APC/C<sub>Cdh1</sub>-dependent proteolysis of USP1 regulates the response to UV-mediated DNA damage

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Introduction

Genotoxic stress, such as exposure to UV light, induces the accumulation of different types of DNA lesions and a wide range of cellular responses (Stokes and Comb, 2008). To maintain cell viability, replication forks that encounter damaged DNA must efficiently bypass these lesions to complete replication of the genome in a timely manner during S phase. DNA synthesis across damaged DNA is achieved by specialized DNA polymerases that incorporate nucleotides opposite to damaged bases in a process known as trans-lesion synthesis (TLS; Lehmann et al., 2007). However, overuse of TLS polymerases can increase mutagenesis because of their highly accommodating active sites and lack of proofreading activity (Waters et al., 2009). The recruitment of TLS polymerases for lesion bypass requires the monoubiquitination of proliferating cell nuclear antigen (PCNA; Kannouche et al., 2004; Watanabe et al., 2004). The model predicts that the blockage of replicative polymerases activates PCNA monoubiquitination at replication-stalled sites ( Hoege et al., 2002; Friedberg et al., 2005). This event in turn promotes the recruitment of TLS polymerases to bypass the lesion and allow continuation of DNA replication. It has been suggested that the switch back from TLS to normal processive polymerases is regulated by ubiquitin-specific protease 1 (USP1), the deubiquitinating enzyme for PCNA (Huang and D’Andrea, 2006; Huang et al., 2006; Ulrich, 2006).

However, recent studies have shown that PCNA monoubiquitination can occur outside of S phase both in mammalian and yeast systems (Frampton et al., 2006; Soria et al., 2006, 2009; Daigaku et al., 2010; Karras and Jentsch, 2010; Ogi et al., 2010). Moreover, Pol-η and other TLS polymerases can be recruited to sites of UV lesions in quiescent or noncycling cells, which is in line with possible roles in gap-filling of DNA damage tracks left behind the replication forks (Lehmann and Fuchs, 2006;...
Lopes et al., 2006). As such, the gap-filling pathway is likely not limited to S phase but also occurs in G0, G1, and G2/M phases. PCNA monoubiquitination and TLS polymerase recruitment to UV lesions have also been recently implicated in nucleotide excision repair (NER), a DNA repair process that can take place outside of S phase (Ogi et al., 2010). It is currently unclear whether specific cell cycle phases, such as G1 or S phase, can dictate the mechanism of how the cell responds to UV-mediated DNA damage through the activation of PCNA monoubiquitination and subsequent DNA repair.

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin ligase that targets many key cell cycle regulators for proteolysis (Qiao et al., 2010). The activation of APC/C is dependent on two WD-40 repeat domain–containing proteins, Cdc20 and Cdh1. Whereas APC/C\(^{\text{Cdc20}}\) principally regulates mitotic progression, APC/C\(^{\text{Cdh1}}\) shows a broad spectrum of G1-specific substrates, including proteins that function beyond cell cycle control (Skaar and Pagano, 2008; Qiao et al., 2010). Previous studies have shown that Cdh1 knockout or depletion in mammalian cells can cause genomic instability, but the precise cause of this instability is unclear (Engelbert et al., 2008; García-Higuera et al., 2008). Recently, however, the stability of several proteins involved in the DNA damage checkpoint response and DNA repair, such as Claspin, Rad17, thymidine kinase 1, and the ribonucleotide reductase subunit RRM2, has been shown to be regulated by APC/C\(^{\text{Cdh1}}\) (Chabes et al., 2003; Ke et al., 2005; Bassermann et al., 2008; Gao et al., 2009; Zhang et al., 2010). Moreover, although APC/C\(^{\text{Cdh1}}\) is active only at the very end of mitosis and during G1, it is reactivated in G2 in response to DNA damage to target the mitotic kinase Plk1 for proteasomal degradation (Bassermann et al., 2008). It is likely that more Cdh1 substrates will be identified with roles in the DNA damage response (DDR) pathways, further underscoring the relevance and importance of APC/C in maintaining cellular genomic integrity.

In humans, protein deubiquitination is controlled by a family of nearly 100 deubiquitinases (DUBs), but the function of many of these ubiquitin proteases is unknown (Nijman et al., 2002). We found that G1-stabilized USP1 was still catalytically active only at the very end of mitosis and during G1, it is reactivated in G2 in response to DNA damage to target the mitotic kinase Plk1 for proteasomal degradation (Bassermann et al., 2008). It is likely that more Cdh1 substrates will be identified with roles in the DNA damage response (DDR) pathways, further underscoring the relevance and importance of APC/C in maintaining cellular genomic integrity.

In this study, we explore whether the control of USP1 levels during cell cycle progression modulates the cellular response to UV DNA damage. We show that during G1, USP1 is targeted for degradation by APC/C\(^{\text{Cdh1}}\) to ensure that USP1 levels are kept in check before S phase entry. Low levels of USP1 enable robust UV-induced PCNA monoubiquitination during G1, which is likely to allow the recruitment of TLS polymerases to UV lesions. These findings suggest that APC/C\(^{\text{Cdh1}}\) plays a direct role in modulating the DNA repair choice in G1 and further solidify the link between cell cycle regulation and DNA repair.

**Results and discussion**

**USP1 levels are low during the G1 phase of the cell cycle**

To investigate whether USP1 activity is cell cycle regulated, we examined whether USP1 protein levels fluctuate in cells progressing through the cell cycle. We synchronized both U2OS and HeLa cells in prometaphase and then let them progress from mitosis into the next cell cycle. We found that USP1 levels are low during G1 and high during S phase (Fig. 1, A and B). We also observed low levels of USP1 in serum-starved and G1 T98G cells, similar to other proteins known to be degraded in G0/G1, such as Plk1 and cyclin A (Fig. 1 C). USP1 DUB activity depends on the interaction with its catalytic cofactor WDR48 (also called UAF1; Cohn et al., 2007). In contrast to USP1, WDR48 levels did not change throughout the cell cycle (Fig. 1 B and not depicted).

**APC/C\(^{\text{Cdh1}}\) regulates USP1 levels in G1**

The down-regulation of USP1 in the G1 phase of the cell cycle led us to ask whether a ubiquitin ligase may be responsible for the degradation of USP1. As the APC/C\(^{\text{Cdh1}}\) complex is critical for promoting the degradation of cell cycle regulators in G1, we tested whether this ubiquitin ligase also regulates the G1 levels of USP1. To this end, we silenced Cdh1 expression in both synchronized T98G and synchronized U2OS cells using well-established siRNA oligonucleotides (Bashir et al., 2004). We found that USP1 accumulated in both asynchronous and G1 cells depleted of Cdh1 (Fig. 2, A and B). This accumulation appears to be caused by USP1 stabilization, as shown by measuring USP1 half-life (Fig. 2 C). As expected (APC/C\(^{\text{Cdh1}}\) is inactive during prometaphase), the accumulation of USP1 after Cdh1 silencing was not as prominent in M phase cells (Fig. 2 B). Similarly, upon Cdh1 silencing, Plk1, a known Cdh1 substrate, was also more accumulated in asynchronous and G1 cells than in M phase synchronized cells (Fig. 2 B).

Next, we determined whether USP1 DUB activity is functional during the G1 phase. To this end, we measured the ability of USP1 to react with a chemically modified ubiquitin suicide substrate probe (ubiquitin vinyl sulfone [UbVS]; Borodovsky et al., 2002). We found that G1-stabilized USP1 was still catalytically active (Fig. 2 D). This suggests that USP1 proteolysis is likely a critical mechanism to inhibit USP1 activity during the G1 phase. Thus, we predict that a stable mutant of USP1 that could not be targeted by APC/C\(^{\text{Cdh1}}\) during G1 would still interact with WDR48 (which is expressed in G1 as shown in Fig. 1 B) and deconjugate specific monoubiquitinated substrates.

To ensure that the Cdh1 knockdown effect on USP1 stability was direct, we addressed whether Cdh1 could interact with USP1 in vivo. We found that both exogenously and endogenously
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Figure 1. USP1 levels are low during the G1 phase of the cell cycle.

(A and B) HeLa (A) and U2OS (B) cells were synchronized in M phase by treating cells with nocodazole (Noc) for 16 h, washed, released, and collected for the indicated time points. (C) T98G human glioblastoma cells were synchronized in G0/G1 by serum deprivation for 72 h and then refed with serum and harvested at the indicated time points. (A–C) Western blot analyses were performed and probed with the indicated antibodies. Separate samples were also collected, fixed, and stained with PI for FACS analysis according to procedures outlined in the Materials and methods section.

Characterization of a USP1 mutant that cannot be degraded via APC/C<sub>Cdh1</sub>

To search for a USP1 mutant that is stable in G1, we attempted to map the degradation motif (degron) in USP1. APC/C<sub>Cdh1</sub>-mediated degradation typically requires the RXXL degron (known as destruction box or D box) in its target proteins (Glotzer et al., 1991). Although human USP1 contains three putative D box motifs and a KEN box (Fig. S1; Pfleger and Kirschner, 2000; Littlepage and Ruderman, 2002; Reis et al., 2006), their mutation, singly or in combination, had no appreciable effect on USP1 stability in G1 (not depicted). Thus, we suspected that USP1 possesses a noncanonical degron, which is similar to those found in Aurora A (Littlepage and Ruderman, 2002), Cdc20 (Reis et al., 2006), or Claspin (Bassermann et al., 2008). To map the USP1 degron, we generated a series of N- and C-terminal deletion mutants (Fig. 3 A) and tested them for their ability to resist degradation in response to Cdh1 overexpression. We found that a region surrounding amino acids 307–330 of human USP1 is required for Cdh1-dependent degradation of USP1 (Fig. 3 B). Accordingly, a near full-length USP1 construct containing an internal deletion of 295–342 amino acids was stable in U2OS cells progressing through G1 (Fig. 3 C), whereas wild-type USP1, a USP1 (C90S) mutant (which is catalytic inactive), or the USP1 (GG670/671AA) diglycine mutant (which is unable to cleave itself) was not (Fig. 3 D). These results show that the catalytic and autocleavage activities of USP1 are not necessary for USP1 to be targeted for degradation by APC/C<sub>Cdh1</sub> during G1. This strongly suggests that the mechanism for degrading USP1 in G1 is completely different from the UV-initiated autocleavage and degradation of USP1 during S phase, as the proteolysis of USP1 during G1 is independent of its own catalytic activity (Huang et al., 2006).

We also generated a double USP1 mutant that contained both the 295–342 deletion and the GG670/671AA mutation, which was also resistant to proteolysis by APC/C<sub>Cdh1</sub> during G1 (Fig. 3 E). To further support that USP1 is ubiquitinated via APC/C<sub>Cdh1</sub>, we reconstituted the ubiquitination of USP1 in vitro. USP1 was efficiently ubiquitinated only when Cdh1 was present and a specific E2 combination was used (see Materials and methods section; Figs. 3 F and S2). Under our conditions, it is unclear why UBCH10-UBCH5 (Aristarkhov et al., 1996; King et al., 1996; Yu et al., 1996) works better than the canonical E2 pair UBCH10-UBE2S (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010) in promoting USP1 polyubiquitination. Interestingly, both UBCH10 and UBE2S are also degraded in G1 by APC/C<sub>Cdh1</sub>, suggesting that other E2 enzymes may be responsible for ubiquitinating G1 versus M phase substrates of APC/C (Williamson et al., 2009). Importantly, Cdh1-dependent ubiquitination was not observed in the USP1 degron mutant (Fig. 3 G). Unexpectedly, the degron mutant was still capable of binding to Cdh1 in vitro, suggesting that the degron region may be more critical for promoting efficient APC/C<sub>Cdh1</sub>-dependent ubiquitination of USP1 (Fig. S3). We have not ruled out the possibility of multiple Cdh1 binding sites on USP1 that may contribute to its direct association with the APC/C<sub>Cdh1</sub> complex. Nevertheless, experiments in cell systems and in vitro show that Cdh1 promotes the ubiquitination and subsequent degradation of USP1 in a manner dependent on its degron region.

Expressed USP1 can interact with members of the APC/C<sub>Cdh1</sub> complex (Fig. 2, E–G). In T98G cells that are accumulating in G0/G1 (serum starvation time course), we also show that endogenous USP1 can interact with Cdh1 before its degradation (Fig. 2 G).
Figure 2. Cdh1 depletion stabilizes USP1 during the G1 phase of the cell cycle. (A) T98G cells were transfected for 48 h with a control (Ctrl) siRNA (AllStars Negative; QIAGEN) or Cdh1 siRNA. Cells were serum starved in culture media containing 0.05% FBS for 24 h to arrest them in G0/G1. 0 indicates cells grown in regular media. (B) U2OS cells were transfected with the indicated siRNAs, synchronized in M phase (M) by incubating with nocodazole for 16 h, washed, and released in fresh media for 3 h for G1 phase (G1). Separate samples were collected for FACS. (C) U2OS cells were transfected with the indicated siRNAs and treated for the indicated time points with cycloheximide (CHX) to inhibit protein synthesis. (D) U2OS cells were transfected with the indicated siRNAs and synchronized in G1 as in B. Samples were collected and lysed according to protocols described for the UbVS DUB activity assay (see Materials and methods). Higher shift in the USP1 protein band indicates active USP1 (covalently modified USP1 by HA-UbVS). (E) Expression constructs were cotransfected in U2OS cells and treated for 6 h with 10 µM of the proteasome inhibitor MG132 before termination. Samples were lysed and collected for immunoprecipitation (IP) or loaded for input. (F) U2OS cells were transfected with Myc-USP1 wild type (WT) for 48 h and treated with MG132 as in E. Samples were lysed and collected for immunoprecipitation. *, heavy chain band. (G) T98G cells were serum starved as in A for the indicated times. Samples were lysed and collected for immunoprecipitation and input (10%) and probed with the indicated antibodies for Western blot analysis.
Figure 3. Identification of a USP1 mutant that cannot be degraded via APC/C^Cdh1. (A) A schematic diagram of critical USP1 domains and Myc-USP1 expression constructs generated for testing of their stability when coexpressed with FLAG-Cdh1. +, degradation of Myc-USP1; −, stabilization of Myc-USP1; GG, diglycine residue for the USP1 autocleavage site; red x’s, deletion or point mutations of the region. (B) U2OS cells were transiently transfected with Myc-USP1 and/or Flag-Cdh1 constructs in the presence or absence of MG132 (3 h). IB, immunoblot. (C) U2OS cells were transfected with Myc-USP1 wild
Degradation of USP1 permits UV-induced monoubiquitination of PCNA during G1

We then investigated the biological significance of USP1 degradation in G1. One possibility is that the repair of UV-induced lesions in G1 requires robust PCNA monoubiquitination. In fact, PCNA monoubiquitination is involved in the recruitment of TLS polymerases to sites of UV damage for repair synthesis (Ogi et al., 2010). To test whether UV-induced DNA damage during G1 induces PCNA monoubiquitination, U2OS cells were synchronized in G1 by releasing them from a prometaphase block. 2 h after release, cells were either left untreated or UV irradiated and followed for up to 10 h. After UV damage, USP1 levels decreased below the G1 levels, whereas in untreated cells, USP1 reaccumulated as they exited G1 and started DNA replication (Fig. 4 A). Accordingly, after UV damage, Cdh1 levels remained elevated, correlating with the loss of USP1 and a delay in S phase entry. Interestingly, robust monoubiquitination of PCNA already occurred by 2 h after UV damage and remained present for up to 10 h (Fig. 4 A). In contrast, detectable PCNA-monoubiquitinated species only started to appear when non-irradiated cells reached S phase. A strong UV-induced PCNA monoubiquitination response was also observed in T98G cells during G1 (Fig. 4 B). Interestingly, the monoubiquitination of FANCDD and FANCI (FA pathway effector proteins) was not induced by UV damage in G1 (unpublished data). This is likely a result of low levels of UBE2T, the E2 conjugating enzyme for the FA pathway, in G1 that prevent activation of the FA pathway outside of S phase (unpublished data). We also noted that the DDR after UV damage is different in G1 versus S phase. For instance, UV irradiation targets the autoleavage and degradation of USP1 in S phase to promote PCNA monoubiquitination, as previously reported (Fig. 4 B; Huang et al., 2006). However, USP1 levels remain low and unchanged after UV damage in G1 (Fig. 4 B), suggesting that the environment in G1 was already conducive for UV-mediated activation of PCNA monoubiquitination. In addition, Cdh1 is degraded after UV damage in S phase cells (Liu et al., 2008) but not in G1. Also, the checkpoint kinase Chk1 is not activated by phosphorylation after UV damage in G1.

We also found that the mechanism controlling PCNA monoubiquitination in G1 is surprisingly similar to the Rad18-dependent mechanism that governs its activation in S phase (Ogi et al., 2010). The knockdown of Rad18 resulted in the inhibition of UV-induced PCNA monoubiquitination during G1 (Fig. 4 C). The monoubiquitination of PCNA is dependent on lysine 164 (unpublished data). Likewise, expression of stable degron mutants of USP1 also reduced the levels of PCNA monoubiquitination and the recruitment of the TLS polymerase Pol-κ to sites of UV lesions in G1 after UV damage (Fig. 4, D and E). Thus, from our study, we provide a model suggesting that degradation of USP1 by the APC/C{sup Cdh1} during G1 is critical for cells to properly recruit TLS polymerases for UV-mediated DNA gap repair (Fig. 4 F).

Conclusion

Proteolytic control of cell cycle regulators by the APC/C{sup Cdh1} ubiquitin ligase complex is responsible not only for a stable G1 phase after mitotic exit but also for allowing accurate preparation for DNA replication in the following S phase (Nakayama and Nakayama, 2006). It is known that some of these APC/C{sup Cdh1} target proteins are frequently up-regulated in tumor cells, and inactivation of human and mouse Cdh1 interferes with genome integrity (Engelbert et al., 2008; García-Higuera et al., 2008). However, it is still unclear what protein targets of APC/C{sup Cdh1} are critical for maintaining genomic stability in human cells. Here, we show that USP1 can be targeted for degradation by the APC/C{sup Cdh1} cell cycle regulator. This mainly occurs in the G1 phase before the start of DNA replication. USP1 is an important negative regulator of monoubiquitination for two major DNA repair pathways (FA and TLS) that control genomic stability. Thus, proper dynamic control of USP1 levels throughout the cell cycle and during DNA damage may strongly impact how the cell deals with certain types of DNA lesions. To further validate this point, experimental evidence has suggested that higher than normal levels of USP1 will inhibit the DNA damage–induced monoubiquitination of USP1 targets, such as FANCDD, FANCI, and PCNA (Huang et al., 2006; Oestergaard et al., 2007), whereas the loss of USP1 has been shown to cause chromosomal instability and elevated perinatal lethality in mice (Kim et al., 2009).

Previous works by several groups have hinted at a new role for TLS that is linked to PCNA monoubiquitination outside of S phase. For example, Soria et al. (2009) has suggested that the TLS polymerase Pol-η can be recruited to UV-induced DNA lesions in cells outside S phase, including cells permanently arrested in G1. Work in DT40 cells has shown that PCNA ubiquitination may not be required to maintain normal fork progression on damaged DNA template but is essential for filling postreplicative gaps (Edmunds et al., 2008). More recently, Ogi et al. (2010) has suggested that ubiquitinated PCNA is involved in the recruitment of the TLS polymerase Pol-κ to sites of UV lesions in the G1 phase. The function of ubiquitinated PCNA outside of S phase is tied to a gap-filling role or repair synthesis, which occurs downstream of NER. From our study, we believe that the cell prefers to maintain a low level of USP1 during G0/G1 to establish a permissive environment and allow for robust PCNA monoubiquitination if necessary (Fig. 4 F). Thus, in the absence of its negative regulator, PCNA can be rapidly activated after...
Figure 4. UV-induced PCNA monoubiquitination during G1 requires prolonged degradation of USP1. (A) U2OS cells were synchronized in M phase with nocodazole (Noc) treatment and released for the indicated time points. Cells were also irradiated with 50 J/m² UV in G1 (2 h after release from nocodazole treatment). Samples were then analyzed by Western blotting. Cells from corresponding samples were pulsed with EdU (BrdU analogue) to label DNA replication. (B) Schematic summary of USP1 proteolysis by APC/C^Cdh1. USP1 is degraded during S phase to allow PCNA monoubiquitination, which is necessary for DNA replication. During G1, USP1 is stabilized, preventing PCNA monoubiquitination and consequently DNA replication.
UV damage to allow repair synthesis to occur downstream of the NER pathway. In contrast, expression of a nondegradable form of USP1 during G1 will lead to less monoubiquitination of PCNA and defective recruitment and repair of UV lesions by the TLS polymerases. The reliance on excision and gap filling–based repair mechanisms becomes more crucial when cells are in quiescence or in the postmitotic phase because of the lack of available sister chromatid for homologous recombination repair (Hoeijmakers, 2001).

Materials and methods

Cell culture and cell cycle synchronization and cell images

T98G, Hela, and U2OS cells were grown in DME with 10% FBS, 1% penicillin/streptomycin, and 1% glucose. Cells were synchronized in G0/G1 by serum deprivation (0.05% FBS) for 72 h. After 72 h, cells were split, plated, and refeed with normal cell culture media. Cells were synchronized in M phase by incubating cells with 0.1 µg/mL nocodazole for 12-16 h. After 12-16 h, mitotic cells were shaken off, collected, and washed twice with 1x PBS and then plated with fresh media. To verify the cell cycle phase for the cell synchronization experiments, cell samples were stained by propidium iodide (PI) according to the following staining procedure: cell pellets were washed and fixed in 70% ethanol overnight and stained with PI staining buffer (50 µg/mL PI and 10 µg/mL RNase in PBS). Flow cytometry analysis was performed using FACSCaliber and analyzed using CellQuest software (BD) and Modfit LT software (V.3.1; Verity Software House). Immunocytochemistry was performed according to protocol as previously described (Colnaghi et al., 2011). In brief, for detection of GFP–Pol–GFP–Pol–released into G1 (3 h), UV irradiated (3 h after nocodazole release), and then collected 3 h after UV exposure (6 h). (E) U2OS cells stably expressing GFP–Pol–GFP–Pol–released for 3 h into the G1 phase. The cells were then irradiated with 50 J/m2 UV and fixed 2 h after UV exposure. Approximately 100 GFP-positive cells were acquired on a flow cytometer (LSR II; BD) using the FACTS DiVa (BD) and Modfit LT softwares.

Transfections, siRNA oligonucleotides, DNA constructs, and antibodies

Transfections with plasmids or siRNA oligonucleotides were performed using Fugene 6 (Roche) or Hiperfect (QIAGEN) reagent. The siRNA oligonucleotide sequences coding for 2× Myc epitope tag.

In vitro ubiquitination, DUB activity assay, and communoprecipitation

USP1 constructs and Cdh1 were in vitro translated using the TNT 17-coupled wheat germ extract system (Promega) and TNT SP6-coupled reticulocyte lysate system (Promega), respectively, following the manufacturer’s protocol. In vitro translation reactions were stopped using 1 mg/mL cycloheximide. Ubiquitination of in vitro–translated USP1 was performed at 30°C with 50 µM Tris, pH 7.6, 5 mM MgCl2, 2 mM ATP, 2.5 mg/mL ubiquitin, 1 µM ubiquitin aldehyde, 1.5 µg/µl UBE1, 25 ng/µl UBCH10, 5 ng/µl UBCH5 (Boston Biochem), and 10 µM GS132 (EMD) in the presence or absence of in vitro–translated HA–Cdh1 (1:10), UBCH10 and UBCH5 with chains as the E2 enzyme pair to use for the in vitro ubiquitination assay after screening several E2 pairs to determine the best E2 combination to use (Fig. S2 A). Cdc2 was used as a positive control for an APC/C-Cdh1-specific ubiquitination substrate (Fig. S2 B), and methyl ubiquitin was used to confirm chain elongation for the in vitro ubiquitination assay (Fig. S2 C). UbVS DUB activity assay was performed according to Borodovsky et al. (2002) with several modifications. Cells were lysed for 1 h on ice with gentle tapping (250 mM Tris, 150 mM NaCl, 3 mM EDTA, and 0.5% NP-40). Supernatants from cell lysis were collected after a 10-min spin of 14,000 rpm at 4°C. Cell extracts and 50 µM HA–UbVS probe (Boston Biochem) were incubated at 25°C for 1.5 h in DUB buffer (50 µM Tris, 50 mM NaCl, 10% glycerol, and 1 mM EDTA). Reactions were terminated with Laemml buffer and boiled for Western blot analysis. Communoprecipitation studies were performed overnight at 4°C with the indicated antibodies according to the protocol previously described (Sims et al., 2007). In brief, cell lysis, antibody incubation, and bead washes were performed using low immunoprecipitation buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 0.5% NP-40 with protease inhibitor cocktail [Roche]).

Induction of DNA damage and measurement of DNA synthesis

T98G and U2OS cells were treated and harvested between 50 and 80% visible conflueny. 254-nm UV-C irradiation was performed (Stratalinker 2400; Agilent Technologies) in the absence of DME media at 50 J/m2. Recovery after UV damage was in complete medium for the times indicated. Replicating cells were detected using a flow cytometry assay kit (Click-IT Edu; Invitrogen) according to the manufacturer’s instructions. Flow cytometric data were acquired on a flow cytometer (LSR II; BD) using the FACTS Dil (BD) and Modfit LT softwares.

Online supplemental material

Fig. S1 shows the sequence alignment of the USP1 degron region. Fig. S2 shows the sequence alignment of the USP1 degron region. Fig. S2 shows APC/C-Cdh1-dependent ubiquitination of USP1 using different E2 pairs.
and methyl ubiquitin. Fig. S3 shows that the degron mutant can still interact with Cdh1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201101062/DC1.

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Figure S1. **Sequence alignment of the USP1 degron region.** (A) USP1 amino acid sequence obtained from the National Center for Biotechnology Information [NCBI Protein database accession no. NP_001017415.1]. Putative APC/C recognition motifs are enclosed in boxes (D box [RxxL], KEN box [KEN], and EEN [Cdh1 interaction motif identified in Claspin]), the deleted region (295–342 amino acids) is underlined, and the putative Cdh1 recognition region (307–330 amino acids) is bold and in between brackets. (B) Alignment of the deleted region 295–342 amino acids from human, mouse, chicken, and frog USP1 using ClustalW2 Multiple Sequence Alignment (European Bioinformatics Institute). Shaded black regions and asterisks signify that the residues in the columns are identical in all sequences in the alignment. Shaded gray regions and colons represent observed conserved amino acid substitutions, and periods represent observed semiconserved substitutions. The bold text in between brackets is the same as described in A. (C) Conservation and consensus of the deleted region 295–342 amino acids shown in bars using the editing program JalView, which is available in the ClustalW2 alignment results. Asterisks are the same as described in B.
Figure S2. APC/C<sup>Cdh1</sup>-dependent ubiquitination of USP1 using different E2 pairs and methyl ubiquitin. (A) In vitro ubiquitination of Myc-USP1 wild type using different E2 ubiquitin-conjugating enzyme combinations in the presence or absence of HA-Cdh1. 25 ng/µl UBCH10, 25 ng/µl Ube2S, 10 ng/µl UBCH5, and 10 ng/µl UBCH3 were purchased from Boston Biochem. (B) In vitro ubiquitination of Cdc6 (a known APC/C<sup>Cdh1</sup> substrate) using the UBCH10 and UBCH5 E2 combination. Cdc6 was produced in wheat germ extract (Promega) by in vitro translation (ivt) and probed with anti-Cdc6 antibody (Santa Cruz Biotechnology, Inc.). Cdc6 cDNA in pCMV6-XL5 was purchased from Origene. (C) In vitro ubiquitination of Myc-USP1 wild type with either ubiquitin or methyl ubiquitin (Boston Biochem) using the UBCH10 and UBCH5 E2 enzyme pair. All aforementioned reactions were performed as described in the Materials and methods section. Molecular mass is indicated in kilodaltons. Rxn, reaction.

Figure S3. The degron mutant can still interact with Cdh1. (A) Myc-USP1 wild type (WT) and degron mutant (Myc-USP1 295–342 del) were transiently co-expressed with Flag-Cdh1 in U2OS cells. Cells were treated with proteasome inhibitor MG132 for 6 h before termination. Cell extracts were used for co-immunoprecipitation (IP; see Materials and methods section) using anti-Flag (M2; Sigma-Aldrich) antibody and were analyzed by Western blotting. Molecular mass is indicated in kilodaltons. IB, immunoblot.