USP11 regulates p53 stability by deubiquitinating p53[*]

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Received June 30, 2014; Revision accepted Nov. 11, 2014; Crosschecked Nov. 26, 2014

Abstract: The p53 tumor suppressor protein coordinates the cellular responses to a broad range of cellular stresses, leading to DNA repair, cell cycle arrest or apoptosis. The stability of p53 is essential for its tumor suppressor function, which is tightly controlled by ubiquitin-dependent degradation primarily through its negative regulator murine double minute 2 (Mdm2). To better understand the regulation of p53, we tested the interaction between p53 and USP11 using co-immunoprecipitation. The results show that USP11, an ubiquitin-specific protease, forms specific complexes with p53 and stabilizes p53 by deubiquitinating it. Moreover, down-regulation of USP11 dramatically attenuated p53 induction in response to DNA damage stress. These findings reveal that USP11 is a novel regulator of p53, which is required for p53 activation in response to DNA damage.

Key words: p53, USP11, Deubiquitination, Stability

1 Introduction

p53 is mutated in more than 50% of human cancers, indicating that p53 is a crucial tumor suppressor (Liu *et al.*, 2014). p53 functions as a sequence-specific transcription factor and through a range of target genes involved in cell cycle arrest (such as *p21*) and apoptosis (such as *puma* and *bax*). p53 maintains genomic integrity and prevents cell transformation and tumor formation (Vogelstein *et al.*, 2000; Riley *et al.*, 2008; Kruse and Gu, 2009). Recent studies indicate that the p53 target gene *TIGAR* (TP53-induced glycolysis and apoptosis regulator), a regulator of glycolysis, also modulates p53 tumor suppressor function (Bensaad *et al.*, 2006; Madan *et al.*, 2012; Won *et al.*, 2012). p53 is kept at very low levels in normal cells and dramatically stabilized in response to a variety of stress signals, including DNA damage, abnormal oncogenic activation, and hypoxia (Harris and Levine, 2005; Kruse and Gu, 2009; Vousden and Prives, 2009). The stability of p53 is primarily regulated by its negative regulator murine double minute 2 (Mdm2), which binds p53 and

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functions as an E3 ubiquitin ligase leading to p53 ubiquitination and degradation (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997; Nag *et al.*, 2013).

It is well documented that ubiquitination of p53 can be reversed by a number of deubiquitinating enzymes (DUBs). The ubiquitin-specific protease USP7 (also known as HAUSP) deubiquitinates and stabilizes p53 (Li *et al.*, 2002). However, USP7 also deubiquitinates and stabilizes Mdm2, which is in fact a better binding partner of USP7 than p53. A modest reduction of USP7 in cells results in an increase in p53 levels (Cummins *et al.*, 2004), while nearly complete ablation of USP7 causes an almost complete loss of Mdm2 and consequently significant stabilization of p53 and cell death (Cummins *et al.*, 2004; Li *et al.*, 2004). USP10, a cytoplasmic ubiquitin-specific protease, is stabilized after DNA damage and translocates to the nucleus to deubiquitinate p53 and counteracts Mdm2-induced p53 nuclear export and degradation (Yuan *et al.*, 2010). USP29 is another DUB involved in the p53 pathway, which deubiquitinates and stabilizes p53 in response to oxidative stress (Liu *et al.*, 2011). Recently two OTU family members, DUB OTUB1 (the ovarian tumor domain-containing Ub aldehyde-binding protein 1) and OTUD5 (OTU deubiquitinase 5), have been shown to positively regulate p53 stability by deubiquitinating p53 (Sun *et al.*, 2012; Luo *et al.*, 2013; Li *et al.*, 2014; Sun and Dai, 2014). Overall, deubiquitination plays an important role in p53 stabilization and activation under both normal and stress conditions.

USP11 is a member of the ubiquitin-specific protease family. USP11 regulates BRCA2 expression levels by deubiquitinating it and participates in the cellular response to DNA damage (Schoenfeld *et al.*, 2004). Recently, USP11 has been shown to deubiquitinate and stabilize promyelocytic leukemia (PML) and inhibit multiple malignant characteristics of brain tumors (Wu *et al.*, 2014). Moreover, USP11 plays a key role in homologous recombination repair of DNA double-strand breaks and may be a novel therapeutic target (Wiltshire *et al.*, 2010). Other substrates deubiquitinated by USP11 include RanBPM (Ideguchi *et al.*, 2002), IκBα (Sun *et al.*, 2010), and type I transforming growth factor β (TGFβ) receptor ALK5 (Al-Salihi *et al.*, 2012). Here we show that USP11 is a novel positive regulator of p53, which deubiquitinates

and stabilizes p53 and is required for p53 induction in response to DNA damage.

2 Materials and methods

2.1 Plasmid construction

Full-length complementary DNA (cDNA) encoding USP11 was obtained by polymerase chain reaction (PCR) and subcloned into pCMV-Flag (Clontech). Pfu polymerase (Promega) was used to generate the point mutation of USP11 (USP11 C275S) through a PCR-based site-directed mutagenesis method. All constructs derived from PCR products were verified by DNA sequencing (Invitrogen).

2.2 Cell culture, transfection, and treatments

Human HEK293, HEK293T, H1299, and U2OS cells were maintained in Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 100 IU penicillin, and 100 µg/ml streptomycin in a 37 °C incubator at 5% CO_2. Cells were transiently transfected using the standard calcium phosphate method. MG132, cycloheximide (CHX), and etoposide (Sigma) were added to subconfluent cells at the indicated concentrations.

2.3 RNA interference

For RNA interference experiments, oligonucleotides targeting USP11 (shUSP11-1 5'-CCAGTGG CGCCAGATAGAA-3') were cloned into lentivirus-based vector pLL3.7. Recombinant lentiviral plasmids and the packaging plasmids VSV-G, RSV-REV, and pMDL were co-transfected into 293T cells. Two days later, the viral supernatants were collected to infect target cells in the presence of 6 µg/ml polybrene (Sigma).

2.4 Western blotting and immunoprecipitation

Cell were collected and washed twice with phosphate buffered saline (PBS) and then cell extracts were prepared in Triton X-100 buffer (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 20 mmol/L β-glycerophosphate, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 10 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mmol/L ethylene diamine tetraacetic acid (EDTA)). The protein samples were boiled for

5 min in the sodium dodecyl sulfate (SDS) buffer and resolved in the SDS polyacrylamide gelelectrophoresis (PAGE) gel. The proteins separated in the gel were transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking with 5% skim milk for 1 h at room temperature, the membranes were probed with antibodies at 4 °C overnight, followed by incubating with horseradish peroxidase-conjugated secondary antibodies (Thermo) for 1 h at room temperature. The amount of proteins was visualized by chemiluminescence. The antibodies used were as follows: anti-Flag M2 and anti-actin monoclonal antibodies (Sigma); hemagglutinin (HA; Y-11), anti-Myc (9E10), USP11, and p53 (DO1) monoclonal antibodies (Santa Cruz Biotechnology).

For the immunoprecipitation experiments, cell extracts were incubated with indicated antibodies overnight at 4 °C. Cell extracts were then incubated with Protein G sepharose beads for another 4 h. The beads were centrifuged and washed five times with lysis buffer. The immunocomplexes were boiled for 10 min in the SDS buffer and subjected to Western blotting analysis.

2.5 Quantitative real-time PCR

Total RNA was extracted using the RNA Simple Total RNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. One microgram of the total RNA was reverse-transcribed to first-strand cDNA by using the First-Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). The expression levels of *p53*, *p21*, *bax*, and *puma* were analyzed by quantitative real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions on ABI 7500 real-time PCR system (Applied Biosystems). The primers of *p53*, p53 target genes *p21*, *bax*, and *puma*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were as follows: human *p53* forward 5'-ACTGCATGGACGATCTGTTG-3' and reverse 5'-GTGACAGGGTCCTGTGCTG-3'; human *p21* forward 5'-TTTCAGCCACAGCGACCATG-3' and reverse 5'-AAAGTTCCACCGTTCTCGGG-3'; human *bax* forward 5'-TGGAGATGAACTGGACAGCA-3' and reverse 5'-GAAGTTGCCATCAGCAAACA-3'; human *puma* forward 5'-GGATGGCGGACGACCTC-3' and reverse 5'-CGGGCAAGGCTGGCAGT-3'; human *GAPDH* forward 5'-GACAT

CAAGAAGGTGGTGAA-3' and reverse 5'-TGTC ATACCAGGAAATGAGC-3'.

2.6 *In vivo* ubiquitination assay

H1299 cells were cotransfected with expression vectors of HA-p53, Myc-ubiquitin, and Flag-USP11. Thirty-six hours after transfection, cells were treated with 5 μmol/L MG132 for 3 h before being harvested. Then cells were lysed with RIPA buffer (0.5% (5 g/L) SDS, 0.5% (5 g/L) sodium deoxycholate, 0.5% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L NaF, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin), followed by incubation with HA antibody overnight at 4 °C and incubated with Protein G sepharose beads for another 4 h. The immunoprecipitates were boiled in the SDS buffer and subjected to Western blotting analysis. p53 proteins were immunoprecipitated by HA antibodies and ubiquitinated p53 was detected by anti-Myc antibody.

3 Results

3.1 p53 interacts with USP11

We detected that the ubiquitin-specific protease USP11 interacts with p53. As shown in Fig. 1a, Flag-USP11 can be observed in the HA-p53 but not in control immunoprecipitates. Since we know p53 is induced rapidly in response to various stresses including DNA damage, we examined the interaction between p53 and USP11 in response to DNA damage caused by the treatment with etoposide. As shown in Fig. 1b, the etoposide treatment significantly enhanced the interaction between endogenous p53 and USP11. These results indicate that p53 interacts with USP11 specifically in mammalian cells, which is consistent with the previous study of Sowa *et al.* (2009).

3.2 USP11 stabilizes p53

Since USP11 is an ubiquitin-specific protease, we explored the possibility that USP11 could function to deubiquitinate and stabilize p53. To this end, we overexpressed increasing amounts of Flag-USP11 in U2OS cells. As shown in Fig. 2a, overexpression of USP11 led to a dose-dependent increase in the level of endogenous p53. We also observed a concomitant

accumulation of *p21*, a p53 target gene. Overexpression of USP11 had no effect on p53 messenger RNA (mRNA) levels as shown in Fig. 2b, suggesting that USP11 might enhance p53 level by enhancing p53 protein stability. To confirm the role of USP11 in regulating p53 protein levels, we treated U2OS control cells and cells overexpressing USP11 with CHX to examine the stability of p53. As shown in Fig. 2c, the half-life of p53 was prolonged in cells transfected

with USP11. These results suggest that USP11 regulates p53 stability.

3.3 USP11 deubiquitinates p53

USP11 can stabilize p53 by deubiquitinating it. We next carried out an *in vivo* ubiquitination assay to examine whether USP11 diminishes p53 ubiquitination. Constant amounts of HA-p53, Myc-Ub together with Flag-USP11 (wild type or C275S) were cotransfected into H1299 cells. Cells were treated with the proteasome inhibitor MG132 before being harvested to observe p53 ubiquitination. HA antibody immunoprecipitated ubiquitin-conjugated proteins and p53-ubiquitin conjugates were detected by Myc antibody. As Fig. 3a shows, the ubiquitination of p53 was markedly reduced by cotransfection of wild type USP11. We also observed that USP11 C275S, the catalytic-inactive mutant, lost the ability to counteract the ubiquitination of p53. Conversely, down-regulation of USP11 increased endogenous p53 ubiquitination (Fig. 3b). U2OS cells stably expressing USP11 shRNA and the control cells were treated with MG132 before being harvested. As Fig. 3b shows, endogenous p53 ubiquitination was markedly increased in USP11 knockdown cells compared with the control cells. These results suggest that USP11 deubiquitinates p53, therefore leading to the stabilization of p53.
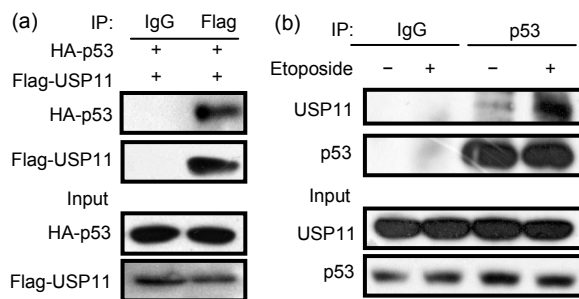

Fig. 1 Interaction of p53 with USP11
(a) HEK293 cells were cotransfected with Flag-USP11 and HA-p53. Cells were collected and lysed 24 h after transfection. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-HA antibody. (b) U2OS cells were treated with or without 20 μmol/L etoposide for 24 h. Cells were harvested and lysed. We adjusted the p53 to the same level and then performed the co-immunoprecipitation assay
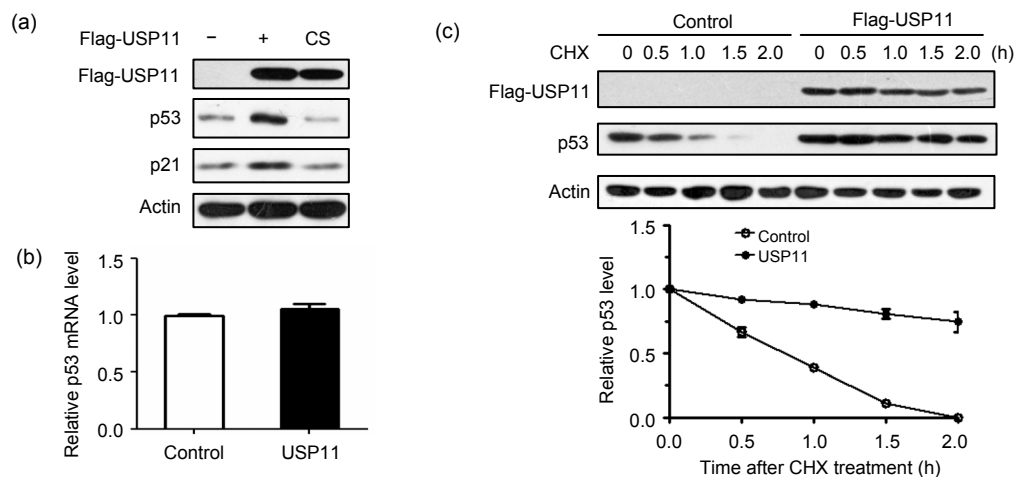
Fig. 2 Regulation of p53 stability by USP11
(a) U2OS cells were transfected with increasing amounts of Flag-USP11. Cell lysates were prepared and analyzed by Western blotting with the indicated antibodies 24 h post-transfection. (b) p53 mRNA levels of U2OS control cells and USP11-expressing cells were measured by quantitative real-time PCR. Results are shown as mean±standard deviation (SD) of triplicate experiments and normalized to the control. (c) Half-life assay of endogenous p53 protein. U2OS cells transfected with vector or Flag-USP11 were treated with 30 μg/ml cycloheximide (CHX) and harvested at the indicated time. Protein levels were detected by Western blotting using the indicated antibodies. The values are obtained from three independent experiments and are normalized to the actin control. For each experimental condition, the signal at the start of the experiment was set to 1. CS: Cys 275 mutants to Ser
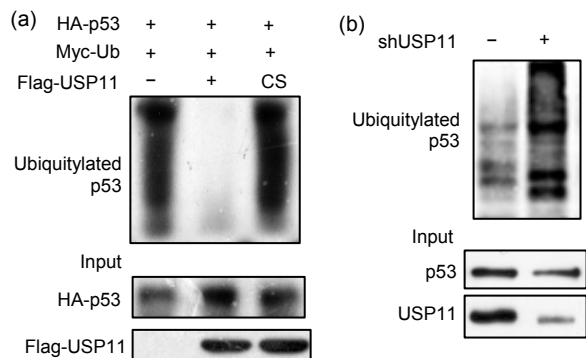
Fig. 3 Deubiquitination of p53 by USP11
(a) H1299 cells transfected with the indicated plasmids were treated with MG132 for 3 h before being harvested. Cell lysates were incubated with HA antibody and the immunoprecipitates were analyzed by immunoblotting with anti-Myc antibody. (b) U2OS cells stably expressing control or USP11 shRNA were treated with MG132 before being harvested. p53 was immunoprecipitated with anti-p53 DO-1 antibody and immunoblotted with anti-ubiquitin antibody. CS: Cys 275 mutants to Ser

3.4 USP11 is required for p53 induction in response to DNA damage

p53 is induced rapidly in response to stress, so we considered whether USP11 is needed for p53 stabilization and activation in response to a DNA damage signal. U2OS cells stably expressing USP11 shRNA or control cells were treated with etoposide. p53 was dramatically stabilized after treatment of cells with etoposide. However, etoposide-induced p53 stabilization was largely impeded in cells where USP11 was silenced (Fig. 4a). In concert with this result, mRNA expression of p53-inducible genes, such as *puma*, *p21*, and *bax*, was also attenuated in USP11 depletion cells (Fig. 4b). Our data show that USP11 is required for the stabilization and activation of p53 in response to DNA damage.

4 Discussion

The tumor suppressor p53 is a short-lived protein and cellular p53 levels are tightly controlled by proteasome-dependent degradation following p53 ubiquitination (Michael and Oren, 2003; Brooks and Gu, 2006). p53 ubiquitination is primarily achieved by E3 ubiquitin ligase Mdm2, which binds p53 with high affinity and efficiently ubiquitinates p53 (Haupt
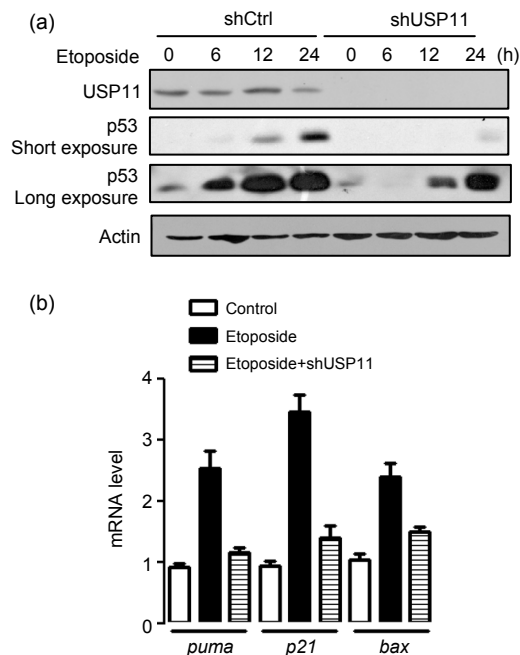
Fig. 4 Role of USP11 on p53 induction in response to DNA damage
(a) U2OS cells stably expressing USP11 shRNA or control cells were treated with 20 μmol/L etoposide for 12 h. Cells were harvested and cell lysates were subjected to immunoblotting analysis with indicated antibodies. (b) Total RNAs were extracted, reverse-transcribed, and analyzed by quantitative real-time PCR for the indicated genes (*p21*, *bax*, and *puma*). Results are shown as mean±SD of triplicate experiments and normalized to the control

et al., 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). Other E3 ubiquitin ligases have been identified to contribute to the degradation of p53 in Mdm2-independent pathways, including COP1, Pirh2, Arf-BP1, TRAF7, and Cullin4B (Leng *et al.*, 2003; Dornan *et al.*, 2004; Chen *et al.*, 2005; Wang *et al.*, 2013; Thirunavukarasou *et al.*, 2014). The ubiquitination of p53 could be reversed by deubiquitination enzymes. Recently, a number of DUBs have been discovered, which control the extent of p53 ubiquitination through deubiquitinating p53 (Cummins *et al.*, 2004; Yuan *et al.*, 2010; Liu *et al.*, 2011; Sun *et al.*, 2012; Luo *et al.*, 2013).

In this study, we report that USP11 is another DUB that functions to deubiquitinate and stabilize p53. We show that (1) USP11 is a novel p53 interacting protein; (2) USP11 stabilizes p53 by deubiquitinating p53; and, (3) USP11 is required for the stabilization and activation of p53 in response to DNA damage. These results provide new insights into

the function of USP11 and reveal a novel mechanism of p53 regulation in response to DNA damage.

USP7, USP10, USP42, USP29, and other DUBs regulate p53 ubiquitination levels under selective conditions. USP7 deubiquitinates both p53 and Mdm2. USP7 prefers to deubiquitinate Mdm2 in unstressed cells, while Mdm2-USP7 interaction is disrupted through ataxia-telangiectasia-mutated kinase (ATM)-dependent phosphorylation in response to DNA damage, tilting the stabilization of p53 (Meulmeester *et al.*, 2005). USP10 deubiquitinates p53 in the cytoplasm and recycles p53 ubiquitinated by Mdm2. ATM-mediated phosphorylation of USP10 promotes its stabilization and translocation to the nucleus to stabilize and activate p53 following DNA damage (Yuan *et al.*, 2010). Here we show another regulation route of p53. We found that USP11, similar to USP42, is required to allow the rapid stabilization and activation of p53 in response to stress. USP11 depletion dramatically inhibits etoposide-induced p53 stabilization (Fig. 4a). It is possible that posttranslational modifications are involved in the regulation of p53-USP11 interaction. USP11 is likely regulated by ATM phosphorylation. Yamaguchi *et al.* (2007) have shown that USP11 regulates p53 levels via the regulation of IκB kinase-α (IKK-α) in response to TNFα. In our study, we found that USP11 could directly interact with p53 and regulate p53 stability in response to DNA damage. Both modes of regulation could potentially coexist to further fine-tune the control of p53 levels in response to a variety of cellular stresses such as DNA damage or TNFα. It has been reported that USP11 could interact with several other DUBs, including USP7, which could stabilize p53 (Sowa *et al.*, 2009). However, the biological function of the interaction between USP7 and USP11 remains unknown. In our studies, we found that knockdown of USP11 largely impeded the activation of p53. We hypothesize that USP11 might cooperate with USP7 to stabilize p53. In conclusion, our work indicated that USP11 plays an important role in p53 regulation in response to DNA damage stress.

Compliance with ethics guidelines

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中文概要：

本文题目：USP11 通过去泛素化 p53 调控 p53 稳定性
　　　　USP11 regulates p53 stability by deubiquitinating p53

研究目的：深入研究 p53 的泛素化及稳定性的调控。

创新要点：发现一个新的调控 p53 去泛素化的酶 USP11，它可以通过与 p53 的结合去泛素化并稳定 p53，从而揭示了一个新的 p53 去泛素化调控的机制。

研究方法：通过免疫共沉淀发现 p53 可以与 USP11 结合（图 1a），通过泛素化检测试验发现 USP11 可以去泛素化 p53（图 3a 和 3b），最后通过逆转录-聚合酶链式反应（RT-PCR）试验发现在 DNA 损伤后，USP11 对 p53 转录活性的提高是非常重要的。

重要结论：USP11 可通过去泛素化 p53 来调控 p53 稳定性。

关键词组：p53 基因；USP11；泛素化；稳定性