Deubiquitylation and stabilization of p21 by USP11 is critical for cell-cycle progression and DNA damage responses

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p21WAF1/CIP1 is a broad-acting cyclin-dependent kinase inhibitor. Its stability is essential for proper cell-cycle progression and cell fate decision. Ubiquitylation by the multiple E3 ubiquitin ligase complexes is the major regulatory mechanism of p21, which induces p21 degradation. However, it is unclear whether ubiquitylated p21 can be recycled.

In this study, we report USP11 as a deubiquitylase of p21. In the nucleus, USP11 binds to p21, catalyzes the removal of polyubiquitin chains conjugated onto p21, and stabilizes p21 protein. As a result, USP11 reverses p21 polyubiquitylation and degradation mediated by SCF, CRL4, and APC/C. In a cell-cycle-independent manner. Loss of USP11 causes the destabilization of p21 and induces the G1/S transition in unperturbed cells. Furthermore, p21 accumulation mediated by DNA damage is completely abolished in cells depleted of USP11, which results in abrogation of the G2 checkpoint and induction of apoptosis. Functionally, USP11-mediated stabilization of p21 inhibits cell proliferation and tumorigenesis in vivo. These findings reveal an important mechanism by which p21 can be stabilized by direct deubiquitylation, and they pinpoint a crucial role of the USP11-p21 axis in regulating cell-cycle progression and DNA damage responses.

Significance

Previous studies have demonstrated that p21 occupies a central position in cell-cycle regulation and DNA damage responses. As an unstable protein, the regulation of p21 stability has been extensively investigated over the past 20 years. Although p21 degradation by the ubiquitin-proteasome pathway has been well characterized, it is unclear whether ubiquitylated p21 can be recycled. Here, we identify USP11 as a deubiquitylase that directly removes p21 polyubiquitylation and stabilizes p21 protein, revealing that cellular p21 protein is finely regulated by a dynamic balance of USP11-mediated stabilization and proteasome-mediated degradation. Meanwhile, we also provide evidence that the USP11-p21 axis plays a crucial role in G1/S transition under physiological conditions and in regulating the balance between cytostasis and apoptosis.


The authors declare no conflict of interest.

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Supporting Online Material

of its substrates (24–27). USP11 dysregulation has been found in a variety of tumors, including colorectal cancer, melanoma, glioma, and cervical cancer (28–30).

In this study, we identified USP11 as the first deubiquitylase that directly reverses p21 polyubiquitylation and stabilizes the p21 protein. We also demonstrated that the USP11-p21 axis is critical for regulating cell-cycle progression and DNA damage-induced G2 arrest. Our findings reveal an important missing piece regarding the regulation of p21 stability and indicate a previously unknown molecular function of USP11 in controlling cell-cycle progression and DNA damage responses.

Results

**USP11 Interacts with p21.** USP11 has been shown to function as a deubiquitylating enzyme that stabilizes multiple cellular proteins by cleaving ubiquitin-protein bonds. To search for cellular proteins that interact with USP11, we expressed Flag-tagged USP11 protein in A549 cells and purified USP11-bound protein complexes using an anti-Flag monoclonal antibody coupled to Dynabeads. USP11-associated proteins were identified by liquid chromatography mass spectrometry/mass spectrometry. Intriguingly, p21 was present in the purified USP11 complexes, but not in the control purifications (Fig. S1A). Given the known cellular feature of p21 that can be rapidly degraded by ubiquitylation, we focused our attention on p21 as an interacting protein with USP11.

To confirm the interaction between USP11 and p21, Flag-USP11 or Myc-p21 plasmid was transfected into A549 cells, and coimmunoprecipitation (co-IP) was performed using an anti-Flag or anti-Myc antibody. The results showed that p21 was detected in the Flag-USP11 immunoprecipitates (Fig. 1A) and that USP11 was present in Myc-p21 immunoprecipitates (Fig. 1B). Meanwhile, the association of endogenously expressed p21 and USP11 was also investigated using co-IP. USP11 and p21 were separately immunoprecipitated from A549 cells, and the reciprocal protein was detected using Western blotting. As shown in Fig. 1C, both USP11 and p21 were detected in their individual immunoprecipitated complexes, but not in the isotype-matched negative control IgG complexes. To determine whether USP11 and p21 directly interact with each other, we generated and purified recombinant USP11 and p21. Purified GST-p21, but not the GST control, was able to bind to GST-USP11 under cell-free conditions (Fig. 1E), demonstrating a direct interaction between USP11 and p21. Similar results were obtained by incubating purified GST-USP11 with extracts from A549 cells (Fig. S1D). Immunofluorescent staining revealed that the colocalization of both USP11 and p21 occurred in the nucleus (Fig. 1F). Collectively, these results suggest that USP11 physically interacts with p21 in vivo and in vitro.

To determine key amino acid residues for the interaction of USP11 with p21, we constructed a catalytically inactive USP11 mutant (C275S/C283S) and multiple p21 mutants with a single point mutation. The results showed that a catalytically inactive USP11 mutant (C275S/C283S) and multiple p21 mutants with a single point mutation. The results showed that a catalytically inactive USP11 mutant and p21 still retained the ability to bind to p21 similar to wild-type (WT) USP11 (Fig. 1G). In contrast, a single point mutation of p21 at T57, S130, T145, or S146 residues resulted in a significant decrease in USP11 binding, indicating that these residues of p21 are essential for USP11 interaction (Fig. 1H). Furthermore, to map the USP11-binding region on p21, a series of p21-deletion mutants was expressed in A549 cells (Fig. S1C). The co-IP assays revealed that the N-terminal region (amino acids 1–90) of p21 was critical for the interaction between USP11 and p21 (Fig. 1I). Conversely, mapping the USP11 region required for p21 binding revealed that the C terminus (amino acids 536–920) was responsible for the interaction with p21 (Fig. S1 E and F).

**USP11 Regulates the Protein Level of p21.** Protein–protein interactions are known to play key roles in regulating p21 levels. Given the identified interaction of USP11 with p21, we next investigated whether USP11 affects the steady-state levels of p21. USP11 was introduced into A549 (p53+/-) as well as two HCT116 cell lines with a p53 WT (HCT116 WT) and null (HCT116 p53−/−) genotype. Interestingly, USP11 overexpression resulted in a significant increase of endogenous p21 levels (Fig. 2A), and increasing USP11 expression caused an elevation of p21 levels in a dose-dependent manner in all cell lines regardless of the p53 status (Fig. 2B and C). In contrast, p53 levels were unaffected by USP11 overexpression, indicating that USP11 increased p21 levels in a p53-independent manner. Notably, overexpression of a catalytically inactive USP11 mutant (C275S/C283S) had no effect on p21 levels (Fig. 2A–C), implying that USP11-mediated up-regulation of p21 may depend on the function of USP11 as a deubiquitylating enzyme. To further confirm the regulation of p21, we performed a loss-of-function analysis using two independent USP11-specific short hairpin RNAs (shRNAs) in the above-mentioned cell
lines. As predicted, USP11 knockdown abolished p21 levels without affecting p53 expression (Fig. 2D). Similar results were obtained using USP11-specific small interfering RNAs (siRNAs) in the A549, H460, and HCT116 cell lines (Fig. S2A).

The effect of USP11 on the p21 steady-state levels was not due to changes in transcription because neither USP11 knockdown nor overexpression affected the p21 mRNA levels (Fig. S2B–D), indicating that USP11 does not regulate p21 expression at the transcriptional level. Furthermore, down-regulation of p21 caused by USP11 knockdown could be blocked by the proteasome inhibitor MG132 and CLL (Fig. S2E and F), suggesting that USP11 maintains the steady-state levels of p21 by blocking its proteasomal degradation.

p21 is one of the most known transcriptional targets of p53. To address whether USP11 is also a target gene of p53 like p21, we transfected HCT116 WT with a plasmid encoding Flag-p53 or an empty vector control. Consistent with previous studies, p21 was obviously up-regulated in the mRNA levels and protein levels. By contrast, the level of USP11 was not affected by p53 overexpression (Fig. S2G and H), suggesting that USP11 is not a p53-inducible gene.

**USP11 Stabilizes p21 by Deubiquitylation.** Because USP11 regulates the protein levels of p21, we questioned whether USP11 stabilizes p21. To this end, in the presence or absence of Flag-USP11, cells were treated with cycloheximide (CHX) to inhibit protein biosynthesis, and protein extracts obtained at indicated time points were analyzed. We found that overexpression of WT USP11 but not catalytically inactive mutant profoundly extended the half-life of the p21 protein (Fig. S3A–C). Conversely, knockdown of USP11 resulted in a significant decrease in the half-life of p21 (Fig. S3D and E). To further understand the underlying mechanism whereby USP11 regulates the stability of p21, we measured the levels of polyubiquitylation of p21 in HCT116 cells. Silencing USP11 expression using two independent shRNAs led to a significant increase in p21 polyubiquitylation (Fig. 2C), suggesting that USP11 maintains the steady-state levels of p21 by directly deubiquitylates p21.

To verify that p21 is a direct substrate of USP11, we purified USP11 WT or USP11mut were analyzed using SDS/PAGE and Coomasie blue staining.

**USP11 Stabilizes p21 in Response to DNA Damage.** p21 can be induced under DNA damage condition via p53-dependent and p53-independent pathways. To explore whether USP11 is involved
in the DNA damage-mediated regulation of p21, we treated cells with genotoxic agents. In agreement with previous reports, etoposide treatment led to the up-regulation of p21 levels in HCT116 WT and HCT116 p53−/− cells (Fig. 3 A and B). Intriguingly, etoposide-induced p21 accumulation was significantly abolished in USP11-depleted cells (Fig. 3 A and B). Similarly, USP11 knockdown also significantly decreased the p21 elevation triggered by doxorubicin (Fig. S5 A and B). Notably, depletion of USP11 did not abolish the induction of p21 mRNA in response to genotoxic treatment (Fig. S5 C–F). Furthermore, we analyzed the binding of USP11 to p21 under DNA damage condition. HCT116 WT cells were treated with genotoxic agents, and total cell lysates were subjected to immunoprecipitation with anti-USP11 or anti-p21 antibody. Interestingly, the amount of USP11-binding p21 was significantly increased in HCT116 WT cells after treatment with genotoxic agents and vice versa (Fig. 3C and Fig. S6G), indicating that DNA damage can enhance the interaction between USP11 and p21. Collectively, these findings suggest that USP11 is indispensable for the expression of p21 under physiological conditions as well as in response to DNA damage (Fig. 3D).

**USP11 Protects p21 from Ubiquitin-Mediated Degradation in a Cell-Cycle-Independent Manner.** Three E3 ubiquitin ligases—SCF, CRL4, and APC/CDC20—have been reported to induce p21 ubiquitylation and degradation at different phases during an unperturbed cell cycle. To assess which E3 ubiquitin ligase complex is regulated by USP11, HCT116 cells stably expressing the indicated shRNAs were synchronized at each phase (Fig. S6A). Strikingly, USP11 knockdown led to a significant decrease of p21 at all phases of the cell cycle, although the p21 protein level varied during the cell cycle (Fig. S6E). Furthermore, we examined whether the effect of USP11 on p21 was associated with SCF, CRL4, or APC/C. Knockdown of USP11 using shRNAs significantly decreased p21 levels with concomitant increases in SKP2 (Fig. 4A), but the levels of CDC2 and CDC20 were unchanged (Fig. 4B and C). Moreover, when SKP2, CDC2, or CDC20 was knocked down by siRNA, USP11 depletion-induced p21 degradation and ubiquitylation was abolished (Fig. 4A–F). Altogether, these results indicate that USP11 stabilizes p21 via the reversible of SCF, CRL4, or APC/C-mediated ubiquitylation and degradation in a cell-cycle-independent manner (Fig. 4G).

**USP11 Regulates Cell-Cycle Progression and the DNA Damage Response in a p21-Dependent Manner.** Because p21 regulates cell-cycle progression at G1 phase, we hypothesized that USP11 may affect cell-cycle progression from G1 to S phase. To test this hypothesis, the percentage of cells in S phase was determined by measuring the DNA content and incorporation of BrdU, as well as by performing double-thymidine block and release. As predicted, the percentage of cells in S phase was increased when USP11 was knocked down in HCT WT and HCT116 p53−/− cells (Fig. 5A–C and Figs. S7 and S8 A and B). In contrast, USP11 depletion in HCT116 p21−/− cells exhibited no effects on the G1/S transition (Fig. 5A and B and Fig. S7A), but USP11-depleted cells transfected with exogenous p21 fully prevented the G1/S transition induced by USP11 ablation (Fig. 5C and Fig. S7B). These results strongly suggest that the USP11-mediated G1/S transition is dependent on p21.

To determine whether p21 is required for the function of USP11 in the G2/M checkpoint after DNA damage, cells were treated with a low dose of doxorubicin. The phospho-histone3 (pH3) at Ser10, an indicator of cells at M phase, was used to monitor the G2/M checkpoint. As shown in Fig. 5D and Fig. S8C, after doxorubicin treatment, the percentage of cells in M phases was significantly increased in HCT WT cells with USP11 knockdown. However, in HCT116 cells lacking p21 (HCT116 p21−/−), silencing USP11 had no effect on the increased percentage of cells in M phase, indicating that USP11 depends on p21 to sustain the DNA damage-induced G2/M checkpoint.

To investigate the effect of USP11 on apoptosis induced by a DNA-damaging agent, HCT116 WT and HCT116 p21−/− cells were treated with either doxorubicin or etoposide. The percentage of cells in sub-G1 phase (apoptotic cells) was measured using flow cytometry with propidium iodide staining. Compared with the control cells, USP11-depleted HCT116 WT cells exhibited a significant increase in the levels of apoptosis after a 24-h treatment with either doxorubicin or etoposide (Fig. 5E). Interestingly, USP11 knockdown did not affect apoptosis triggered by either doxorubicin or etoposide in cells lacking p21 (Fig. 5F). Collectively, these data show that USP11 knockdown sensitizes cells to DNA damage-induced apoptosis by abolishing p21 accumulation.

**USP11 Functions as a Tumor Suppressor by Regulating p21.** Generally, p21 acts as a tumor suppressor in the nucleus. Given that USP11 regulates p21 stability in the nucleus, we hypothesized that USP11 might affect cell proliferation via acting on p21. To address this, we conducted a cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The results indicated that USP11 depletion promoted the proliferation of A549 and HCT116 WT cells and that p21 restoration completely reversed the effect of USP11 depletion (Fig. S9A). However, USP11 knockdown showed no effect on the proliferation of HCT116 p21−/− cells (Fig. S9B). Conversely, overexpression of USP11, but not of the catalytically inactive mutant of USP11, inhibited the proliferation of A549 and HCT116 WT cells (Fig. S9B). Likewise, overexpression of USP11 had no effect on the proliferation of HCT116 p21−/− cells (Fig. S9B).

To investigate the role of USP11 in nonsmall-cell lung cancer (NSCLC) cells in vivo, USP11-depleted A549 cells were implanted into nude mice, and tumor growth was monitored at the indicated time points. Compared with mice implanted with control shRNA-infected cells, mice bearing USP11-shRNA-expressing A549 cells showed increased tumor growth throughout the experiment (Fig. S10A). At 45 d after tumor cell implantation, the volume and weight of the tumors formed by USP11-depleted A549 cells significantly increased. (Fig. S10A–C). Notably, restoring p21 expression fully reversed the tumor-promoting effect of USP11 shRNA (Fig. S10A–C). Western blot analysis confirmed that the effect of USP11
Discussion

In the present study, we identified USP11 as a p21 deubiquitylase. Our results indicate that USP11 and p21 interact with each other and colocalize in the nucleus. Overexpression of USP11 stabilizes p21 by removing its ubiquitin chain, whereas USP11 down-regulation decreases p21 levels, which is accompanied by increasing ubiquitylation. Thus, p21 can be stabilized by direct deubiquitylation mediated by a deubiquitylase.

p21 is a well-known transcriptional target of p53. In response to various stresses including DNA damage and oxidative stress, activation of p53 induces p21 protein expression by binding its promoter. A recent study revealed that USP11 deubiquitylates and stabilizes p53 (31). However, our results indicated that USP11 had no effect on p53. Overexpression or knockdown of USP11, failed to affect p53 levels, which is consistent with a previous study demonstrating that USP11 does not interact with p53 and does not exhibit any effect on the levels of p53 ubiquitylation or stabilizing p53 (32). Furthermore, we found that USP11 exerts its function on p21 both in p53 WT and null cell lines, suggesting that USP11 regulates p21 levels in a p53-independent manner. In response to genotoxic treatment, p21 was accumulated depletion on p21 was retained in these tumors (Fig. S10D).

Furthermore, overexpression of WT USP11, but not the catalytically inactive mutant, inhibited tumor growth, and USP11-mediated inhibition on tumor growth could be fully reversed by knockdown of p21 (Fig. S10 E–H). Taken together, our data demonstrate that USP11 has a p21-dependent tumor-suppressing function.

To determine the relevance between USP11 and p21 abundance in NSCLC, we performed immunohistochemical staining of p21 and USP11 in the 35 NSCLC tissues. A significant positive correlation ($R = 0.79, P = 0.016$) between USP11 and p21 protein levels was observed in these NSCLC tissues (Fig. S10 I and J), in which 71% (25 of 35) of total tumors with low USP11 expression were accompanied by low p21 expression. Thus, these results suggest that loss of USP11 may contribute to the loss of p21 in NSCLC.

Fig. 4. USP11 protects p21 from ubiquitin-mediated degradation. (A–C) HCT116 WT cells infected with the indicated shRNAs were transfected with scrambled, SKP2 (A), CD2 (B), or CDC20 (C) siRNA for 48 h, and then the cell lysates were harvested and analyzed using Western blotting. (D–F) HCT116 cells infected with the indicated shRNAs were transfected with scrambled, SKP2 (D), CD2 (E), and CDC20 (F) siRNA for 48 h and treated with 20 μM of the proteasome inhibitor MG132 (Sigma) for another 6 h. p21 was immunoprecipitated with an anti-p21 antibody, and the immunoprecipitates were probed with anti-p21 or anti-Ub antibody. (G) A proposed working model that illustrates how USP11 reverses p21 ubiquitination in a cell-cycle-independent manner.

Fig. 5. USP11 regulates the G1/S transition and DNA damage-induced G2 checkpoint in a p21-dependent manner. (A) HCT116 WT, HCT116 p53−/−, and HCT116 p21−/− cells infected with the indicated lentiviral shRNAs were stained with propidium iodide and analyzed using flow cytometry. (B) HCT116 WT and HCT116 p21−/− cells transfected with the indicated shRNAs were labeled with BrdU for 60 min before harvesting and then analyzed using flow cytometry. The error bars represent the mean ± SD of three independent experiments. *P < 0.05. (C) HCT116 WT cells infected with the indicated lentiviral shRNAs were transfected with the indicated constructs for 24 h. Cells were stained with propidium iodide and analyzed using flow cytometry. The error bars represent the mean ± SD of three independent experiments. *P < 0.05. (D) HCT116 WT and HCT116 p21−/− cells infected the indicated lentiviral shRNAs were pretreated with 0.2 μM doxorubicin (Dox) for 2 h, followed by synchronization with nocodazole (100 ng·mL−1) for 16 h. The mitotic index was determined using pH3 staining as a marker of mitosis. The error bars represent the mean ± SD of three independent experiments. **P < 0.01. (E and F) HCT116 WT (E) and HCT116 p21−/− (F) cells were infected with the indicated lentiviral shRNAs. Cells were then treated with either 0.2 μM doxorubicin (Dox) or 5 μM etoposide (Etop) for 48 h, followed by flow cytometry analysis of the sub-G1 fraction. The error bars indicate the mean ± SD of three independent experiments. *P < 0.05.
in p53 WT and null cell lines. Strikingly, USP11 knockdown completely abolished p21 elevation induced by genotoxic agents, but not p21 mRNA induction. This finding reveals the interesting fact that the stability of p21 mediated by USP11 is indispensable for both p53-dependent and p53-independent transactivation of p21.

p21 is an unstable protein with a relatively short half-life that can respond to rapid intrinsic and extrinsic alterations. Its stability is regulated mainly by posttranslational modifications such as phosphorylation and ubiquitylation. For the ubiquitin-dependent pathway, three E3 ubiquitin ligase complexes—SCF

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\text{SCF}^\text{SKP2}, \text{CRL4}^\text{CDT2}, \text{and APOC}^\text{CDC20}
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—have been identified to promote p21 ubiquitylation and degradation at specific stages of the cell cycle. SCF

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\text{SCF}^\text{SKP2}
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is necessary for p21 degradation at the G1/S transition as well as during S phase of the cell cycle (33), whereas CRL4

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\text{CRL4}^\text{CDT2}
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specifically targets p21 for degradation in S phase (34). During mitosis, the APOC

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\text{APOC}^\text{CDC20}
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complex primarily drives p21 degradation (13). Here, we showed that USP11 protected p21 from ubiquitin-mediated degradation by abolishing the action of the above E3 ubiquitin ligase complex. Loss of p21 expression upon USP11 knockdown was significantly ameliorated by depleting SKP2, CDT2, and CDC20, indicating that USP11-mediated protection of p21 is independent of the cell cycle. Of note, p21 levels oscillate during mitosis, the APC/C

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\text{APC/C}^\text{Cdt2}
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subunit Cdh1 interacts with the RanGTP-associated protein RanBPM.

Materials and Methods

See SI Materials and Methods for additional methods.

Acknowledgments. We thank Dr. Han You (Xiamen University) for generously providing the HCT116 p21

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\text{p21}^\text{−/−}
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and HCT116 p53

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cells and Dr. Tiejang Kang (Sun Yat-sen University) and Dr. Zhouwei Hu (Chinese Academy of Medical Sciences) for their fruitful advice and discussion. This work was supported by grants from the National Natural Science Foundation of China (81171950, 81272220, 81402504, and 81672670), the National Basic Research Program of China (2013CB932702), and Hunan Provincial Natural Science Foundation of China (2016JJ3048).

References


10. Wang W, Nacuci L, Sheaff RJ, Liu X (2005) Ubiquitin-mediated degradation by abolishing the action of the above E3 ubiquitin ligase complex. Loss of p21 expression upon USP11 knockdown was significantly ameliorated by depleting SKP2, CDT2, and CDC20, indicating that USP11-mediated protection of p21 is independent of the cell cycle. Of note, p21 levels oscillate with high levels occurring during G1 and G2 (33). However, our result showed that USP11 levels gradually increased with cell-cycle progression from G1 to M phase (Fig. S6d), which is consistent with a previous study (36). Interestingly, USP11 levels do not result in consistent change of p21 levels during cell-cycle progression. We speculate that other factors may be involved in the regulation of p21 by USP11 during the cell cycle. Further studies are needed to fully understand the detailed mechanism.

It has been reported that p21 can function as a tumor suppressor as well as an oncosene. This dual behavior of p21 depends primarily on its subcellular location. The tumor-suppressive activities of p21 are associated with its nuclear localization, whereas cytoplasmic p21 contributes to its oncogenic effects. Our results show that USP11 acts as tumor suppressor, as overexpression of USP11 inhibited cell proliferation, whereas cells with USP11 depletion exhibited increased proliferation. This is consistent with previous reports that USP11 functions as a tumor suppressor (30, 37, 38). Furthermore, silencing USP11 in HCT116 p21

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\text{p21}^\text{−/−}
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cells had no effect on the proliferation, indicating that USP11 exerts its function via p21. Given that USP11 interacts with p21 in the nucleus, we speculated that the biological function of USP11 is associated with the tumor-suppressive activities of nuclear p21. Further studies are necessary to establish a detailed association between USP11 and a variety of human cancers, which will provide clues as to how to utilize USP11 as a potential cancer therapeutic target.
Supporting Information

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SI Materials and Methods

Immunofluorescence Staining. Cells were cultured in Lab-Tek chambers for 24 h, washed three times with PBS, and fixed with 4% paraformaldehyde for 15 min. The fixed cells were then washed twice with PBS, permeabilized for 10 min with 0.2% Triton X-100, blocked for 1 h in 5% BSA, and incubated with the appropriate primary antibodies overnight at 4 °C. The USP11 (1:200) and p21 (1:200) antibodies were used to detect endogenous protein expression. Cells were labeled for 1 h using secondary antibodies conjugated to either FITC (Thermo Fisher Scientific catalog no. 20-58-060111) or Texas Red (Thermo Fisher Scientific catalog no. 19-183-062312) at room temperature followed by three washes with PBS. Cells were then counterstained with DAPI at room temperature for 5 min to visualize nuclear DNA. Fluorescence images were acquired using a confocal microscope (Zeiss LSM510).

Western Blotting and Immunoprecipitation. Western blotting and co-IP were performed as described previously. Briefly, cells were lysed in M-PER buffer (Thermo Fisher Scientific catalog no. 78501) containing protease inhibitors (Biotool catalog no. B14001), and the clarified lysates were resolved on 12% gels using SDS/PAGE and transferred to nitrocellulose membranes for Western blotting using ECL detection reagents (Advansta catalog no. 160625-66). Alternatively, 3 mg of clarified lysates was incubated overnight at 4 °C with 3 μg of either relevant primary antibodies or an isotype-matched negative control IgG. Subsequently, the samples were incubated for 1 h with 30 μL of magnetic beads conjugated with protein G (Invitrogen catalog no. 10004D) and then washed four times with co-IP/wash buffer. Precipitated proteins were dissolved in 2× SDS sample buffer, boiled, and subjected to Western blot analysis.

GST Pulldown Assays. Bacterially expressed GST-USP11 was retained on glutathione Sepharose beads (Promega catalog no. V8611) and incubated for 1 h at 4 °C with 5A49 cell extracts.

Protein Half-Life Assays. Cells were treated with cycloheximide (50 μg·mL⁻¹) for various periods of time to block protein synthesis. Crude extracts were prepared, and the protein levels were assessed using Western blot analysis.

Synchronization of HCT116 WT Cells. To synchronize HCT116 WT cells at the G1/S border, cells were treated with 2 mM thymidine (Millipore catalog no. 6060) for 18 h. Cells were released from the block by washing with PBS followed by the addition of complete growth medium. After 9 h, thymidine was added to the medium to a final concentration of 2 mM, and the cells were cultured for an additional 18 h. Cells were then rinsed twice with PBS, cultured in complete growth medium for 3 h (S-phase cells), 6 h (G2-phase cells), or treated with culture media containing 100 ng·mL⁻¹ of nocodazole for 11 h (M-phase cells). Cells were collected and analyzed using flow cytometry and Western blotting.

Plasmids. Full-length USP11 was PCR-amplified from human cDNA and subcloned into the pCMV-Tag2B vector (containing a Flag tag) to create a Flag-tagged USP11 expression plasmid. Various USP11 deletion mutants were generated using PCR. GST-USP11 was constructed by inserting USP11 cDNA into the pGEX-4T-1 vector. GST-p21, myc-p21, and various p21 deletion mutants with an HA tag were gifts from Tiebang Kang, State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Guangzhou, China. All of the constructs were sequenced before use.

Antibodies. Anti-p53 (DO-1, catalog no. sc-126), anti-CDC20 (E7, catalog no. sc-13162), anti-USP11 (catalog no. sc-365528), and anti-MDM2 (catalog no. sc-965) antibodies were purchased from Santa Cruz. Anti-p21 (BD Pharmingen catalog no. 556430) Antibody Catalog (catalog no. A300-947A) antibodies were purchased from Abcam. Anti-Flag (catalog no. M185-3L), anti-Myc (catalog no. M192-3), and anti-HA (catalog no. M180-3) antibodies were purchased from Medical & Biological Laboratories. Anti-GAPDH (catalog no. KC-5G4) antibodies were purchased from Kangchen Bio-tech.

Real-Time PCR. Total RNA was extracted using Eastep (Promega catalog no. LS1040). RNA (1 μg) was reverse-transcribed in a 20-μL reaction using a FastQuant RT Super Mix Kit (TIANGEN catalog no. KR106-02). After reverse-transcribing the RNA at 42 °C for 15 min followed by inactivation of the polymerase at 95 °C for 3 min, the real-time (RT) PCR reaction was diluted. The resulting cDNA was used for RT-PCR using the following primer sequences: USP11-F AGGTGTCAGGTCGCATTGAG; USP11-R TGAGAGCCGGTACATCGAGG; GAPDH-F AAGGTGAAGGTCGGAGTCAA; GAPDH-R CTGTGAAAAGGTGAAGGTCGGAGTCAA; and p21-R CTGTGAAAAGGTGAAGGTCGGAGTCAA. Real-time PCR was performed according to the manufacturer’s protocol. After 48 h, the cells were washed with PBS and lysed directly into M-PER lysis buffer, after which the protein levels were assessed by Western blot analysis. To stably knock down endogenous USP11 expression, we used a lentiviral packaging shRNA expression vector (purchased from GenePharma) to transduce the cells. Target cells were infected with lentivirus for 24–48 h according to the manufacturer’s instructions. The following shRNA target sequences were used: USP11#1: 5′-AATGAGAACATCGAGATGTC-3′; USP11#2: 5′-AAGCAGCCAGCTATGCTCCTT-3′; and p21: 5′-TTTCCAGAGTGCTGATGAC; and control sequence, 5′-TTTCCAGAGTGCTGATGAC.

RNA Interference and Lentivirus Transduction. The sequences of the USP11 siRNAs have been previously reported (siUSP11#1: 5′-GGACCCAGCCGATGCAGAGG-3′; siUSP11#2: 5′-ACCAGCCCTAGCCAGAGG-3′; and p21: 5′-GACCCAGCCGATGCAGAGG-3′). siRNA transfection was performed according to the manufacturer’s instructions. After 48 h, the cells were washed with PBS and lysed directly into M-PER lysis buffer, after which the protein levels were assessed by Western blot analysis. To stably knock down endogenous USP11 expression, we used a lentiviral packaging shRNA expression vector (purchased from GenePharma) to transduce the cells. Target cells were infected with lentivirus for 24–48 h according to the manufacturer’s instructions. The following shRNA target sequences were used: USP11#1: 5′-CCGTGATGATATCTTCGTCT-3′; USP11#2: 5′-CCGTGATGATATCTTCGTCT-3′; p21: CTTCGAGTTCTTCTTCT-3′; and control sequence, 5′-TTTCCAGAGTGCTGATGAC.

In Vivo Ubiquitination Assay. HCT116 WT cells were infected with the indicated lentiviruses or transfected with specified plasmids followed by treatment with 20 μM MG132 for 6 h, washed with PBS, and lysed in RIP buffer (Beyotime, catalog no. P0013B) containing 2% SDS, protease inhibitors, and 10 mM N-ethylmaleimide. The lysates were transferred into a 1.5-mL Eppendorf tube and placed on a hot plate immediately to boil for 10 min. Next, 900 μL of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton) was added and samples were incubated at 4 °C for 30 min with rotation. Then, the lysates were centrifuged to obtain the cytosolic protein fraction, which
was incubated with anti-p21 or anti-Myc antibodies overnight followed by protein A/G agarose beads for an additional 1 h at room temperature. Then, the beads were washed three times with wash buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% Nonidet P-40). The proteins were released from the beads by boiling the SDS/PAGE sample buffer, and they were analyzed using immunoblotting with anti-Ub or anti-HA monoclonal antibodies.

**In Vitro Ubiquitylation of p21.** To prepare p21 as the substrate for the in vitro deubiquitylation assay, HCT116 WT cells were transfected with Myc-p21 with or without cotransfected HA-ubiquitin and treated with 20 μM MG132 for 6 h. Non-ubiquitylated or ubiquitylated Myc-p21 was purified from the cell extracts using an anti-Myc antibody. Either GST-tagged USP11 or the GST-tagged USP11mut was expressed in the BL21 *Escherichia coli* strain. After the cells were induced with 0.4 mM isopropyl-β-d-1-thiogalactopyranoside, they were lysed, and GST-USP11 was purified using glutathione agarose (Pierce, Thermo Fisher catalog no. 16100) and eluted with elution buffer (50 mM Tris, 150 mM NaCl, pH 8.0) containing 10 mM deduced glutathione (Sigma catalog no. G4251). Nonubiquitylated or ubiquitylated Myc-p21 was incubated with purified USP11 in deubiquitylation buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 5% glycerol) for 2 h at 37 °C.

**BrdU Labeling.** Cells stably expressing the indicated shRNAs for 48 h were washed twice with PBS and incubated with 20 μM BrdU for 1 h, after which they were prepared for flow cytometry according to the manufacturer’s protocol (BD Biosciences catalog no. 559619).

**G2/M Checkpoint Assay.** Cells stably expressing the indicated shRNAs were pretreated with 0.2 μM doxorubicin for 2 h, synchronized with nocodazole (100 ng/mL) for 16 h, harvested, and then fixed with 70% ethanol at −20 °C. Thereafter, the cells were resuspended in 1 mL of 0.25% Triton X-100 in PBS and incubated at 4 °C for 15 min with gentle rocking. After the cells were centrifuged, the cell pellet was resuspended in 200 μL of PBS containing 1% BSA and 1.5 μg of a polyclonal antibody that specifically recognizes the phosphorylated form of histone H3 (Abcam) and incubated for 1.5 h at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with an FITC-conjugated goat anti-rabbit antibody (CMTAG catalog no. AT0118) diluted at a ratio of 1:50 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, the cells were stained with propidium iodide, and cellular fluorescence was measured using flow cytometry.

**Cell Proliferation Assay.** A549, HCT116 WT, and HCT116 p21−/− cells that were infected with the indicated lentiviral shRNA constructs for at least 48 h were seeded into six-well plates at an optimized density of 3 × 10^5 cells per well. Then p21 was transfected into the indicated cells for an additional 24 h. Cells were then seeded into a 96-well plate at an optimized density of 8 × 10^3 cells per well. At the indicated time points, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Beyotime Biotechnology, catalog no. ST316) was added to each well and incubated at 37 °C for 4 h. The supernatants were aspirated carefully, and 100 μL of DMSO was added. The absorbance of the individual wells was determined at 490 nm. Each condition was conducted in quadruplicate, and the experiment was repeated at least three times.

**In Vivo Tumorigenesis Study.** To establish lung cancer xenografts in nude mice, a total of 6 × 10^6 A549 cells expressing the indicated shRNA or constructs were stably transfected with either control, or the indicated shRNAs targeting USP11 were harvested, washed twice with PBS, suspended in 100 μL of PBS, and injected into the right flank of each mouse (n = 5 per group). The tumor size was measured every 2 d for 45 d using a caliper, and the tumor volume was calculated based on the following formula: \[ V = 0.5 \times L \times W^2 \], where \( L \) is the longest diameter and \( W \) is the shortest diameter.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded samples were sectioned at 5 μM. Sections were treated with antigen retrieval buffer. Specifically, USP11 or p21 antibody was applied overnight at room temperature at a dilution of 1:100. Slides were then incubated with secondary antibody. After staining, slides were scanned by Pannoramic MIDI digital slide scanner (3DHISTECH).

Levels of USP11 and p21 expression in lung cancer tissue specimens from NSCLC patients were reviewed and scored. The USP11 and p21 expression was quantified by a visual grading system (0–3) based on the intensity of cytoplasm and nuclear staining as follows: grade 0, no immunoreactivity; grade 1, weak immunoreactivity slightly stronger than background staining; grade 2, clear immunoreactivity in more than half of the cancer cells; and grade 3, strong immunoreactivity as dark as nuclear counterstain in the majority of cancer cells. Finally, the cases were classified into two different groups: low expression (grade 0 and 1) and high expression (grade 2 and 3). The χ^2 test was used for statistical analysis of the correlation between USP11 and p21.
Fig. S1. USP11 is associated with p21. (A) A549 cells transfected with either empty vector or a vector expressing Flag-USP11 were subjected to immunoprecipitation with an anti-Flag antibody for mass spectrometry. (B) GST or GST-USP11 was retained on glutathione Sepharose beads, incubated with extracts of A549 cells, and then immunoblotted with an antibody targeting endogenous p21. GST and recombinant GST-USP11 were purified from bacteria and analyzed using SDS/PAGE and Coomassie blue staining. (C) Schematic representation of the HA-tagged full-length p21 (FL) and its various deletion mutants. (D) A549 cells transfected with the indicated constructs were lysed. The cell lysates were subjected to immunoprecipitation with anti-HA antibodies, and the immunoprecipitates were then probed with anti-USP11, anti-CDK2, or anti-HA antibody. (V: vector). (E) Schematic representation of the Flag-tagged full-length USP11 (FL) and its various deletion mutants. (F) A549 cells transfected with the indicated constructs were lysed. The cell lysates were subjected to immunoprecipitation with the anti-Flag antibodies, and the immunoprecipitates were then probed with anti-Flag or anti-p21 antibody. (V, vector).
Fig. 5G. USP11 regulates the protein level of p21. (A) A549, H460, and HCT116 WT cells were transfected with either scrambled or USP11 siRNAs for 48 h. The resulting cell extracts were analyzed by Western blotting with anti-USP11, anti-p21, or anti-GAPDH antibody. (B) A549 cells were transfected with either scrambled or USP11 siRNAs for 48 h, and then total RNA was isolated and subjected to qRT-PCR. The error bars represent the SD of triplicate measurements. (C and D) Total RNA either from cells infected with the indicated lentiviral shRNAs (C) or from cells transfected with the indicated constructs (D) was isolated and subjected to qRT-PCR. The error bars represent the SD of triplicate measurements. (E) HCT116 WT and HCT116 p53−/− cells infected with the indicated lentiviral shRNAs were treated with DMSO, MG132 (20 μM), or Clasto-Lactacystin β-lactone (CLL) (10 μM) for 6 h, and the indicated proteins were analyzed using Western blotting. (F) A549 cells transfected with either scrambled or USP11 siRNA were treated with DMSO or MG132 (20 μM) for 6 h, and the indicated proteins were analyzed using Western blotting. (G and H) HCT116 WT cells were transfected with the Flag-p53 or control vector. Cell lysates were then extracted and subjected to Western blotting (G). Total RNA from cells was isolated and subjected to qRT-PCR (H).
**Fig. S3.** USP11 stabilizes p21 protein. (A and B) HCT116 WT cells transfected with the indicated constructs were treated with 50 μg mL⁻¹ CHX, collected at the indicated time points, and immunoblotted with anti-Flag, anti-p21, and anti-GAPDH antibodies. Quantification of the p21 levels relative to GAPDH expression is shown. (C) A549 cells transfected with the indicated constructs were treated with 50 μg mL⁻¹ CHX and collected at the indicated times for preparation for Western blotting. Quantification of the p21 levels relative to GAPDH expression is shown. (D) HCT116 WT cells infected with the indicated lentiviral shRNAs were treated with 50 μg mL⁻¹ CHX, collected at different time points, and then immunoblotted with anti-USP11, anti-p21, or anti-GAPDH antibody. Quantification of the p21 levels relative to GAPDH expression is shown. (E) A549 cells infected with the indicated lentiviral shRNAs were treated with 50 μg mL⁻¹ CHX and then collected at the indicated time points for Western blot analysis. Quantification of the p21 levels relative to GAPDH expression is shown.
Fig. S4. USP11 removes K48-linked poly-Ub in p21. Myc-p21 and various HA-ubiquitin mutants were transfected into HCT116 cells infected with the indicated lentiviral shRNA for 24 h. The cells were treated with 20 μM of the proteasome inhibitor MG132 (Sigma) for 6 h. Myc-p21 was immunoprecipitated with an anti-Myc antibody, and the immunoprecipitates were probed with anti-HA and anti-Myc antibodies.
Fig. S5. USP11 knockdown abolishes p21 elevation triggered by genotoxic agents. (A and B) HCT116 WT (A) and HCT116 p53−/− (B) cells infected with the indicated lentiviral shRNAs were treated with 0.2 μM doxorubicin (Dox) for either 8 or 16 h. Cell lysates were then extracted and subjected to Western blotting. (C–F) Total RNA from HCT116 WT (C and E) and HCT116 p53−/− (D and F) cells infected with the indicated lentiviral shRNAs and treated with 5 μM etoposide (C and D) or 0.2 μM doxorubicin (E and F) for either 8 or 16 h was isolated and subjected to qRT-PCR. The error bars represent the SD of triplicate measurements. (G) Lysates of HCT116 cells with or without treatment with 5 μM etoposide or 0.2 μM doxorubicin (Dox) for 16 h were subjected to immunoprecipitation with control IgG and anti-USP11 antibody. The immunoprecipitates were then probed with anti-USP11, anti-p53, or anti-p21 antibody.
Fig. S6. USP11 regulates p21 in a cell-cycle-independent manner. (A and B) HCT116 WT cells infected with the indicated lentiviral shRNAs were synchronized using a double-thymidine block and released at the indicated phase. Cells were collected and stained with propidium iodide for flow cytometry analysis. (B) The remaining cells were lysed and subjected to Western blotting using anti-USP11, anti-cyclinB1, anti-pH3, anti-p21, or anti-GAPDH antibody.
Fig. S7. USP11 regulates the G1/S transition in a p21-dependent manner. (A) HCT116 WT, HCT116 p53\(^{-/-}\), and HCT116 p21\(^{-/-}\) cells infected with the indicated lentiviral shRNAs were stained with propidium iodide and analyzed using flow cytometry. (B) HCT116 cells infected with the indicated lentiviral shRNAs with or without ectopic expression of p21 were stained with propidium iodide and analyzed using flow cytometry.
Fig. S8. USP11 affects cell-cycle progression. (A) HCT116 cells stably expressing indicated USP11 shRNA were synchronized by a double-thymidine block. The released cells were then harvested at the indicated time points and analyzed by flow cytometry. (B) The percentage of S-phase cells is shown. The error bars represent the mean ± SD of three independent experiments. *P < 0.05. (C) HCT116 WT and HCT116 p21−/− cells infected with the indicated lentiviral shRNAs were pretreated with 0.2 μM doxorubicin for 2 h, followed by synchronization with nocodazole (100 ng/mL) for 16 h. The mitotic index was determined using pH3 staining as a marker of mitosis.
Fig. S9. USP11 affects cell proliferation via p21. (A) A549, HCT116 WT, and HCT116 p21−/− cells were infected with the indicated lentiviral shRNAs and then transfected with the indicated constructs. Cell proliferation was monitored using MTT assays at the indicated time points. Statistical significance was determined by a two-tailed, unpaired Student’s t test. (B) A549, HCT116 WT, and HCT116 p21−/− cells were transfected with the indicated constructs, and cell proliferation was monitored using MTT assays at the indicated time points.
Fig. S10. USP11 functions as a tumor suppressor by regulating p21. (A–C) The 6 × 10^6 indicated shRNA-transduced A549 cells with or without ectopic expression of p21 were subcutaneously injected into mice. Tumor growth (A), tumor weight (B), and tumor images (C) were shown. (D) A549 cells implanted into nude mice were lysed and analyzed using Western blotting. (E–G) Tumor growth (E), tumor weight (F), and tumor images (G) of mice with s.c. injection of 6 × 10^6 A549 cells or p21-depleted A549 cells transfected with USP11 or USP11mut are shown. (H) A549 cells implanted into nude mice were lysed and analyzed using Western blotting. (I) Representative images from immunohistochemical staining of USP11 and p21 in NSCLC tissues. (Scale bars, 20 μm.) (J) Summary of staining results from 35 NSCLC tissues. The χ² test was used for statistical analysis of the correlation between USP11 and p21.