Rather than accumulating and then being maintained at a near constant level for many hours (Figure 3B, blue plots), cyclin B1-YFP oscillated more smoothly—sometimes almost sinusoidally—with a period of ~2.5 h (Figure 3B, red plots). Thus, the cyclin B1-YFP probe corroborated the rapid cycles seen with the MBS and lamin A1 probes, and in addition revealed a qualitative change in the character of cyclin dynamics from slow switch-like cycles to rapid, more sinusoidal cycles.

The cyclin B1-YFP flashes sometimes continued until the end of the experiment (Figure 3B, cell 5), but they often damped and then terminated with cyclin B1-YFP fluorescence remaining at a level intermediate between normal G1 phase and G2/M phase levels (Figure 3B, cell 7), or they just quit abruptly (Figure 3B, cell 10). Less than 5% of the mitotic CDK1AF-CFP-expressing cells successfully completed karyokinesis and cytokinesis, whereas 92% of the CDK1WT-CFP-transfected cells and 85% of the control cells did (Figure 3D). Overexpressing cyclin B1-YFP seemed to cause mitotic arrest in some cells irrespective of what CDK1 construct was cotransfected (compare Figures 1C and 3C). However, flashes of cyclin B1-YFP were rare in the CDK1WT-CFP-cotransfected cells and common in the CDK1AF-CFP-cotransfected cells (Figure 3, C and D).

The Transition from Normal Cycles to Rapid Cycles

The rapid cycles seen in the CDK1AF-CFP-expressing cells were at first surprising given that previous studies reported no changes in the timing of mitosis in CDK1AF-expressing HeLa cells released from a double thymidine block (Jin *et al.*, 1996, 1998; Blasina *et al.*, 1997). To attempt to reconcile our findings with this previous work, we carried out transfection studies on synchronized HeLa cells. HeLa cells were blocked at the G1/S boundary by thymidine treatment. They were then released from the block by washing out the thymidine and transfected with the MBS to monitor NEB and NER and with YFP-tagged histone H2B, another mitotic probe, to monitor chromatin condensation (Kanda *et al.*, 1998). Cells were subjected to a second thymidine block, released, and transfected with CDK1WT-CFP or CDK1AF-CFP. Eight hours later, imaging was begun. Cells were then followed for 16 h.

Figure 4, A and B, shows the timing of mitotic entry and exit from 142 cells transfected with CDK1WT-CFP and the same number transfected with CDK1AF-CFP. In agreement with previous studies, there was no obvious effect of CDK1AF-CFP expression on the timing of NEB (Figure 4A) (Jin *et al.*, 1996; Blasina *et al.*, 1997). Mitosis was slightly prolonged in the CDK1AF-CFP-transfected cells (Figure 4B), with the interval between NEB and NER being 60 ± 3 min (SEM) in CDK1WT-CFP-transfected cells and 78 ± 4 min in CDK1AF-CFP-transfected cells.

However, although the first mitosis in the CDK1AF-CFP-transfected cells was normal with respect to the timing of NEB and nearly normal with respect to the timing of mitotic exit, progression through subsequent phases of the cell cycle was markedly abnormal. Within a few hours of completing mitosis, most of the CDK1AF-CFP-transfected daughter cells reentered a mitotic-like state and carried out one or more abortive mitoses at intervals of 3–6 h (Figure 4, D and E). One example of repeated rapid cycles in a CDK1AF-CFP-transfected cell is shown in Figure 4D. In contrast, none of the CDK1WT-CFP-transfected cells exited interphase prematurely (Figure 4, C and E).

Thus, when CDK1AF-CFP-transfected cells are released from a double-thymidine block, they undergo a relatively normal first mitosis. However, they are unable to maintain the next interphase for a normal period. They enter an M phase-like state grossly prematurely, fail to successfully complete this mitosis, and then alternate between interphase and M phase states every ~3–6 h until eventually the cycles cease. This reconciles our findings with the previous studies that found near normal mitotic timing in CDK1AF-CFP-expressing cells, and it shows that the rapid-cycling phenotype can be seen with several mitotic probes, with or

without cyclin B1-cotransfection, and with or without synchronization (Figures 1–4).

CDK1AF Shortens G1 and S Phases

We next set out to determine whether S phase was initiated prematurely in the CDK1AF-CFP-transfected cells. We transfected unsynchronized cells with two sensors: an S phase biosensor consisting of PCNA fused to a C-terminal YFP tag (Leonhardt *et al.*, 2000) and the MBS. During G1 phase, the PCNA sensor is distributed diffusely throughout the nucleoplasm (Figure 5A, left). At the onset of DNA replication, the probe's fluorescence becomes punctate (Figure 5A, right; and Supplemental Movie 5) (Leonhardt *et al.*, 2000). Therefore, the duration of G1 phase can be determined from the time interval between cytokinesis and the onset of punctate PCNA-YFP fluorescence. Likewise, the end of S phase can be determined by the disappearance of PCNA-YFP puncta.

Figure 5B shows the timing of G1 phase and S phase in 50 individual CDK1WT-CFP-transfected cells; Figure 5C shows the cumulative percentage of the cells that had entered S phase as a function of time. In addition, montages of one CDK1WT-CFP-transfected cell and one CDK1AF-CFP-transfected cell are shown as Supplemental Figure 2. The average duration of G1 phase was 9 h, and all of the cells had completed G1 phase and entered S phase by 13 h after cytokinesis (Figure 5, B and C). None of the CDK1WT-CFP-transfected cells completed S phase by the end of the video, and none reentered M phase.

The behavior of the CDK1AF-CFP-transfected cells was strikingly different. Most of the cells reentered mitosis before the end of the video, i.e., within a time interval of <16 h (Figure 5, D and E, and Supplemental Movie 6). The median length of G1 phase was shortened by 3.6 h compared with the CDK1WT-CFP-transfected cells (Figure 5, B–E). These abbreviated G1 phases were then followed by S phase in 84% (42/50) of the cells. The S phases typically lasted ~3 h, and they were followed or interrupted by a second M phase (M2) (Figure 5, D and E). The remaining 16% of the postcytokinesis cells entered M2 directly from G1 phase, without any evidence of DNA replication. In both cases, M2 lasted up to several hours (Figure 5, D and E), and it was generally followed by a return to interphase without successful completion of mitosis or cytokinesis, and then sometimes entry into a third mitosis (M3). Thus, most of the CDK1AF-CFP-expressing cells went through a short G1 phase followed by a very short S phase, and then progression into a mitotic-like state without the completion of S phase or the occurrence of G2 phase.

These findings imply that many of the mitotic-like CDK1AF-CFP-expressing cells should have less than a fully replicated 4N DNA content. To test this hypothesis, we subjected CDK1WT-CFP- and CDK1AF-CFP-expressing cells to flow cytometry after staining for DNA content and phosphohistone H3 (pHH3) immunoreactivity. The latter is a marker for M phase cells. As shown in Figure 5F, virtually all of the pHH3-positive CDK1WT-CFP-expressing cells had a 4N DNA content. In contrast, a substantial fraction of the pHH3-positive CDK1AF-CFP-expressing cells had a 2N or an intermediate DNA content (Figure 5F). This confirms that many CDK1AF-CFP cells enter a mitotic-like state without completing DNA replication.

Wee1 Knockdowns Phenocopy CDK1AF Expression

If the effects of CDK1AF-CFP expression on cell cycle dynamics are the result of a short-circuiting of the CDK1/Cdc25/Wee1/Myt1 system, then knocking down Wee1

Figure 4. CDK1AF-CFP-expressing cells released from a double-thymidine block carry out one normal mitosis and then cycle rapidly. HeLa cells were treated with thymidine, released from this block, and then transfected with MBS and GFP-histone H2B. After a second thymidine block, cells were released and then transfected with either CDK1WT-CFP or CDK1AF-CFP. Beginning 8 h after release, cells were imaged for 16 h. (A) Plot of cumulative percentage of NEB in cells released from a double thymidine block. A total of 142 CDK1WT-CFP- and 142 CDK1AF-CFP-transfected cells were analyzed. (B) Histograms of mitotic duration (the interval between NEB and NER) in cells transfected with CDK1WT-CFP or CDK1AF-CFP. (C and D) Montages of cells expressing either CDK1WT-CFP (C) or CDK1AF-CFP (D) after release from a double thymidine block. White arrowheads indicate which of the two daughter cells is being tracked. Montages of the individual cells do not start at the same time after transfection; cytokinesis is taken to be $t = 0$ for both montages. (E) Cell cycle phases in 25 CDK1WT-CFP-transfected cells (left) and 25 CDK1AF-CFP-transfected cells (right) after cytokinesis. After cytokinesis, CDK1WT-CFP-transfected cells remained in interphase for the duration of the imaging while CDK1AF-CFP-transfected cells entered a precocious M phase.

Figure 5. CDK1AF-CFP-expressing cells enter M phase from either early S phase or G1 phase. (A) The S phase biosensor. This PCNA-YFP chimera exhibits smooth nuclear fluorescence during G1 phase (left), and then it becomes punctate as cells enter S phase (right). (B–E) Cell cycle progression in unsynchronized HeLa cells transfected with PCNA-YFP and MBS plus either CDK1WT-CFP or CDK1AF-CFP. The time of completion of the first (normal) cytokinesis is taken to be $t = 0$. B and D show the time courses for 50 cells expressing CDK1WT-CFP and 50 expressing CDK1AF-CFP, respectively. C and E show the cumulative percentage of cells that had entered S phase, entered a second mitosis (M2), exited that second mitosis, and entered a third mitosis (M3) as a function of time after the completion of the first cytokinesis. (F) Flow cytometry analysis of DNA content (as assessed by propidium iodide staining) and phospho-histone H3 staining in CDK1WT-CFP- and CDK1AF-CFP-expressing HeLa cells at various times after transfection.

might cause similar effects. To test this idea, cells were synchronized by thymidine treatment, transfected with cyclin B1-YFP and the MBS during the first release, treated with thymidine again, released, and then transfected with d-siRNAs produced from a 601-base pair fragment of the human somatic Wee1 cDNA or from firefly (*Photinus pyralis*) luciferase (GL3). Cells were then followed by epifluorescence time-lapse microscopy or, alternatively, they were lysed at various times to assess Wee1 protein levels. As shown in Figure 6A, Wee1 levels fell within 8 h of release and continued to decline over the next 16 h. Most of the cells transfected with the Wee1 d-siRNA underwent a qualita-

tively normal first mitosis, but the daughter cells began to undergo rapid cycles of cyclin B1 accumulation/destruction, cell rounding/flattening, and NEB/NER without successful completion of karyokinesis or cytokinesis (Figure 6, B and C). Most of the daughters flashed once or twice; a few flashed four or five times (Figure 6B). Many of the rapidly cycling cells exhibited damped oscillations in their cyclin B1 levels (Figure 6, C and D), and many cells arrested or died after a few cycles (data not shown). Rapid cycles were also seen in unsynchronized HeLa cells treated with Wee1 d-siRNAs (Figure 6E). Rapid cycles were not seen in HeLa cells transfected with control GL3 d-siRNAs. Thus, knocking

Figure 6. Rapid cycles in cells treated with Wee1 d-siRNAs. HeLa cells were blocked in thymidine, released, and then transfected with cyclin B1-YFP and MBS. After a second thymidine block, cells were released and then transfected with either Wee1 d-siRNAs or control GL3 luciferase d-siRNAs. (A) Wee1 levels in cells transfected with GL3 or Wee1 d-siRNAs. (B) Numbers of cyclin flashes in Wee1 knockdown cells. Data are from the 200 daughters of 100 cell divisions. The total time of the video microscopy was 14 h. (C) Cyclin B1-YFP fluorescence in a Wee1 knockdown cell undergoing a relatively normal first mitosis, and in its two daughter cells, which undergo rapid cyclin flashes. (D) Montage of the same cells whose cyclin B1-YFP levels are quantified in B. Time stamps represent the time in minutes after release from double thymidine block. (E) Montage of an asynchronous cell transfected with Wee1 d-siRNAs, showing rapid cyclin flashes.

down Wee1 (Figure 6) has similar consequences to expressing CDK1AF-CFP (Figures 1–4). This supports the hypothesis that the rapid cycles are a specific, consistent consequence of short-circuiting the CDK1/Cdc25/Wee1/Myt1 system.

The DNA Damage Checkpoint

One potential mechanism to explain the rapid cycles and cyclin flashes is suggested by the fact that CDK1AF-CFP is unable to be restrained by checkpoints, like the DNA replication and DNA damage checkpoints, that are mediated by Chk1 regulation of Cdc25 and Wee1 (Zhou *et al.*, 2000; Nigg, 2001). This raises the possibility that DNA checkpoint responses are required to normally prevent the activation of mitotic CDK complexes during S phase, with the rapid cycles and cyclin flashes being simply due to loss of this checkpoint. However, the checkpoint inhibitor caffeine (Sarkaria *et al.*, 1999) did not produce flashes in CDK1WT-CFP-transfected cells (Supplemental Figure 3, lanes 1 and 2), although it did block Chk1 phosphorylation (Lopez-Girona *et al.*, 2001) in UV-treated, CDKWT-transfected cells (Supplemental Figure S1, lanes 3 and 4). Therefore, abrogating the checkpoint does not, in and of itself, cause rapid cycles and cyclin flashes.

The Spindle Assembly Checkpoint

As discussed in the introduction, the presence of a spindle assembly checkpoint could render the CDK1/Cdc25/Wee1/

Myt1 toggle switch less critical for sustained oscillations in the somatic cell cycle than it is in the embryonic cell cycle. However, the rapidly cycling CDK1AF-CFP-expressing cells seemed to activate the APC during their abortive mitoses—cyclin levels began falling shortly after the cells underwent NEB (Figure 3)—despite the fact that the cells had not aligned their chromosomes on the metaphase plate. This suggested that the CDK1AF-CFP-transfected cells were not capable of mounting a normal spindle assembly checkpoint response during these rapid cycles. Both the failure to achieve metaphase and the apparent failure to generate a spindle assembly checkpoint signal could be explained if the cells do not assemble functional kinetochores and/or replicate and separate their centrosomes by the time they enter their first abortive mitoses (Chan *et al.*, 2005; Musacchio and Salmon, 2007).

To explore this issue further, we asked whether CDK1AF-CFP-transfected cells would arrest in response to the spindle poison nocodazole during their first (relatively normal) mitosis and during their second (abortive) mitosis. We arrested HeLa cells by thymidine block, transfected them with cyclin B1-YFP, MBS, and either CDK1WT-CFP or CDK1AF-CFP, released the cells from G1/S arrest, allowed them to progress into a first mitosis, and then treated them with nocodazole to block spindle formation. We followed the cells by time-lapse microscopy and compared cells that had not yet completed their first mitosis at the time of the nocodazole treatment (large cells that entered mitosis early on

Figure 7. The spindle assembly checkpoint in CDK1AF-CFP-expressing cells. Asynchronous HeLa cells were blocked in thymidine, released, and then transfected with cyclin B1-YFP and MBS. After a second thymidine block, cells were released and then transfected with either CDK1WT-CFP or CDK1AF-CFP. Ten hours after release, nocodazole was added, and cells were imaged for 16 h. (A) Montage of a CDK1AF-CFP-transfected cell that had not yet undergone mitosis at the time of nocodazole treatment. (B) Montage of two CDK1AF-CFP-transfected cells that had undergone mitosis before the time of nocodazole treatment. (C) Table of percentage of M phase arrested and percentage of cyclin B1-YFP flashers after nocodazole treatment.

during the video) to those that had (smaller cells, some of which were still immediately adjacent to their mirror-image sibling cell).

Premittotic cells were found to undergo a normal spindle assembly checkpoint arrest in response to nocodazole treatment. One typical CDK1AF-CFP-transfected premitotic cell is shown in Figure 7A. In the presence of nocodazole, the cell rounded up, condensed its chromatin, entered mitosis, and remained in mitosis for the duration of the video. In contrast, cells that had already completed mitosis before the nocodazole treatment were not capable of mounting a normal spindle assembly checkpoint arrest (Figure 7B). Rather than entering their next precocious mitosis and arresting, they went through one or more cycles of cell rounding and flattening and of cyclin accumulation and destruction (Figure 7B). All of the CDK1WT-CFP-transfected cells arrested in mitosis after nocodazole treatment, whereas nearly one third of CDK1AF-CFP-transfected cells continued to rapidly cycle despite nocodazole treatment (Figure 7C), presumably because these were daughter cells like the cell shown in Figure 7B. These findings indicate that CDK1AF-CFP-expressing cells can mount a normal spindle assembly checkpoint response during their first (relatively normal) posttransfection mitosis, but not during the subsequent (grossly abnormal) M phase-like states.

CDK1AF Expression Impairs APC-Cdc20 and APC-Cdh1 Function

The shortening of G1 phase and the disruption of other cell cycle phases in the CDK1AF-CFP-transfected cells suggested that CDK1AF-CFP compromises the activation of APC-Cdc20 or APC-Cdh1. The precocious accumulation of cyclin B1-YFP in cells cotransfected with CDK1AF-CFP and cyclin B1-YFP is consistent with this hypothesis as well. APC-Cdc20 is thought to be largely responsible for the degradation of the earliest APC substrates during metaphase and anaphase, with APC-Cdh1 taking over during the latest stages of mitosis and G1 phase (Hagting *et al.*, 2002; Pines, 2006). Both of these APC complexes are active toward cyclin B1 and toward securin, a regulator of sister chromatid cohesion (Cohen-Fix *et al.*, 1996; Uhlmann *et al.*, 1999; Waizenegger *et al.*, 2000).

To assess whether there was a defect in APC-Cdc20 or APC-Cdh1 regulation in the CDK1AF-CFP-transfected cells, we made use of cyclin B1-YFP plus two fluorescent derivatives of securin: wild-type securin fused to red fluorescent protein (WT-securin-tDimer2), which is a substrate for both APC-Cdc20 and APC-Cdh1; and a destruction box (D-box) mutant of securin (R61A-securin-tDimer2) that can be ubiquitinated by APC-Cdh1 but not by APC-Cdc20 (Hagting *et al.*, 2002). In principle these proteins could affect

the processes being monitored, either by adding to the total securin pool or by competing for the APC's access to other targets. In practice, they seem to be reasonably benign reagents for monitoring the successive activation of APC-Cdc20 and APC-Cdh1 (Hagting *et al.*, 2002). Cells were transfected with one of the two securin constructs, plus cyclin B1-YFP and either CDK1WT-CFP or CDK1AF-CFP.

CDK1WT-CFP-expressing cells degraded both cyclin B1-YFP and WT-securin-tDimer2 nearly simultaneously (Figure 8, A and E), and R61A-securin-tDimer2 was degraded ~20–30 min after cyclin B1-YFP (Figure 8, B and E), consistent with previous work (Hagting *et al.*, 2002). The situation was markedly different in CDK1AF-CFP-transfected cells, particularly with respect to the degradation of R61A-securin-tDimer2. Notably, the proteolysis of R61A-securin-tDimer2 was not sufficient to bring the R61A-securin-tDimer2 levels down to normal interphase levels before the next cycle of R61A-securin-tDimer2 accumulation began (Figure 8, D and F). In addition, there was an increase in the time lag between cyclin B1 proteolysis and R61A-securin proteolysis (Figure 8E).

The proteolysis of both cyclin B1 and R61A-securin also seemed more sluggish in the CDK1AF-CFP-transfected cells than in the CDK1WT-CFP-transfected cells (Figure 8, B and D). To quantify this, we examined six rounds of cyclin B1-YFP and R61A-securin-tDimer2 proteolysis in CDK1WT-CFP-transfected cells and nine rounds in CDK1AF-CFP-transfected cells. We then plotted cyclin B1-YFP and R61A-securin-tDimer2 levels as a function of time. We began the plots at the time point when the rate of protein destruction was highest in the hope that the destruction kinetics could be fitted to simple exponential decay curves. As shown in Figure 8, G and H, this was the case. The half-time for cyclin B1-YFP in the CDK1WT-CFP-transfected cells was 6.3 ± 0.4 min (avg. $\pm$ SEM) and in the CDK1AF-CFP-transfected cells, 15.7 ± 1.1 min (Figure 8G). Assuming Cdc20 was largely responsible for cyclin B1-YFP polyubiquitylation, this increased half-time corresponded to a 60% decrease in the apparent activity of Cdc20. The half-time for R61A-securin-tDimer2 in CDK1WT-CFP-transfected cells was 7.9 ± 0.7 min, and in the CDK1AF-CFP-transfected cells, 35.5 ± 2.9 min (Figure 8H). This was a decrease of 78% in the apparent activity of Cdh1. Therefore, the activation of both APC-Cdc20 and APC-Cdh1 was compromised in the CDK1AF-CFP-transfected cells.

DISCUSSION

Previous experimental work established that the phosphorylation and dephosphorylation of CDK1 at Thr 14 and/or Tyr 15 is required for sustained CDK1 oscillations in cycling *Xenopus* egg extracts (Pomerening *et al.*, 2005). However, these phosphorylations could be completely dispensable in somatic cells due to the presence of additional control mechanisms and checkpoints that are not operative in the early embryo.

In the present work, CDK1AF-CFP was used to investigate whether Thr 14/Tyr 15 phosphorylation is required in the somatic cell cycle in HeLa cells. In agreement with previous reports, we found no obvious effect of CDK1AF-CFP expression on the timing of the first NEB after release of arrested cells from a double thymidine block (Jin *et al.*, 1996, 1998; Blasina *et al.*, 1997) (Figure 4), presumably because of the requirement for cyclin A2-dependent accumulation of CDK1 in the nucleus for NEB (Jin *et al.*, 1998; Gong *et al.*, 2007). The duration of the first M-phase was somewhat lengthened in the CDK1AF-CFP-transfected cells, from 60 to

78 min (Figure 4D), but otherwise mitotic entry, progression, and exit were qualitatively normal.

However, the subsequent cell cycle phases were markedly abnormal. Cycling HeLa cells typically spend 6.5 h in G1 phase, followed by a 9-h S phase and a 3.3-h G2 phase, for a total interphase duration of 18.8 h, and then enter mitosis (Hahn, personal communication). By comparison, the CDK1AF-CFP-expressing cells reentered mitosis within 3–6 h of cytokinesis (Figures 2, 4, and 5). Most of the CDK1AF-CFP-expressing cells transitioned from G1 phase to S phase prematurely, remained in S phase for only a few hours, and then directly entered an M phase-like state without completing DNA replication or going through a recognizable G2 phase (Figure 5). A minority of the cells proceeded directly from G1 phase to M phase (Figure 5). CDK1AF-CFP-expressing cells typically remained in M phase for ~2 h, returned to interphase without carrying out karyokinesis or cytokinesis, and then cycled into M phase one or more additional times at intervals of 3–6 h (Figures 2–4, 5). The fact that the cells exited mitosis without dividing suggested that the spindle assembly checkpoint was not functioning properly – possibly because mitosis had been entered so prematurely that functional kinetochores and spindle poles were not yet present—and direct assessment of the cells' responses to the spindle poison nocodazole confirmed that the checkpoint functioned during the first postrelease M phase but not in subsequent M phases (Figure 7). Rapid cycles were also seen in cells treated with Wee1 d-siRNAs (Figure 6), consistent with the hypothesis that the phenotype arises specifically from defects in the CDK1/Cdc25/Wee1/Myt1 system rather than from some idiosyncratic effect of the CDK1AF-CFP mutant or CDK overexpression.

The rapid cell cycles seen in CDK1AF-CFP-expressing cells and Wee1 knockdown cells were characterized by smooth, relatively sinusoidal oscillations in the concentration of cyclin B1-YFP (Figure 3, 6), in contrast to the abrupt, switch-like changes in cyclin levels seen in CDK1WT-CFP-transfected cells (Figure 3). APC-Cdc20 and APC-Cdh1 activities were compromised during the rapid cycles, both in terms of the maximal rates of cyclin B1 and securin R61A destruction and the length of time during which APC-Cdh1 remained active (Figure 8). This could explain the cells' premature exit from G1 phase and entry into mitosis. The inability to properly lock the cell into G1 phase could be due to a requirement for CDK1 tyrosine phosphorylation during late G1 phase to keep APC-Cdh1 from being turned off prematurely; from a requirement for CDK1 tyrosine phosphorylation during mitotic exit to properly initiate the activation of APC-Cdh1; or from a requirement for abrupt CDK1 activation at the onset of mitosis in order to ultimately achieve normal levels of APC-Cdc20 and APC-Cdh1 activation.

Why Does CDK1AF Expression and Wee1 Knockdown Affect Only the Second Cycle?

As has been observed previously, the effects of CDK1AF-CFP expression on the progression of synchronized HeLa cells into the first postrelease mitosis are surprisingly subtle (Jin *et al.*, 1996, 1998; Blasina *et al.*, 1997). In contrast, the effects on subsequent cycles are dramatic (this study). One trivial explanation for this difference could be that during the first postrelease cycle the majority of cyclin B1 is bound to endogenous CDK1, leaving CDK1AF-CFP inactive. However, this explanation is difficult to reconcile with Jin and Morgan's observation that vast amounts of cyclin B1 expression fail to dramatically accelerate postrelease mitotic entry in CDK1AF-CFP-expressing cells (Jin *et al.*, 1998). Instead,

Figure 8. APC-Cdc20 and APC-Cdh1 activities in individual cells expressing recombinant CDK1WT-CFP and CDK1AF-CFP. Asynchronous HeLa cells were transfected with cyclin B1-YFP, WT-, or R61A-securin-tDimer2, and either CDK1WT-CFP or CDK1AF-CFP, and then they were imaged for 13 h. (A) Cyclin B1 and WT-securin destruction in a typical CDK1WT-CFP-transfected cell. (B) Cyclin B1 and R61A-securin destruction in a typical CDK1WT-CFP-transfected cell. (C) Oscillations of cyclin B1-YFP and WT-securin-tDimer2 in a CDK1AF-CFP-expressing cell. (D) Oscillations of cyclin B1-YFP and R61A-securin-tDimer2 in a CDK1AF-CFP-expressing cell. (E) Time lag between the degradation of cyclin B1-YFP and WT-securin (gray) or R61A-securin (black) in cells expressing CDK1WT-CFP or CDK1AF-CFP. Data are shown as means $\pm$ SEM. (F) Percentage of completion of cyclin B1-YFP (light gray), WT-securin-tDimer2 (dark gray), and R61A-securin-tDimer2 (black) proteolysis in successive cycles. Data are presented as means $\pm$ SEM. (G and H) Time course of cyclin B1-YFP destruction (G) and R61A-securin-tDimer2 destruction (H) in CDK1WT-CFP- and CDK1AF-CFP-expressing cells. Data are from six rounds of cyclin B1-YFP and R61A-securin-tDimer2 proteolysis in CDK1WT-CFP-transfected cells and nine rounds in CDK1AF-CFP-transfected cells.

we hypothesize that the CDK1 tyrosine phosphorylation is of lesser importance in suppressing M phase entry subsequent to the thymidine arrest point, and of greater importance during G1 phase. As Jin and Morgan showed, nuclear

mitotic events such as chromatin condensation depend upon both the activation of cyclin B1-CDK1 and the accumulation of the complex in the nucleus (Jin *et al.*, 1998). Perhaps the ability to exclude cyclin B1-CDK1 from the nucleus is not

Figure 9. Schematic view of cyclin-CDK1 regulation. (A) Normal HeLa cells. (B) HeLa cells expressing CDK1AF or after Wee1 knock-down.

properly established in G1 phase. Indeed, the localization of cyclin B1-YFP in some CDK1AF-expressing cells (Figure 3) and Wee1 knockdown cells (Figure 6) is consistent with this hypothesis.

Switches and Oscillations in HeLa Cells

Figure 9 shows a simplified schematic of the protein network that regulates mitotic entry and exit in somatic cells. The activation of CDK1 is regulated by three interlinked positive feedback loops (CDK1 $\rightarrow$ Cdc25 $\rightarrow$ CDK1; CDK1 $\rightarrow$ Wee1 $\rightarrow$ CDK1; CDK1 $\rightarrow$ Myt1 $\rightarrow$ CDK1) that we hypothesize function as a bistable switch. In G2 phase, the switch is "off": CDK1 and Cdc25 are low in activity and Wee1 and Myt1 are high. At the onset of M phase, the switch toggles to "on," with CDK1 and Cdc25 high in activity and Wee1 and Myt1 low. One consequence of CDK1 activation is alignment of the chromosomes on the metaphase plate. This turns off the spindle assembly checkpoint and allows a negative feedback loop from CDK1 to APC-Cdc20 to be engaged. Once CDK1 activity has fallen sufficiently, APC-Cdc20 turns back off and APC-Cdh1 turns on, locking the cell into a stable interphase state.

The present findings emphasize that the CDK1/Cdc25/Wee1/Myt1 toggle switch is essential for cycling in HeLa cells. We propose that when this toggle switch is intact, the CDK1 system runs like relaxation oscillator (Nave, 2005), with the system abruptly switching between alternative states (Figure 9A). However, when this toggle switch is compromised by the expression of CDK1AF-CFP or by knocking down Wee1, the system runs more like a simple negative feedback oscillator or phase-shift oscillator. The characteristics of this oscillator are revealed when the toggle switch is compromised: it generates rapid, sinusoidal oscillations in the level of cyclin B1 and correspondingly rapid cycles between M phase-like and interphase states (Figure 9B). In addition, this negative feedback oscillator is not at all robust; the oscillations often damped out or ceased abruptly. Evidently, the CDK1/Cdc25/Wee1/Myt1 toggle switch is important for keeping successive cell cycle events like CDK1 activation and APC activation properly separated in time, just as it is in *Xenopus* egg extracts. The double-negative feedback loop between CDK1 and Cdh1 (Figure 9), which also has the potential to act as a bistable toggle switch, seems to be insufficient to sustain the switch-like oscillations characteristic of a relaxation oscillator if the CDK1/Cdc25/Wee1/Myt1 system is not functioning properly.

It should be kept in mind that HeLa cells are transformed, and although they have been an exceedingly useful system

for studies of mitosis and mitotic control, it is possible that nontransformed cells possess mechanisms that render the CDK1/Cdc25/Wee1/Myt1 system less essential. Nonetheless, the present studies are significant in showing that a properly functioning CDK1/Cdc25/Wee1/Myt1 system can be sufficient to convert unreliable, rapid cell cycle oscillations into reliable, switch-like oscillations with a period typical of the somatic cell cycle.

The Role of Positive Feedback in Cell Cycle Oscillations in Budding Yeast

Our studies are strongly reminiscent of Cross's work on the role of positive feedback in the *Saccharomyces cerevisiae* cell cycle (Cross, 2003). In that system, the CDK1 (Cdc28)/Cdc25 (Mih1)/Wee1 (Swe1) circuit seems to be relatively unimportant for normal cell cycle progression; instead, a bistable trigger is thought to be provided by two interlinked double-negative feedback loops: Clb-CDK1 $\rightarrow$ Sic1 $\rightarrow$ Clb-CDK1 and Clb-CDK1 $\rightarrow$ Cdh1 $\rightarrow$ Clb-CDK1. Cross showed that when both of these double-negative feedback loops are abrogated by the deletion of Sic1 and Cdh1, under some circumstances (overexpression of Cdc20) viable yeasts can be obtained (Cross, 2003). The same was true when an additional possible double-negative feedback loop (between CDK1 and the N-terminal inhibitory region of Cdc6) was abrogated as well. Conversely, mutant APC subunits that have compromised APC-Cdc20 function but not APC-Cdh1 function render Cdh1 essential (Cross, 2003). This demonstrates that there is striking redundancy in the budding yeast cell cycle oscillator. In contrast, there seems to be little redundancy in the HeLa cell cycle; compromising one pair of interlinked positive feedback loops through CDK1AF-CFP expression, or one of the two interlinked loops through Wee1 knock-down, is sufficient to dramatically alter the dynamics of the cell cycle.

Positive Feedback as a Recurring Motif in Biological Oscillators

The simplest biological oscillator circuit is a negative feedback loop. A three-component negative feedback loop, or a shorter negative feedback loop with time lags built into one or more of its components, is sufficient to generate oscillations in principle, and in practice a synthetic oscillating gene circuit of this sort was successfully expressed in *E. coli* eight years ago (Elowitz and Leibler, 2000). However, many of the best-characterized biological oscillators have positive feedback loops as well as negative feedback loops. These include the Hodgkin-Huxley oscillator in cardiac pacemaker cells,

where the positive feedback is provided by the voltage-sensitive sodium channel (Hodgkin and Huxley, 1952); calcium oscillations, where the positive feedback is provided by calcium-induced calcium release (Berridge, 2001; Lewis, 2001); and several cell cycle oscillators (Novak and Tyson, 1993; Novak *et al.*, 2001; Tyson and Novak, 2001; Cross *et al.*, 2002; Pomerening *et al.*, 2003, 2005; Sha *et al.*, 2003), including the somatic cell oscillator being studied here. p53 oscillations (Harris and Levine, 2005) and circadian oscillations in both cyanobacteria and animal cells (Reppert and Weaver, 2002; Rust *et al.*, 2007) may depend upon positive feedback loops as well. Modeling studies will be important for understanding what the design advantages might be of positive-plus-negative feedback oscillators over negative feedback oscillators, and experimental studies, like those presented here, will be critical for testing whether these possible design advantages are in fact achieved.

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