Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells

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We have found that key mitotic regulators show distinct patterns of degradation during exit from mitosis in human cells. Using a live-cell assay for proteolysis, we show that two of these regulators, polo-like kinase 1 (Plk1) and Aurora A, are degraded at different times after the anaphase-promoting complex/cyclosome (APC/C) switches from binding Cdc20 to Cdh1. Therefore, events in addition to the switch from Cdc20 to Cdh1 control the proteolysis of APC/C Cdh1 substrates in vivo. We have identified a putative destruction box in Plk1 that is required for degradation of Plk1 in anaphase, and have examined the effect of nondegradable Plk1 on mitotic exit. Our results show that Plk1 proteolysis contributes to the inactivation of Plk1 in anaphase, and that this is required for the proper control of mitotic exit and cytokinesis. Our experiments reveal a role for APC/C-mediated proteolysis in exit from mitosis in human cells.

Introduction

In animal cells, the regulated proteolysis of cyclin A, cyclin B1, and securin during mitosis are all essential for the proper timing of events leading up to separation of sister chromatids at the onset of anaphase (den Elzen and Pines, 2001; Geley et al., 2001; Stemmann et al., 2001; Hagting et al., 2002; Leismann and Lehner, 2003). Proteolysis of these key mitotic regulators is mediated by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase and requires the activating subunit Cdc20/fizzy (Morgan, 1999; Peters, 2002). Cdc20 activity is replaced by that of Cdh1/fizzy-related during mitotic exit, and the role of Cdh1 in suppressing mitotic cyclins is essential to establish the G1 phase of the cell cycle (for review see Peters, 2002). However, this switch from Cdc20 to Cdh1 is thought to allow degradation of many additional substrates because APC/C Cdh1 has been shown to have broader substrate specificity than APC/C Cdc20 (Fang et al., 1998; Pfleger and Kirschner, 2000; Hagting et al., 2002; Zur and Brandeis, 2002). Amongst the regulators degraded during mitotic exit in mammalian cells are Cdc20, the polo-like kinase 1 (Plk1), the Aurora kinases, and the CENP-E motor protein (Brown et al., 1994; Weinstein, 1997; Ferris et al., 1998; Honda et al., 2000). However, it has not been shown whether these substrates are all degraded as soon as the APC/C switches from its Cdc20- to Cdh1-activated form, or whether they are degraded at distinct times, perhaps to coordinate exit from mitosis. In budding yeast, mitotic exit is under the tight control of the mitotic exit network (Morgan, 1999), a signaling cascade required for the activation of Cdh1 by the Cdc14 phosphatase that can be restrained by a Bub2-dependent checkpoint that monitors the position of the spindle (Li, 1999; Pereira et al., 2000). An equivalent network in mammalian cells has yet to be identified, although homologues of some of the components, such as Cdc14, have been identified. In human cells, a homologue of the spindle checkpoint protein Mad2 (Mad2B) has been shown to inhibit Cdh1 in vitro (Chen and Fang, 2001), but the role of Mad2B in mitotic exit, if any, is not known.

Here, we have begun to examine the role and regulation of proteolysis during mitotic exit in mammalian cells, through studying fluorescent protein (FP)–tagged substrates in living cells. We find that different mitotic regulators are degraded at different times, indicating that APC/C Cdh1 activity may be modulated to coordinate mitotic exit and cytokinesis.

Results

Initially, we investigated whether it was possible to identify differences in the degradation patterns of substrates by analyzing immunoblots of endogenous protein levels in HeLa

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; D-box, destruction box; DIC, differential interference contrast; FP, fluorescent protein; Plk1, polo-like kinase 1.
The disappearance of mitotic regulators at exit from mitosis is not concerted. (A and B) HeLa cells were synchronized in prometaphase by a nocodazole block after a thymidine/aphidicolin double block/release (see Materials and methods). Mitotic cells were harvested by shake-off, washed, and replated in the absence of nocodazole. At the time points indicated, samples were processed for immunoblot analysis of the indicated proteins. (A) Parallel samples were processed for analysis of DNA content by flow cytometry, and the M-phase/G1 distribution of each cell population is indicated. Protein loading was assayed by Ponceau S staining and is equivalent for each time point. (B) Extracts were probed with antibodies against p55\(^{Cdc20}\) and Plk1, and with \(\beta\)-tubulin as a loading control. We found that the time required for recovery from nocodazole varied between each of the three experiments we performed (compare Plk1 degradation in A and B), but that Cdc20 levels always fell before those of Plk1. ns, extracts from nonsynchronized cells. Note that comparing levels in unsynchronized cells to mitotic cells in A indicates that PRC1 (but not BubR1) levels are cell cycle regulated.

cell extracts. Analysis of extracts from cells synchronized through mitotic exit by release from nocodazole-induced arrest showed that the degradation of APC/C\(^{Cdh1}\) substrates was not concerted (Fig. 1). Plk1 disappeared from cell extracts earlier than Aurora A and CENP-E, whereas Aurora B appeared to be degraded later and was still detectable in G1 cell extracts (Fig. 1 A). In further experiments, p55\(^{Cdc20}\) appeared to be degraded earlier than Plk1 (Fig. 1 B). PRC1 and BubR1, proposed to be late mitotic substrates (Jiang et al., 1998; Chan et al., 1999), were not significantly degraded after release from nocodazole (Fig. 1 A). Although this analysis confirmed that specific mitotic regulators were degraded during exit from mitosis and indicated that they were degraded with different kinetics, it did not show exactly when degradation of the substrates began. Moreover, we found that the rate of recovery from the nocodazole block varied between cells. Therefore, we tagged substrates with FPs to use as markers for the endogenous substrates in single-cell analyses of degradation.

We began by analyzing Plk1. A GFP-Plk1 chimera had previously been validated as a marker for the localization of endogenous human Plk1 (Arnaud et al., 1998), so we examined whether it was also a suitable marker for the mitotic degradation of Plk1. We injected G2 phase HeLa cells with a cDNA encoding Plk1 tagged at its amino terminus with EYFP (YFP-Plk1), and recorded the fluorescence of mitotic cells by time-lapse imaging. YFP-Plk1 fluorescence started to decline at the beginning of anaphase (Fig. 2), timing that is clearly distinguishable from the decrease in cyclin B1-CFP in metaphase (Clute and Pines, 1999; Hagting et al., 2002; Fig. 2 A; Video 1 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). The onset of Plk1 degradation correlated with its relocalization from the kinetochores to the midzone of the mitotic spindle (Arnaud et al., 1998; Fig. 2 A, time point 3). The decline in YFP-Plk1 fluorescence was indeed a result of proteolysis because it was blocked by the proteasome inhibitor MG132 (Fig. 2 B). The onset and rate of YFP-Plk1 degradation were highly reproducible in this assay (Fig. 2 C), and in almost all cells, 60–75% of YFP-Plk1 was degraded during mitotic exit. Cells expressing very high levels of YFP-Plk1 were not able to degrade the protein properly and were unable to exit normally from mitosis (see Fig. 5). Using a U2OS cell line stably expressing YFP-Plk1 (Jackman et al., 2003), we have estimated that the usual levels of expression of YFP-Plk1 achieved during injection experiments varied from less than that of endogenous Plk1 to approximately threefold the endogenous level. The timing of YFP-Plk1 degradation was identical in U2OS cells (unpublished data).

The time at which YFP-Plk1 degradation began appeared to reflect the switch from APC/C\(^{Cdh1}\) to APC/C\(^{Cdh1}\) at anaphase (Hagting et al., 2002). Therefore, we compared the proteolysis of YFP-Plk1 with an in vivo marker for APC/C\(^{Cdh1}\). We used a CFP-tagged destruction box (D-box) mutant of securin (db-securin-CFP) that was a substrate for APC/C\(^{Cdh1}\) but not APC/C\(^{Cdc20}\) in vitro, and whose degradation in vivo depended on the KEN box motif present in the amino terminus, and on a decline in cyclin B-Cdk1 activity (Hagting et al., 2002). db-securin-CFP degradation preceded that of YFP-Plk1 by several minutes (Fig. 3 A), indicating that the activation of APC/C\(^{Cdh1}\) is not sufficient to initiate YFP-Plk1 destruction. Our finding that APC/C\(^{Cdh1}\) was active before Plk1 degradation began was consistent with our observation that another APC/C\(^{Cdh1}\) substrate, p55\(^{Cdc20}\), appeared to be degraded earlier than Plk1 after release from a nocodazole arrest (Fig. 1 B). However, we were...
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unable to verify this in vivo because FP-Cdc20 was a very poor substrate for APC/C
Cdh1 (unpublished data).

We were interested in comparing the timing of destruction of other APC/C
Cdh1 substrates with Plk1, and selected Aurora A, which was recently shown to be an APC/C
Cdh1 substrate both in vitro and in vivo (Castro et al., 2002; Littlepage and Ruderman, 2002; Taguchi et al., 2002). Degradation of Aurora A-YFP began 6–12 min after the start of anaphase, always after that of CFP-Plk1 as in A. Its degradation curve has been superimposed on that of a control cell in the dish, which had completed mitosis before addition of MG132. (C) YFP fluorescence in cells expressing different levels of YFP-Plk1. Degradation of YFP-Plk1 at anaphase was measured in 20 cells, with or without coexpression of cyclin B1-CFP, in 10 separate experiments. Here, natural log values of fluorescence from representative cells have been plotted to show that the rate of degradation does not vary between cells, and is not affected by coexpression of cyclin B1. The vertical line in C indicates the average time of anaphase onset and is accurate within 90 s for each cell.

Figure 2. Plk1 degradation begins at the start of anaphase and is proteasome dependent. (A) HeLa cells synchronized in late G2 phase were coinjected with cDNAs encoding YFP-Plk1 and cyclin B1-CFP, and fluorescence in mitotic cells was recorded by time-lapse imaging. Fluorescence in whole cells was measured and plotted as pixel values. The degradation curves shown are representative of at least seven cells in at least two separate experiments. YFP-Plk1-associated fluorescence at the various stages of mitosis is shown (images taken from Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). 1, prophase; 2, prometaphase; 3, early anaphase; 4, late anaphase; 5, telophase. (B) MG132 at a final concentration of 50 μM was added to a cell in anaphase during time-lapse imaging of cells expressing YFP-Plk1 as in A. Its degradation curve has been superimposed on that of a control cell in the dish, which had completed mitosis before addition of MG132. (C) YFP fluorescence in cells expressing different levels of YFP-Plk1. Degradation of YFP-Plk1 at anaphase was measured in 20 cells, with or without coexpression of cyclin B1-CFP, in 10 separate experiments. Here, natural log values of fluorescence from representative cells have been plotted to show that the rate of degradation does not vary between cells, and is not affected by coexpression of cyclin B1. The vertical line in C indicates the average time of anaphase onset and is accurate within 90 s for each cell.

full/jcb.200309035/DC1). In contrast to Plk1 degradation, which stopped once cells entered G1 phase, Aurora A-YFP degradation continued to completion (Fig. 3 B, see also Fig. 2 C). The distinct patterns of disappearance of Plk1 and Aurora A indicate that the degradation of different APC/C
Cdh1 substrates is tied to specific events during mitotic exit.

Having found that there was an ordered proteolysis of APC/C
Cdh1 substrates during anaphase, we wished to determine whether APC/C
Cdh1-mediated proteolysis played a role in mitotic exit by blocking degradation of one of its substrates. We examined the Plk1 sequence for motifs that might direct its mitotic degradation and identified an RxxL D-box-like motif (Glotzer et al., 1991) between the kinase and polo-box domains of Plk1 (Fig. 4 A). This motif is conserved in all mammalian orthologues of Plk1, in Xenopus
Plx1, and in *Drosophila* polo, but is not present in fission yeast *plo1*, whose levels do not fluctuate during the cell cycle (Mulvihill et al., 1999). Moreover, this motif is not conserved in mammalian Plk3/Prk that is not degraded at mitosis (Ouyang et al., 1997; Ferris et al., 1998; Fig. S1 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). Therefore, this motif appeared to be a good candidate to act as a Plk1 D-box. We made alanine substitutions at the conserved RxxL positions of the putative D-box sequence (R337A, L340A), and found that this mutant of Plk1 (YFP-db-Plk1) was stable in anaphase (Fig. 4 B), and the dynamic localization of this protein during mitosis was indistinguishable from that of the wild-type protein (Video 4 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). Moreover, this motif is not conserved in mammalian Plk3/Prk that is not degraded at mitosis (Ouyang et al., 1997; Ferris et al., 1998; Fig. S1 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). Therefore, this motif appeared to be a good candidate to act as a Plk1 D-box. We made alanine substitutions at the conserved RxxL positions of the putative D-box sequence (R337A, L340A), and found that this mutant of Plk1 (YFP-db-Plk1) was stable in anaphase (Fig. 4 B), and the dynamic localization of this protein during mitosis was indistinguishable from that of the wild-type protein (Video 4 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1). Mutating an adjacent sequence (E348A, N349A) had no effect (unpublished data). Immunofluorescence analysis confirmed that the untagged version of the R337A, L340A mutant (db-Plk1) was stable in mitosis because it was present at high levels in newly divided cells (Fig. S2 and supplemental data). We conclude that R337 defines a D-box that directs Plk1 destruction in anaphase. YFP-db-Plk1 did not prevent Aurora A-YFP being degraded, showing that the degradation of later substrates did not depend on prior degradation of Plk1, and confirming that the stabilization of Plk1 was not due to a general inhibition of APC/C/Cdc14 (Fig. 4 C).

Nondegradable Plk1 frequently interfered with exit from mitosis. Furthermore, cells expressing high levels of YFP-Plk1 frequently were unable to degrade the protein properly, and in these cells mitotic exit was perturbed (Fig. 5). In these cells, anaphase and cytokinesis were slowed down; spindle elongation was impaired and cleavage furrow ingress was delayed, with many cells exhibiting extensive blebbing of the plasma membrane during this delay (Fig. 5 A). Although spindle elongation was frequently slow, maximum elongation (measured as the extent of sister chromatid separation observed by differential interference contrast [DIC] microscopy) was usually unaffected. There was frequently a delay after maximum spindle elongation before the cleavage furrow began to ingress, whereas these events occurred concurrently in uninjected cells. We did not observe chromosome decondensation during this delay; therefore, both mitotic exit and cytokinesis appeared to be delayed. The delay in mitotic exit in cells unable to degrade Plk1 was frequently accompanied by abnormal movement of the anaphase spindle during prolonged cleavage furrow ingress (Videos 5 and 6 and supplemental data, available at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1), indicating that the coordination between microtubule and actin microfilaments might be compromised in these cells. Overexpression of Plk1 or YFP-Plk1 also delayed mitosis before anaphase, and this delay was aggravated by a constitutively active form of the kinase, but not by nondegradable forms. However, the delay in mitotic exit was independent of the total time spent in mitosis (unpublished data).
We sought to quantify the delay in mitotic exit by measuring the total time \( t \) taken from sister chromatid separation to the completion of cleavage in cells injected with either YFP-tagged or untagged versions of Plk1 (Fig. 5 A). Injecting cells with YFP alone had no effect on mitotic exit (unpublished data). We found that there was a large variation in exit times in cells injected with nondegradable Plk1, best illustrated by box plots (Fig. 5, B and C), with some cells taking three times longer than the control mean value. Untagged, nondegradable Plk1 (Fig. 5 B; db-Plk1, untagged) consistently had a more pronounced effect on mitotic exit than wild-type Plk1, indicating that Plk1 proteolysis might be required for normal mitotic exit.

In budding yeast, redundant pathways have been demonstrated to inactivate mitotic cyclin-Cdns after mitosis: APC\(^{Cdh1}\)-mediated degradation is not required for cell viability as long as the Cdk inhibitor Sic1 is present (Schwab et al., 1997; Visintin et al., 1997). Thus, we considered the possibility that there might be parallel pathways to inactivate APC\(^{Cdh1}\) targets during exit from mammalian mitosis. Because Plks are activated in mitosis by phosphorylation at a threonine residue in the activation loop (Kelm et al., 2002), we inserted the activating T210D mutation (Qian et al., 1999) at this site to generate a version of Plk1 that could not be inactivated by dephosphorylation. We found that, like nondegradable db-Plk1, the constitutively active T210D-Plk1 delayed mitotic exit compared with wild-type Plk1. Furthermore, a double mutant of Plk1 that was constitutively active and nondegradable, dbT210D-Plk1, significantly increased the frequency with which we observed this phenotype (Fig. 5 B, see \( P \) values).

We thought that the large variation in mitotic exit times (Fig. 5 B) might arise from variability between cells in the level of Plk1, which we were unable to measure because the proteins were not fluorescently tagged. Therefore, we examined mitotic exit times in cells expressing YFP-tagged Plk1, where we could select cells expressing comparable levels of the different constructs. In these cells, we observed similar delays in mitotic exit, but also saw a similar variation in mitotic exit times (Fig. 5 C). Additionally, we found an increased delay in cells expressing wild-type YFP-Plk1 compared with untagged Plk1. This indicated that the YFP tag could reduce the rate of degradation of the protein. Perhaps because of this, mutating the D-box alone in YFP-Plk1 had
Nondegradable Plk1 causes a delay in mitotic exit. (A) Time-lapse DIC images of mitotic exit for a normal (uninjected) HeLa cell and for one injected in G2 phase with a YFP-Plk1 expression plasmid. These cells were filmed concurrently in the same dish (see Materials and methods). (B and C) Box plot distributions of mitotic exit times for uninjected cells and cells injected with cDNAs encoding untagged wt-, db-, T210D-, dbT210D-Plk1 (B), or YFP-tagged versions of the same constructs (C). A YFP expression plasmid was used as a marker for cells injected with untagged constructs (B). In these distributions, the maximum and minimum values, the interquartile range (marked by boxes), and the median value (marked by horizontal line) for mitotic exit times in each population are shown. Suspected outliers are shown as open circles, an outlier as a filled circle. P values for each population of cells expressing a Plk1 mutant, compared with that expressing the wild-type version, are indicated. The software used is available online upon request. (D) Degradation curves (plotted as natural log values) for YFP-Plk1 and YFP-T210D-Plk1 in cells showing impaired mitotic exit. Cells expressing either YFP-Plk1 (filled circles) or YFP-T210D-Plk1 (open circles) were compared from the same experiment. Cells in which mitotic exit took >24 min are indicated by arrowheads. This figure is representative of three separate experiments.
a less significant effect on mitotic exit times (Fig. 5 C). To confirm the link between Plk1 degradation and mitotic exit times in cells expressing YFP-tagged Plk1, we examined the rates of YFP-Plk1 and YFP-T210D-Plk1 degradation and correlated this with the time taken to exit mitosis. We found that degradation was systematically impaired in cells displaying very prolonged mitotic exit times (Fig. 5 D). This occurred more frequently in YFP-T210D-Plk1 cells than in YFP-Plk1 cells, indicating that inactivation of Plk1 in ana-phase may be required for its proper degradation.

Discussion

We have presented evidence that normal exit from mitosis requires the inactivation of Plk1, and that this can be either by degradation or by dephosphorylation. Thus, APC/C\(^{Cdh1}\) dependent proteolysis appears to play an important role in the proper exit from mitosis. However, because there are likely to be parallel pathways to inactivate mitotic kinases, there may not be any one mitotic regulator whose degradation is essential for mitotic exit. Indeed, in early embryos, mitotic cycles occur in the absence of Cdh1 expression (Sigrist and Lehner, 1997; Lorca et al., 1998). We suggest that degradation directed by APC/C\(^{Cdh1}\) increases the efficiency with which mitotic regulators are inactivated at the end of mitosis. Consistent with this suggestion, Cdh1\(^{-/-}\) DT40 cells, although viable, show prolonged mitotic exit (Sudo et al., 2001).

Why should there be a requirement for Plk1 inactivation during anaphase? This appears to contradict the well-documented requirement for Plk activity in cytokinesis and mitotic exit (for review see Bahler et al., 1998; Carmena et al., 1998; Heitz et al., 2001; Lee et al., 2001; Song and Lee, 2001; Mulvihill and Hyams, 2002; Seong et al., 2002). We propose that in addition to being required for assembly of the contractile ring, Plk1 may inhibit cleavage furrow ingression in mammalian cells, perhaps to ensure that ingestion cannot occur before anaphase (Shuster and Burgess, 2002). According to the model most recently proposed (Dechant and Glotzer, 2003), this inhibition could be achieved by regulating microtubule bundling in the central spindle. Work published by Neef et al. (2003) during the preparation of this manuscript allows us to identify the kinesin-like protein MKLP-2 as a candidate to mediate the inhibitory effect of Plk1 on cell cleavage. MKLP-2 localizes to the central spindle during anaphase in a complex with Plk1, and has microtubule bundling activity in vitro that can be negatively regulated by Plk1.

Although previous work by others has described the appearance of multinucleated cells after transfection of Plk1 (Mundt et al., 1997), a failure of cytokinesis is a rare event under our experimental conditions, perhaps reflecting the lower levels of expression achieved with microinjection. We find that almost all cells attempt cytokinesis within 50 min of anaphase. This 50-min interval corresponds to the previously described period of "cortical contractility," or C-phase (Martineau et al., 1995; Canman et al., 2000). C-phase can be extended by inhibiting proteolysis with MG132 in the presence of blebbistatin, an inhibitor of myosin II (Straight et al., 2003), indicating that the length of C-phase is regulated by proteolysis. Our results show that the relevant substrate does not appear to be Plk1 because nondegradable Plk1 delays cytokinesis without altering the length of C-phase (Fig. 5, B and C). Moreover, cells starting with excess Plk1 almost always eventually perform cytokinesis. Thus, we suggest that C-phase is not just the window of opportunity during which cytokinesis can occur, but also represents the maximum period for which cytokinesis can be delayed in response to inappropriate conditions.

Our preliminary analyses indicate that the delay in cytokinesis correlates with delayed recruitment of cleavage furrow components (unpublished data). An understanding of the pathways that coordinate cytokinesis with mitotic exit remains an important challenge, and our finding that there is ordered degradation of mitotic regulators as cells exit from mitosis may provide important clues to these pathways.

Materials and methods

Cell culture and synchronization

HeLa cells were cultured and synchronized for microinjection in G2 phase as described previously (Clute and Pines, 1999). Mitotic cells for the experiment shown in Fig. 1 were prepared by a modified version of the synchronization regime, where cells were released from aphidicolin (Sigma-Aldrich) into medium containing 400 ng/ml nocodazole (Sigma-Aldrich) and incubated for 12 h before harvesting by shake-off. Mitotic cells were washed three times in ice-cold PBS and replated in medium prewarmed to 37°C. Cell cycle distributions of cell populations at different time points were calculated by analysis of DNA content on a FACSsort™ Flow Cytometer (Becton Dickinson) after propidium iodide staining as described previously (Lindon et al., 2000). MG132 was obtained from Calbiochem.

Immunoblotting

Extracts were made from cell populations at the indicated time points after nocodazole release. Cells were washed with PBS. Extracts prepared by addition of boiling SDS sample buffer directly to culture dishes (for reattached cells in G1), and to cells pelleted from the culture medium (for unattached mitotic cells), were pooled. Samples were heated at >95°C for 3 min and then sheared through 21G needles. Approximately 5 µg of each sample was blotted by the standard semi-dry transfer technique onto Immobilon™-P (Millipore). Filters were processed for immunoblotting using standard techniques. Rabbit polyclonal antisera were used for raising (1) human Plk1 amino-terminal peptide (Upstate Biotechnology); (2) cyclin B1 (Hagting et al., 1998); (3) human AURKA (Aurora A) and mouse AURK2 amino terminus (Aurora B; both gifts of Peter Donovan, Thomas Jefferson University, Philadelphia, PA); (4) BubR1 (a gift of Gordon Chan, Fox Chase Cancer Center, Philadelphia, PA); (5) CENP-E (a gift of Tim Yen, Fox Chase Cancer Center, Philadelphia, PA); and (6) PRC1 (a gift of Tony Hunter, Salk Institute, La Jolla, CA). Goat polyclonal anti-human p53 was obtained from Santa Cruz Biotechnology, Inc.

Construction of cDNA plasmids

Plk1 cDNA was cloned from pCMX-GFP10c-Plk1 (Arnaud et al., 1998) into pcYFP-C3 (CLONTECH Laboratories, Inc.) for expression as a fusion protein with YFP at the amino terminus, and into pcDNA3 for expression as an untagged protein. New point mutations were constructed by whole-plasmid PCR using Pfu DNA polymerase (Stratagene) and confirmed by automated sequencing. Constitutively active versions of Plk1 were constructed by swapping in sequences from pRcCMV-Plk1-T210D (a gift of Erich Nigg, Max Planck Institute, Martinsried, Germany).

Human Aurora A sequence was generated by PCR from pCRUZ-myc-Aurora A (a gift of Claude Prigent, University of Rennes, Rennes, France) and was cloned into pcYFP-N1, and is expressed as a fusion protein linked at its carboxy terminus to YFP. pECFP-N1-cyclin B1 and pECFP-N1-securinΔ61-68 have been described previously (Hagting et al., 2002). Histone 2B-YFP was the gift of Claire Acquaviva (Wellcome Trust/ Cancer Research UK Institute, Cambridge, UK). Further details of all constructs used are available upon request.

Microinjection and time-lapse imaging and analysis

Cells were injected and analyzed using time-lapse DIC fluorescence microscopy with different filter cubes to distinguish YFP- and CFP-associated
fluorescence as described previously (Clute and Pines, 1999; Haging et al., 1999, 2002), but with the addition of a programmable XY stage (iPrior Scientific Instruments Ltd.) to allow concurrent filming of several fields of cells. Images were collected every 2 or 3 min and saved in IP Lab Spectrum (Scancoanalytics) format as 16-bit data using a reference look-up table with a preset linear pixel intensity scale. Image software (National Institutes of Health; modified by Jean-Yves Thuret) was used for quantifying CFP and YFP fluorescence. Fluorescence levels in whole cells were measured as pixel values within a region of interest (ROI) drawn around each cell and applied to all images in a series. The ROI drawn in each case was large enough to allow for changing cell shape during mitotic exit. Because we subtracted background pixel values from our measured values, this metric gave accurate measurements of total cell fluorescence. DIC images were used to determine the onset of anaphase. Images were then converted to PICT format and exported to Adobe Photoshop®, or processed via ImageJ to make QuickTime® movies.

Online supplemental material
A sequence alignment of human Plk family members, showing the position of the nonconserved D-box motif, is shown in Fig. S1. Fig. S2 shows anti-Plk1 staining of G1 cells injected with untagged versions of Plk1, and confirms that untagged db-Plk1 is not degraded in mitosis and/or G1 phase. Videos available online show examples of cells degrading Plk1 (Video 1), Aurora A (Video 2), or both (Video 3), cells expressing nondegradable Plk1 (Video 4), and cells exhibiting delayed mitotic exit in response to nondegraded Plk1 (Videos 5 and 6). All supplemental videos, supplemental figures, and an associated Materials and methods section are available online at http://www.jcb.org/cgi/content/full/jcb.200309035/DC1.

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