Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry

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Oscillations in cyclin-dependent kinase (CDK) activity drive the somatic cell cycle. After entry into mitosis, CDKs activate the anaphase-promoting complex (APC), which then promotes cyclin degradation and mitotic exit. The re-accumulation of cyclin A causes the inactivation of APC and entry into S phase, but how cyclin A can accumulate in the presence of active APC has remained unclear. Here we show that, during G1, APC autonomously switches to a state permissive for cyclin A accumulation. Crucial to this transition is the APC^{Cdh1}-dependent autoubiquitination and proteasomal degradation of the ubiquitin-conjugating enzyme (E2) UbcH10. Because APC substrates inhibit the autoubiquitination of UbcH10, but not its E2 function, APC activity is maintained as long as G1 substrates are present. Thus, through UbcH10 degradation and cyclin A stabilization, APC autonomously downregulates its activity. This indicates that the core of the metazoan cell cycle could be described as a self-perpetuating but highly regulated oscillator composed of alternating CDK and APC activities.

The machinery of the somatic cell cycle integrates external signals with a basic oscillator, thereby coordinating events required for cell growth and division. Central to this machinery are cyclins, which activate specific kinases after association. During G1, D- and E-type cyclins convey exogenous signals to the basic cell cycle machinery by phosphorylating the retinoblastoma protein (pRb), leading to the activation of E2F transcription factors and entry into S phase. Surprisingly, neither D- or E-type cyclins nor the Cdk2 kinase is required for cell cycle progression in mammals[1,2]. In contrast, cyclin A, expressed from late G1 until mitosis, is essential for both the G1/S and G2/M transitions[3,4].

Cyclin A binds and activates Cdk1 and Cdk2. Among its critical substrates at the G1/S transition is the anaphase-promoting complex/cyclosome (APC). APC is a multiprotein ubiquitin ligase that initiates sister chromatid separation at the metaphase/anaphase transition (reviewed in ref. 5). It also triggers exit from mitosis and is thought to remain active throughout G1. Because APC controls the degradation of proteins essential for DNA replication, its activity is downregulated before entry into S phase[6,7]. APC inactivation also allows the accumulation of mitotic cyclins during S and G2, and thereby enables entry into the next mitosis. APC can be inhibited at the G1/S transition either by binding the Emi1 inhibitor or by phosphorylation of the APC activator Cdh1 by cyclin A/Cdk2 (refs 8–10). The expression of a non-phosphorylatable Cdh1 mutant, the ablation of cyclin A function in late G1 or the downregulation of Emi1 delay entry into S phase[3,8,11]. Accordingly, heterologous expression of either cyclin A or Emi1 overcomes a G1 arrest mediated by constitutively active pRb[8,9]. These results indicate that APC inactivation is required for S-phase entry and that it can be mediated by either cyclin A or Emi1.

Although cyclin A inactivates APC in G1, it is itself an APC substrate that is efficiently degraded in prometaphase[12–16]. Degradation of cyclin A is required for proper chromosome congression, progression beyond metaphase, and establishment of G1 during *Drosophila* development. This raises a mechanistic paradox: if APC remains active throughout G1, how does cyclin A accumulate in late G1 and inhibit APC? Here we show that cyclin A can begin to accumulate only after the APC-specific ubiquitin-conjugating enzyme (E2) UbcH10 has been degraded. The cause of the degradation of UbcH10 is its autoubiquitination, which is promoted by APC^{Cdh1}. We find that APC substrates inhibit the autoubiquitination of UbcH10 but not its E2 function; hence APC remains active until its substrates have been destroyed. APC therefore downregulates its activity through an autonomous mechanism centred on the degradation of a specific E2 enzyme. This mechanism couples the mitotic activation of APC to its inactivation before S-phase entry and suggests that the metazoan cell cycle is built around a self-perpetuating but highly regulated oscillator.

Cyclin A is stabilized after UbcH10 is degraded in G1

To study the activity of APC in G1, we prepared extracts from synchronized cells. The APC substrates securin, geminin and cyclin B1 were stable in extracts from nocodazole-arrested cells, which is consistent with the inhibition of APC by the spindle checkpoint (Supplementary Fig. S1a). In extracts from cells synchronized in G1, securin, geminin and cyclin B1 were degraded efficiently, and their degradation was inhibited by the competitive APC inhibitor N-cyclin B (NcycB). These extracts therefore recapitulate stage-specific APC activity. In contrast with the rapid and complete degradation of other APC substrates, cyclin A degradation was inefficient in these G1 extracts.

Because cyclin A is ubiquitinated by APC^{Cdh1} (ref. 11), it was assumed that cyclin A is unstable throughout G1. However, previous experiments did not distinguish between early and late G1 (refs 12, 17). To analyse whether cyclin A was stabilized at a specific stage during G1, we prepared extracts from cells that had been released from nocodazole arrest for various durations (Fig. 1a). Cyclin A was degraded in extracts from nocodazole-arrested cells in the presence of an active spindle checkpoint, although with lower efficiency (0 h; refs 12–14). When extracts were prepared from cells in late mitosis or early G1 (2 h), cyclin A rapidly turned over in an NcycB-sensitive and thus APC-dependent manner. Intriguingly, cyclin A degradation gradually diminished in later G1 extracts, whereas the APC^{Cdh1} substrates securin, geminin and Cdc20 were all still degraded. Thus, cyclin A degradation, which occurs rapidly in mitosis and in early G1, was blocked as cells progress through G1.

To identify limiting components for cyclin A degradation in mid/late G1, we supplemented G1 extracts with proteins known to

function with APC. Both E2 enzymes that interact with APC (UbcH5α and UbcH10), but not their inactive mutants (UbcH5α^{C85S} and UbcH10^{C114S}), promoted cyclin A degradation, even in late-G1 extracts (Fig. 1b). Cyclin A degradation was only minimally stimulated by UbcH10 in extracts of cells arrested in S phase, when APC is inactive (Fig. 1b). Thus, E2 enzymes are limiting for cyclin A degradation in mid/late G1.

In the reciprocal experiment we asked whether cyclin A degradation in early-G1 extracts is reduced by lowering E2 activity. Immunodepletion of one of these E2 enzymes, UbcH10, inhibited cyclin A degradation almost as effectively as the APC inhibitor NcycB (Fig. 1c). The addition of UbcH10 to depleted extracts restored cyclin A degradation, just as it had supported cyclin A degradation in mid/late-G1 extracts. By contrast, UbcH10 depletion only weakly affected the degradation of other APC substrates, such as securin, indicating that the remaining UbcH5 is sufficient to catalyse securin but not cyclin A ubiquitination. Consistent with these observations was our finding that UbcH10 supported cyclin A

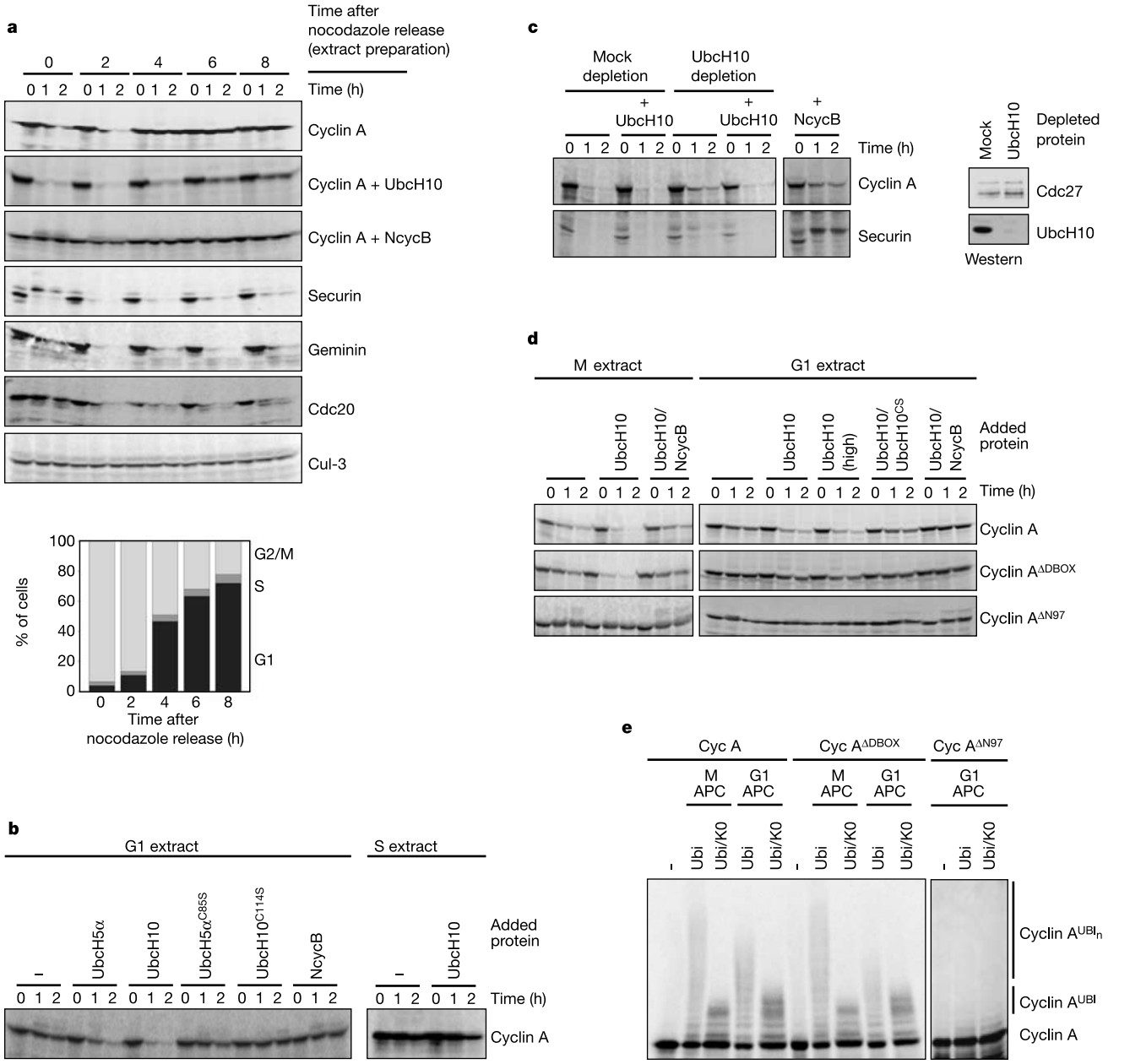

Figure 1 Cyclin A degradation is specifically impaired at low concentrations of UbcH10. **a**, The timing of cyclin A degradation in G1. Extracts were prepared at indicated times after release from arrest with nocodazole. The degradation of ^{35}S-labelled proteins was analysed over time by SDS–PAGE and autoradiography. The cell-cycle stage of the cells used for extract preparation as determined by fluorescence-activated cell sorting analysis is depicted below (light grey, G2/M; mid-grey, S; black, G1). **b**, Cyclin A degradation in G1 extracts (4 h after nocodazole release) is accelerated by the addition of UbcH10 or UbcH5α, but not of inactive UbcH10^{C114S} or UbcH5α^{C85S}. Only minimal effects are observed in extracts of cells synchronized in S phase. **c**, Immunodepletion of UbcH10 in early G1 extracts (2 h after nocodazole release). Mock- or UbcH10-immunodepleted extracts were analysed for degradation of ^{35}S-labelled cyclin A and securin. Immunoblots against the APC subunit Cdc27 and UbcH10 are shown on the right. **d**, Degradation of cyclin A, cyclin A$^{\Delta DBOX}$ and cyclin A$^{\Delta N97}$ in mitosis and in G1. The degradation of ^{35}S-labelled proteins was analysed in extracts of cells arrested with nocodazole (M) or in G1 cells (G1). UbcH10 and NcycB were added as indicated. **e**, Ubiquitination of cyclin A, cyclin A$^{\Delta DBOX}$ and cyclin A$^{\Delta N97}$ by APC^{Cdc20} (M APC) and APC^{Cdh1} (G1 APC). ^{35}S-labelled substrates were incubated with UbcH10, APC and either ubiquitin (Ubi) or a ubiquitin mutant lacking all lysines (Ubi/K0). The reaction products were resolved by SDS–PAGE and autoradiography.

ubiquitination by APC^{Cdh1} more efficiently than UbcH5 (Supplementary Fig. S1b). Taken together, these results indicate that cyclin A ubiquitination by APC^{Cdh1} is highly sensitive to the concentration of UbcH10.

The G1-specific ubiquitination of cyclin A by APC^{Cdh1} is different from its mitotic ubiquitination by APC^{Cdc20}. In mitotic extracts activated by UbcH10, a mutation of an amino-terminal D-box (cyclin A$^{R47A/L50V}$; referred to as cyclin A$^{\Delta DBOX}$) had no effect on cyclin A stability (Fig. 1d; refs 12–14). However, in G1 the situation was different. Cyclin A$^{\Delta DBOX}$ (but not cyclin A) was stable in UbcH10-supplemented G1 extracts and not efficiently multiubiquitinated by APC^{Cdh1} (Fig. 1d, e). The multiubiquitination and degradation of cyclin A in G1 therefore require a conserved D-box and a threshold concentration of APC-specific E2 enzymes.

Because the degradation of cyclin A was very sensitive to the UbcH10 concentration in extracts, we analysed the concentrations of UbcH5 and UbcH10 throughout the cell cycle by western blotting and immunofluorescence (Fig. 2). In both experiments we found that UbcH10 concentration fluctuated, whereas UbcH5 concentration remained constant. UbcH10 accumulated with cyclin B1 and Plk1 as cells entered mitosis, and therefore its concentration was high at the time of cyclin A degradation (Fig. 2a). UbcH10 remained stable during mitosis and in early G1 (Fig. 2b). Its concentration decreased only later in G1 with a significant delay compared with the APC^{Cdh1} substrates Cdc20 and Plk1, which were degraded in anaphase and telophase, respectively (Fig. 2b, c). The concentration of cyclin A began to increase shortly after UbcH10 was degraded. UbcH10 is therefore present in G1 extracts that degrade cyclin A, but its concentration declines before cyclin A accumulates late in G1. Together, these results indicate that the loss of UbcH10 in G1 stabilizes cyclin A, allowing it to accumulate in late G1.

APC-dependent regulation of UbcH10

Most importantly, UbcH10 is itself regulated by APC. APC^{Cdh1} purified from G1 cells, but not mitotic APC^{Cdc20}, efficiently catalysed UbcH10 multiubiquitination (Fig. 3a). Consistently, UbcH10 was rapidly degraded in G1 extracts by the proteasome but was stable in extracts of mitotic cells that were activated to degrade APC^{Cdc20} substrates by the addition of UbcH5 or UbcH10 (Fig. 3g). The multiubiquitination and degradation of UbcH10 in G1 were inhibited by both unlabelled UbcH10 and UbcH10^{C114S} in a concentration-dependent manner, which is consistent with a requirement for APC binding (Fig. 3a, b; Supplementary Fig. S2a). UbcH5 also inhibited APC-dependent UbcH10 ubiquitination.

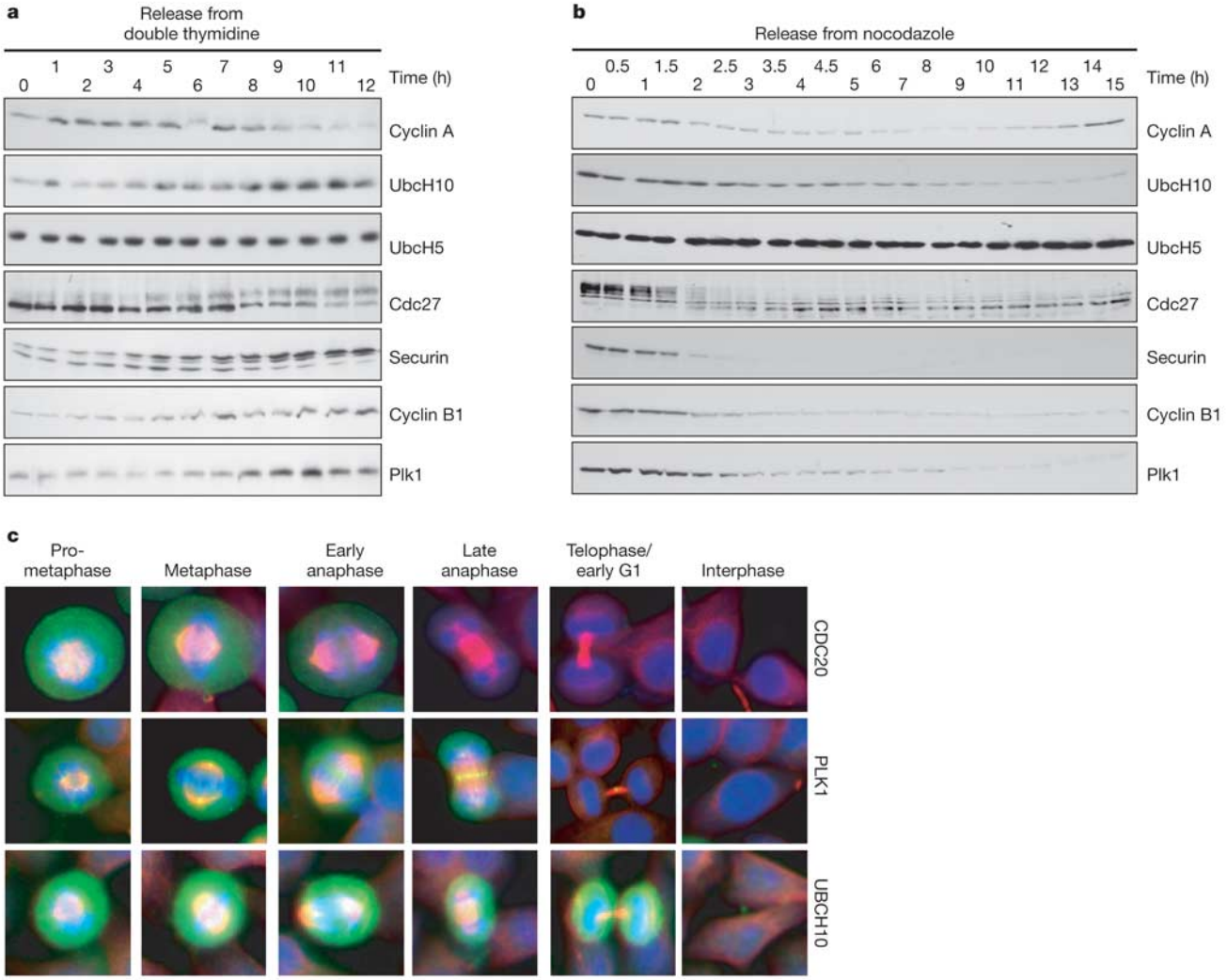

Figure 2 Cell-cycle regulation of E2 enzymes and cyclin A. **a**, Release of HeLa S3 cells from a double-thymidine arrest (early S phase) into nocodazole (mitosis). The expression of the depicted proteins was analysed by western blotting. **b**, Release of HeLa S3 cells from a nocodazole arrest, and analysis of protein expression by western blotting. **c**, Analysis of UbcH10, Cdc20 and Plk1 expression (green) in asynchronous HeLa cells by immunofluorescence. Single representative cells of subsequent mitotic stages are depicted. β-Tubulin staining (red) indicates the formation of mitotic spindles. DNA is stained with 4,6-diamidino-2-phenylindole (blue).

This probably reflects the inability of APC to interact functionally with two different E2 enzymes at the same time, because both E2 enzymes require the APC11 subunit for ubiquitination[18]. In line with this interpretation, but in contrast with a previous report[19], we found that UbcH10 multiubiquitination was strictly dependent on the activity of UbcH10 itself. Inactive UbcH10^{C114S} was not multiubiquitinated *in trans* by active UbcH10 or UbcH5 (Fig. 3a). Accordingly, UbcH10^{C114S} was not degraded in G1 extracts, but accumulated as a monoubiquitinated protein (Fig. 3g; data not shown). In summary, these results show that UbcH10 undergoes autoubiquitination *in cis* catalysed by APC^{Cdh1}, leading to its degradation in G1.

As mentioned above, there is a profound delay in the disappearance of UbcH10 relative to the onset of APC^{Cdh1} activity. At least two mechanisms contribute to this; intriguingly, both are intrinsic to APC. First, UbcH10 autoubiquitination *in cis* is significantly slower than geminin and cyclin A ubiquitination, both of which occur *in trans* (Fig. 3c). Second, other APC^{Cdh1} substrates interfere strongly with UbcH10 autoubiquitination. Increasing amounts of NcycB and securin, both of which are APC^{Cdh1} substrates, inhibited the autoubiquitination of UbcH10 in a concentration-dependent manner, whereas non-substrate proteins had no effect (Fig. 3d; Supplementary Fig. S2b). Accordingly, in extracts both APC^{Cdh1} substrates stabilized UbcH10 (Fig. 3g). APC substrates therefore inhibit the slow autoubiquitination of UbcH10 and its degradation.

Surprisingly, these effects are not reciprocal. Whereas cyclin B and securin inhibited UbcH10 autoubiquitination, UbcH10 in turn did not block the ubiquitination of canonical APC substrates even when added at a 1,000-fold excess (Fig. 3e). Instead, increasing concentrations of UbcH10 further promoted the ubiquitination of APC^{Cdh1} substrates. By contrast, cyclin B and securin, both of which contain a well-characterized D-box, competed with APC^{Cdh1} substrates such as geminin in this assay (Fig. 3e). Consistently, high concentrations of UbcH10 did not impair APC-dependent degradation in G1 extracts, but stimulated it (data not shown). UbcH10 also did not interfere with substrate binding to APC^{Cdh1}; similarly,

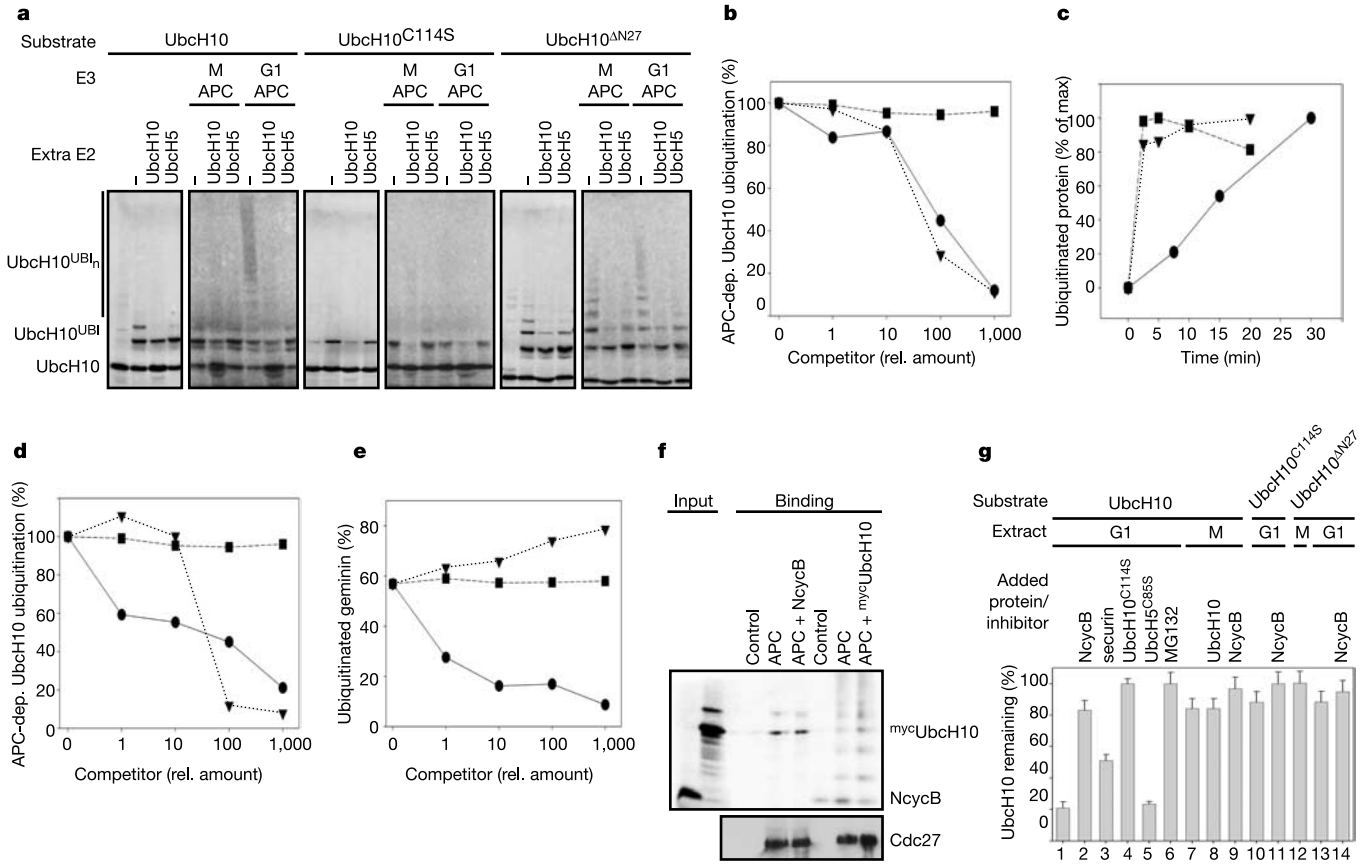

Figure 3 Degradation of UbcH10 in G1 is mediated by APC-dependent autoubiquitination. **a**, Analysis of UbcH10 autoubiquitination. ^{35}S-labelled UbcH10, UbcH10^{C114S} and UbcH10$^{\Delta N27}$ were incubated with buffer alone, with E1 (–) or with APC from nocodazole-arrested (M APC) or G1 (G1 APC) cells. Unlabelled E2 was added as indicated, and reaction products were analysed by autoradiography. Monoubiquitination of all UbcH10 mutants (UbcH10^{UBI}) is observed after the addition of E1. Multiubiquitination (UbcH10^{UBIn}) depends on UbcH10 activity and on G1 APC. **b**, Regulation of UbcH10 autoubiquitination by APC binding. Autoubiquitination of ^{35}S-labelled UbcH10 was analysed as described, and increasing concentrations of unlabelled UbcH10 (circles), UbcH10^{C114S} (triangles) or Mad2 (squares) were added. **c**, Kinetics of UbcH10 autoubiquitination. UbcH10 autoubiquitination (circles) was performed in the presence of G1 APC, and aliquots were removed at the indicated times. Similarly, ubiquitination of geminin (triangles) and cyclin A (squares) by APC^{Cdh1} in the presence of UbcH10 was analysed. **d**, APC substrates inhibit UbcH10 autoubiquitination. ^{35}S-labelled UbcH10 was incubated with G1 APC in the presence of increasing amounts of unlabelled APC substrates N-cyclin B1 (circles) and securin (triangles), and of the control protein Mad2 (squares). **e**, UbcH10 does not inhibit degradation of APC substrates in G1. ^{35}S-labelled geminin was incubated with APC^{Cdh1}, UbcH5 and increasing concentrations of unlabelled NcycB (circles), UbcH10 (triangles) or Mad2 (squares). The percentage of ubiquitinated geminin is indicated. **f**, UbcH10 and substrates bind APC simultaneously. The amount of ^{35}S-labelled UbcH10 and NcycB bound to IgG beads (control) or APC^{Cdh1} in the presence or absence of a 1,000-fold excess of the respective other unlabelled protein was determined by autoradiography. APC was detected by western blotting against Cdc27. **g**, Degradation of UbcH10, but not of UbcH10^{C114S} and UbcH10$^{\Delta N27}$, occurs in G1. Untagged ^{35}S-labelled proteins were generated from $^{ProA/TEV}$UbcH10, $^{ProA/TEV}$UbcH10^{C114S} and $^{ProA/TEV}$UbcH10$^{\Delta N27}$ by cleavage with TEV protease and incubated in extracts prepared from nocodazole-arrested cells (M) or from cells released into G1 for 4 h. The protein concentrations after 120 min were quantified and compared with the initial concentrations. Unlabelled proteins or the proteasome inhibitor MG132 were added as indicated. Results are means ± s.e.m. for three independent experiments.

substrates did not affect the binding of UbcH10 to APC^{Cdh1} (Fig. 3f). UbcH10 therefore has little affinity for the substrate-binding site on APC. This enables substrates to selectively inhibit the autoubiquitination of UbcH10, but not its E2 function, thereby preventing premature APC inactivation.

The autoubiquitination of UbcH10 is regulated by sequence motifs within the N-terminal extension of UbcH10, which is unique among E2 enzymes. Its deletion (UbcH10$^{\Delta N27}$) impaired the formation of ubiquitin chains by APC^{Cdh1} but simultaneously allowed some ubiquitination of UbcH10 by mitotic APC^{Cdc20} (Fig. 3a). However, these ubiquitin chains did not promote the proteasomal degradation of UbcH10$^{\Delta N27}$ in extracts (Fig. 3g). A similar misregulation of both ubiquitination and degradation was observed with N-terminally tagged UbcH10 (data not shown). The integrity of its N terminus is therefore required for the APC-dependent autoubiquitination of UbcH10 in G1 and the prevention of its multiubiquitination in mitosis. The N-terminal extension of UbcH10 is conserved in all metazoan homologues but not in yeast. Yeasts also lack a clear cyclin A homologue, raising the interesting possibility that cyclin A and UbcH10 might have coevolved.

APC therefore promotes the autoubiquitination of UbcH10 *in cis*. This is in contrast with a report that implied that UbcH10 is a D-box-dependent APC substrate[19]. However, these experiments were performed mainly with N-terminally tagged UbcH10, which as shown here interferes with the regulation of UbcH10 autoubiquitination. In addition, mutation of the proposed D-box produces an inactive protein incapable of catalysing its own ubiquitination[19].

Regulation of cyclin A accumulation by UbcH10 *in vivo*

To study the effects of UbcH10 on cyclin A accumulation *in vivo* we expressed ^{myc}UbcH10, which is stabilized because of its N-terminal tag. We could not synchronize cells by mitotic arrest, because UbcH10 overexpression itself abrogated the checkpoint (data not shown). When cells expressing ^{myc}UbcH10 were instead synchronized at the G1/S transition, the concentration of cyclin A was decreased (Fig. 4a). This could not be due solely to effects on cyclin A transcription, because cyclin A expressed from a constitutive

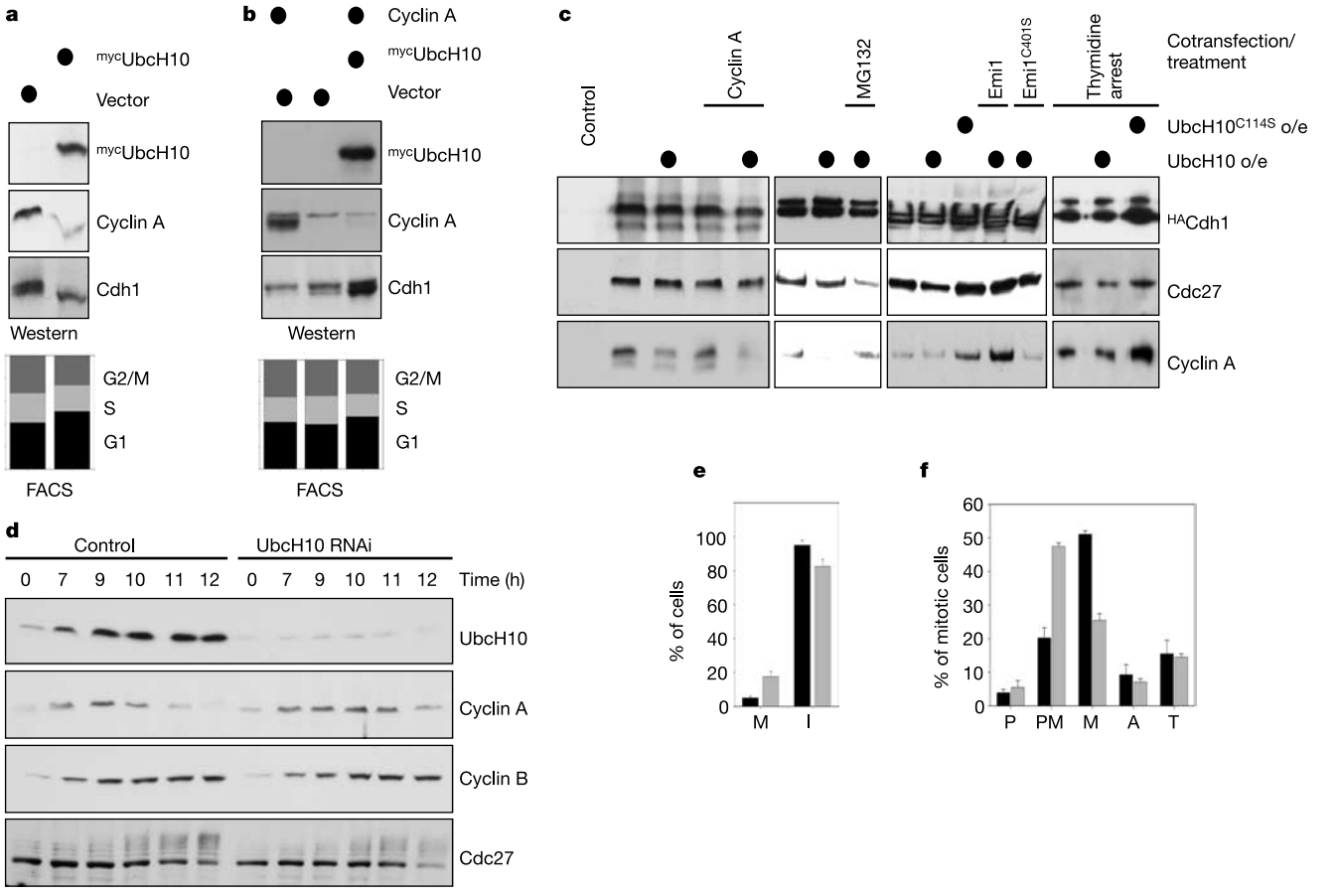

Figure 4 Misregulation of UbcH10 degradation interferes with cyclin A accumulation. **a**, Overexpression of stable ^{myc}UbcH10 impairs cyclin A accumulation at the G1/S transition. Cells were transfected with a plasmid expressing ^{myc}UbcH10 and subjected to a double-thymidine arrest. Cyclin A and ^{myc}UbcH10 were detected by western blotting. The cell-cycle stage of transfected cells was determined by sorting with green fluorescent protein (GFP) and fluorescence-activated cell sorting (FACS). **b**, Coexpression of cyclin A and ^{myc}UbcH10 interferes with cyclin A accumulation in early S phase. Cells were transfected with the indicated constructs, synchronized in early S phase by a double-thymidine arrest, and analysed by western blotting. The cell cycle stage of transfected cells was determined by GFP sorting and FACS. **c**, Overexpression (o/e) of UbcH10 inhibits the binding of cyclin A to Cdh1. Cells were transfected with ^{HA}Cdh1, ^{myc}UbcH10, ^{myc}UbcH10^{C114S}, ^{myc}Emi1 and ^{myc}Emi1^{C401S} as indicated. Cdh1-containing complexes were purified by anti-HA immunoprecipitation from asynchronously grown cells or from cells arrested in S phase by thymidine treatment. The proteasome inhibitor MG132 was added to cultures 2 h before harvesting when indicated. Cyclin A and Cdc27 were detected in ^{HA}Cdh1 precipitates by western blotting. **d**, Depletion of UbcH10 by RNAi delays cyclin A degradation in prometaphase. Cells were treated with control siRNA or siRNA against UbcH10, arrested in early S phase, and released into mitosis in the presence of nocodazole. Samples were taken at the indicated times and analysed by western blotting. **e**, HeLa cells were treated with either control siRNA (dark bars) or siRNA against UbcH10 (light bars), and mitotic (M) and interphase (I) cells were counted. **f**, Mitotic cells of HeLa cells either treated with control siRNA (dark bars) or siRNA against UbcH10 (light bars) were categorized into prophase (P), prometaphase (PM), metaphase (M), anaphase (A) and telophase (T) by cell morphology and by immunostaining against cyclin A, cyclin B, Cdc20 and Plk1. Results in **e** and **f** are means ± s.e.m. for three independent experiments.

promoter was equally downregulated by ^{myc}UbcH10 (Fig. 4b). However, these effects are transient. When the cyclin A-expressing cells were released from the arrest at the G1/S transition into the next cell cycle, cyclin A reaccumulation was delayed, but not blocked, by ^{myc}UbcH10 (Supplementary Fig. S3a). Consistently, the expression of ^{myc}UbcH10 caused a decrease in the fraction of S-phase cells in an asynchronous population, but it did not permanently arrest cells in G1 (Supplementary Fig. S3b). These experiments show that UbcH10 promotes cyclin A degradation *in vivo*, but also imply that backup mechanisms exist that drive cells into S phase in the event of UbcH10 misregulation.

We repeated these experiments and directly measured the binding of cyclin A to tagged Cdh1. In asynchronous cells, the expression of ^{myc}UbcH10, but not that of inactive ^{myc}UbcH10^{C114S}, decreased the amount of cyclin A co-precipitating with Cdh1, reflecting the decreased cyclin A concentration (Fig. 4c). By contrast, cyclin A concentration remained high in Cdh1 precipitates, when the proteasome was inactivated by MG132, or in S-phase cells, in which APC is inactive. Similarly, coexpression of Emi1 counteracted any effects of UbcH10 overexpression. UbcH10 therefore induced the degradation of cyclin A *in vivo* in an APC^{Cdh1}- and proteasome-dependent manner. Importantly, after UbcH10 has been degraded, cyclin A can bind Cdh1 stably.

To study the consequences of UbcH10 depletion, we decreased UbcH10 concentration by using short interfering RNA (siRNA). Significant effects occur in prometaphase, in which cyclin A degradation was significantly delayed (Fig. 4d). Accordingly, UbcH10 depletion led to a threefold increase in the mitotic index and in the percentage of cyclin A-positive prometaphase figures, which is indicative of a delay in mitotic progression at prometaphase (Fig. 4e, f). This caused mitotic aberrations, as judged by a fourfold increase in multinuclear cells and the presence of cells with multipolar spindles and misaligned DNA in the siRNA-treated samples (Supplementary Fig. S4a). Similar phenotypes were reported after overexpression of cyclin A or Emi1, suggesting that the depletion of UbcH10 could have caused these mitotic aberrations by inhibiting cyclin A degradation[12,13,16].

However, unlike the situation in G1, the decrease in UbcH10 concentration in mitosis did not permit the selective degradation of other APC substrates, while maintaining cyclin A stability. In UbcH10-depleted mitotic cells, securin, cyclin B, Cdc20 or Plk1 were stable until cyclin A had finally been degraded (Supplementary Fig. S4b). This might be due to differences between cyclin A degradation during mitosis and G1, or to a requirement for cyclin A degradation to permit full APC activation towards other substrates.

Taken together, these experiments show conclusively that UbcH10 is rate-limiting for cyclin A degradation both *in vitro* and *in vivo*. The increase in UbcH10 concentration in mitosis is required for efficient cyclin A degradation in prometaphase. The degradation of UbcH10 in G1 accordingly stabilizes cyclin A and eventually allows the complete inactivation of APC.

Emi1, UbcH10, and S-phase entry

Cells commit to a complete round of cell division during G1 in response to exogenous growth factors. The autonomous inactivation of APC through UbcH10 degradation should therefore be coupled to external signals. A potential mediator of this coupling is Emi1, which is transcribed at the G1/S transition in an E2F-dependent manner and can inactivate APC^{Cdh1} independently of Cdh1 phosphorylation[8]. Indeed, Emi1 also inactivates APC in the presence of high concentrations of UbcH10. As shown in Fig. 5a, when the Zn-binding domain of Emi1, but not that of inactive Emi1^{C401S}, was added to G1 extracts supplemented with UbcH10, cyclin A degradation was blocked. Similarly, Emi1, but not Emi1^{C401S}, Mad2 or Mad2L2, inhibited the APC^{Cdh1}-dependent ubiquitination of cyclin A and geminin in the presence of UbcH10 (Fig. 5b). Both Emi1 and Emi1^{C401S} also inhibited the autoubiquitination of UbcH10. Finally, Emi1 counteracts UbcH10 *in vivo*, as shown by the increased amount of Cdh1-bound cyclin A in cells

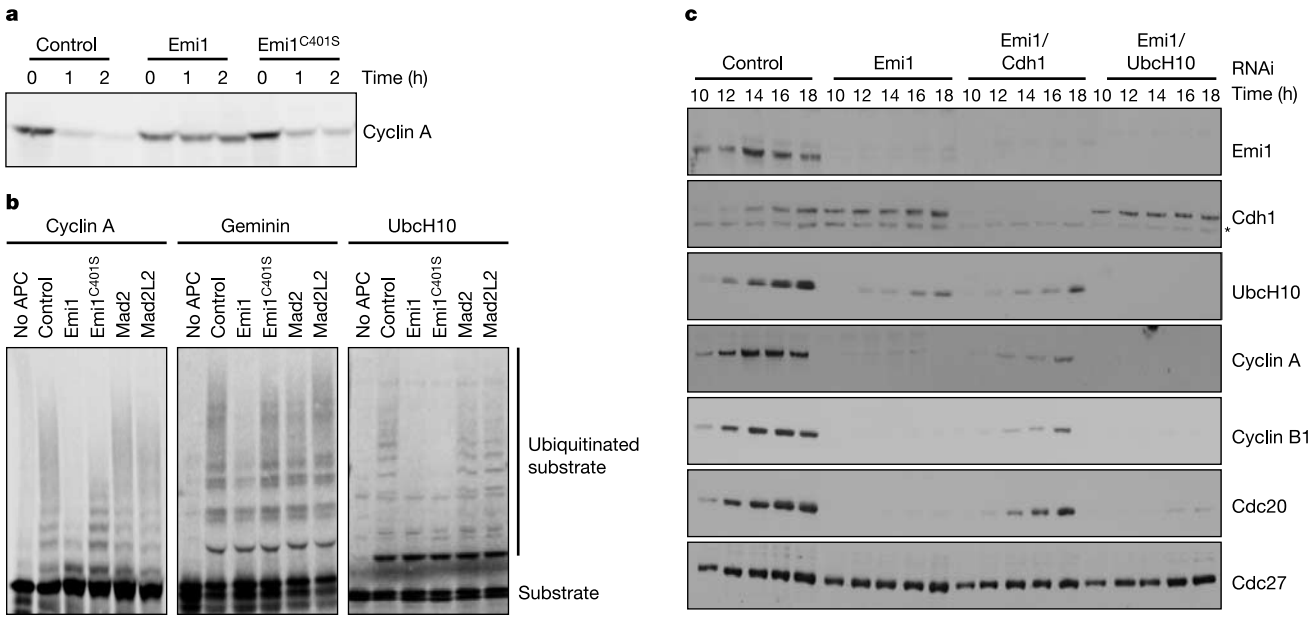

Figure 5 Control of autonomous APC inactivation by Emi1. **a**, Emi1 inhibits cyclin A degradation in the presence of high concentrations of UbcH10. G1 extracts were supplemented with UbcH10 and with a C-terminal fragment of Emi1 or of inactive Emi1^{C401S}. The degradation of ^{35}S-labelled cyclin A was analysed as before. **b**, Emi1 inhibits both substrate ubiquitination and UbcH10 autoubiquitination. APC^{Cdh1} from G1 cells was analysed for its capacity to catalyse the ubiquitination of either cyclin A or geminin, or the autoubiquitination of UbcH10, respectively. C-terminal fragments of Emi1 and Emi1^{C401S}, or Mad2 and Mad2L2, were added as indicated. **c**, Emi1 is required for entry into S phase in the absence of Cdh1. Cells treated with siRNAs against Emi1, Cdh1 and UbcH10, as indicated, were released from a nocodazole arrest. Samples were taken after the indicated durations, and entry into S phase was measured by western blotting against the indicated proteins. The asterisks mark nonspecific bands of the respective antibodies.

expressing Emi1 and UbcH10 (Fig. 4c). Emi1 is therefore a general APC inhibitor that could inactivate APC in the event of UbcH10 misregulation, or during S and G2, when UbcH10 concentrations recover.

However, the role of Emi1 at the G1/S transition might be more complex. When Emi1 was depleted by siRNA, cells were arrested at the G1/S transition (Fig. 5c; ref. 8). This might be caused partly by an unexpected increase in Cdh1 concentration, because elevated Cdh1 concentrations would overwhelm the low cyclin A concentrations present before entry into S phase[11,20]. Most significantly, however, when both Cdh1 and Emi1 are depleted by siRNA, cells were still arrested in G1 (Fig. 5c). Thus, even when APC has been inactivated by UbcH10 degradation and Cdh1 depletion, Emi1 is required for S-phase entry. This indicates that there might be an additional, hitherto unknown, S-phase-promoting function of Emi1, which is independent of APC^{Cdh1} and connects the final steps of S-phase entry to complete E2F activation. APC therefore first downregulates its activity towards cyclin A by UbcH10 autoubiquitination, allowing cyclin A to accumulate. This is followed by the E2F-dependent transcription of cyclin A and Emi1, which ensures complete inactivation of APC^{Cdh1} and promotes S phase by additional means.

Discussion

Cyclin A is the only essential cyclin for S-phase entry in unperturbed cell cycles[1–3]. It inactivates APC by phosphorylating Cdh1, and it regulates DNA replication. However, cyclin A is also a substrate of APC, and the cyclin-binding motif of Cdh1 is pivotal for both Cdh1 phosphorylation and cyclin A ubiquitination[10,11]. An explanation of why, early in G1, cyclin A is ubiquitinated by APC^{Cdh1} but stably binds and inactivates APC^{Cdh1} late in G1 has long remained elusive. Here we show that the decision between cyclin A degradation and APC inactivation is determined by the availability of UbcH10. UbcH10 is rate-limiting for the G1-specific ubiquitination of cyclin A. The degradation of UbcH10 consequently stabilizes cyclin A, which then can bind to Cdh1 without being degraded. APC^{Cdh1} itself controls this transition by promoting the autoubiquitination of UbcH10 *in cis*. Other substrates of APC act as timers and inhibit the autoubiquitination of UbcH10 but not its E2 activity. This ensures that mitotic proteins are degraded before APC is inactivated. Once activated in mitosis, APC therefore autonomously downregulates its activity through the ubiquitination and degradation of UbcH10.

These findings have important implications for our understanding of the eukaryotic cell cycle. The autonomous inactivation of APC strongly suggests the existence of a self-perpetuating oscillator at the core of eukaryotic cell cycles. This oscillator would be composed only of the APC ubiquitin ligase and two cyclin/CDKs. In this simplified scheme, cyclin A/Cdk1 and cyclin B/Cdk1 promote entry into mitosis and activate APC[21]; APC in turn inactivates mitotic cyclins and triggers exit from mitosis; degradation of its remaining substrates is followed by the autoubiquitination and degradation of UbcH10; subsequent accumulation of cyclin A completely inactivates APC, promotes S-phase entry and allows the reaccumulation of mitotic cyclins and UbcH10.

In multicellular organisms, this self-perpetuating oscillator is tightly coupled to external controls. In addition to the degradation of UbcH10 and the stabilization of cyclin A, transcriptional steps are required for entry into S phase. These include an increased transcription of not only cyclin A but also Emi1 (refs 8, 9). Emi1 combines an uncharacterized APC^{Cdh1}-independent activity, essential for S-phase entry, with its role as an APC inhibitor. Why the cell requires a further means of inhibiting APC after UbcH10 degradation and Cdh1 phosphorylation seems surprising. One possibility, in addition to the need for triple assurance, is that inactivation of APC by Emi1 is needed in S and G2, when UbcH10 and activators of APC reaccumulate. Furthermore, Emi1 could assist in Cdh1 inactivation in case cyclinA/Cdk2 activity is restrained by CDK inhibitors or UbcH10 is misregulated. The cell cycle therefore seems to be composed of an endogeneous oscillator driven largely by post-translational mechanisms, namely phosphorylation and degradation, and of multiple transcriptional and translational controls that couple this oscillator to exogenous signals. It will be interesting to examine variations on this theme in different organisms. □

Methods

Plasmids and antibodies

UbcH10 and $UbcH10^{C114S}$ were cloned into $pCS2^{ProA/TEV}$ for *in vitro* transcription/translation, or into pET28 for purification. Cdh1 was cloned into haemagglutinin-tagged pCS2 to generate ^{HA}Cdh1. Securin, geminin, Cdc20 and cyclin A were all in pCS2. ^{myc}Emi1 and $^{myc}Emi1^{C401S}$ were in pCS2. Carboxy-terminal fragments of Emi1 and $Emi1^{C401S}$ were cloned into pET28. Anti-UbcH10 and anti-UbcH5 antibodies were purchased from Boston Biochem. Anti-Cdc27, anti-HA, anti-cyclin A, anti-cyclin B1 and anti-Cdc20 antibodies were purchased from Santa Cruz. Anti-securin antibody was purchased from MBL, and anti-Plk1 antibody from Upstate. Anti-Emi1 antibody was a gift from P. Jackson. Alexa 488-labelled and Alexa 546-labelled secondary antibodies were purchased from Molecular Probes, and Cy3-labelled anti-β-tubulin antibody from Sigma.

Tissue culture and cell synchronization

HeLa, HeLa S3 and 293T cells were synchronized in prometaphase by treatment with nocodazole, in G1 by a release from nocodazole arrest, and in early S by double-thymidine arrest. Cells were incubated in thymidine-containing (2 mM) medium for 24 h. Cells were released into fresh medium for 6 h, followed by a second thymidine arrest for 18 h or a nocodazole arrest (0.1 μg ml^{-1}) for 12 h. For G1 cells, nocodazole-arrested cells were released into fresh medium for 4 h unless noted otherwise. Cells were harvested, washed with PBS, and either processed for extraction or lysed in SDS buffer for analysis by SDS–polyacrylamide-gel electrophoresis (SDS–PAGE) and western blotting.

Protein purification

^{His}UbcH10, $^{His}UbcH10^{C114S}$, His-tagged Zn-binding domains of Emi1 and $Emi1^{C401S}$, and ^{His}Mad2 were purified from BL21(DE3) pRIL cells (Stratagene) by Ni^{2+}-nitrilotriacetate agarose chromatography in accordance with the manufacturer's protocol (Qiagen). ^{MBP}Mad2L2 was purified after expression in BL21(DE3) pRIL cells on amylose resin according to the manufacturer's protocol (NEB).

Degradation assays

In vitro degradation assays were performed with extracts of synchronized HeLa S3 cells. Synchronized cells were harvested, washed with PBS, lysed in SB buffer (25 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM dithiothreitol, 1 × complete protease inhibitors (Roche), 15 mM creatine phosphate, 2 mM ATP) and homogenized by freeze–thawing and passage through a needle. Extracts were cleared by subsequent centrifugations (5 min at 5,000 r.p.m.; 60 min at 14,000 r.p.m.). Extract (20 μl) was supplemented with degradation cocktail (1.5 mg ml^{-1} ubiquitin (Boston Biochem), 7.5 mM creatine phosphate, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mg ml^{-1} cycloheximide) and ^{35}S-labelled substrate at 23 °C. Aliquots were removed at 0, 1 and 2 h, and resolved by SDS–PAGE (5–15%) and autoradiography. To analyse the degradation of UbcH10, it was synthesized as $^{ProA/TEV}$UbcH10, and 1 U of tobacco etch virus (TEV) protease was included in the degradation assay.

Ubiquitination assays

In vitro ubiquitination assays were performed with APC purified from extracts of synchronized HeLa S3 cells. For 10 ubiquitination reactions, 1 ml of extract was incubated with 10 μg of anti-Cdc27 antibodies for 4 h at 4 °C, and with ProG-agarose (Roche) for 2 h. Precipitates were washed three times with SB buffer containing 0.1% Triton X-100 and twice with SB buffer. E1 (Boston Biochem), E2 (as indicated), 20 mM ATP, 1.5 mg ml^{-1} ubiquitin and 10 mM dithiothreitol in UBAB buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) were added. Reactions were started by the addition of ^{35}S-labelled substrate, incubated for 60 min at 30 °C and resolved by SDS–PAGE and autoradiography.

Immunodepletion of UbcH10

Synchronized HeLa S3 cells (500 ml) were used for extraction as described above. The extract was incubated for 2 h with 10 μg of anti-UbcH10 antibody coupled to ProG-agarose. Beads were removed by centrifugation, and supernatants were used in degradation assays as described above.

Immunofluorescence

HeLa cells were grown on coverslips in DMEM supplemented with 10% FCS and 1 × penicillin/streptomycin in 5% CO_2 at 37 °C. Cells were washed with PBS and fixed in 4% formaldehyde, 0.1% Tween 20 in PBS. Cells were incubated for 15 min in TBS containing 2% BSA and 0.5% Tween 20 and for a further 60 min in TBS containing 2% BSA, 0.1% Tween 20, followed by incubation with primary and secondary antibodies at 23 °C. S-phase cells were identified by bromodeoxyuridine staining using the bromodeoxyuridine labelling and detection kit I (Roche).

RNAi

For RNA-mediated interference (RNAi) against UbcH10, cells were arrested with thymidine, released for 2 h and transfected with siRNA-oligos against UbcH10. After 4 h serum was added, and cells were again arrested with thymidine. Cells were washed, then released into fresh medium. Nocodazole was added after 4 h and samples were taken at times indicated. For RNAi against Emi1, Emi1/Cdh1 and Emi1/UbcH10, cells were arrested with thymidine, released for 1 h and treated with siRNA. Nocodazole was added with serum after 4 h. After a further 6 h, mitotic cells were collected by shake-off, then washed and released into fresh medium. All siRNAs were Smartpools from Dharmacon. Transfections were performed with Oligofectamine.

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