UbcH10 has a rate-limiting role in G1 phase but might not act in the spindle checkpoint or as part of an autonomous oscillator

Adam Walker*,‡, Claire Acquaviva*,§, Takahiro Matsusaka*, Lars Koop and Jonathon Pines¶

Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Zoology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK

*These authors contributed equally to this work
‡Present address: Domantis Limited, Cambridge, UK
§Present address: Centre de Recherche en Cancerologie de Marseille, UMR 891, Marseille, France
¶Author for correspondence (e-mail: jp103@cam.ac.uk)

Accepted 24 April 2008
Journal of Cell Science 121, 2319-2326 Published by The Company of Biologists 2008
doi:10.1242/jcs.031591

Summary

Ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase lies at the heart of the cell cycle. The APC/C targets mitotic cyclins for destruction in mitosis and G1 phase and is then inactivated at S phase, thereby generating the alternating states of high and low cyclin-Cdk activity required for the alternation of mitosis and DNA replication. Two key questions are how the APC/C is held in check by the spindle-assembly checkpoint to delay cells in mitosis in the presence of improperly attached chromosomes, and how the APC/C is inactivated once cells exit mitosis. The ubiquitin-conjugating protein UbcH10 has been proposed to be crucial in the answers to both questions. However, here we show that the behaviour of UbcH10 is inconsistent with both a crucial role in the spindle checkpoint and in inactivating the APC/C as part of an autonomous oscillator. Instead, we find that the rate-limiting role of UbcH10 is only at the end of G1 phase, just before DNA replication begins.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/14/2319/DC1

Key words: APC/C, Mitosis, Cyclin, Cell cycle, Ubiquitin

Introduction

UbcH10 (also known as UBE2C) is one of three ubiquitin-conjugating proteins that can be used by the anaphase-promoting complex/cyclosome to target mitotic regulators – such as cyclin A, cyclin B and securin – to the proteasome for degradation (Aristarkhov et al., 1996; King et al., 1995; Rodrigo-Brenni and Morgan, 2007). Cyclin A ubiquitylation in particular has been reported to be sensitive to the level of UbcH10 (Rape and Kirschner, 2004). Although most substrate specificity is conferred by the APC/C, UbcH10 has been proposed to be crucial in the regulation of APC/C activity. Certainly *vihar*, the *Drosophila* homologue of UbcH10, is an essential gene, and cells that lack vihar arrest in mitosis with elevated levels of cyclin B1 (Mathe et al., 2004). In mammalian cells, UbcH10 has been suggested to act in concert with the APC/C to generate an autonomous oscillator that inactivates the APC/C after mitosis and to allow cyclin A to accumulate and DNA replication to begin (Rape and Kirschner, 2004). This oscillator relies on two described properties of UbcH10. First, the destruction of cyclin A has been reported to be particularly sensitive to the level of UbcH10. Second, UbcH10 triggers its own destruction by auto-ubiquitylation when the APC/C runs out of substrates in G1 phase. To inactivate the APC/C, the autonomous oscillator model itself requires that UbcH10 accumulates to a high level in mitosis to enable cyclin A destruction, and then declines in G1 phase after other APC/C substrates have been degraded.

Recently, UbcH10 has also been proposed to be required to inactivate the spindle-assembly checkpoint (Reddy et al., 2007). In the presence of improperly attached chromosomes in early mitosis the checkpoint protein Mad2 inactivates Cdc20, which is required for APC/C activity, to delay cells before anaphase. The crystal structure of Mad2 in a complex with a Cdc20-mimicking peptide shows that Mad2 should bind tightly to full-length Cdc20, with part of Mad2 forming a 'seat-belt' to lock Cdc20 in place (Sironi et al., 2002). This structure implies that Mad2 and Cdc20 must be actively dissociated. UbcH10 has been proposed to dissociate the Mad2-Cdc20 complex by ubiquitylating Cdc20, thereby releasing the cells from mitotic arrest (Reddy et al., 2007). However, most of the evidence used to support this role for UbcH10 was based on experiments that assayed the behaviour of proteins in mammalian cell extracts in vitro, and it is unclear whether these extracts truly recapitulate the spindle checkpoint, especially because they do not contain or depend upon unattached kinetochores for their properties. Nevertheless, overexpressing UbcH10 in HeLa cells did result in a twofold increase in the number of bi-nucleate cells in a population of cells arrested in mitosis with nocodazole (Reddy et al., 2007), implying that UbcH10 forced these cells to exit mitosis. Moreover, a number of studies have linked the overexpression of UbcH10 to cancer: these studies found that UbcH10 levels are increased in some cancer cell lines (Berlingieri et al., 2007; Okamoto et al., 2003; Pallante et al., 2005) and tumours (Okamoto et al., 2003; Wagner et al., 2004), and that the locus encoding the UbcH10 gene (20q13.1) is amplified in some tumours (Wagner et al., 2004). Small interfering RNA (siRNA) targeting UbcH10 was also reported to reduce the proliferation rate of both normal and cancer cells

(Berlingieri et al., 2007; Pallante et al., 2005; Wagner et al., 2004). Thus, UbcH10 has been proposed to be a potential target for anti-cancer therapy.

Given the potential importance of UbcH10 to the human cell cycle, we have analysed the role of UbcH10 in vivo in both normal diploid cells (hTert-RPE) and transformed cells (HeLa). We find that, increasing UbcH10 levels by more than threefold does not inactivate the spindle checkpoint in vivo, although it does promote mitotic slippage, and its properties in vivo are inconsistent with the autonomous oscillator model. Instead, we find that cells lacking UbcH10 have a shorter G1 phase and begin DNA replication slightly earlier than control cells, which might conceivably have an impact on long-term genomic stability.

Results

The amount of UbcH10 protein is cell-cycle-regulated (Rape and Kirschner, 2004; Yamanaka et al., 2000), rising before cells enter mitosis and declining upon mitotic exit, and this has been proposed to set up an autonomous oscillator controlling the cell cycle (Rape and Kirschner, 2004). One key component of this model is that UbcH10 should auto-ubiquitylate and trigger its own degradation once the APC/C runs out of substrates in G1 phase. However, we found that UbcH10 is degraded much earlier than allowed by this model. We compared the levels of UbcH10 with two APC/C substrates: cyclin B1, which is degraded in metaphase (Clute and Pines, 1999), and Aurora A, which is degraded in anaphase (Lindon and Pines, 2004). On immunoblots of RPE cells synchronised by release from a mitotic block induced by colcemid (Fig. 1A), UbcH10 levels accumulated to a peak in mid-mitosis after which UbcH10 was degraded. Similar results were seen in HeLa cells synchronised by release from a mitotic block induced by nocodazole (Fig. 1B). The reduction in UbcH10 levels before the end of mitosis was also apparent by immunofluorescence analysis (Fig. 1C and supplementary material Fig. S1), when the level of fluorescence had detectably decreased by telophase in untreated RPE and HeLa cells (Fig. 1C and supplementary material Fig. S1, the signal in mitotic cells before and after anaphase was significantly different (Student's *t*-test, $P<0.001$). This decrease was consistent whether we fixed and permeabilised cells by paraformaldehyde-detergent or by methanol-acetone.

To measure more accurately when UbcH10 was degraded we used a live-cell assay (Clute and Pines, 1999) where we attached UbcH10 to the Venus yellow fluorescent protein (YFP) tag (Nagai et al., 2002). The Venus-UbcH10 fusion protein retained its UBC activity because it was able to bind ubiquitin in a manner that depended on the catalytic cysteine (supplementary material Fig. S2), and an N-terminal-tagged UbcH10 is able to work with the APC/C to conjugate ubiquitin to securin in vitro (J. Nilsson, M. Yekezare, J. Minshull and J.P., unpublished results). Monitoring the fluorescence of Venus-UbcH10 showed that the construct was stable in early mitosis but began to be degraded just after cells entered anaphase (Fig. 1D). This is the point when other anaphase APC/C substrates such as Plk1 and Aurora A kinase also begin to be degraded, and before Aurora B is degraded (Lindon and Pines, 2004). Indeed, co-injection experiments showed that UbcH10 began to be degraded just before Aurora A (data not shown). Thus, the timing of UbcH10 destruction was not consistent with the proposal that destruction begins when the APC/C has run out of substrates in G1 phase (Rape and Kirschner, 2004), but does coincide with the time when *vihar*, the *Drosophila* homologue of UbcH10, is degraded (Mathe et al., 2004). However, unlike *vihar*, mutation of the 'destruction-box' (R129A mutation) in UbcH10 did not stabilise the protein nor did it block cells in anaphase (Mathe et al., 2004). Instead, the mutation inactivated UbcH10 – as previously described (Rape and Kirschner, 2004) (supplementary material Fig. S2) – and made it unstable (supplementary material Fig. S3). Investigating the means by which UbcH10 is recognised for proteolysis, we confirmed that UbcH10 required its catalytic cysteine (C114) to be degraded (Rape and Kirschner, 2004), indicating that its degradation was likely to be through auto-ubiquitylation in *cis* (supplementary material Fig. S3), as demonstrated for the Ubc7 protein (Ravid and Hochstrasser, 2007). The degradation of UbcH10 was prevented by blocking cells in metaphase with a non-degradable version of cyclin B1 (data not shown), indicating that UbcH10 destruction required a drop in cyclin B1-Cdk1 kinase activity, possibly to generate the anaphase form of the APC/C.

Our finding that UbcH10 disappeared in mitosis at the same time as the APC/C substrates Plk1 and Aurora A, and before others, such as Aurora B, was not consistent with UbcH10 acting as an autonomous regulator to inactivate the APC/C. This prompted us to determine when UbcH10 reappeared in the subsequent interphase. We analysed HeLa cells that had been released from a prometaphase block and found that UbcH10 did not completely disappear in G1 phase and began to increase at approximately the same time as cyclin A (Fig. 2A). To confirm our results in normal cells and to obtain better temporal resolution we turned to human retinal pigment epithelial (RPE) cells. Serum-starved control RPE cells had undetectable levels of UbcH10 and cyclin A. After adding back serum, UbcH10 levels began to rise detectably, 2 hours before cyclin A as cells progressed towards S phase (Fig. 2B), which was inconsistent with low levels of UbcH10 being required for cyclin A to be stabilised. Overall, we conclude that the disappearance of UbcH10 – which starts in mitosis – and its reappearance, together with or before cyclin A, in G1 phase are inconsistent with the autonomous APC/C oscillator model (Rape and Kirschner, 2004).

If UbcH10 is not part of an autonomous APC/C oscillator, what is its function in the cell cycle? UbcH10 has recently been proposed to antagonise the spindle-assembly checkpoint by ubiquitylating Cdc20 in order to release it from a complex with Mad2 that inactivates Cdc20 in the presence of improperly attached chromosomes. Overexpression of UbcH10 has been reported to overcome the spindle checkpoint in the presence of nocodazole (Rape and Kirschner, 2004; Reddy et al., 2007; Salic et al., 2004), and siRNA targeting UbcH10 delayed the cells progress through mitosis by ~30 minutes (Reddy et al., 2007). However, when we overexpressed wild-type UbcH10 tagged with Venus or co-expressed untagged wild-type UbcH10 with GFP [under an internal ribosome entry site (IRES)] to measure the transfection efficiency (supplementary material Fig. S4), we found that overexpressed UbcH10 was unable to override the spindle-assembly checkpoint. We assayed the response to the spindle checkpoint by using time-lapse microscopy and counting the number of cells that arrested in mitosis over time (Fig. 3A). In the presence of 50 ng/ml nocodazole, cells that overexpressed UbcH10 arrested in mitosis with a frequency similar to that of control cells (Fig. 3A), and 90% remained arrested until the end of the experiment (>18 hours; $n=224$). Similarly, all cells that overexpressed UbcH10 remained in mitosis when treated with 5 μg/ml taxol ($n=21$). In nocodazole-treated cells, a small minority of control (24 out of 584 cells) and slightly more UbcH10-expressing cells (24 out of 224 cells) did eventually exit mitosis after several hours (Fig. 3B), probably

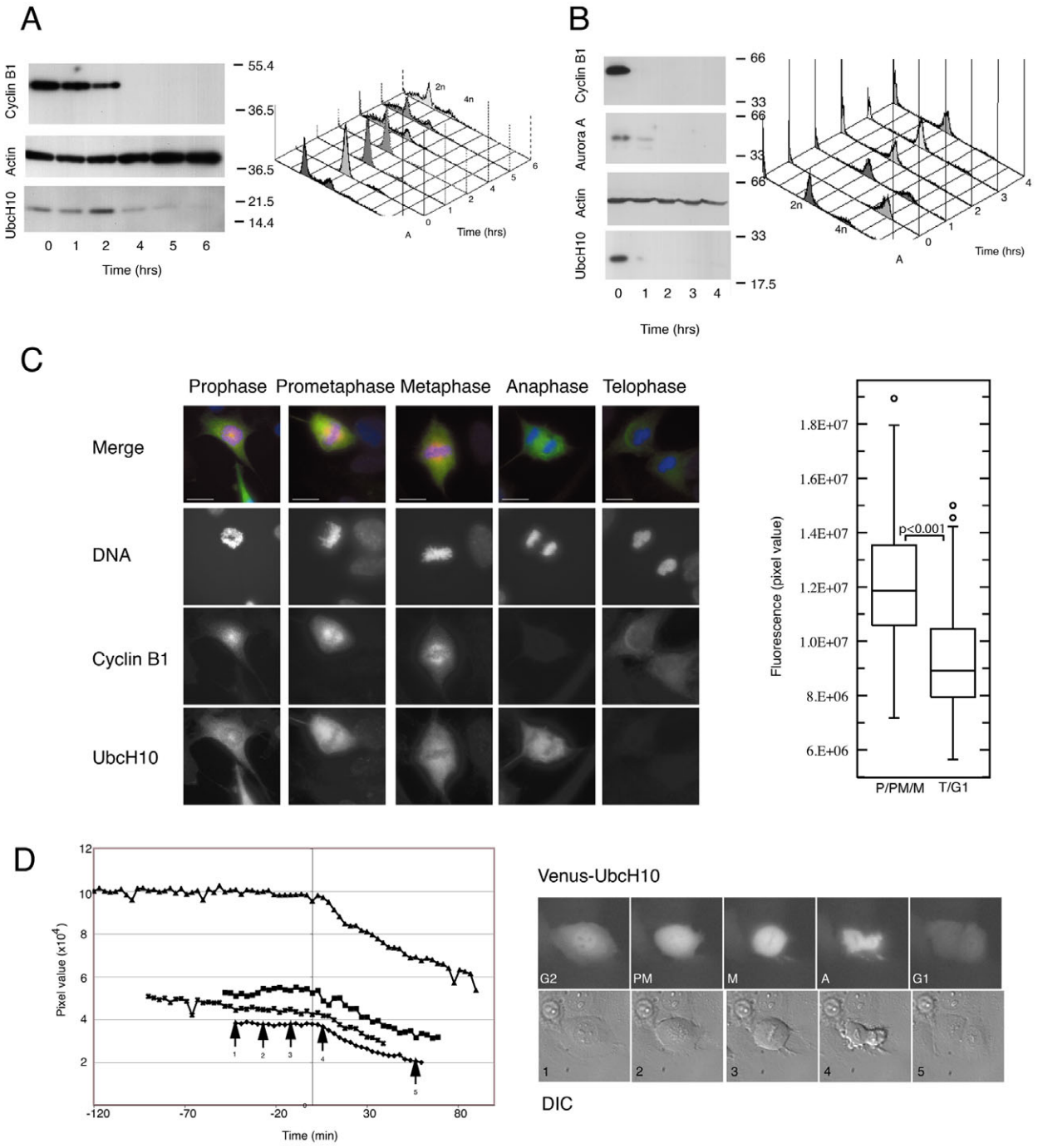


Fig. 1. UbcH10 is degraded in mitosis. (A) hTert-RPE cells cells were arrested in mitosis with 15 µM colcemid, collected by mitotic shake-off and released into fresh medium. Samples were taken at the indicated times and analysed by propidium iodide staining and flow cytometry (right panel) and for protein levels by immunoblotting with the relevant antibodies (left panel, molecular mass markers in kDa on the right). (B) HeLa cells were arrested in mitosis with 20 ng/ml nocodazole, collected by mitotic shake-off and released into fresh medium. Samples were taken at the indicated times and analysed using propidium iodide staining and flow cytometry (right panel), and using immunoblotting with the relevant antibodies to evaluate protein levels (left panel, molecular mass markers in kDa on the right). (C) Main panel (left). Asynchronous hTert-RPE cells were fixed and stained for immunofluorescence with anti-UbcH10 and anti-cyclin B1 antibodies and with Hoechst 33342 to visualise the DNA. Merged images on the top. Scale bar, 30 µm. The dot plot shows the levels of UbcH10 fluorescence in various mitotic cells measured using ImageJ software (arbitrary units). The small bars in the dot plot show the minimum and maximum values, and the box shows the first and third quartiles. The horizontal bar in the box is the median value. Suspected outliers (open circles) as determined by statistical analysis are shown. The two samples were compared applying Student's *t*-test and are significantly different from each other ($P<0.001$). P/PM/M, prophase + prometaphase + metaphase cells; T/G1, telophase + G1 cells that had not yet completed separation. More than 200 cells were analysed in three separate experiments. (D) Venus-tagged UbcH10 begins to be degraded in anaphase. (Top) Graph showing G2-phase HeLa cells that had been injected with an expression construct encoding venus-tagged UbcH10 (5 ng/µl). Degradation profiles of Venus-UbcH10 in four different cells (▲, ■, ✕,◆) representative of 38 cells analysed. Venus-UbcH10 fluorescence was measured at 3-minute intervals as cells progressed through mitosis. Time 0 was set to nuclear envelope breakdown (NEBD). (Bottom) DIC and fluorescence images (taken every 3 minutes) of the cell represented by ◆ in the graph above. The cell is shown at different points of the cell cycle: PM, prometaphase; M, metaphase; A, anaphase.

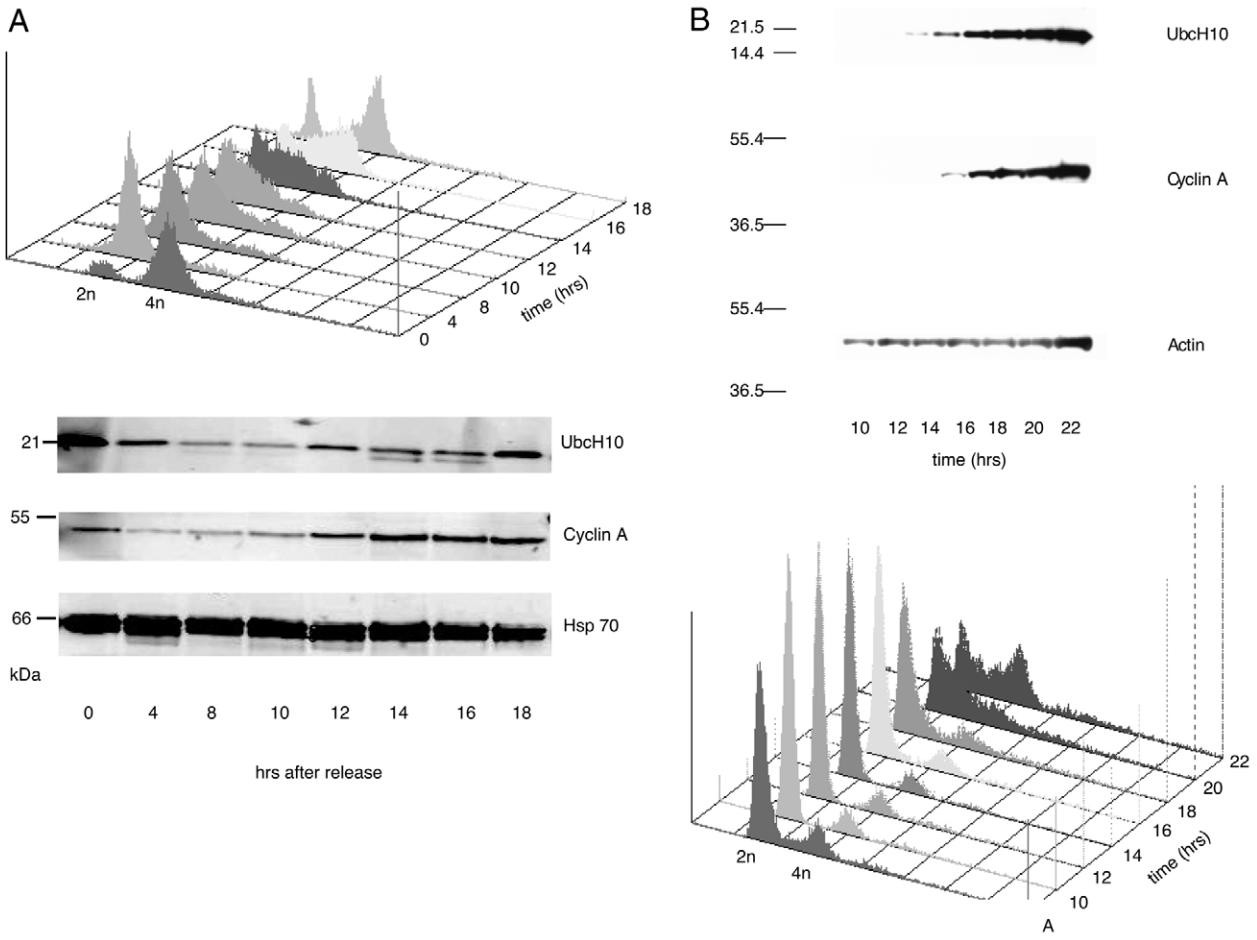


Fig. 2. UbcH10 levels increase at a similar time to cyclin A in late G1 phase. (A) HeLa cells were released from a dimethylenastron-induced prometaphase block and samples taken at the indicated times (hrs). Samples were analysed by propidium iodide staining and flow cytometry (top) and by immunoblotting with the indicated antibodies (bottom). Results are representative of two independent experiments. (B) Serum-starved hTert-RPE cells were released into the cell cycle by adding 20% serum and harvested at the indicated time points (hrs). Samples were analysed by propidium iodide staining and flow cytometry (bottom) and by immunoblotting with the indicated antibodies (top). Results are representative of five independent experiments.

through 'mitotic slippage' caused by the slow degradation of cyclin B1 in the presence of an active checkpoint (Brito and Rieder, 2006). By contrast, eliminating the checkpoint by using Mad2-targeting siRNA prevented cells from arresting in mitosis when treated with nocodazole, and cells completed mitosis within 22 minutes (n=56) (Fig. 3C). In support of our conclusion that UbcH10 does not affect the spindle-assembly checkpoint, there was no significant difference in the timing from nuclear envelope breakdown (NEBD) to anaphase (which is set by the checkpoint) in HeLa cells that overexpress UbcH10 (n=30, in three independent experiments) compared with untreated cells (n=98).

If UbcH10 were an important antagonist of the spindle checkpoint then reducing its levels should delay progress through mitosis, as previously reported (Reddy et al., 2007). To address this we used siRNA to reduce the levels of UbcH10 in both transformed and normal diploid cells. Three different oligonucleotides substantially reduced the level of UbcH10 by at least 90%, which is below that normally found at its nadir in G1 phase (supplementary material Fig. S5). We first analysed the effect of reducing UbcH10 levels on the behaviour of cells in mitosis. We used time-lapse DIC microscopy to assay the progress of cells through mitosis, and found that cells depleted of UbcH10 resembled control cells and exhibited no delay in progressing through mitosis. The time taken from NEBD to anaphase in control cells was 27±8 minutes (median, 26 minutes; n=36), and in UbcH10-depeleted cells it was 24±7 minutes (median, 22 minutes; n=43). There was no significant difference between these samples (Student's t-test, P=1.52). This indicated both that the spindle checkpoint was unaffected by depleting UbcH10 by more than 90%, and that UbcH10 is not essential to degrade APC/C substrates in mitosis, because the persistent presence of cyclin A, or securin and cyclin B1 would have delayed anaphase (Clute and Pines, 1999; den Elzen and Pines, 2001; Geley et al., 2001; Hagting et al., 2002; Murray et al., 1989). Therefore, we assayed APC/C activity by measuring the destruction of GFP-tagged cyclin-A (cyclin-A–GFP), the first APC/C substrate in mitosis and the one that has been reported to be most sensitive to a variation in UbcH10 levels. We found there was no difference in the timing or rate of cyclin-A–GFP destruction in UbcH10-depleted cells compared with control cells (Fig. 4A).

Since it did not appear to have a rate-limiting role in mitosis we asked whether UbcH10 was required in interphase. UbcH10 levels began to rise with or before cyclin A at the end of G1 phase. We therefore reasoned that UbcH10 might help to regulate the timing of DNA replication by destabilising cyclin A at this time, because

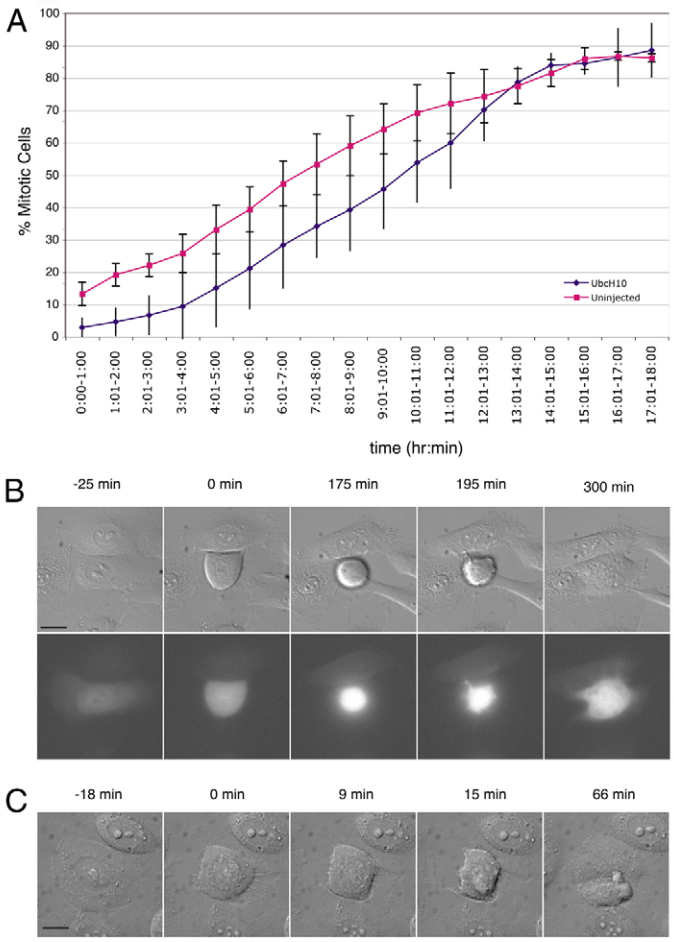


Fig. 3. Overexpressing UbcH10 does not overcome the spindle-assembly checkpoint. (A) HeLa cells that overexpress UbcH10 arrest in mitosis in response to nocodazole at a frequency similar to that of control cells. Cells were treated with 50 ng/ml nocodazole and entry into and exit from mitosis was monitored by time-lapse DIC microscopy with images taken every 15 minutes (not shown). The graph shows the mean values and ±s.e.m. of mitotic cells counted at hourly intervals from three independent experiments are shown. (B) A minority of cells that overexpress UbcH10 undergo mitotic slippage. HeLa cells that had been transfected to express UbcH10 and GFP under an IRES were incubated in 50 ng/ml nocodazole and monitored by fluorescence and DIC microscopy. Images were taken at 5-minute intervals. The cell shown is representative of 24 cells out of 224, and exits mitosis after more than 3 hours. Bar, 10 µm. (C) Cells with reduced levels of Mad2 cannot arrest in mitosis in response to nocodazole. HeLa cells were transfected with 200 nM siRNA targeting Mad2 and 48 hours later monitored by time-lapse DIC microscopy in the presence of 50 ng/ml nocodazole. The cell shown is representative of 56 cells out of 564 cells analysed. Bar, 10 µm.

raising the level of cyclin A in G1 phase accelerates the entry of cells into S phase (Resnitzky et al., 1995; Rosenberg et al., 1995). To investigate this we used serum-starved RPE cells to obtain better temporal resolution when cells entered S phase. We released serum-starved RPE cells into the cell cycle by adding serum, and assayed the levels of cyclin A and UbcH10 over time in control and UbcH10 siRNA-treated cells. Cyclin A accumulated slightly earlier in UbcH10 siRNA-treated RPE cells than in control cells, and we found that cells that lack UbcH10 entered S phase 2 hours earlier than control cells, as measured by flow cytometry and incorporation of BrdU (Fig. 4B). To determine whether the increase in cyclin A levels was required for the early initiation of DNA replication, we used siRNA to deplete UbcH10 and cyclin A together. Consistent with our hypothesis, depletion of cyclin A together with UbcH10 prevented premature DNA replication (Fig. 4B).

Discussion

Here, we have shown that human UbcH10 does not appear to be important to inactivate the spindle checkpoint, at least when present at levels similar to those in normal cells, nor is its behaviour consistent with UbcH10 forming an autonomous oscillator with the APC/C in G1 phase. Moreover, we find that UbcH10 does not have a rate-limiting role in the destruction of APC/C substrates in mitosis: instead we find that it is rate-limiting in late G1 phase where it is required to destabilise cyclin A and prevent premature DNA replication.

How can we reconcile our results with previous studies on the role of UbcH10? To start with its proposed role in the spindle checkpoint, one possibility is that the checkpoint is affected when UbcH10 is expressed to levels that are much higher than the endogenous level. We have expressed UbcH10 to levels at least 3.5 times above its endogenous level – and this is an underestimate because not all cells were transfected with the plasmid. However, in a previous study UbcH10 was added to yield a final concentration of 12.5 µM within in vitro extracts, which is 100 times more than UbcH10 endogenous levels of 100 nM (Reddy et al., 2007). Which of these experimental conditions is more closely related to a tumour cell is unclear because the extent to which individual cells overexpress UbcH10 in tumours has not been measured. Although at tissue level there is certainly a large increase in the amount of UbcH10, it is unclear how much of this is caused by the increase in the percentage of dividing cells in the tumour (Berlingieri et al., 2007; Pallante et al., 2005; Wagner et al., 2004). A previous study concluded that overexpressing UbcH10 abrogated the spindle checkpoint by arresting cells in mitosis with nocodazole and, counting the number of binucleate cells 18 hours later, they found a twofold increase in multi-nucleated cells compared with controls (Reddy et al., 2007). However, an alternative explanation is that more cells eventually exit mitosis trough mitotic slippage because of the slow destruction of cyclin B1 (Brito and Rieder, 2006). Using live-cell imaging we observed that all cells that overexpress UbcH10 arrest for many hours in mitosis, and only a very small minority (10%) eventually exit mitosis. This phenotype is more likely to represent mitotic slippage than a real abrogation of the checkpoint, but definitive proof would require an analysis of the kinetics of cyclin B1 degradation.

It is more difficult to reconcile our results on the cell-cycle stability of UbcH10 with those of the previous report, which claims that UbcH10 forms part of a cell-cycle oscillator (Rape and Kirschner, 2004). First, our experiments using RPE cells re-entering the cell cycle argue against the suggestion that UbcH10 levels must decrease in order for cyclin A to accumulate. In RPE cells, we reproducibly find that UbcH10 levels rise before those of cyclin A after the re-addition of serum. Second, UbcH10 levels rise before, or at the same time as, cyclin A levels in RPE and HeLa cells cycling through G1 phase. With respect to declining UbcH10 levels, we find that this begins in anaphase, inconsistent with the previous suggestion that it begins when the APC/C runs out of substrates. Both this previous study and ours used the same reagent (anti-UbcH10 from Boston Biochemical), and very similar synchronisation protocols for HeLa cells, except that Rape and Kirschner presynchronised cells with a single thymidine block followed 6 hours later by the addition of nocodazole for 12 hours.

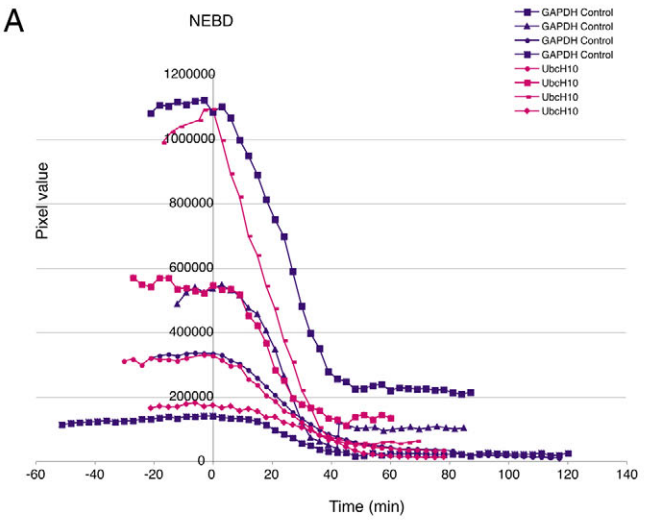
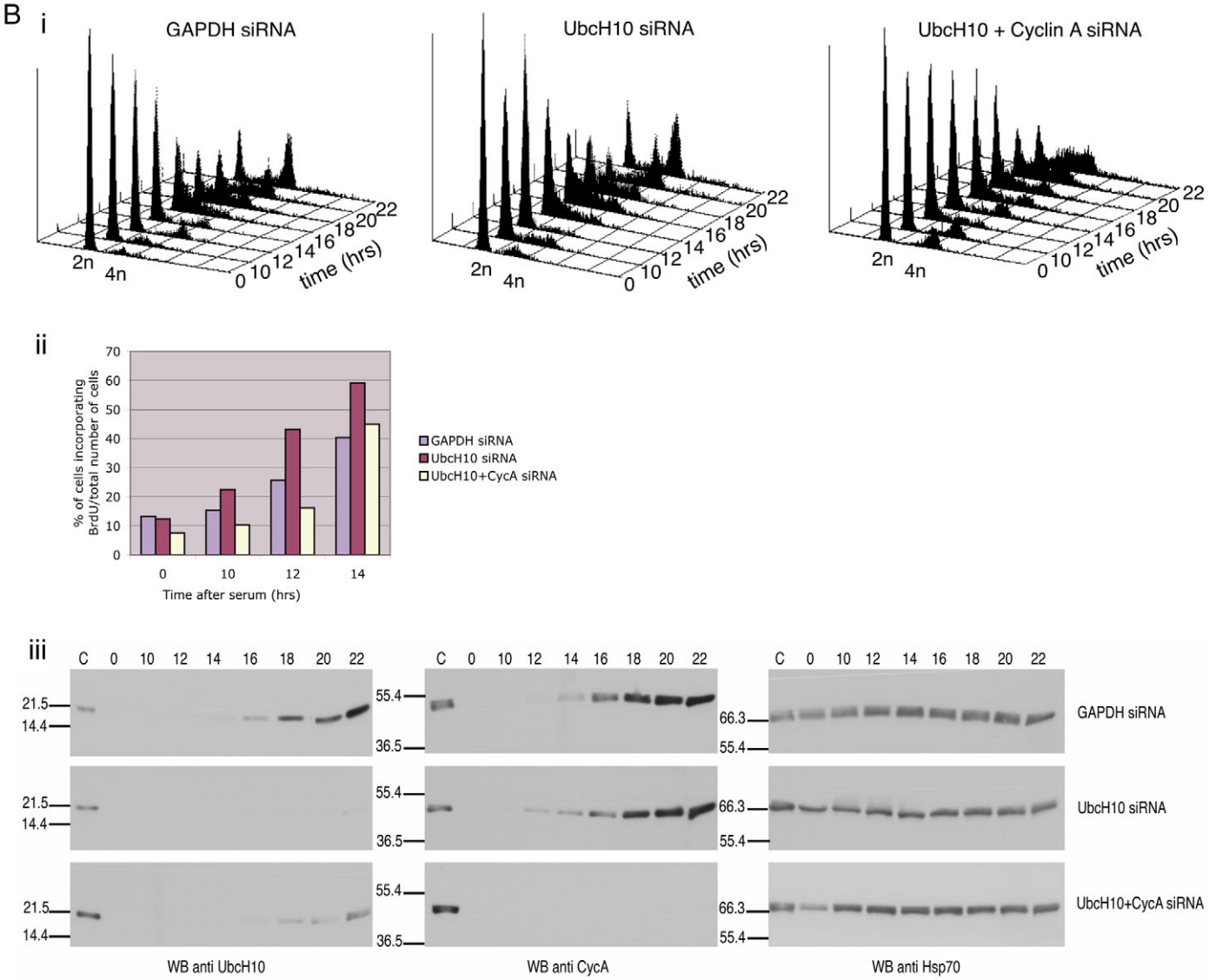


Fig. 4. Cells depleted of UbcH10 degrade cyclin A correctly in mitosis but not in the following interphase. (A) Cells depleted of UbcH10 degrade cyclin A with normal kinetics. HeLa cells treated with 40 nM of GAPDH (control) or UbcH10 siRNA were injected in late G2 phase with a plasmid encoding cyclin A tagged with Venus (5 ng/μl) and followed by time-lapse fluorescence and DIC microscopy. Images were taken every 3 minutes (exposure 200 mseconds), and the total fluorescence minus background was measured for each cell in successive images of a time series and plotted against time. The degradation curves shown are representative of nine control cells and 16 UbcH10 siRNA-treated cells in three independent experiments. Cells expressing low and high levels of cyclin-A–GFP were analysed to exclude any effects caused by overexpressing cyclin A. Four control cells expressing similar levels of cyclin A to four UbcH10-depleted cells are shown. Time 0 represents time of nuclear envelope breakdown. (B) Depleting UbcH10 causes cells to prematurely begin DNA synthesis, which requires cyclin A. hTERT-RPE cells were transfected with 40 nM control siRNA (GAPDH), and siRNA targeting UbcH10 or UbcH10+CycA, and synchronised in G0 by serum starvation for 24 hours. Cells were stimulated to re-enter the cell cycle by adding serum and harvested at the indicated time points. (i) Progression through the cell cycle was assayed by propidium iodide staining and flow cytometry analysis. (ii) Incorporation of BrdU that had been added 30 minutes before each time point. (iii) Protein levels were assayed by immunoblotting with the indicated antibodies. C, asynchronous cells. Results are representative of three independent experiments.



In our study we used a thymidine-aphidicolin pre-synchronisation regime followed 8 hours later by the addition of nocodazole for 10 hours. In both cases the majority of cells would have experienced a 6-8 hour delay in mitosis. In our hands, HeLa cells exhibit a very slow rate of mitotic slippage and remain arrested for 18 hours or more in nocodazole. Thus, it is unlikely that the difference in protein levels was caused by the mitotic arrest. Another possibility are differences in the kinetics with which the cells exited mitosis in the two studies. Our flow cytometry profiles show that ~100% of HeLa cells had exited mitosis by 2 hours (Fig. 1B), and more than 90% of RPE cells had done so by 6 hours (Fig. 1A). By comparison, ~70% of the HeLa cells were still in G2 or mitosis 6 hours after release from the nocodazole block in the earlier study (Rape and Kirschner, 2004, see figure 1A within). This would lead to an apparent delay in the disappearance of UbcH10.

Unfortunately, we cannot compare the analysis of UbcH10 levels by immunofluorescence in the two studies because only one cell at each stage of mitosis is shown in the study by Rape and Kirschner (Rape and Kirschner, 2004), and immunofluorescence as an assay for protein levels is inherently ambiguous because it cannot show the amount of protein the cell contained before it was fixed. In our hands, we find that there is a statistically significant ($P<0.001$) 33% decrease in the mean fluorescence of UbcH10 in a population of telophase and early G1 phase cells compared with a population of mitotic cells before anaphase, which strongly indicates that degradation must begin before telophase; and this agrees with our live-cell studies using a GFP-fusion protein whose degradation begins in anaphase.

We also found differences in the effects of depleting UbcH10 levels. In our hands, depleting UbcH10 by more than 90%, which reduced it to less than the level normally found in G1 phase, had no effect on APC/C activity in mitosis. We found no delay in the time taken for cells to progress from NEBD to anaphase and chromosomes correctly separated, indicating that neither cyclin A, nor cyclin B1 nor securin had been stabilised. These conclusions were supported by the degradation of a cyclin-A–GFP marker, which was degraded with normal kinetics in prometaphase in UbcH10-depleted cells. We are forced to conclude that in mammalian cells UbcH10 is either not essential for the APC/C to ubiquitylate its substrates in mitosis – for example, the APC/C may be able to use UbcH5 and E2-25K, as it can in vitro (Rodrigo-Brenni and Morgan, 2007) – or UbcH10 is not rate limiting and the low levels remaining in the cells are sufficient for the normal kinetics of the APC/C. Completely eliminating UbcH10 might inactivate the APC/C because cells of *vihar* mutants in *Drosophila* arrest in mitosis with defective spindles (Mathe et al., 2004), and fission yeast cells lacking the UbcH10 orthologue UbcP4/Ubc11 are unable to degrade cyclin B (Cdc13) (Seino et al., 2003).

Our observation that depleting UbcH10 does not affect progress through mitosis apparently contradicts a previous study in which RNAi was used to gain a similar reduction in UbcH10 that showed a delay in the degradation of cyclin A on immunoblots of synchronised cells (Rape and Kirschner, 2004). However, these data were also consistent with a delay caused by cells entering more slowly into mitosis. They also contradict another study (Reddy et al., 2007), in which cells were treated with siRNA to deplete UbcH10, and which found using time-lapse microscopy that this caused cells to delay anaphase by about 20 minutes. However, these authors used cells expressing histone H2B-GFP, which in our hands are more sensitive to photo-damage in mitosis, especially after siRNA treatment, and this can delay progress to anaphase.

The most reproducible effect we find in cells that lack UbcH10 is that they begin DNA replication about 2 hours earlier than control cells. This correlates with premature stabilisation of cyclin A, and the artificial increase of cyclin A levels in G1 phase has been demonstrated to cause cells to begin DNA replication early (Resnitzky et al., 1995; Rosenberg et al., 1995). Thus, we suggest that UbcH10 is required in late G1 phase for the APC/C to depress cyclin A levels, and this agrees with our observation that UbcH10 levels rise before those of cyclin A in RPE cells before they enter S phase. Why UbcH10 should be rate-limiting at this stage is not clear, although there is an increase in cyclin A mRNA transcription at this time (Huet et al., 1996; Liu et al., 1998; Pines and Hunter, 1990); therefore, the consequent increase in the rate of cyclin A synthesis may overcome the activity of the APC/C if only UbcH5 and E2-25K are present. We also conclude that the degradation of UbcH10 is unlikely to be the key event that inactivates the APC/C after mitosis. APC/C^{Cdh1} is more likely to be inactivated by the E2F-stimulated increase in Emi1 levels in G1 phase. Indeed, we and others have shown that in the absence of Emi1, or its *Drosophila* homologue Rca1, the APC/C remains active in S phase and neither cyclin A nor cyclin B1 can accumulate (Di Fiore and Pines, 2007; Grosskortenhaus and Sprenger, 2002; Machida and Dutta, 2007).

Materials and Methods

Antibodies

Antibodies against the following proteins were used: actin (mAb clone AC-40, Sigma) used at1:1000; cyclinA (mAb clone AT10.3, Cancer Research UK) used at 1:1000; cyclin A (BF683, Cell Signalling) used at 1:1000; cyclin B1 (mAb clone GNS1, BD Biosciences) against human cyclin B1 used at 1:1000 (immunoblots) and 1:500 (immunofluorescence); Emi1 (mAb clone 3D2D6, Zymed laboratories) against human Emi1 used at1:100; GFP (rabbit polyclonal, Clontech) used at 1:500; $(His)_4$, mAb against tetra His (Qiagen) used at 1:1000; Hsp70 (mAb clone brm-22, Sigma) used at 1:1000, Myc-epitope tag (mAb clone 9E10, Abcam Plc.) used at 1:500; UbcH10 (Boston Biochem) used at 1:1000 immunoblotting and 1:500 for immunofluorescence. Alexa-fluor-488, Alexa-fluor-568 and Alexa-fluor-680 conjugates, goat polyclonal antibodies against mouse IgG or rabbit IgG (Molecular Probes) used at 1:5000; HRP conjugates: goat polyclonal antibody against rabbit IgG or mouse IgG (DAKO) used at 1:5000.

Cell culture and synchronisation

HeLa cells were cultured in Advanced Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% foetal bovine serum (FBS), glutamax-I (200 μM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (250 ng/ml) at 37°C, 10% CO_2. hTERT-RPE cells were cultured in DMEM Nutrient Mixture F-12 HAM supplemented with 10% FBS, glutamax-I (200 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium bicarbonate (0.25%) and fungizone (250 ng/ml) at 37°C, 5% CO_2. HeLa cells were synchronised using a thymidine/aphidicolin regime as previously described (Pines and Hunter, 1989) with the omission of cytidine and thymidine in the medium used to release cells from aphidicolin. To block cells in mitosis, the culture medium was supplemented with nocodazole (20 ng/ml final concentration) or 10 μM dimethylenastron (Muller et al., 2007) (kind gift of Athanassios Giannis, University of Leipzig, Germany) and cells incubated for a further 12 hours after release from aphidicolin. Cells were released by mitotic shake off and washed three times before replating. hTERT-RPE cells were synchronised using serum withdrawal for 24 hours. To release cells from G0 cell culture medium supplemented with 20% FBS was added. To block hTERT-RPE cells in mitosis, unsynchronised cells were grown to 70% confluence and 15 μM colcemid was added for 16 hours. Cell synchrony was assayed by staining cells with propidium iodide as previously described (Pines and Hunter, 1989) for analysis by flow cytometry on a Becton Dickinson FACSCalibur.

Transfection of cell lines with plasmid DNA and siRNA

Cells were transfected with DNA-Lipofectamine 2000 complexes according to the manufacturers instructions. Pre-annealed control (recognising GAPDH, Ambion) or UbcH10 siRNA (UbcH10 Smart pool, Dharmacon, and individual oligonucleotides from Dharmacon and Ambion) oligonucleotides were complexed with oligofectamine and added to culture medium lacking serum as advised by the manufacturers.

Immunoblotting

Cells were pelleted three times by centrifugation and boiled in 2×SDS sample loading buffer (without reducing agents) syringed repeatedly using a fine gauge needle to

reduce viscosity, and loaded on either 15% acrylamide gels, or precast 4-12% NuPage Bis Tris gradient gels, as appropriate. For Fig. 4, cells were lysed in RIPA buffer and protein levels quantified before mixing with 2×SDS sample buffer. Proteins resolved by SDS-PAGE were transferred to Immobilon P polyvinylidene difluoride (PVDF) membrane (for ECL) or low fluorescence PVDF membrane (for LiCOR) by semi-dry western blotting. Protein transfer and molecular mass markers were assayed by staining the membranes in Ponceau S. Membranes were blocked in 5% milk in PBS, (plus 0.2% Tween-20 for ECL) rotating for one hour at room temperature and incubated with primary antibody in blocking solution rotating for one hour at room temperature. Membranes were washed three times for 5 minutes in PBS, 0.2% Tween-20 and incubated with secondary antibody, diluted as required in blocking buffer (plus 0.02% SDS for LiCOR), rotating for 30 minutes at room temperature (in the dark for LiCOR), and washed three times for 5 minutes in PBS, 0.2% Tween-20. Membranes were processed for standard western blotting by ECL^{plus} according to manufacturer's protocol, or for quantitative western blotting using the LiCor Odyssey gel scanner.

In vitro ubiquitylation assay

HeLa cells were harvested by trypsinisation, washed first in culture medium and then in PBS Mg^{2+}/Ca^{2+} and resuspended in ice-cold 1× lysis buffer (50 mM Tris.HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, plus EDTA free protease inhibitors (Roche) and 1 μg/ml cytochalasin B, 0.1 μg/ml okadaic acid and 2 μg/ml microcystin-LR at a concentration of 10^7 cells/ml. After 15 minutes incubation on ice, cells were spun at 20,000 ***g***, 4°C for 15 minutes, and supernatants harvested. Ubiquitin binding assays were assembled by addition of 1.75 μl of each of the following to a 60 μl volume of supernatant; 200 mM $MgCl_2$, 40 mM phosphocreatine, 80 mM ATP, 750 U/ml phosphocreatine kinase. The reaction was initiated by addition of 6 μl of 10 mg/ml bovine $(His)_6$ ubiquitin, and reactions incubated for 60 minutes at room temperature. Samples were processed by SDS-PAGE, following addition of 30 μl LDS-Sample buffer containing 4 M urea and boiling.

Immunofluorescence

Cells were fixed and stained in paraformaldehyde or methanol:acetone (50:50 v/v) as previously described (Pines, 1997) except that 100 mM lysine hydochloride was added to the blocking solution to reduce the background. Cells were stained with anti-UbcH10 (Boston Biochem, 1:500) and anti-cyclin B1 (GNS1 mAb, BD, 1:500) and mounted with Prolong anti-fade mounting medium (Molecular Probes). Cells were analyzed by epifluorescence microscopy using a Deltavision image restoration system (Applied Precision) and softworx software, to generate 3 dimensional projections from *z*-stacks. Adobe Photoshop software was used to analyse TIFF files generated from raw output files with no in-software adjustment applied, setting the output of all images to the same level for direct comparison. Data were plotted and analysed using statistical software at http://www.physics.csbsju.edu/.

Microinjection and time-lapse imaging

Cells were microinjected and filmed in Leibovitz L-15 medium with L-glutamine, without Phenol Red, supplemented with 10% FBS, penicillin (10 U/ml) and streptomycin (10 μg/ml), on a Bioptechs ΔT heating staged attached to a Leica DMIRBE microscope. Cells were microinjected with cDNAs (diluted at 5-50 ng/μl in TE, pH 8,0) and assayed by time-lapse fluorescence and DIC microscopy as previously described (Karlsson and Pines, 1998). Parameters used for all images captured were exposure time 200 mseconds, 40× oil objective lens with a numerical aperture of 1.0 and an image size of 206×207 pixels. All images were saved in 16 bit IP lab native format and analysed using Image J software (http://rsb.info.nih.gov/ij/index.html) as previously described (Lindon and Pines, 2004).

We thank Athanassios Giannis (University of Leipzig, Germany) for dimethylenastron. We thank all members of the lab for discussions and criticisms.

References

Aristarkhov, A., Eytan, E., Moghe, A., Admon, A., Hershko, A. and Ruderman, J. V. (1996). E2-C, a cyclin-selective ubiquitin carrier protein required for the destruction of mitotic cyclins. *Proc. Natl. Acad. Sci. USA* **93**, 4294-4299.

Berlingieri, M. T., Pallante, P., Guida, M., Nappi, C., Masciullo, V., Scambia, G., Ferraro, A., Leone, V., Sboner, A., Barbareschi, M. et al. (2007). UbcH10 expression may be a useful tool in the prognosis of ovarian carcinomas. *Oncogene* **26**, 2136-2140.

Brito, D. A. and Rieder, C. L. (2006). Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr. Biol.* **16**, 1194-1200.

Clute, P. and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat. Cell Biol.* **1**, 82-87.

den Elzen, N. and Pines, J. (2001). Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J. Cell Biol.* **153**, 121-136.

Di Fiore, B. and Pines, J. (2007). Emi1 is needed to couple DNA replication with mitosis but does not regulate activation of the mitotic APC/C. *J. Cell Biol.* **177**, 425-437.

Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J.-M. and Hunt, T. (2001). APC/C-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J. Cell Biol.* **153**, 137-148.

Grosskortenhaus, R. and Sprenger, F. (2002). Rca1 inhibits APC-Cdh1(Fzr) and is required to prevent cyclin degradation in G2. *Dev. Cell* **2**, 29-40.

Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J. M. and Pines, J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J. Cell Biol.* **157**, 1125-1137.

Huet, X., Rech, J., Plet, A., Vie, A. and Blanchard, J. M. (1996). Cyclin a expression is under negative transcriptional control during the cell cycle. *Mol. Cell. Biol.* **16**, 3789-3798.

Karlsson, C. and Pines, J. (1998). Green fluorescent protein. In *Cell Biology: A Laboratory Handbook*. Vol. 4 (ed. J. Celis), pp. 246-252. San Diego: Academic Press.

King, R. W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81**, 279-288.

Lindon, C. and Pines, J. (2004). Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *J. Cell Biol.* **164**, 233-241.

Liu, N., Lucibello, F. C., Engeland, K. and Muller, R. (1998). A new model of cell cycle-regulated transcription: repression of the cyclin A promoter by CDF-1 and anti-repression by E2F. *Oncogene* **16**, 2957-2963.

Machida, Y. J. and Dutta, A. (2007). The APC/C inhibitor, Emi1, is essential for prevention of rereplication. *Genes Dev.* **21**, 184-194.

Mathe, E., Kraft, C., Giet, R., Deak, P., Peters, J. M. and Glover, D. M. (2004). The E2-C vihar is required for the correct spatiotemporal proteolysis of cyclin B and itself undergoes cyclical degradation. *Curr. Biol.* **14**, 1723-1733.

Muller, C., Gross, D., Sarli, V., Gartner, M., Giannis, A., Bernhardt, G. and Buschauer, A. (2007). Inhibitors of kinesin Eg5: antiproliferative activity of monastrol analogues against human glioblastoma cells. *Cancer Chemother. Pharmacol.* **59**, 157-164.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**, 280-286.

Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K. and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87-90.

Okamoto, Y., Ozaki, T., Miyazaki, K., Aoyama, M., Miyazaki, M. and Nakagawara, A. (2003). UbcH10 is the cancer-related E2 ubiquitin-conjugating enzyme. *Cancer Res.* **63**, 4167-4173.

Pallante, P., Berlingieri, M. T., Troncone, G., Kruhoffer, M., Orntoft, T. F., Viglietto, G., Caleo, A., Migliaccio, I., Decaussin-Petrucci, M., Santoro, M. et al. (2005). UbcH10 overexpression may represent a marker of anaplastic thyroid carcinomas. *Br. J. Cancer* **93**, 464-471.

Pines, J. (1997). Localization of cell cycle regulators by immunofluorescence. In *Methods Enzymol.* Vol. 283 (ed. W. G. Dunphy), pp. 99-113. San Diego: Academic Press.

Pines, J. and Hunter, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. *Cell* **58**, 833-846.

Pines, J. and Hunter, T. (1990). Human cyclin A is adenovirus E1A-associated protein p60, and behaves differently from cyclin B. *Nature* **346**, 760-763.

Rape, M. and Kirschner, M. W. (2004). Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. *Nature* **432**, 588-595.

Ravid, T. and Hochstrasser, M. (2007). Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nat. Cell Biol.* **9**, 422-427.

Reddy, S. K., Rape, M., Margansky, W. A. and Kirschner, M. W. (2007). Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* **446**, 921-925.

Resnitzky, D., Hengst, H. and Reed, S. I. (1995). Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by $p27^{Kip1}$. *Mol. Cell. Biol.* **15**, 4347-4352.

Rodrigo-Brenni, M. C. and Morgan, D. O. (2007). Sequential E2s drive polyubiquitin chain assembly on APC targets. *Cell* **130**, 127-139.

Rosenberg, A. R., Zindy, F., Le Deist, F., Mouly, H., Metezeau, P., Brechot, C. and Lamas, E. (1995). Overexpression of human cyclin A advances entry into S phase. *Oncogene* **10**, 1501-1509.

Salic, A., Waters, J. C. and Mitchison, T. J. (2004). Vertebrate shugoshin links sister centromere cohesion and kinetochore microtubule stability in mitosis. *Cell* **118**, 567-578.

Seino, H., Kishi, T., Nishitani, H. and Yamao, F. (2003). Two ubiquitin-conjugating enzymes, UbcP1/Ubc4 and UbcP4/Ubc11, have distinct functions for ubiquitination of mitotic cyclin. *Mol. Cell. Biol.* **23**, 3497-3505.

Sironi, L., Mapelli, M., Knapp, S., De Antoni, A., Jeang, K. T. and Musacchio, A. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. *EMBO J.* **21**, 2496-2506.

Wagner, K. W., Sapinoso, L. M., El-Rifai, W., Frierson, H. F., Butz, N., Mestan, J., Hofmann, F., Deveraux, Q. L. and Hampton, G. M. (2004). Overexpression, genomic amplification and therapeutic potential of inhibiting the UbcH10 ubiquitin conjugase in human carcinomas of diverse anatomic origin. *Oncogene* **23**, 6621-6629.

Yamanaka, A., Hatakeyama, S., Kominami, K., Kitagawa, M., Matsumoto, M. and Nakayama, K. (2000). Cell cycle-dependent expression of mammalian E2-C regulated by the anaphase-promoting Complex/Cyclosome. *Mol. Biol. Cell* **11**, 2821-2831.