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

Tanggang Deng[a,b,1], Guobei Yan[a,b,1], Xin Song[c,1], Lin Xie[a,b], Yu Zhou[a,b], Jianglin Li[a,b], Xiaoxiao Hu[a,b], Zhen Li[c], Jun Hu[a,b], Yibin Zhang[d], Hui Zhang[a,b], Yang Sun[a,b], Peifu Feng[a,b], Dong Wei[a,b], Bin Hu[a,b], Jing Liu[d,2], Weihong Tan[a,b,2], and Mao Ye[a,b,2]

[a]Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Biology, Hunan University, Changsha, 410082 Hunan, China; [b]Aptamer Engineering Center of Hunan Province, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082 Hunan, China; [c]Department of Cancer Biotherapy Center, The Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province), Kunming, 650118 Yunnan, China; and [d]Molecular Biology Research Center & Center for Medical Genetics, School of Life Sciences, Central South University, Changsha, 410078 Hunan, China

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$p21^{WAF1/CIP1}$ 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^{SKP2}, $CRL4^{CDT2}$, and APC/C^{CDC20} 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.

ubiquitin | deubiquitylation | cell cycle | p21 | USP11

The cyclin-dependent kinase (CDK) inhibitor p21 (also known as $p21^{WAF1/Cip1}$) is a key negative regulator of cell-cycle progression, which mediates cell-cycle arrest at the G1 or G2 phase in response to a variety of stress stimuli (1). p21 contributes to the G1 arrest primarily by inhibiting cyclin E and cyclin A/CDK2 activity (2), which results in the hypo-phosphorylation of the retinoblastoma protein (pRb) and inhibits the release and activation of the transcription factor E2F—a protein required for S-phase entry (3). p21 sustains cell-cycle arrest at the G2 phase by blocking the interaction between CDK1 and CDK-activating kinase, thus inhibiting the activating phosphorylation of CDK1 at Thr-161 (4). Moreover, several studies have reported that p21 also mediates arrest at G2 by retaining the cyclin B1-CDK1 complex in the nucleus, degrading cyclin B, and decreasing the expression of early mitotic inhibitor 1 (Emi1) (5–7).

Under normal growth conditions, p21 is an unstable protein with a relatively short half-life (8, 9). Its degradation is controlled primarily through the ubiquitin-proteasome pathway (9). Three E3 ubiquitin ligase complexes—SCF^{SKP2}, $CRL4^{CDT2}$, and APC/C^{CDC20}—have been reported to promote p21 ubiquitylation and degradation in the nucleus. During the G1/S transition, the SCF^{SKP2} complex promotes the ubiquitylation and degradation of p21 after it is phosphorylated at Ser130 by CDK2 (10, 11), whereas the $CRL4^{CDT2}$ complex mediates the ubiquitin-dependent proteolysis of p21 only when p21 is bound to proliferating cell nuclear antigen and phosphorylated at Ser-114 during the S phase (12). When bound to CDK1/cyclin B during prometaphase, p21 is degraded by the APC/C^{CDC20} complex (13). In contrast, p21 stability can be positively regulated by various mechanisms. Phosphorylation of p21 by p38 alpha, JNK1, AKT, and NDR has been reported to enhance its stability (14–16). Wisp39, nucleophosmin/B23, hSSB1, and TRIM39 were found to stabilize p21 by engaging in protein–protein interactions (17–20). Cables1 stabilizes p21 by antagonizing PSMA3-mediated proteasomal degradation (21). However, it remains unclear whether ubiquitylated p21 can be recycled.

The removal of ubiquitin from a target protein by deubiquitylase has emerged as an important regulatory mechanism of many cellular functions. The human genome encodes ~98 deubiquitylases that can be subdivided into six families (22). USP11 is a deubiquitylase that belongs to the ubiquitin-specific processing protease (USP) family, which is primarily localized to the nucleus and possesses multiple highly conserved domains including Cys box, Asp, KRF, and His box (23). Growing evidence has shown that USP11 plays an important role in signal transduction, apoptosis, DNA repair, and viral replication by regulating the stability

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.

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[1]T.D., G.Y., and X.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: goldleaf@hnu.edu.cn, tan@chem.ufl.edu, or jingliucsu@hotmail.com.

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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. S1*A*). 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. 1*A*) and that USP11 was present in Myc-p21 immunoprecipitates (Fig. 1*B*). 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. 1 *C* and *D*, 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. 1*E*), demonstrating a direct interaction between USP11 and p21. Similar results were obtained by incubating purified GST-USP11 with extracts from A549 cells (Fig. S1*B*). Immunofluorescent staining revealed that the colocalization of both USP11 and p21 occurred in the nucleus (Fig. 1*F*). 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 mutant of USP11 still retained the ability to bind to p21 similar to wild-type (WT) USP11 (Fig. 1*G*). 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. 1*H*). Furthermore, to map the USP11-binding region on p21, a series of p21-deletion mutants was expressed in A549 cells (Fig. S1*C*). 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. S1*D*). 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

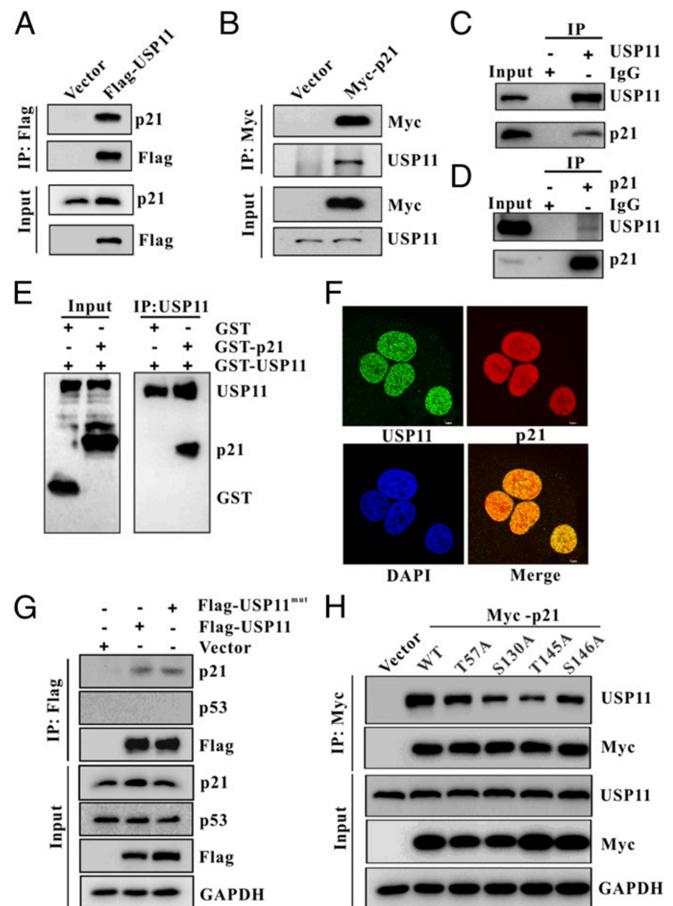

Fig. 1. USP11 interacts with p21. (*A* and *B*) A549 cells were transfected with plasmids expressing either Flag-USP11 or Myc-p21. Total cell lysates were subjected to immunoprecipitation with anti-Flag (*A*) or anti-Myc antibody (*B*). The immunoprecipitates were then probed with anti-p21, anti-Flag, anti-Myc, or anti-USP11 antibody. (*C* and *D*) A549 cell lysates were subjected to immunoprecipitation with control IgG, anti-USP11 (*C*), or anti-p21 (*D*) antibody. The immunoprecipitates were then probed with anti-USP11 or anti-p21 antibody. (*E*) GST, GST-p21, and GST-USP11 produced from bacteria were assessed using Western blotting, and anti-USP11 antibodies were used to immobilize purified GST-USP11 protein onto protein A beads and were then incubated with purified GST or GST-p21. The bound proteins were eluted and analyzed by Western blotting using anti-GST antibodies. (*F*) The subcellular localization of endogenous USP11 (green) and p21 (red) in A549 cells was visualized using immunofluorescence with anti-USP11 and anti-p21 antibodies. DNA was stained with DAPI, and a merged view of the red and green channels within the same field is shown (merge). (*G*) A549 cells were transfected with plasmids expressing either Flag-USP11 or the catalytically inactive mutant. Total cell lysates were subjected to immunoprecipitation with anti-Flag antibody. (*H*) The A549 cells transfected with the indicated Myc-p21 mutants were subjected to immunoprecipitation with anti-Myc antibody.

(HCT116 $p53^{-/-}$) genotype. Interestingly, USP11 overexpression resulted in a significant increase of endogenous p21 levels (Fig. 2*A*), 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. 2 *B* 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. 2 *A*–*C*), implying that USP11-mediated upregulation 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

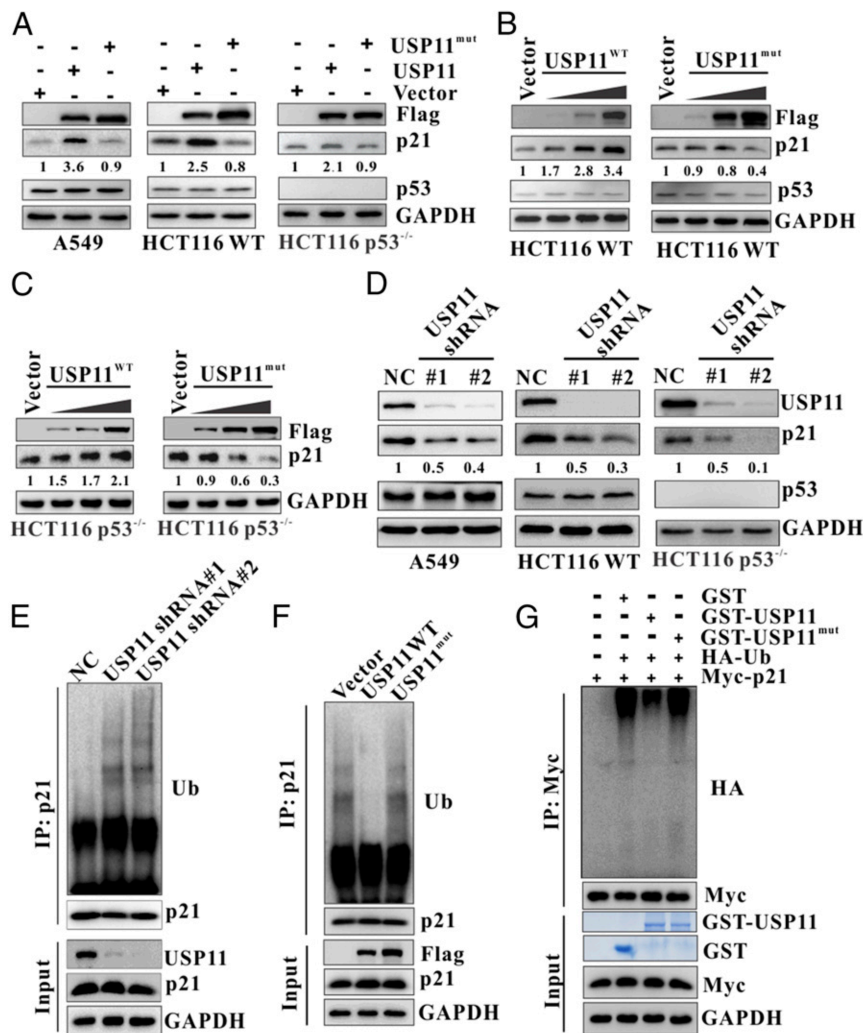

Fig. 2. USP11 regulates the protein level of p21. (*A*) A549, HCT116 WT, and HCT116 p53$^{-/-}$ cells were transfected with the indicated constructs. Total protein was extracted and subjected to Western blotting using anti-Flag, anti-p53, anti-p21, or anti-GAPDH antibody. (*B* and *C*) Increasing amounts of USP11 WT or USP11^{mut} were transfected into HCT116 WT (*B*) and HCT116 p53$^{-/-}$ (*C*) cells, and total protein was extracted from these cells and subjected to Western blotting using anti-Flag, anti-p53, anti-p21, or anti-GAPDH antibody. (*D*) A549, HCT116 WT, and HCT116 p53$^{-/-}$ cells were infected with the indicated lentiviral constructs. The resulting cell extracts were analyzed using Western blotting with anti-USP11, anti-p53, anti-p21, or anti-GAPDH antibody. (*E* and *F*) HCT116 WT cells either infected with the indicated lentiviral shRNAs (*E*) or transfected with the indicated constructs (*F*) were treated with MG132 (20 μM) for 6 h before harvest. p21 was immunoprecipitated with an anti-p21 antibody, and the immunoprecipitates were probed with anti-Ub or anti-p21 antibody. (*G*) Ubiquitylated Myc-p21 was incubated with GST, GST-tagged USP11 WT, or GST-tagged USP11^{mut} purified from bacteria using glutathione agarose. After coincubation, Myc-p21 was immunoprecipitated using an anti-Myc antibody, and the immunoprecipitates were probed using antibodies against HA and Myc. Recombinant GST, GST-tagged USP11 WT, or GST-tagged USP11mut were analyzed using SDS/PAGE and Coomassie blue staining.

lines. As predicted, USP11 knockdown abolished p21 levels without affecting p53 expression (Fig. 2*D*). Similar results were obtained using USP11-specific small interfering RNAs (siRNAs) in the A549, H460, and HCT116 cell lines (Fig. S2*A*).

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. S2 *B–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. S2 *E* 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. S2 *G* 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. S3 *A–C*). Conversely, knockdown of USP11 resulted in a significant decrease in the half-life of p21 (Fig. S3 *D* 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. 2*E*), whereas the overexpression of WT USP11 reduced the levels of polyubiquitylated p21 (Fig. 2*F*). In contrast, the catalytically inactive mutant failed to protect p21 from ubiquitylation (Fig. 2*F*), suggesting that the enzymatic activity of USP11 is essential for the USP11-dependent deubiquitylation of p21. To verify that p21 is a direct substrate of USP11, we purified USP11 and ubiquitylated p21 and incubated these two proteins in a cell-free system. As expected, WT USP11 but not the catalytically inactive mutant decreased p21 polyubiquitylation in vitro (Fig. 2*G*). These data indicate that USP11 directly deubiquitylates p21.

To investigate the type of poly-Ub chain on p21 that is removed by USP11, we transfected HCT116 cells with Myc-tagged p21, together with HA-tagged ubiquitin mutants in which all lysines, except only one lysine (K6, K11, K27, K29, K33, K48, or k63), were mutated into arginines. As shown in Fig. S4, USP11 knockdown significantly increased K48-linked poly-Ub but not any other isopeptide-linked (K6, K11, K27, K29, K33, or K63) poly-Ub. The result suggests that USP11 removes K48-linked poly-Ub in p21.

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. 3*C* and Fig. S5*G*), 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. 3*D*).

USP11 Protects p21 from Ubiquitin-Mediated Degradation in a Cell-Cycle–Independent Manner. Three E3 ubiquitin ligase complexes—SCF^{SKP2}, CRL4^{CDT2}, and APC/C^{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. S6*A*). 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. S6). Furthermore, we examined whether the effect of USP11 on p21 was associated with SCF^{SKP2}, CRL4^{CDT2}, or APC/C^{CDC20}. Knockdown of USP11 using shRNAs significantly decreased p21 levels with concomitant increases in SKP2 (Fig. 4*A*), but the levels of CDT2 and CDC20 were unchanged (Fig. 4 *B* and *C*). However, when SKP2, CDT2, or CDC20 was knocked down by siRNA, USP11 depletion-induced p21 degradation and ubiquitylation was abolished (Fig. 4 *A–F*). Altogether, these results indicate that USP11 stabilizes p21 via the reversal of SCF^{SKP2}-, CRL4^{CDT2}-, or APC/C^{CDC20}-mediated ubiquitylation and degradation in a cell-cycle–independent manner (Fig. 4*G*).

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. 5 *A–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. 5 *A* and *B* and Fig. S7*A*), but USP11-depleted cells transfected with exogenous p21 fully prevented the G1/S transition induced by USP11 ablation (Fig. 5*C* and Fig. S7*B*). 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 phopho-histone3 (pH3) at Ser10, an indicator of cells at M phase, was used to monitor the G2/M checkpoint. As shown in Fig. 5*D* and Fig. S8*C*, 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

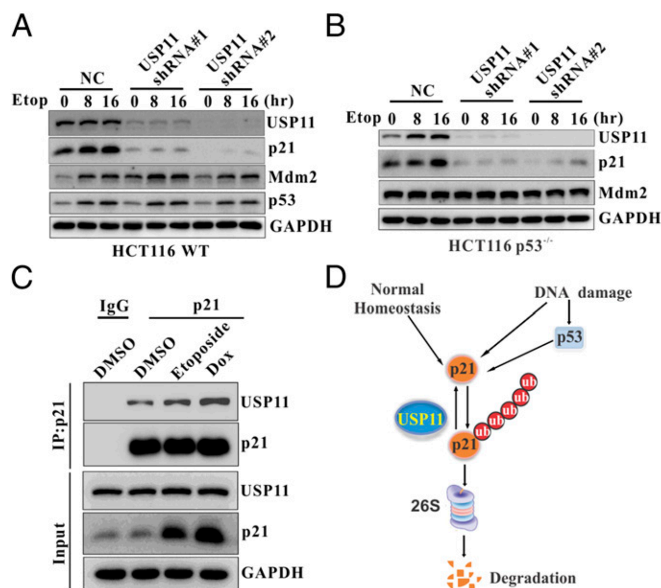

Fig. 3. USP11 knockdown abolishes p21 elevation triggered by genotoxic agents. (*A* and *B*) HCT116 WT (*A*) and HCT116 p53$^{-/-}$ (*B*) cells infected with the indicated lentiviral shRNAs were treated with 5 μM etoposide (Etop) for either 8 or 16 h. Cell lysates were then extracted and subjected to Western blotting. (*C*) Lysates of HCT116 cells with or without treatment with 5 μM etoposide (Etop) or 0.2 μM doxorubicin (Dox) for 16 h were subjected to immunoprecipitation with control IgG or anti-p21 antibody. The immunoprecipitates were then probed with anti-USP11 or anti-p21 antibody. (*D*) A proposed working model for p21 regulation by USP11 in response to DNA damage.

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. 5*E*). Interestingly, USP11 knockdown did not affect apoptosis triggered by either doxorubicin or etoposide in cells lacking p21 (Fig. 5*F*). 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. S9*A*). However, USP11 knockdown showed no effect on the proliferation of HCT116 p21$^{-/-}$ cells (Fig. S9*A*). Conversely, overexpression of USP11, but not of the catalytically inactive mutant of USP11, inhibited the proliferation of A549 and HCT116 WT cells (Fig. S9*B*). Likewise, overexpression of USP11 had no effect on the proliferation of HCT116 p21$^{-/-}$ cells (Fig. S9*B*).

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. S10*A*). At 45 d after tumor cell implantation, the volume and weight of the tumor formed by USP11-depleted A549 cells significantly increased. (Fig. S10 *A–C*). Notably, restoring p21 expression fully reversed the tumor-promoting effect of USP11 shRNA (Fig. S10 *A–C*). Western blot analysis confirmed that the effect of USP11

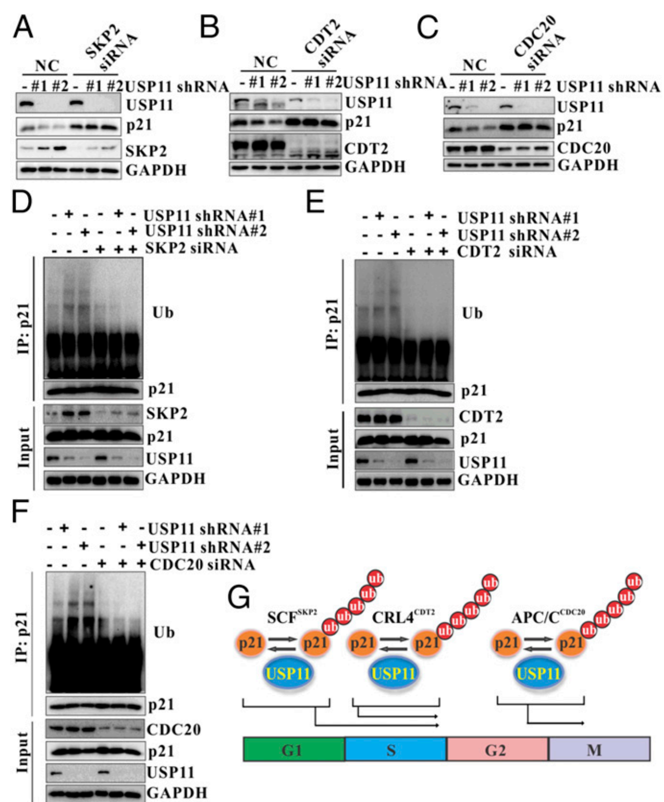

Fig. 4. USP11 protects p21 from ubiquitin-mediated degradation. (*A*–*C*) HCT116 WT cells infected with the indicated shRNAs were transfected with scrambled, SKP2 (*A*), CDT2 (*B*), or CDC20 (*C*) siRNA for 48 h, and then the cell lysates were harvested and analyzed using Western blotting. (*D*–*F*) HCT116 cells infected with the indicated shRNAs were transfected with scrambled, SKP2 (*D*), CDT2 (*E*), and CDC20 (*F*) siRNA as indicated 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.

depletion on p21 was retained in these tumors (Fig. S10*D*). 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.

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

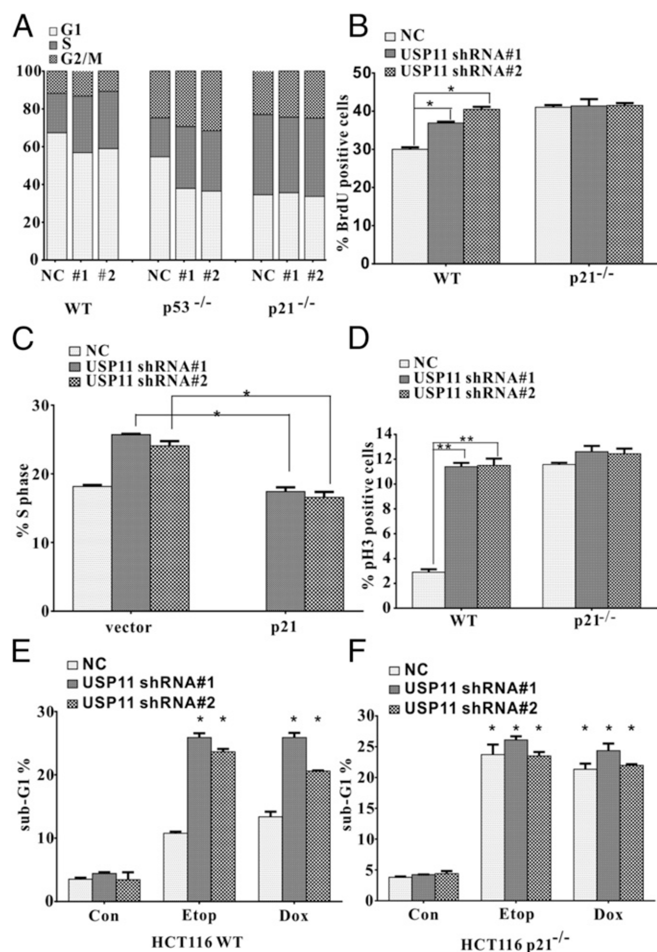

Fig. 5. USP11 regulates the G1/S transition and DNA damage-induced G2 checkpoint in a p21-dependent manner. (*A*) HCT116 WT, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells infected with the indicated lentiviral shRNAs were stained with propidium iodide and analyzed using flow cytometry. (*B*) HCT116 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 for 2 h, followed by synchronization with nocodazole (100 $ng \cdot mL^{-1}$) 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^{SKP2}, $CRL4^{CDT2}$, and APC/C^{CDC20}—have been identified to promote p21 ubiquitylation and degradation at specific stages of the cell cycle. SCF^{SKP2} is necessary for p21 degradation at the G1/S transition as well as during S phase of the cell cycle (33), whereas $CRL4^{CDT2}$ specifically targets p21 for degradation in S phase (34). During mitosis, the APC/C^{CDC20} 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 with high levels occurring during G1 and G2 (35). However, our result showed that USP11 levels gradually increased with cell-cycle progression from G1 to M phase (Fig. S6*B*), 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 oncogene. 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 $p21^{-/-}$ 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.

Materials and Methods

See *SI Materials and Methods* for additional methods.

HEK293 cells were cultured in DMEM (GIBCO catalog no. 8116490) supplemented with 10% (vol/vol) FBS; A549 cells were cultured in RPMI 1640 (GIBCO catalog no. 8116491) supplemented with 10% FBS; and HCT116 WT, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells were cultured in McCoy's 5A medium (Invitrogen catalog no. 16600082) supplemented with 10% FBS. The following reagents were used: MG132 (Sigma catalog no. C2211-5MG), nocodazole (Sigma catalog no. M1404-10MG), GSH-Sepharose (Thermo Fisher Scientific catalog no. 16,100), doxorubicin (Sigma catalog no. D1515-10MG), and etoposide (Sigma catalog no. E1383-25MG).

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