LETTERS

Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation

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Eukaryotic cells rely on a surveillance mechanism known as the spindle checkpoint to ensure accurate chromosome segregation. The spindle checkpoint prevents sister chromatids from separating until all kinetochores achieve bipolar attachments to the mitotic spindle[1–3]. Checkpoint proteins tightly inhibit the anaphase-promoting complex (APC), a ubiquitin ligase required for chromosome segregation and progression to anaphase. Unattached kinetochores promote the binding of checkpoint proteins Mad2 and BubR1 to the APC-activator Cdc20, rendering it unable to activate APC. Once all kinetochores are properly attached, however, cells inactivate the checkpoint within minutes, allowing for the rapid and synchronous segregation of chromosomes[4]. How cells switch from strong APC inhibition before kinetochore attachment to rapid APC activation once attachment is complete remains a mystery. Here we show that checkpoint inactivation is an energy-consuming process involving APC-dependent multi-ubiquitination. Multi-ubiquitination by APC leads to the dissociation of Mad2 and BubR1 from Cdc20, a process that is reversed by a Cdc20-directed de-ubiquitinating enzyme[5]. The mutual regulation between checkpoint proteins and APC leaves the cell poised for rapid checkpoint inactivation and ensures that chromosome segregation promptly follows the completion of kinetochore attachment. In addition, our results suggest a mechanistic basis for how cancer cells can have a compromised spindle checkpoint without corresponding mutations in checkpoint genes[6].

Cells exposed to spindle poisons such as nocodazole or taxol arrest in prometaphase owing to activation of the spindle checkpoint by unattached kinetochores[6]. In previous studies on APC, we found that cells overexpressing the APC-specific ubiquitin-conjugating enzyme (E2), UbcH10, showed a compromised arrest in mitosis in response to nocodazole[7]. To characterize this phenotype more precisely, we transfected HeLa cells with wild-type UbcH10, a catalytically dead active-site mutant—UbcH10^{C114S}—or empty vector, and subsequently challenged them with nocodazole. Whereas cells transfected with empty vector or UbcH10^{C114S} were efficiently arrested in mitosis, nearly 70% of cells overexpressing wild-type UbcH10 were in interphase (Fig. 1a). Cells with a defective spindle checkpoint can exit mitosis even in the presence of spindle poisons, generating multinucleated cells with decondensed interphase-like chromatin. Multinucleated cells were rarely observed among taxol-treated HeLa cells overexpressing UbcH10^{C114S} or empty vector (Fig. 1b). Under the same conditions, nearly half of the interphase cells overexpressing UbcH10 were multinucleated. These data strongly suggest that cells expressing high levels of UbcH10 can enter mitosis, but are unable to maintain spindle checkpoint activity.

To biochemically characterize the effect of UbcH10, we prepared checkpoint extracts (CP-extracts) from HeLa cells arrested in mitosis by nocodazole treatment. As reported previously[8], CP-extracts failed to degrade substrates of APC (Supplementary Fig. 1a). Addition of UbcH10 to CP-extracts, however, inactivated the spindle checkpoint, as indicated by the degradation of substrates (geminin, securin and cyclin B1) of the APC^{Cdc20} complex (Fig. 1c and Supplementary Fig. 1a). This degradation was APC-dependent because it could be reversed by the addition of the APC inhibitor Emi1, or by additional Mad2 (Supplementary Fig. 1a, b). APC purified from CP-extracts (CP-APC) was also inactivated by the spindle checkpoint. CP-APC was unable to ubiquitinate substrates in the absence of exogenous E2 but addition of either UbcH10 or the other APC-interacting E2, UbcH5α, to CP-APC promoted the ubiquitination of APC^{Cdc20} substrates (Supplementary Fig. 1c, d). Together, these results demonstrate

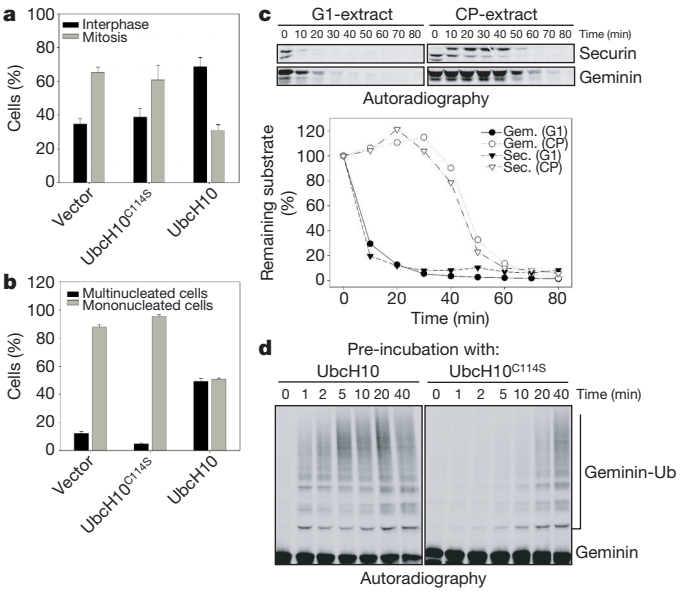

Figure 1 | UbcH10 activates CP-APC for substrate ubiquitination and degradation. a, UbcH10 overrides the spindle checkpoint in nocodazole-arrested cells. Cells were transfected with indicated plasmids and treated with nocodazole 4 h later. After 16 h the mitotic index was determined in transfected cells. Error bars, s.e.m. ($n = 3$). **b**, Overexpression of UbcH10, but not of inactive UbcH10^{C114S}, causes multinucleation. Error bars, s.e.m. ($n = 3$). **c**, APC-substrates are degraded rapidly in G1-extracts but display a lag before degradation in CP-extracts. G1-extracts or CP-extracts were incubated with UbcH10, and the abundance of ^{35}S-labelled substrates was analysed by autoradiography. Gem., geminin; sec., securin. **d**, Pre-incubation with UbcH10 but not with UbcH10^{C114S} eliminates the lag in substrate ubiquitination by purified CP-APC.

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that UbcH10 can directly override inhibition of APC by the spindle checkpoint *in vivo*, in extracts and in the purified system.

APC substrates such as securin and geminin are rapidly degraded in extracts prepared from G1 cells (G1-extracts)[8] (Fig. 1c). In contrast, these substrates demonstrated biphasic kinetics in UbcH10-treated CP-extracts, with little substrate degradation in the first 40 minutes of reaction followed by rapid degradation thereafter. We observed a similar lag phase for substrate ubiquitination by purified CP-APC (Supplementary Fig. 1e). These results indicated that UbcH10 may have two roles on CP-APC, an initial role in checkpoint inactivation, which occurs during the lag phase, and a subsequent role in ubiquitin transfer to substrates. To test this, we pre-incubated CP-extracts with either wild-type UbcH10 or inactive UbcH10^{C114S} to allow any inactivation of the checkpoint to occur. We then purified APC from these extracts, washed away the UbcH10 variants, and added fresh, wild-type UbcH10 along with ubiquitination components to observe the kinetics of substrate ubiquitination. Markedly, pre-incubation of CP-extracts with wild-type UbcH10 but not UbcH10^{C114S} completely eliminated the lag in substrate ubiquitination (Fig. 1d). These results strongly suggest that UbcH10 has a direct role in overriding checkpoint inhibition of APC and that this requires UbcH10's catalytic activity.

Because the stability of Mad2, BubR1, Bub3 and Mad1 in CP-extracts was unaffected by UbcH10 addition, the initial inactivation of the checkpoint is unlikely to involve the destruction of checkpoint proteins (Supplementary Fig. 2a). Previous studies had shown that association of Mad2 and BubR1 with APC is necessary for proper checkpoint function[9–11]. Therefore, we examined whether UbcH10 affected this association. In the absence of UbcH10, the interaction of Mad2 and BubR1 with CP-APC was stable, with a half-life of several hours (Supplementary Fig. 2b). Furthermore, in CP-extracts incubated with UbcH10^{C114S}, Mad2 and BubR1 co-migrated with APC as analysed by sucrose gradient centrifugation (Fig. 2a). In contrast, incubation of CP-extracts with wild-type UbcH10 led to the complete loss of Mad2 and BubR1 from APC (Fig. 2a and Supplementary Fig. 2c). Similarly, purified CP-APC incubated with UbcH10 and ubiquitination components E1 and ubiquitin, showed complete dissociation of Mad2 from APC as well as dissociation of BubR1 and Cdc20 (Fig. 2b). Finally, UbcH10 promoted the dissociation of checkpoint proteins *in vivo*. In cells depleted of UbcH10 by RNA interference (RNAi), dissociation of Mad2 from Cdc20 was delayed after release from nocodazole (Supplementary Fig. 2d). This corresponds with a delay in completing the metaphase to anaphase transition as observed by live-cell imaging of UbcH10-depleted cells that had not been exposed to spindle toxins (Fig. 2c and Supplementary Fig. 3). Taken together, these results demonstrate that catalytically active UbcH10 can promote the release of checkpoint proteins from APC.

We next explored the detailed requirements for UbcH10 activity during checkpoint inactivation. When CP-extracts were incubated with UbcH10 and wild-type ubiquitin, checkpoint proteins dissociated from APC as expected (Fig. 3a). In contrast, incubation of CP-extracts with UbcH10 and methylubiquitin or lysine-free ubiquitin failed to promote dissociation of checkpoint proteins from APC (Fig. 3a and Supplementary Fig. 4b). Both methylubiquitin and lysine-free ubiquitin are competent to mono-ubiquitinate substrates but not to form multi-ubiquitin chains, suggesting that multi-ubiquitination by APC is required for checkpoint inactivation. Multi-ubiquitination usually marks substrates for proteasomal proteolysis; however, whether extracts were treated with UbcH10 alone or UbcH10 in the presence of the proteasome inhibitor MG132, Mad2 and BubR1 still dissociated from APC (Fig. 3b). Furthermore, MG132 treatment prevented the partial proteolysis of Cdc20 observed on UbcH10 addition, but did not interfere with checkpoint complex disassembly. Thus, proteasomal proteolysis does not seem to be essential for the disassembly of checkpoint complexes, although we cannot rule out a role for Cdc20 degradation at steps subsequent to Mad2 dissociation. We conclude that release of checkpoint proteins requires multi-ubiquitination of one or more components of the APC–Cdc20–checkpoint protein complex.

While examining Mad2 and BubR1 release from purified CP-APC, we noticed that co-purifying Cdc20 showed a characteristic ladder of multi-ubiquitination that was dependent on UbcH10 (Supplementary Fig. 4c). We also detected ubiquitinated Cdc20 *in vivo* from nocodazole-arrested cells. This ubiquitination was maintained even after short interfering (si)RNA-mediated depletion of Cdh1, the APC activator responsible for Cdc20 ubiquitination in late mitosis (Supplementary Fig. 4d). These results suggest that CP-APC directly ubiquitinates Cdc20 in early mitosis. Importantly, multi-ubiquitination of Cdc20 in UbcH10-treated CP-extracts coincided with lower amounts of co-purifying Mad2 (Fig. 3c). Furthermore, in reactions with purified CP-APC, Cdc20 ubiquitination preceded the onset of substrate ubiquitination (Fig. 3d and Supplementary Fig. 1e). Thus, Cdc20 ubiquitination is an early event that may contribute to disassembly of checkpoint complexes by CP-APC. In a parallel study, a Cdc20-directed de-ubiquitinating enzyme, USP44, was shown to counteract UbcH10-mediated checkpoint inactivation both *in vitro* and *in vivo*[5]. Purified USP44 directly promotes de-ubiquitination of Cdc20 (ref. 5). Additionally, when USP44 is depleted from cells, Cdc20 ubiquitination is increased and the amount of Mad2 associated with Cdc20 is markedly reduced. Co-depletion of UbcH10 reverses both phenotypes[5]. Taken together, these results strongly suggest that APC-dependent ubiquitination of Cdc20 decreases its association with Mad2 and thus frees APC to ubiquitinate substrates.

Mad2 and Cdc20 are intimately associated in an energetically stable complex when the checkpoint is active[12–14]. Ubiquitination

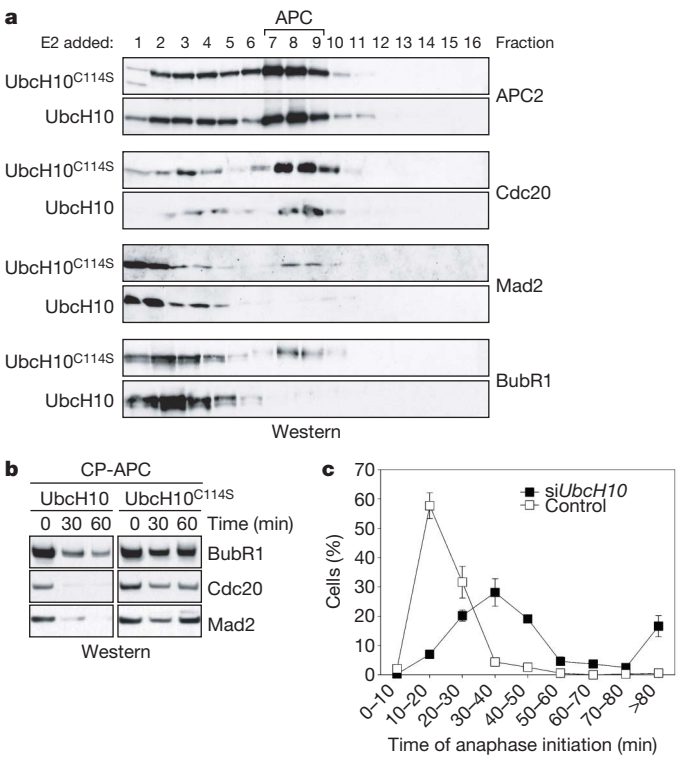

Figure 2 | UbcH10 catalyses the dissociation of checkpoint components from APC. a, Checkpoint proteins dissociate from APC in the presence of UbcH10. CP-extracts were incubated with UbcH10 or UbcH10^{C114S} along with ubiquitination components and centrifuged through sucrose gradients. Indicated proteins were detected by western analysis. **b**, UbcH10 promotes dissociation of checkpoint proteins from purified CP-APC. **c**, Depletion of UbcH10 delays the onset of anaphase. HeLa cells expressing GFP–histone-2B were treated with control or with *UBCH10* siRNA and monitored by fluorescence microscopy. Error bars, s.e.m. ($n = 3$).

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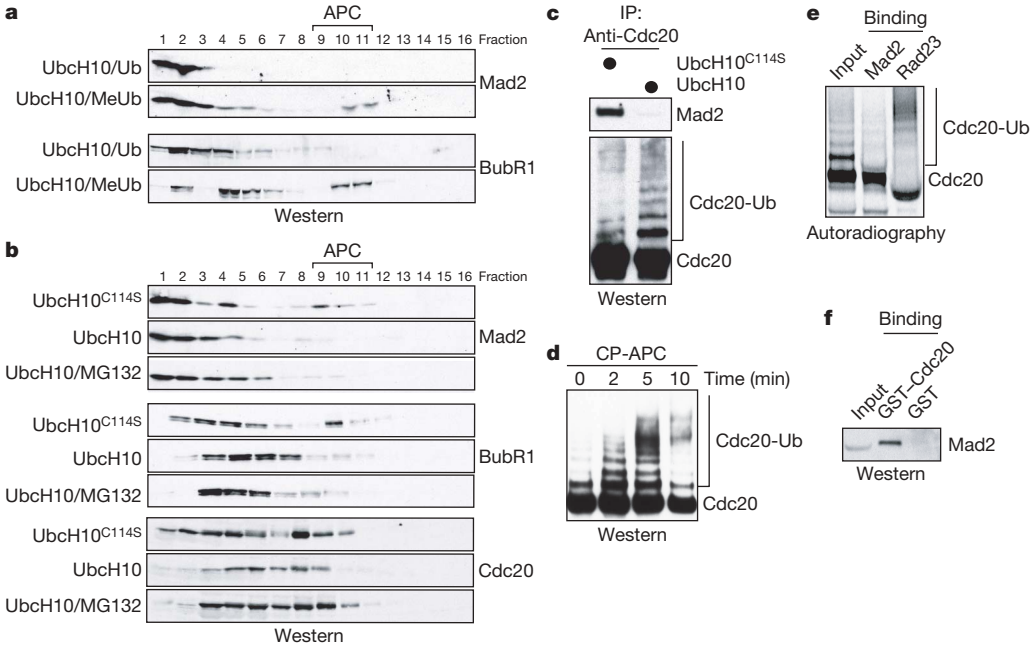

Figure 3 | Disassembly of checkpoint complexes requires APC-dependent multi-ubiquitination. a. UbcH10-dependent dissociation of checkpoint proteins requires multi-ubiquitination. CP-extracts were incubated with ubiquitin (Ub) or methylubiquitin before sucrose gradient centrifugation. **b**, Dissociation of checkpoint proteins does not require proteolysis. MG132 is a proteasome inhibitor. **c**, UbcH10 but not UbcH10^{C114S} promotes Cdc20 ubiquitination and dissociation of Mad2 from Cdc20. CP-extracts were incubated with UbcH10 or UbcH10^{C114S}. Cdc20 ubiquitination and the amount of co-purifying Mad2 were determined by western analysis. **d**, Cdc20 ubiquitination occurs during the lag-phase of substrate ubiquitination seen with CP-APC. Purified CP-APC was incubated with UbcH10 and ubiquitination components to determine kinetics of ubiquitination of co-purifying Cdc20. **e**, Ubiquitinated Cdc20 cannot bind Mad2. ^{35}S-labelled Cdc20 was exchanged into CP-APC and ubiquitinated *in vitro*. Cdc20 was then incubated with recombinant Mad2- or Rad23-conjugated Ni-NTA-agarose beads. Bound Cdc20 was analysed by autoradiography. **f**, Mad2 released from CP-APC seems to undergo a conformational change. Mad2 was released from CP-APC by UbcH10 addition and subsequently incubated with glutathione sepharose beads pre-conjugated with GST–Cdc20 peptide or GST alone. Bound Mad2 was detected by western analysis.

of Cdc20 might be expected to interfere with Mad2 binding by eliciting a conformational change in Cdc20 or by direct steric occlusion of the Mad2 binding surface. Consistent with this hypothesis, we found that purified Mad2 failed to bind ubiquitinated Cdc20, though it efficiently bound unmodified Cdc20 (Fig. 3e). Additionally, Mad2 liberated from CP-APC by the addition of UbcH10 was efficiently bound by a GST-tagged peptide of Cdc20 (Fig. 3f). Because Mad2 in CP-APC is locked in a closed or N2′ conformation, and because this confomer is incapable of binding the Cdc20 peptide[15,16], APC-dependent multi-ubiquitination seems to induce a substantial conformational change in Mad2. Together, these results suggest that APC-dependent multi-ubiquitination leads to the release of Mad2 from Cdc20 and blocks rebinding of Mad2 to ubiquitinated Cdc20.

In earlier work p31^{comet}, a protein that binds Cdc20-bound Mad2, was implicated in spindle checkpoint inactivation[17,18]. We found that, as with UbcH10, addition of p31^{comet} to CP-extracts promoted the degradation of APC substrates (Fig. 4a). Addition of both UbcH10 and p31^{comet} markedly accelerated the rate of substrate degradation beyond that observed with either protein alone. Furthermore, during substrate ubiquitination by CP-APC, p31^{comet} lowered the threshold for UbcH10 action to the physiological concentrations of UbcH10 in mitosis (100 nM; Fig. 4b). Thus, p31^{comet} and UbcH10 work in concert during checkpoint inactivation.

Importantly, p31^{comet} strongly promoted Cdc20 ubiquitination by CP-APC, indicating that it may contribute to disassembly of checkpoint complexes (Fig. 4c). Indeed, when CP-extracts were treated with p31^{comet} alone, some Mad2 dissociated from Cdc20 (Fig. 4d). This dissociation required endogenous UbcH10 in the extracts, as addition of UbcH10^{C114S} along with p31^{comet} blocked Mad2 dissociation. In contrast, when both p31^{comet} and wild-type UbcH10 were added to CP-extracts, Mad2 was completely removed from Cdc20 (Fig. 4d). Similarly, both UbcH10 and p31^{comet} are necessary for the dissociation of checkpoint proteins in intact cells. In cells treated with control short interfering RNAs (siRNAs) and released from nocodazole, Mad2 was readily lost from Cdc20-immunoprecipitates, whereas this dissociation was delayed in cells depleted of UbcH10 or p31^{comet} alone (Fig. 4e). Depletion of both UbcH10 and p31^{comet} had a strongly additive effect, with substantial Mad2 remaining on Cdc20 90 minutes after release from nocodazole (Fig. 4e). Depleted cells were correspondingly delayed in degrading cyclin B1 and progressing through mitosis (Supplementary Fig. 5). Together, these results demonstrate that p31^{comet} lowers the threshold for UbcH10-activity on CP-APC, thereby allowing CP-APC to promote dissociation of checkpoint components and subsequent progression to anaphase.

Our data indicate that APC itself can drive the disassembly of checkpoint complexes and the consequent inactivation of the checkpoint (Fig. 4f). While bound to checkpoint proteins, APC ubiquitinates Cdc20 and possibly other components of the APC–Cdc20–checkpoint protein complex, leading to the release of Mad2 (Fig. 3c, d). While unattached kinetochores are available, released Mad2 may once again capture Cdc20. However, once all kinetochores are properly attached, disassembly of checkpoint complexes predominates. Newly liberated APC could bind and inactivate additional Mad2–Cdc20 complexes leading to a rapid, switch-like transition from metaphase to anaphase. This mechanism is controlled by UbcH10 and p31^{comet}, which promote APC-dependent ubiquitination and by de-ubiquitinating enzymes such as USP44, which antagonize it. The high level of UbcH10 observed in many cancer cells is likely to disrupt this control and promote checkpoint inactivation[19–21]. Because spindle checkpoint activity has been shown to enhance tumour cell killing by anti-mitotic drugs[6,22], we expect agents that lower UbcH10 activity to potentiate the action of these drugs, and thus expand our armamentarium of cancer chemotherapeutics.

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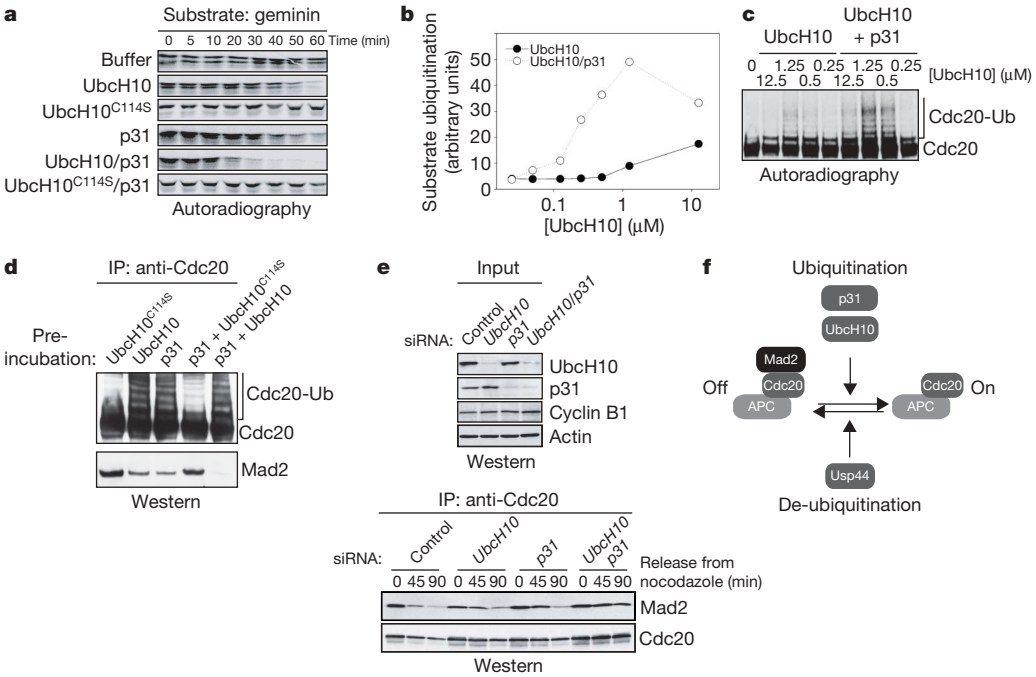

Figure 4 | p31^{comet} lowers the threshold for UbcH10-mediated checkpoint inactivation. **a**, p31^{comet} (p31) and UbcH10 synergistically promote substrate degradation in CP-extracts. CP-extracts were incubated with indicated proteins and degradation of ^{35}S-labelled geminin was detected by autoradiography. **b**, p31^{comet} lowers the threshold for UbcH10-dependent substrate ubiquitination by CP-APC. CP-APC was incubated with UbcH10 alone or in the presence of p31^{comet}. The intensity of ubiquitinated ^{35}S-labelled geminin was quantified by densitometry. **c**, p31^{comet} promotes Cdc20 ubiquitination. ^{35}S-labelled Cdc20 was exchanged into CP-APC, which was then incubated with UbcH10 alone or in the presence of p31^{comet}. Cdc20 ubiquitination was analysed by autoradiography. **d**, UbcH10 and p31^{comet} synergistically promote Mad2 dissociation from Cdc20. CP-extracts were incubated with indicated proteins before Cdc20 immunoprecipitation. Cdc20 and co-purifying Mad2 were detected by western analysis. **e**, UbcH10 and p31^{comet} are both required for efficient inactivation of the checkpoint *in vivo*. HeLa cells were treated with the indicated siRNAs before synchronization. Cdc20 was immunoprecipitated at the indicated times after nocodazole washout. The amount of co-precipitating Mad2 was visualized by western blotting (lower panel). **f**, APC-dependent ubiquitination drives checkpoint complex disassembly and inactivation of the checkpoint. Ubiquitination is promoted by UbcH10 and p31 and is opposed by Usp44.

METHODS

Dissociation assays. For dissociation of checkpoint proteins from purified APC, APC-immunoprecipitates were incubated with 12.5 μM UbcH10 or UbcH10^{C114S} in the presence of ubiquitination components: E1 (Boston Biochem), 20 mM ATP, 1.5 mg ml^{-1} ubiquitin (Boston Biochem), and 10 mM dithiothreitol in UBAB buffer[7] supplemented with 150 mM NaCl and 1 mg ml^{-1} BSA. Reactions were carried out at 23 °C with shaking at 1,000 r.p.m. APC beads were washed extensively and boiled in sample buffer. Bound proteins were examined by SDS–PAGE and western analysis. For dissociation of checkpoint proteins from APC or from Cdc20 in extracts, extracts were incubated with 1.25 μM E2 in the presence of degradation cocktail[7] for 1 h at 23 °C. Extracts were subsequently incubated with anti-Cdc27- or anti-Cdc20-conjugated ProG beads and processed as described above. Alternatively, extracts were centrifuged through gradients of 15–40% sucrose for 18 h at 30,000 r.p.m. in a SW-40 rotor. Fractions were subjected to TCA precipitation, and proteins were subsequently resuspended in sample buffer and analysed by SDS–PAGE and western blotting.

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