USP2 is an SKP2 deubiquitylase that stabilizes both SKP2 and its substrates

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Fengwu Zhang, Yongchao Zhao, and Yi Sun

From the 1 Cancer Institute of the Second Affiliated Hospital, 2 Institute of Translational Medicine, 3 Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; and 4 Cancer Center, Zhejiang University, Hangzhou, China

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The stability of a protein is regulated by a balance between its ubiquitylation and deubiquitylation. S-phase kinase-associated protein 2 (SKP2) is an oncogenic F-box protein that recognizes tumor suppressor substrates for targeted ubiquitylation by the E3 ligase SKP1-Cullin1-F-box and degradation by proteasome. SKP2 is itself ubiquitylated by the E3 ligases APC/C<sup>Cdh1</sup> and SCF<sup>BxW2</sup>, and deubiquitylated by deubiquitylases (DUBs) USP10 and USP13. Given the biological significance of SKP2, it is likely that the other E3s or DUBs may also regulate its stability. Here, we report the identification and characterization of USP2 as a new DUB. We first screened a panel of DUBs and found that both USP2 and USP21 bound to endogenous SKP2, but only USP2 deubiquitylated and stabilized SKP2 protein. USP2 inactivation via siRNA knockdown or small-molecule inhibitor treatment remarkably shortened SKP2 protein half-life by enhancing its ubiquitylation and subsequent degradation. Unexpectedly, USP2-stabilized SKP2 did not destabilize its substrates p21 and p27. Mechanistically, USP2 bound to SKP2 via the leucine-rich repeat substrate-binding domain on SKP2 to disrupt the SKP2-substrate binding, leading to stabilization of both SKP2 and these substrates. Biologically, growth suppression induced by USP2 knockdown or USP2 inhibitor is partially mediated via modulation of SKP2 and its substrates. Our study revealed a new mechanism of the cross-talk among the E3–DUB substrates and its potential implication in targeting the USP2–SKP2 axis for cancer therapy.

The stability of a protein is precisely regulated by a fine balance between ubiquitylation, a process that destabilizes it, and deubiquitylation, a process that stabilizes it (1). Ubiquitylation is catalyzed by a three-enzyme cascade, consisting of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligases, leading to a covalent attachment of ubiquitin molecule to a substrate protein, which is recognized by the proteasome system for degradation (2). Deubiquitylation is a reversed process, catalyzed by deubiquitylase, which binds to ubiquitylated substrates and removes/cleaves the ubiquitin from proteins to disassemble polyubiquitylation chains from substrates before proteasomal degradation (3). Through the highly regulated processes of ubiquitylation and deubiquitylation, the ubiquitin–proteasome system precisely controls the fate of substrate protein (4).

The E3 ubiquitin ligases can be categorized into four classes: N-end rule; homology to E6AP C terminus; really interesting new gene (RING); and anaphase-promoting complex/cyclosome (APC/C) (5, 6). Among 600 different types of E3 ligases, SKP1-Cullin1-F-box (SCF) under the RING class category is the largest family, consisting of four components: scaffold cullin, adaptor SKP1, RING RBX/ROC, and substrate receptor, F-box proteins (7, 8). In mammalian cells, there are 69 different F-box proteins and among them, S-phase kinase-associated protein 2 (SKP2, also known as F-box and leucine-rich repeat protein 1) is one of the best-studied proteins (9, 10). It has been well documented that SKP2 is overexpressed in numerous human cancers and SKP2 overexpression is positively associated with poor survival of patients with cancer (9). Mechanistically, SKP2 acts as an oncogene by recognizing and promoting the ubiquitylation and degradation of tumor suppressor proteins, such as p21 (11, 12), p27 (13–15), p57 (16), and FOXO1 (17). By doing so, SKP2 accelerates the S phase entry to promote cell proliferation, migration, and invasion. Thus, SKP2 has been well validated as a promising cancer target for anticancer drug discovery (18).

Deubiquitylases (DUBs) are categorized into seven subfamilies, including ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases, ovarian tumor proteases, Machado–Joseph domain–containing proteases, motif-interacting with ubiquitin-containing novel DUB family (MINDDYs), ZUP1, and JAB1/MPN/MOV34 metalloenzymes (19). USPs are the most abundant DUBs among all subfamilies (20). USP2 was first identified as an inducible USP enzyme in rat testes (21). USP2 was characterized as a bona fide oncogene via stabilizing many oncoproteins, including TGF-β serine/threonine kinase receptors 1 (22), mouse double minute 2 homolog (MDM2) (23), mouse double minute 4 homolog (24), and fatty acid synthase (25). USP2 was overexpressed in several human cancers and is crucial for tumorigenesis (3). Previous studies have shown that SKP2 is subjected to ubiquitylation by APC/C<sup>Cdh1</sup> E3 ligase for proteasome
**USP2 stabilizes SKP2 and SKP2 substrates**

degradation (26), leading to accumulation of its substrate p27 to ensure that the G1 to S phase progression is timely controlled, when cells are ready. Our recent study showed that SKP2 is also subjected to ubiquitylation by SCF^FBXW2 to ensure again precisely controlling of cell cycle progression in a manner independent of APC/C^CDH1 E3 ligase (27). On the other hand, USP10 (28) and USP13 (29) were reported to promote SKP2 deubiquitylation. USP10 stabilized SKP2 and subsequently activated break-point cluster region-Abelson (BCR-ABL) via SKP2-mediated K63-linked polyubiquitylation (28), whereas USP13 counteracts the CDH1–SKP2–p27 axis to regulate the endoplasmic reticulum stress response (29).

In this study, we investigated a panel of DUBs for their potential binding of endogenous SKP2 by a conventional pull-down assay and found that both USP2 and USP21 bind to SKP2, but only USP2 stabilized SKP2. Unexpectedly, stabilized SKP2 did not destabilize its substrates by promoting their ubiquitylation and degradation. This is due to USP2 binding to SKP2 via the leucine-rich repeat (LRR) domain on SKP2, the domain that mediates SKP2 substrate binding. The USP2–SKP2 binding disrupts the SKP2 substrate binding. Thus, USP2 stabilizes both SKP2 and SKP2 substrates. Biologically, growth suppression induced by USP2 knockdown or USP2 inhibitor appears to be partially mediated via modulating SKP2 and its substrates. Our study reveals a new mechanism of the cross-talk among the E3, DUBs, and substrates.

**Results**

**USP2 bound to and stabilized SKP2**

To identify DUBs with potential to deubiquitylate SKP2, we used the conventional pull-down assay to determine which DUB will bind to endogenous SKP2. A panel of 9 DUBs (USP2, USP3, USP7, USP8, USP11, USP18, USP21, USP30, and USP33) was transiently transfected into HEK293 cells, followed by FLAG pull-down and Western blotting for SKP2. Among the 9 DUBs, USP2 and USP21 selectively bound to SKP2 (Fig. 1A). However, ectopic expression of USP2, but not USP21, extended protein half-life of SKP2 (Fig. 1B). Consistently, knockdown of USP2, but not USP21, reduced endogenous levels of SKP2 in three lung cancer cell lines (Fig. 1C), suggesting USP2, but not USP21, is likely an SKP2 deubiquitylase. We further confirmed that ectopic expression of WT USP2, but not of its catalytic-inactive mutant USP2–C276A, caused accumulation of endogenous SKP2 in a dose-dependent manner (Fig. 1D), and endogenous USP2 and SKP2 bound to each other under a physiological condition detected in a reciprocal immunoprecipitation (IP) assay (Fig. 1E). Collectively, these results showed that USP2 interacts and stabilizes SKP2.

**USP2 is an SKP2 deubiquitylase**

We next excluded the possibility that USP2 may affect SKP2 mRNA. The siRNA-based USP2 knockdown indeed reduced the USP2 mRNA level, but had no effect on SKP2 mRNA, indicating that USP2 regulates SKP2 not occurring at the transcription level (Fig. 2A). We further found that USP2 knockdown indeed reduced SKP2 protein levels, which is fully rescued by proteasome inhibitor, MG132 (Fig. 2B), indicating that the regulation occurs at the post-translational level. Likewise, USP2 knockdown shortened the protein half-life of SKP2 (Fig. 2C) and significantly increased the polyubiquitylation of SKP2 (Fig. 2D). Conversely, ectopic expressed USP2 almost completely removed the ubiquitin chains from SKP2 (Fig. 2E) in a matter dependent of its deubiquitylase activity because a catalytic-inactive mutant, USP2–C276A, completely abrogated this effect (Fig. 2F). Expectedly, unlike WT USP2, this mutant failed to extend the protein half-life of SKP2 (Fig. 2G). Taken together, USP2 is a bona fide SKP2 deubiquitylase that negatively regulated SKP2 degradation.

**ML364, a small-molecule inhibitor of USP2, accelerates SKP2 degradation**

ML364, a small-molecule inhibitor of USP2, was used to understand the biological functions of USP2 (30). We next used ML364 as a pharmacological approach to determine the effect of USP2 on SKP2. Indeed, in multiple lung cancer cell lines, ML364 reduced the levels of SKP2, as well as MDM2 and Aurora-A, two known substrates of USP2 (23, 31), serving as the positive controls, in a dose- (Fig. 3A) and time-dependent manner (Fig. 3B), which can be completely rescued by MG132 (Fig. 3C). Likewise, ML364 shortened the protein half-life of SKP2 (Fig. 3D) and significantly increased SKP2 polyubiquitylation (Fig. 3E). Finally, we investigated possible contribution of CDH1 and FBXW2, two E3 ubiquitin ligases known to ubiquitylate SKP2 (26, 27), to ML364-induced SKP2 polyubiquitylation. We knocked down CDH1 or FBXW2, alone or in combination, and found that either siFBXW2 or siCDH1 significantly inhibited, whereas combinational knockdown completely abolished SKP2 polyubiquitylation induced by ML364 (Fig. 3F). The results demonstrated that SKP2 polyubiquitylation is triggered by CDH1 and FBXW2, and deubiquitylated by USP2, which is inhibited by ML364. Thus, ML364-induced SKP2 polyubiquitylation is in a manner dependent of both CDH1 and FBXW2. Collectively, we have validated that SKP2 is a substrate of USP2 using both genetic (siRNA) and pharmacological (ML364) approaches.

**USP2 stabilizes SKP2 substrates**

We then investigated the effect of USP2 on SKP2 substrates with expectation that USP2 would reduce the levels of SKP2 substrates, given its SKP2 stabilization activity. Surprisingly, ectopic USP2 expression caused the dose-dependent accumulation of both SKP2 and SKP2 substrates, p21 (11, 12) and p27 (13–15) in H1299 and H2170 cells (Fig. 4A). USP2 overexpression also increased the levels of two additional SKP2 substrates, cyclin D1 (12) and cyclin E2 (32) in H2170 cells with the low basal levels, but not in H1299 cells with high basal levels (Fig. 4A). Likewise, USP2 knockdown reduced the protein levels of these SKP2 substrates, including p21, p27, cyclin A2 (33), cyclin D1, and cyclin E2 (Fig. 4B), without affecting the mRNA levels of p21 and p27 (Fig. 4C). Consistently, USP2 knockdown shortened the protein half-life of SKP2 as well as...
Figure 1. USP2 bound to and stabilized SKP2. A, USP2 and USP21 specifically bind to SKP2. HEK293 cells were transfected with the mock vector or FLAG-tagged DUBs as indicated for 48 h, followed by immunoprecipitation (IP) with FLAG beads and then IB with the indicated Abs. The asterisk indicates the specific band. B, USP2 overexpression, not USP21, extends the protein half-life of SKP2. HEK293 cells were transfected with indicated plasmids for 48 h, treated with 100 mg/ml CHX for the indicated time periods, and then subjected to IB with the indicated Abs. Densitometry quantification was determined using ImageJ, and the decay curves are shown (right). C, USP2 silencing, not USP21, downregulates SKP2. Cells were transfected with siRNA targeting USP2 or USP21 for 48 h and then subjected to IB with the indicated Abs. D, USP2 overexpression increases the protein levels of SKP2 in dose- and deubiquitylase-dependent manners. H1299 and H2170 cells were transfected with an increasing amount of FLAG-USP2 or FLAG-USP2-C276A, followed by IB with indicated antibodies. E, binding of endogenous USP2 to SKP2. Cell lysates from H2170 cells were immunoprecipitated using Ab against USP2 (top) or SKP2 (bottom), along with a normal IgG control, followed by IB with the indicated Abs. Abs, antibodies; CHX, cycloheximide; DUBs, deubiquitylases; IB, immunoblotting; SKP2, S-phase kinase-associated protein 2; WCE, whole-cell extracts; USPs, ubiquitin-specific proteases.
**USP2 stabilizes SKP2 and SKP2 substrates**

**Figure 2. USP2 is an SKP2 deubiquitylase.**

A. USP2 silencing does not affect the levels of SKP2 mRNA. H1299 and H2170 cells were transfected with the indicated siRNA oligos for 48 h and then subjected to qRT-PCR (mean ± SEM, n = 3, ***p < 0.001).

B. Proteasome inhibitor MG132 blocks the decrease of SKP2 induced by USP2 knockdown. Cells were transfected with the indicated siRNA oligos for 48 h, treated with 20 μM MG132 for 6 h, and then subjected to IB with the indicated Abs.

C. USP2 knockdown shortens the protein half-life of SKP2. Cells were transfected with indicated siRNA oligos for 48 h, treated with 100 μg/ml CHX for the indicated time periods, and then subjected to IB with the indicated Abs. Densitometry quantification was determined using ImageJ, and the decay curves are shown (right).

D and E. USP2 knockdown promotes SKP2 ubiquitylation (D), whereas USP2 overexpression suppresses SKP2 ubiquitylation (E). HEK293 cells were transfected with the indicated plasmids or siRNA oligos for 48 h, treated with 20 μM MG132 for 6 h, and then lysed under denaturing conditions. The ubiquitylated proteins were pulled down by Ni-NTA beads and then subjected to IB with the indicated Abs.

F and G. WT USP2, but not catalytic-inactive mutant USP2(C276A), blocks the ubiquitylation of SKP2 (F) and extends the protein half-life of SKP2 (G). HEK293 cells were transfected with the indicated plasmids for 48 h, treated with 20 μM MG132 for 6 h, and then lysed under denaturing conditions. The ubiquitylated proteins
its substrates, p21 and p27 (Fig. 4D). Thus, USP2 stabilized both SKP2 and SKP2 substrates.

**USP2 interacts with SKP2 via the LRR domain of SKP2 to disrupt the SKP2 substrate binding**

We next investigated the underlying mechanism of this unexpected finding. We first confirmed that USP2 did not directly bind to SKP2 substrates, excluding the possibility that USP2 stabilizes SKP2 substrates by directly deubiquitylating them (Fig. 5A). We then found that SKP2 knockdown (a) caused the accumulation of its substrates, as expected, and (b) fully rescued SKP2 substrates reduced by USP2 knockdown (Fig. 5B). Thus, SCF^SKP2^ is a functional E3 ligase in these lung cancer cells, and USP2 regulation of SKP2 substrate stability is in a manner dependent on SKP2. We then mapped the USP2-binding domain on SKP2 and found that it is mediated by the LRR domain–containing C terminus, but not the F-box domain–containing N terminus of SKP2 (Fig. 5C). Given that the LRR domain is known to mediate SKP2 substrate binding (34, 35), we hypothesized that USP2 may compete with SKP2 substrates for SKP2 binding. Indeed, ectopic USP2 expression decreased the SKP2 binding with its substrates, p21, cyclin D1, and cyclin E2 (Fig. 5D), whereas USP2 knockdown enhanced the SKP2 binding with its substrates, p21 and cyclin D1 (Fig. 5E). It is well known that the SCF E3 substrate binding requires prephosphorylation of the substrate. We, therefore, investigated whether the SKP2–USP2 binding is also phosphorylation dependent. We treated cell lysates with λPPase and found a significant attenuation of the SKP2 substrate binding, as expected, but without much affecting the SKP2–USP2 binding (Fig. 5F). Thus, the SKP2–USP2 binding appears to be phosphorylation independent.

It has been previously reported that USP2 catalytic-inactive mutant USP2–C276A (36) interacts with the substrates but did not regulate substrate stability. Using an in vivo ubiquitylation assay in both HEK293 and H1299 cells, we found that p21 polyubiquitylation by SKP2 can be blocked effectively by either USP2–WT or USP2–C276A mutant (Fig. 5G), indicating that USP2 regulation of SKP2 substrate stability is independent on its DUB activity. Finally, we knocked down endogenous USP2, followed by ectopically expressing USP2–WT or USP2–C276A, to measure the effect on protein levels of SKP2 and SKP2 substrates. We found that while both USP2–WT and USP2–C276A rescued the levels of SKP2 substrates, only USP2–WT restored SKP2 protein levels (Fig. 5H). Taken together, these results clearly showed that USP2 has no direct effect on SKP2 substrates, rather it blocked the SKP2 interaction with its substrates, leading to substrate stabilization.

**Growth suppression by USP2 inactivation is partially mediated via modulating SKP2 and its substrates**

Finally, we determined the biological significance of the USP2–SKP2 interaction. USP2 knockdown via siUSP2 suppressed growth of both H358 and H2170 lung cancer cells, which can be rescued by simultaneous ectopic expression of SKP2 (Fig. 6A). Given that USP2 knockdown reduced the levels of both SKP2 and SKP2 substrates, p21 and p27 (Fig. 6B), this growth inhibitory effect appears to be mediated by other substrates of USP2 in a manner largely independent of SKP2. Interestingly, we did not see much growth-stimulating effect upon ectopic SKP2 expression under siCtrl-transfected condition, in which the levels of SKP2 substrates p21 and p27 were reduced by ~2-fold (lanes 3 versus 1). However, ectopic SKP2 overexpression in combination with USP2 knockdown caused more than 5-fold reduction of SKP2 substrates, p21 and p27 (lanes 4 versus 1). This result strongly suggests that under siUSP2 condition, “free” SKP2, even at lower amount (compare lanes 4 versus 3), was effectively promoting degradation of its substrates, p21 and p27 (~3-fold reduction, lanes 4 versus 3) to abrogate their cell suppressive function, leading to the rescue effect. Thus, SKP2 appears to play a role at least in part in mediating USP2 function on cell growth.

Similar growth suppression effect was also observed upon USP2 inactivation by ML364, which was also partially rescued by simultaneous SKP2 overexpression, but to a lesser extent (Fig. 6C). We reasoned that while ML364 inactivated USP2 catalytic activity, this small molecule is unlikely to disrupt the USP2–SKP2 binding, leading to less “free” SKP2 to degrade its substrates. Indeed, only 2-fold reduction in the levels of p21 and p27 was observed upon SKP2 overexpression in ML364-treated cells (Fig. 6D, lanes 4 versus 3). This result further supports the notion that USP2 competes with SKP2 for its substrate binding.

**Discussion**

Post-translational modifications play an important role in maintaining protein homeostasis through controlling subcellular localization, stability, and activity of a modified protein (37). The ubiquitylation–deubiquitylation axis is one of the major post-translational modifications that regulates protein stability. We reported here that USP2 is a new deubiquitylase of SKP2 E3 ligase, which interestingly stabilized both SKP2 and SKP2 substrates.

Two DUBs, USP10 and USP13, were previously reported to deubiquitylate SKP2. In chronic myeloid leukemia cells, USP10 deubiquitylated SKP2 to stabilize SKP2. The accumulated SKP2 then promoted BCR-ABL polyubiquitylation via the K63 linkage, leading to BCR-ABL activation and resistance of chronic myeloid leukemia cells to imatinib, a tyrosine kinase inhibitor, which is abrogated by USP10 inhibition (28). In HeLa cells, the ubiquitin-recognition protein Ufd1 was reported as a scaffold protein for SKP2–USP13 interaction to facilitate SKP2 deubiquitylation by USP13. Prolonged endoplasmic reticulum stress downregulated Ufd1 to attenuate the USP13–SKP2 interaction, leading to SKP2 destabilization and USP2 stabilizes SKP2 and SKP2 substrates
Figure 3. ML364, a small-molecule inhibitor of USP2, accelerates SKP2 degradation. A and B, ML364 treatment reduces the protein levels of SKP2 in both dose- and time-dependent manners. Cells were treated with various concentrations of ML364 for 24 h (A), or with 10 μM ML364 for (B) indicated time periods, followed by IB with indicated Abs. C, proteasome inhibitor MG132 blocks the decrease of SKP2 induced by ML364. Cells were pretreated with 10 μM ML364 for 6 h, cotreated with 20 μM MG132 for 6 h, followed by IB with indicated Abs. Densitometry quantification was determined using ImageJ, and the decay curves were shown (right). E, ML364 treatment promotes SKP2 ubiquitylation. HEK293 cells were transfected with the indicated plasmids for 48 h, pretreated with 10 μM ML364 for 6 h, and then cotreated with 20 μM MG132 for 6 h, followed by in vivo polyubiquitylation assay. F, ML364 evaluated SKP2 polyubiquitylation mediated by SCFFBXW2 and SCFCDH1. HEK293 cells were transfected with plasmids or/and siRNA oligos for 48 h, pretreated with 10 μM ML364 for 6 h, and then co treated with 20 μM MG132 for 6 h, followed by in vivo polyubiquitylation assay. Abs, antibodies; CHX, cycloheximide; IB, immunoblotting; SCF, SKP1-Cullin1-F-box; SKP2, S-phase kinase-associated protein 2; USPs, ubiquitin-specific proteases; WCE, whole-cell extracts.
**USP2 stabilizes SKP2 and SKP2 substrates**

*Figure 4. USP2 stabilizes SKP2 substrates.* A, USP2 overexpression increases the protein levels of both SKP2 and SKP2 substrates. H1299 and H2170 cells were transfected with an increasing amount of FLAG-USP2, followed by IB with indicated antibodies. B and C, USP2 silencing decreases the protein levels of both SKP2 and its substrates (B) but does not decrease the mRNA levels of its substrates (C). Cells were transfected with siRNA targeting USP2 for 48 h and then subjected to IB with the indicated Abs (B) or subjected to qRT-PCR (mean ± SEM, n = 3) (C). D, USP2 silencing shortens the protein half-life of the substrates of SKP2. Cells were transfected with siRNA targeting USP2 for 48 h, treated with 100 mg/ml CHX for the indicated time periods, and then subjected to IB with the indicated Abs. Denitometry quantification was determined using ImageJ, and the decay curves are shown (bottom). Abs, antibodies; CHX, cycloheximide; IB, immunoblotting; NS, not significant; qRT-PCR, quantitative real-time PCR; SKP2, S-phase kinase-associated protein 2; USPs, ubiquitin-specific proteases.
USP2 stabilizes SKP2 and SKP2 substrates

Figure 5. USP2 interacts with SKP2 via the LRR domain of SKP2 to disrupt the SKP2 substrate binding. A, USP2 does not bind to the substrates of SKP2. HEK293 cells were transfected with the mock vector or FLAG-USP2 for 48 h, followed by immunoprecipitation (IP) with FLAG beads and then IB with the indicated Abs. B, simultaneous silencing of SKP2 abrogates the reduction of the substrates of SKP2 by USP2 knockdown. Cells were transfected with siRNAs targeting USP2 and SKP2 for 48 h and then subjected to IB with the indicated Abs. C, USP2 binds to the LRR domain of SKP2. HEK293 cells were transfected with the indicated plasmids for 48 h, followed by IP with HA beads and then IB with the indicated Abs. The asterisk indicates the specific band. D, USP2 overexpression inhibits the interaction of SKP2 with its substrates. HEK293 cells were transfected with the indicated plasmids for 48 h, and then treated with 20 μM MG132 for 6 h, followed by IP with HA beads and then IB with the indicated Abs. E, USP2 silencing enhances the interaction of SKP2 with its substrates. Cells were transfected with siRNA targeting USP2 for 48 h and then treated with 20 μM MG132 for 6 h. Cell lysates were immunoprecipitated using SKP2 Ab, along with a normal IgG control, followed by IB with the indicated Abs. F, USP2 interacts with SKP2 in a phosphorylation-independent manner. Cell lysates from H2170 cells were pretreated with or without λPPase for 1 h and then immunoprecipitated with the antibody against SKP2, followed by IB with the indicated Abs. G, both WT and catalytic-inactive mutant USP2 overexpression inhibit the ubiquitylation of p21. Cells were transfected with the indicated plasmids for 48 h, treated with 20 μM MG132 for 6 h, and then lysed under denaturing conditions. The ubiquitylated proteins were pulled down by Ni-NTA beads and then subjected to IB with the indicated Abs. H, overexpression of both WT and catalytic-inactive mutant USP2 abrogates the reduction of p21 by USP2 knockdown. Cells infected with shRNA targeting USP2 were transfected with the indicated plasmids for 48 h and then subjected to IB with the indicated Abs. Abs, antibodies; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation; LRR, leucine-rich repeat; WCE, whole-cell extracts; SKP2, S-phase kinase-associated protein 2; USPs, ubiquitin-specific proteases.
Figure 6. Growth suppression by USP2 inactivation is partially mediated via modulating SKP2 and its substrates. A and B, SKP2 ectopic expression rescues the suppressed growth by USP2 knockdown. H358 and H2170 cells were transfected with siRNA oligos or plasmids for 48 h, and cell growth was measured by the CCK8 assay (A) or subjected to IB with the indicated Abs (B). C and D, SKP2 ectopic expression partially rescues the suppressed growth by ML364. Cells were transfected with indicated plasmids for 48 h and treated with ML364 or DMSO, and cell growth was measured by CCK8 assay (C) or subjected to IB with the indicated Abs (D). E, a model for USP2 stabilization of both SKP2 and SKP2 substrates. USP2 stabilizes SKP2 by directly deubiquitylating SKP2. On the other hand, USP2 stabilizes SKP2 substrates by competitively binding to the LRR domain of SKP2 to disrupt the interaction of SKP2 with its substrates. Abs, antibodies; CCK-8, Cell Counting Kit-8; IB, immunoblotting; LRR, leucine-rich repeat; SKP2, S-phase kinase-associated protein 2; USPs, ubiquitin-specific proteases.

USP2 stabilizes SKP2 and SKP2 substrates.

J. Biol. Chem. (2021) 297(4) 101109 9
USP2 stabilizes SKP2 and SKP2 substrates

p27 accumulation. Accumulated p27 then triggered G1 phase arrest to facilitate the clearance of misfolded proteins (29). However, in both studies, the authors did not define the binding domains of USP10 or USP13 on SKP2.

The DUBs participate in many key cellular signaling pathways and were emerged as novel anticancer targets (38). USP2 is overexpressed in various human cancers, which is positively correlated with tumor malignancy (3, 39). USP2 acts as an oncogenic protein by deubiquitylating and stabilizing oncogenic proteins. For example, USP2 stabilized (a) MDM2 to promote p53 degradation to block apoptosis (23); (b) fatty acid synthase to regulate prostate cancer cell survival (25); (c) Aurora-A to accelerate cell cycle progress (31); and (d) TGF-β serine/threonine kinase receptors 1 to promote metastasis (22). Thus, USP2 appears to be an attractive anticancer target, and indeed, small-molecule inhibitors of USP2 have shown to inhibit growth and metastasis of cancer cells (22, 40).

The DUB–substrate interaction in some cases is not simple and straightforward. For example, USP7 deubiquitylated both MDM2 and p53, a pair of negative regulators of each other. Interestingly, USP7 deubiquitylated p53 through MDM2 without direct p53 binding (41). In another case, USP28 regulated the stability of both F-box/WD repeat-containing protein 7 (FBXW7) and FBXW7 substrates in a dose-dependent manner. While heterozygous deletion of USP28 stabilized FBXW7 to trigger substrate degradation, its homozygous deletion destabilizes FBXW7 to cause substrate accumulation. On the other hand, USP28 overexpression stabilized FBXW7 or its substrates also in a dose-dependent manner: USP28 ectopic overexpression at a low or high level stabilized FBXW7 or FBXW7 substrates, respectively, because FBXW7 substrates were recruited to USP28 via FBXW7 and USP28 preferred to binding and deubiquitylating FBXW7 (42).

In this study, we showed that USP2 is a new bona fide deubiquitylase of SKP2, which is supported by the following lines of evidence: (1) SKP2 binding with USP2 under physiological conditions; (2) the levels of endogenous SKP2 are directly regulated by USP2 manipulation with USP2 knockdown to decrease and USP2 overexpression to increase SKP2; (3) USP2 stabilized SKP2 by cleaving SKP2 polyubiquitylation chains to extend SKP2 half-life; (4) pharmacologic inhibition of USP2 significantly impaired SKP2 stability; and (5) USP2 catalytic-inactive mutant USP2–C276A had no effect on SKP2. Thus, stabilization of oncogenic SKP2 could be yet another mechanism by which USP2 acts as an oncogenic protein.

What is biochemical consequence of USP2 stabilization of SKP2? This is a much more complicated issue to address, given the fact that (1) USP2 has multiple substrates in addition to SKP2 and (2) USP2 positively regulates both SKP2 and SKP2 substrates, which either promotes or inhibits cell growth, respectively. Nevertheless, we found that USP2 inactivation, via either siRNA-based knockdown or small-molecule inhibitor ML364, significantly inhibited growth of lung cancer cells, which appears to be independent of SKP2 because the levels of both SKP2 and SKP2 substrates, p21 and p27, were reduced. However, the observation that growth suppression induced by USP2 inactivation can be rescued at least in part by SKP2 ectopic expression suggests a role played by SKP2.

In summary, we reported an unexpected mechanism among the interactions of a DUB, an E3 ligase, and its substrates, which extended DUB–substrate regulatory model. Our study fits the following working model that USP2 has a dual role in stabilizing both SKP2 and SKP2 substrate through binding to the LRR domain on SKP2: (a) when SKP2 is free of USP2 binding, it promotes the degradation of p21 and p27, and (b) when SKP2 binds to USP2, p21 and p27 are freed up and stabilized (Fig. 6E).

Experimental procedures

Cell culture

All cell lines used in this study were obtained from the American Type Culture Collection. H358 and H2170 cells were maintained in the RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. H1299 and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin.

Plasmids, siRNAs, and reagents

FLAG-tagged USP2, USP3, USP7, USP8, USP11, USP18, USP21, USP30, and USP33 plasmids were a kind gift from Dr lingqiang Zhang. FLAG-SKP2 and HA-SKP2 were previously described (43). The catalytically inactive mutant USP2–C276A was generated by the QuikChange Site-Directed mutagenesis kit (#200522, Agilent Technologies). The primers used for USP2–C276A were as follows: 5′-AAC CTT GGG AAC ACG GCC TTC ATG AAC TCA ATT-3′ and 5′-AAT TGA GTT CAT GAA GGC CGT GTT CCC AAG GTT-3′. Truncated SKP2 mutants were subcloned into pCDNA3.1-3HA. The primers used for PCR were as follows: 5′-AGA TCT CTC GGC GCC GCA TGC ACA GGA GGA ACC GCC TTC ATG AAC TCA ATT-3′ and 5′-AGA TCT CTC GAG TCA CAC ATC CGG GTG CAG ATT TT-3′ for N-terminal SKP2 and 5′-AGA TCT GCG GCC CGC CGC CGC TGA CGT GTC GTG TGC TGT CTC-3′ and 5′-AGA TCT CTC GAG TCA CAC ATC CGG GTG CAG ATT TT-3′ for C-terminal SKP2.

The sequences of siRNA oligonucleotides were as follows: siUSP2-1: 5′-CGG CAG TCG TCT TAT TCT TAT-3′ and siUSP2-2: 5′-ACG TCG TCT TAT TCT TAT-3′. For lentivirus-based shRNA silencing, short hairpins were cloned
into the pLKO.1-puro vector. The targeting sequence for USP2 was as follows: 5′-ACAGACGGACGGAGCAGAATCTTTTGG-3′.

The small-molecular inhibitor of USP2, ML364, was purchased from TargetMol. Cycloheximide was purchased from Sigma. MG132 and Cell Counting Kit-8 (CCK-8) were purchased from MedChemExpress.

**Transfection and lentiviral infection**

Cells were transfected with plasmids or siRNA oligos, using Lipofectamine 2000, according to the manufacturer’s instructions, followed by various assays 48 h after transfection. For lentivirus-based shRNA silencing, lentiviral shRNA viruses were packaged in 293T cells and then infected cells for 48 h along with 8 μg/ml polybrene.

**Immunoblotting and IP**

For direct immunoblotting (IB), cells were harvested and lysed in the RIPA lysis buffer containing protease inhibitors (#11873580001, Roche) and phosphatase inhibitors (#04906837001, Roche). The same amounts of whole-cell lysates were subjected to IB after the protein concentration was measured using the BCA protein assay kit (#23225, Thermo). For IP, cells were lysed in the co-IP lysis buffer (50 mM Tris HCl, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, pH 8.0) with protease inhibitors, and the whole-cell lysates were incubated with bead-conjugated HA antibody (#A2095; Sigma), or SKP2 bead-conjugated FLAG antibody (#A2220; Sigma), or USP2 antibody followed by protein-G beads (#17061801, GE Healthcare) in a rotating incubator overnight at 4°C. The immunoprecipitates were washed with the co-IP lysis buffer for four times and then subjected to IB. Primary antibodies were used as follows: SKP2 (#2652, 1:1000), p21 (#2947, 1:1000), p27 (#2955, 1:1000), cyclin A2 (#4656, 1:1000), cyclin D1 (#2978, 1:1000), cyclin E2 (#4132, 1:1000), Aurora-A (#91590, 1:1000) (Cell Signaling Technology), HA antibody (#11867423001, 1:2000) (Roche Life Science), FLAG (#F1804, 1:2000), CDH1 (#7855, 1:1000) (Sigma), SKP2 (#32-3300, 1:500) (Invitrogen), USP2 (#AP2131c, 1:2000) (Abgent), MDM2 (#OP46, 1:500) (Calbiochem), FBXW2 (#11499-1-AP, 1:1000) (Proteintech), and β-actin (sc-47778, 1:10,000) (Santa Cruz Biotechnology).

**Quantitative real-time PCR**

Cells were harvested in the TRIzol reagent (#15596018, Invitrogen), and total RNA was isolated and then reverse-transcribed to cDNA using PrimeScript 1st Strand cDNA Synthesis kit (RR037A, Takara). The cDNA levels were examined by Applied Biosystems 7900HT Real-Time PCR System using Power SYBR Green Master Mix and normalized to GAPDH. The primer sequences used were as follows: 5′-GTC TCC TCT GAC TCC ACG G-3′ and 5′-ACC ACC CTG TTT CTG TAG CCA A-3′ for GAPDH; 5′-CCA AGA AGA GGC AGC CCA TG-3′ and 5′-TAG TGG GCA TCT GTG TAG CG-3′ for SKP2; 5′-CGA CTC CAG CGT CAC TCC C-3′ and 5′-GAC ACA GTT GTT TCT GAC ACA TAG GA-3′ for USP2; 5′-CTG TCA CTG TCT TGT ACC CTT GT-3′ and 5′-GGT AGA AAT CTG TGC TGG T-3′ for p21; 5′-ATA AGG AAG CGA CCT GCA ACC G-3′ and 5′-TTG GTC GGC GTC TGC TCC ACA G-3′ for p27.

**The in vivo polyubiquitylation assay**

HEK293 or H1299 cells were transfected with the indicated plasmids or/siRNA oligos for 48 h and treated with 20 μM MG132 for 6 h before being harvested. Then, the cells were lysed in the guanidine denaturing solution (6 M guanidinium-HCl, 10 mM Tris HCl pH 8.0, 0.1 M Na2HPO4/NaH2PO4, 10 mM β-mercaptoethanol). After completely sonicated, the whole-cell lysates were incubated with Ni-NTA agarose (#1018244; Qiagen) for 4 h at room temperature (RT), as described previously (44). Ni-NTA agarose was then successively washed once with each of denaturing solution, buffer A (8 M urea, 10 mM Tris HCl, pH 8.0, 0.1 M Na2HPO4/NaH2PO4, 10 mM β-mercaptoethanol), buffer B (8 M urea, 10 mM Tris HCl, pH 6.3, 0.1 M Na2HPO4/NaH2PO4, and 10 mM β-mercaptoethanol) with 0.2% Triton X-100, and buffer B with 0.1% Triton X-100 for 5 min in each step at RT. Ubiquitylated proteins were eluted from Ni-NTA agarose with the elution buffer (200 mM imidazole, 0.15 M Tris HCl, pH 6.7, 0.72 M β-mercaptoethanol, 5% SDS, 30% glycerol) for 30 min at RT and then subjected to IB.

**CCK8 assay**

Cells were transfected with the indicated plasmids or/siRNA oligos for 48 h, then equal cells were seed into a 96-well plate in triplicate, and cell viability was detected by CCK-8.

**Statistical analyses**

Statistical analyses between the two groups were performed by two-tailed Student’s t-tests with the data from three independent biological replicates. Differences were considered statistically significant at p < 0.05.

**Data availability**

All data are contained within the article.

**Author contributions—**F. Z. writing–original draft; Y. Z. and Y. S. supervision; Y. Z. and Y. S. funding acquisition.

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**Conflict of interest—**The authors declare that they have no conflicts of interest with the contents of this article.

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**USP2 stabilizes SKP2 and SKP2 substrates**

AGA GGC AGC CCA TG-3

GGT AGA AAT CTG TCA TGC TGG T-3′ for p21; 5′-ATA AGG AAG CGA CCT GCA ACC G-3′ and 5′-TTG GTC GGC GTC TGC TCC ACA G-3′ for p27.

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**CCK8 assay**

Cells were transfected with the indicated plasmids or/siRNA oligos for 48 h, then equal cells were seed into a 96-well plate in triplicate, and cell viability was detected by CCK-8.
**Abbreviations**—The abbreviations used are: APC/C, anaphase-promoting complex/cyclosome; BCR-ABL, break-point cluster region-Abelson; CCK-8, cell counting kit-8; DUB, deubiquitylase; FBXW7, F-box/WD repeat–containing protein 7; IB, immunoblotting; IP, immunoprecipitation; LRR, leucine-rich repeat; MDM2, mouse double minute 2 homolog; RING, really interesting new gene; SCF, Skp1-Cullin1-F-box; SKP2, S-phase kinase-associated protein 2; USP2, ubiquitin-specific proteases 2.

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