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Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1

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The ubiquitin (Ub)–proteasome system plays a pivotal role in the regulation of p53 protein stability and activity. p53 is ubiquitinated and destabilized by MDM2 and several other Ub E3s, whereas it is deubiquitinated and stabilized by Ub-specific protease (USP)7 and USP10. Here we show that the ovarian tumour domain-containing Ub aldehyde-binding protein 1 (Otub1) is a novel p53 regulator. Otub1 directly suppresses MDM2-mediated p53 ubiquitination in cells and *in vitro*. Overexpression of Otub1 drastically stabilizes and activates p53, leading to apoptosis and marked inhibition of cell proliferation in a p53-dependent manner. These effects are independent of its catalytic activity but require residue Asp88. Mutation of Asp88 to Ala (Otub1^{D88A}) abolishes activity of Otub1 to suppress p53 ubiquitination. Further, wild-type Otub1 and its catalytic mutant (Otub1^{C91S}), but not Otub1^{D88A}, bind to the MDM2 cognate E2, UbcH5, and suppress its Ub-conjugating activity *in vitro*. Overexpression of Otub1^{D88A} or ablation of endogenous Otub1 by siRNA markedly impaired p53 stabilization and activation in response to DNA damage. Together, these results reveal a novel function for Otub1 in regulating p53 stability and activity.

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Introduction

The p53 tumour suppressor plays a key role in maintaining genomic integrity and preventing cells from malignant transformation (Levine, 1997; Vogelstein *et al*, 2000; Oren, 2003). Inactivation of p53 function by mutations in the *p53* gene or other defects in the p53 signalling pathways is associated with most human cancers (Vogelstein *et al*, 2000; Oren, 2003). p53 is activated in response to diverse stress and induces expression of various genes, whose protein products mediate cell cycle arrest, apoptosis, senescence, autophagy, angiogenesis inhibition, and regulation of energy metabolism (Vogelstein *et al*, 2000; Oren, 2003; Levine and Oren, 2009; Vousden and Prives, 2009; Vousden and Ryan, 2009).

Under physiological conditions, p53 is maintained at low levels primarily by the oncoprotein MDM2. MDM2 binds to the N-terminal transactivation domain (TAD) of p53 (Chen *et al*, 1993; Oliner *et al*, 1993), directly inhibiting its transcriptional activity (Momand *et al*, 1992; Chen *et al*, 1993). As a Ring-finger-containing ubiquitin (Ub) ligase (E3) (Honda *et al*, 1997; Fang *et al*, 2000), MDM2 also promotes p53 ubiquitination and degradation through the proteasome system (Haupt *et al*, 1997; Kubbutat *et al*, 1997). Further, MDM2 mediates p53 nuclear export (Freedman and Levine, 1998; Roth *et al*, 1998; Li *et al*, 2003). Together, MDM2 suppresses p53-mediated cell growth arrest and apoptosis. Consistently, MDM2 is overexpressed in several types of human cancers, such as soft tissue sarcomas, leukaemia, and breast cancers (Bueso-Ramos *et al*, 1993; Cordon-Cardo *et al*, 1994; Momand *et al*, 1998; Deb, 2003; Dworakowska *et al*, 2004). As MDM2 is transcriptionally induced by p53, the two proteins form an elegant autoregulatory feedback loop (Barak *et al*, 1993; Picksley and Lane, 1993; Wu *et al*, 1993). Genetic disruption of the *p53* gene rescues the lethal phenotype of *mdm2* knockout mice, firmly validating the notion of the MDM2–p53 feedback loop (Jones *et al*, 1995; Montes de Oca Luna *et al*, 1995). Mice that are homozygous for a knock-in of an MDM2 E3-inactive mutant, C462A, are also embryonic lethal and can be rescued by deleting p53 as well, providing compelling evidence that the Ub E3 function of MDM2 is indispensible for its suppression of p53 *in vivo* (Itahana *et al*, 2007). p53 can also be ubiquitinated by a number of other Ub E3s (Dai *et al*, 2006), including Pirh2 (Leng *et al*, 2003), COP1 (Dornan *et al*, 2004), and ARF-BP1 (Chen *et al*, 2005), although their exact function in regulating p53 *in vivo* remains unknown.

Similar to most posttranslational modifications, ubiquitination of p53 can be reversed by counteraction of deubiquitinating enzymes (DUBs). Human genome encodes approximately 95 putative DUBs, categorized into five classes: Ub-specific protease (USP), Ub C-terminal hydrolase (UCH), ovarian tumour (OTU) domain-containing protease, Machado–Joseph disease (MJD) protease, and JAB1/MPN/Mov34 metalloenzyme (JAMM; Nijman *et al*, 2005). The UCH, USP, OTU, and MJD families are cysteine proteases, whereas the JAMMs are zinc metalloproteases (Nijman *et al*, 2005; Komander *et al*, 2009). Several USP family members have been shown to regulate the MDM2–p53 pathway. USP7 (also called HAUSP) deubiquitinates p53, leading to p53 stabilization and activation (Li *et al*, 2002). USP7 also deubiquitinates MDM2 and MDMX, an MDM2 homologue also known as MDM4. Interestingly, partial knockdown of USP7 destabilizes p53, whereas substantial knockdown of USP7 stabilizes p53 through destabilization of MDM2 (Cummins *et al*, 2004; Li *et al*, 2004). DNA-damage-induced phosphorylation of MDMX disrupts its binding to USP7, contributing to the destabilization of MDMX following DNA damage

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(Meulmeester *et al*, 2005). Thus, a proper level of USP7 is required for maintaining the molecular ratio of p53–MDM2–MDMX axis. Most recently, USP10 has been shown to specifically deubiquitinate p53, but not MDM2 and MDMX (Yuan *et al*, 2010). DNA damage triggers ATM-dependent phosphorylation and nuclear translocation of USP10, providing another mechanism for the regulation of p53 stability and activity by deubiquitination. Importantly, USP10 is required for efficient p53 activation in response to DNA damage (Yuan *et al*, 2010). Similarly, USP29 has recently been shown to deubiquitinate and stabilize p53 in response to oxidative stress (Liu *et al*, 2011). In contrast, USP2 deubiquitinates MDM2 (Stevenson *et al*, 2007) and MDMX (Allende-Vega *et al*, 2010), but not p53, leading to suppression of p53 activity. Knockdown of USP2 results in p53-dependent cell cycle arrest (Stevenson *et al*, 2007). Thus, deubiquitination plays a crucial role in finely tuning normal homeostasis of the p53–MDM2–MDMX loop as well as its response to stress.

However, it is not known whether p53 is regulated by DUBs other than USP family members. Here we show that the ovarian tumour domain-containing Ub aldehyde-binding protein 1 (Otubain 1, Otub1 thereafter), an OTU family member DUB, is a novel p53 regulator. Otub1 directly suppresses MDM2-mediated p53 ubiquitination in cells and *in vitro* independent of its catalytic activity. It does so primarily by suppressing the activity of the MDM2 cognate Ub-conjugating enzyme (E2) UbcH5. Overexpression of Otub1 results in marked apoptosis and inhibition of cell proliferation in a p53-dependent manner. Inhibition of Otub1 markedly impaired p53 activation induced by DNA damage. Together, our results reveal a novel and critical role for Otub1 in regulating p53 stability and activity in response to DNA damage.

Results

Otub1 stabilizes p53

In search for DUBs other than USP family members that regulate p53, we were initially interested in the OTU family members and found that ectopic expression of Otub1, but not its close homologue Otub2, drastically induced the levels of p53 as well as its targets p21 and MDM2 in p53-proficient U2OS cells (Figure 1A). Doxycycline (Dox)-induced expression of Otub1, but not Otub2, in tetracycline (tet)-inducible T-Rex-U2OS clones (two clones from T-Rex-U2OS-Flag-Otub1 and T-Rex-U2OS-Flag-Otub2, respectively) also drastically induced the levels of p53, p21, and MDM2 (Figure 1B). The induction of p53 by Otub1 was dose-dependent in U2OS cells with either transient (Supplementary Figure S1A) or Dox-induced expression of Otub1 (Supplementary Figure S1B), as increasing expression of Otub1, but not Otub2, leads to increasing levels of p53, MDM2, and p21. The effect of p53 induction by Otub1 is similar to that by USP7 (Supplementary Figure S1C). Lentiviral-mediated expression of Otub1 induced p53 in human normal fibroblast WI38 cells (Figure 1C) and human p53-proficient lung cancer H460 cells (Supplementary Figure S1D), suggesting that the induction of p53 by Otub1 is not a cell-type-specific effect. The induction of p53 was not due to an increase in *p53* mRNA levels (Supplementary Figure S1E). To test if Otub1 affects p53 degradation, H1299 cells were transfected with p53 and MDM2 in the presence or absence of Otub1. As shown in Figure 1D, expression of Otub1 drastically alleviated MDM2-mediated p53 degradation (compare lane 4 with lane 3). This effect was specific as expression of Otub2 failed to do so (compare lane 5 with lane 3, Figure 1D). Consistently, overexpression of Otub1 markedly prolonged the half-life of p53 (Figure 1E and F), but not p21 (Supplementary Figure S1F and G), as compared with control. All together, these results indicate that Otub1, but not Otub2, stabilizes p53 in cells.

Otub1 induces p53 transcriptional activity and inhibits cell proliferation

To test if Otub1 stimulates p53 activity, reverse transcriptase-quantitative polymerase chain reaction (RT–qPCR) assays were conducted to examine the expression of p53 target genes. Overexpression of Otub1, but not Otub2, significantly induced the levels of *p21*, *mdm2*, and *Bax* mRNAs (Supplementary Figure S2A). The induction of these transcripts was p53-dependent, as knocking down endogenous p53 by siRNA completely abolished the induction of these mRNAs by Otub1 in T-Rex-U2OS-Flag-Otub1 cells (Figure 2A). These results indicate that Otub1 stimulates the transactivation activity of p53. Next, we examined whether Otub1 induces p53-dependent cell cycle arrest and/or apoptosis. The T-Rex-U2OS-Flag-Otub1, T-Rex-U2OS-Flag-Otub2, or control T-Rex-U2OS clones were cultured with or without Dox for 24 h. The cells were harvested and stained with FITC-conjugated anti-Annexin V antibody or propidium iodide (PI) followed by flow cytometry analysis. As shown in Figure 2B, induced expression of Otub1, but not Otub2, significantly induced apoptosis as evidenced by the marked increase in Annexin V-positive cells (top panel, Figure 2B) and sub-G1 populations (Supplementary Figure S2B) as well as the presence of the caspase 3-cleaved PARP product (bottom panels, Figure 2B), an apoptotic marker (Tewari *et al*, 1995). To test if this effect was p53-dependent, U2OS or p53-null Saos2 cells were transfected with GFP or GFP–Otub1. Forty-eight hours posttransfection, the cells were harvested and GFP-positive cells were analysed. As shown in Figure 2C, expression of GFP–Otub1 significantly increased apoptosis in U2OS, but not Saos2, cells as compared with that of GFP. Similarly, induced expression of Otub1 also failed to induce apoptosis in Saos2 (Supplementary Figure S2C) and H1299 (Supplementary Figure S2D) cells. These results indicate that Otub1 induces p53-dependent apoptosis. To test the effect of Otub1 on cell proliferation, colony formation assays were conducted using tet-inducible U2OS and Saos2 cells. As shown in Figure 2D and Supplementary Figure S2E, induced expression of Otub1 completely inhibited colony formation in U2OS, but not Saos2, cells. Also, induced expression of Otub1 significantly inhibited colony formation in isogenic HCT116$p53^{+/+}$, but not HCT116$p53^{-/-}$, cells (Supplementary Figure S2G and H). Similarly, induced expression of Otub1 did not inhibit cell proliferation in H1299 cells (Supplementary Figure S2F). Altogether, these data show that overexpression of Otub1 drastically induces p53-dependent apoptosis and inhibition of cell proliferation.

Otub1 interacts with p53

To examine whether Otub1 physically interacts with p53, co-immunoprecipitation (co-IP) assays were conducted in H1299 cells. As shown in Figure 3A, p53 specifically

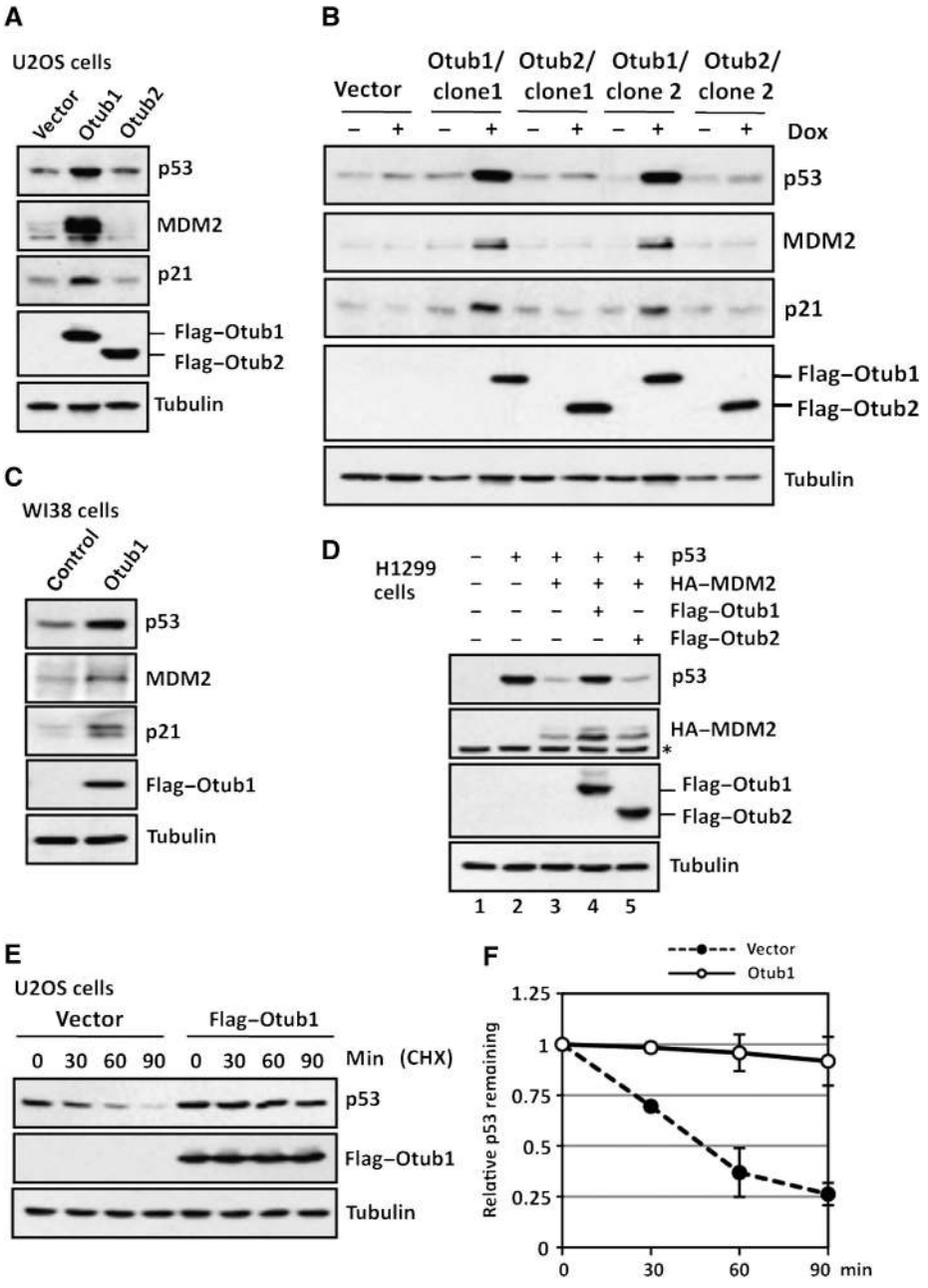


Figure 1 Otub1 stabilizes p53. (**A**) Overexpression of Otub1, but not Otub2, induces p53. U2OS cells transfected with Flag–Otub1, Flag–Otub2, or control vector were assayed for the expression of indicated proteins by IB. (**B**) Induced expression of Otub1 induces p53. Two representative clones from T-Rex-U2OS-Flag-Otub1, T-Rex-U2OS-Flag-Otub2 stable cell lines and the control U2OS-tet-Flag cells were cultured in the absence or presence of 2 μg/ml of doxycycline (Dox) for 24 h. The cell lysates were immunoblotted with the indicated antibodies. (**C**) Otub1 induces p53 in WI38 cells. WI38 cells were transduced with lentiviruses encoding Flag–Otub1 or control viruses for 48 h. The protein expression was analysed using IB. (**D**) Otub1 suppresses MDM2-mediated p53 degradation. H1299 cells transfected with the indicated plasmids were subjected to IB analysis using antibodies as indicated. The asterisk indicates a nonspecific antibody-reacting band. (**E**, **F**). Otub1 stabilizes p53. U2OS cells were transfected with control vector or Flag–Otub1 for 48 h and then treated with 50 μg/ml cycloheximide (CHX). The cells were harvested at different time points and assayed for the levels of p53 and tubulin by IB (**E**). The bands were quantified and the levels of p53 were normalized with the levels of tubulin, and results from three independent experiments were plotted in **F**.

co-immunoprecipitated with Otub1 by the anti-Flag antibody (right panels) and Otub1 specifically co-immunoprecipitated with p53 by the anti-p53 antibody (left panels) in cells co-expressed with both Flag–Otub1 and p53, but not expressed individually. By contrast, we did not observe any detectable co-IP between Otub2 and p53 (Supplementary Figure S3A). Further, endogenous p53 was specifically co-immunoprecipitated with endogenous Otub1 in U2OS cells by anti-Otub1 antibodies, but not control IgG (Figure 3B). This specific interaction was abolished when Otub1 was knocked down by siRNA (lane 6, Figure 3B). Further, glutathione *S*-transferase (GST)-fusion protein–protein association assays showed that His–Otub1 purified from bacteria was specifically bound by purified GST–p53 protein, but not GST alone (Figure 3C). Hence, Otub1 directly interacts with p53 in cells and *in vitro*.

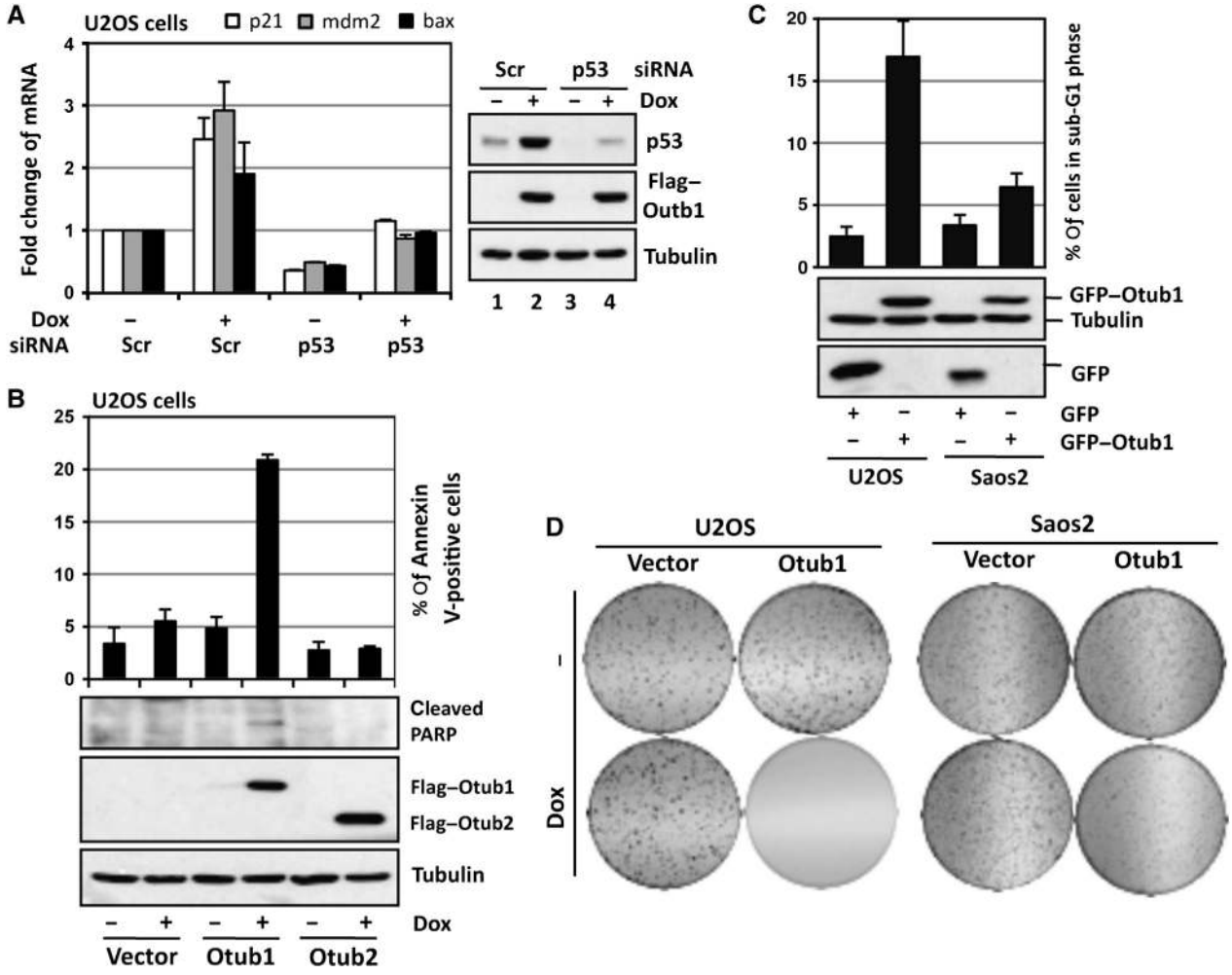


Figure 2 Otub1 stimulates p53 activity and induces p53-dependent cell growth inhibition. (**A**) Otub1 induces p53 activity. T-Rex-U2OS-Flag-Otub1 cells were transfected with scrambled or p53 siRNA. At 24-h posttransfection, the cells were treated with or without 2 μg/ml Dox for another 24 h. The cells were then subjected to RT–qPCR analysis for detection of the *p21*, *mdm2,* and *bax* mRNA, normalized to the expression of *GAPDH*. The expression of indicated proteins are shown in the right panel. (**B**) Otub1, but not Otub2, induces apoptosis. T-Rex-U2OS-Flag, T-Rex-U2OS-Flag-Otub1, or T-Rex-U2OS-Flag-Otub2 cells were cultured in medium containing 2 μg/ml Dox for 24 h. The cells were then stained with anti-Annexin V-FITC followed by flow cytometry analysis. The average percentages of Annexin V-positive cells from three independent experiments are shown in the top panel. The cleaved PARP and Otub1 or Otub2 proteins were detected using IB analysis and are shown in the bottom panels. (**C**) Otub1 induces p53-dependent apoptosis. U2OS or Saos2 cells were transfected with GFP or GFP–Otub1 for 48 h and stained with PI followed by flow cytometry analysis for the DNA content. The GFP-positive cells were gated for analysis of the sub-G1 population of cells. The percentages of sub-G1 cells from four separate experiments are shown in the top panel and protein expression is shown in the bottom panels. (**D**) Otub1 inhibits cell proliferation. Colony formation assays were performed in T-Rex-U2OS-Flag, T-Rex-U2OS-Flag-Otub1, T-Rex-Saos2-Flag, or T-Rex-Saos2-Flag-Otub1 cells in the absence or presence of 2 μg/ml Dox for up to 3 weeks. The colonies were visualized by staining with crystal violet blue. The relative colony number counted from three independent experiments is plotted in Supplementary Figure S2E.

Otub1 binds to the DNA-binding domain of p53 via its N and C termini

To define which domain of p53 is required for binding to Otub1, H1299 cells were co-transfected with V5-Otub1 and a panel of Flag-tagged deletion mutants of p53, followed by co-IP assays using anti-Flag antibodies. As shown in Figure 3D, DNA-binding domain (DBD)-containing p53 mutants (lanes 9–11), but not the C-terminal regulatory regions (lane 12) bound to Otub1, suggesting that Otub1 binds to the DBD of p53 (bottom diagram, Figure 3D). Interestingly, mutants with deletion of C-terminal region (lanes 9 and 10) showed stronger Otub1 binding activity compared with wild-type (wt) p53 (lane 8). The reason for this increased binding is likely due to a 'substrate-trapping' mechanism known for other transient interaction between substrates and enzymes, such as protein phosphotases (Flint *et al*, 1997; Blanchetot *et al*, 2005). The major lysines subjected to ubiquitination are located within the C-terminus of p53 (Rodriguez *et al*, 2000). Deletion of this region renders p53 unable to be ubiquitinated and thus likely unable to be regulated by Otub1, thereby inhibiting the release of Otub1 from p53. Alternatively, deletion of the C-terminal region would change p53 to a conformation facilitating the Otub1–p53 binding. Reciprocal mapping was also conducted using H1299 cells transfected with p53 together with a set of Flag-tagged deletion mutants of Otub1 using co-IP assays. As shown in Figure 3E, both the N-terminal (lane 8) and C-terminal (lane 10) fragments of Otub1 bound to p53. These results

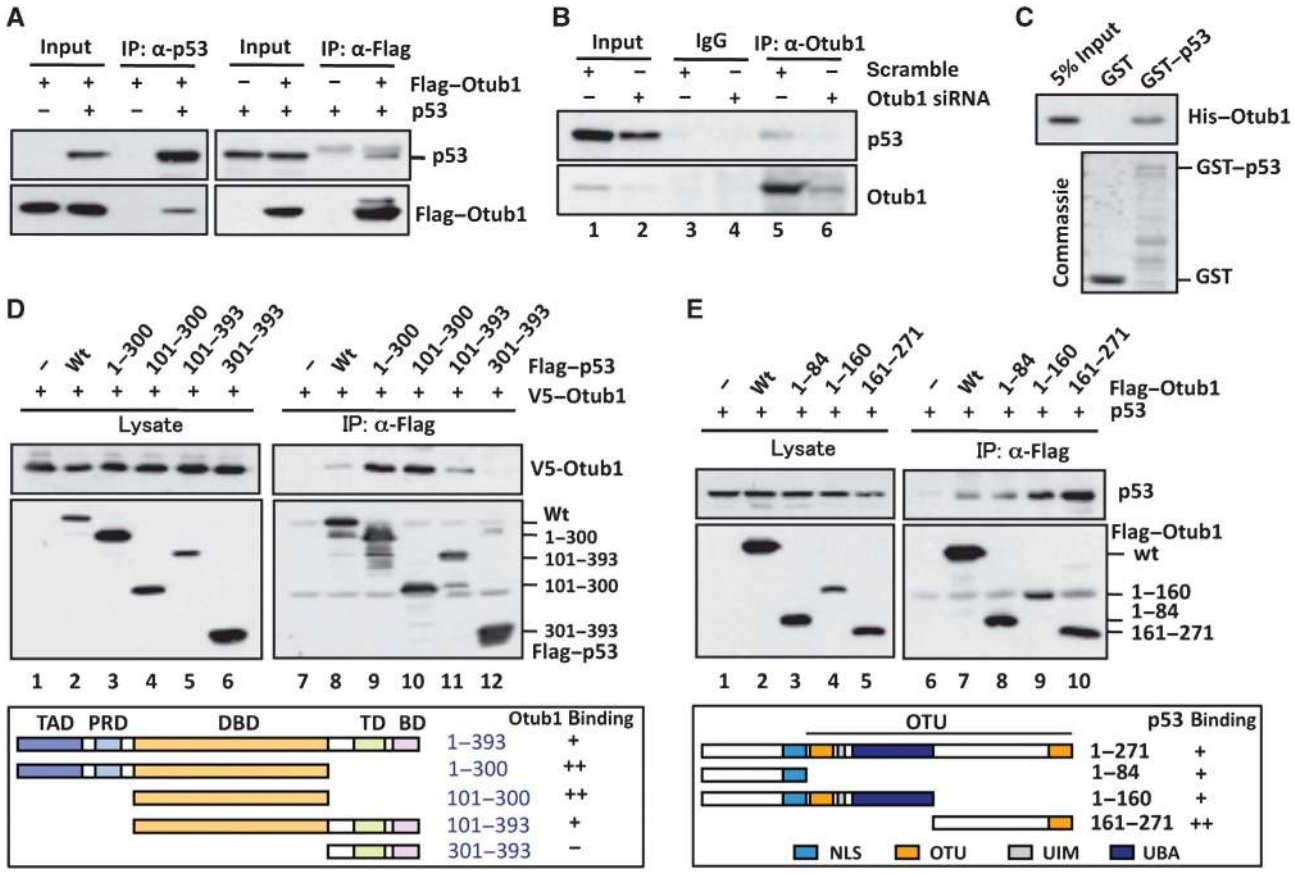


Figure 3 Otub1 interacts with p53. (**A**) Otub1 interacts with p53 in cells. H1299 cells were transfected with Flag–Otub1 and p53 individually or together. The cell lysates were immunoprecipitated with monoclonal anti-p53 (DO-1) or anti-Flag antibodies followed by IB with polyclonal anti-p53 and anti-Flag antibodies. (**B**) Co-IP between endogenous Otub1 and p53. Lysates from U2OS cells transfected with scrambled or Otub1 siRNA were immunoprecipitated with anti-Otub1 antibodies or control immunoglobulin-G (IgG), followed by IB with anti-p53 and anti-Otub1 antibodies. (**C**) Otub1 directly interacts with p53 *in vitro*. Purified GST or GST–p53 immobilized on glutathione beads was incubated with purified His–Otub1. Bound proteins were assayed using IB with anti-Otub1 antibodies. Commassie staining of the GST and GST–p53 proteins is shown in the bottom panel. (**D**). Otub1 binds to p53 DNA-binding domain. H1299 cells were transfected with V5–Otub1 alone or together with Flag–p53 or different deletion mutants as diagramed at the bottom panel. The cell lysates were immunoprecipitated with anti-Flag antibodies followed by IB with anti-V5 or anti-Flag antibodies. The results are summarized in the bottom panel. TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; TD, tetramerization domain; BD, basic domain. (**E**) p53 binds to both the N-terminal and C-terminal domains of Otub1. H1299 cells were transfected with p53 alone or together with Flag–Otub1 or its deletion mutants as diagramed. The cell lysates were immunoprecipitated with anti-Flag antibodies followed by IB with anti-p53 or anti-Flag antibodies. The results are summarized in the bottom panel. NLS, nuclear localization signal; OTU, ovarian tumour, UIM, ubiquitin interaction motif; UBA, ubiquitin-associated domain.

indicate that p53 binds to both the N-terminal and C-terminal domains of Otub1.

Otub1 suppresses MDM2-mediated p53 ubiquitination in cells

To test whether Otub1 regulates p53 ubiquitination in cells, *in vivo* ubiquitination assays were conducted in H1299 cells using Ni^{2+}-NTA purification method (Dai *et al*, 2004, 2008). As shown in Figure 4A, expression of Otub1 completely abolished the MDM2-mediated polyubiquitination of p53 (compare lane 5 with lane 4). Alternative co-IP-immunoblot (IB) assays also showed that Otub1 drastically alleviated MDM2-mediated p53 ubiquitination in H1299 cells (compare lane 5 with lane 4, Figure 4B). To examine whether Otub1 deubiquitinates endogenous p53, U2OS cells were transfected with V5-Ub in the absence or presence of Otub1. The cell lysates were immunoprecipitated with anti-p53 antibodies followed by IB with anti-V5 antibody. As shown in Figure 4C, overexpression of Otub1 markedly suppressed ubiquitination of endogenous p53 (compare lane 3 with lane 2). Again, Otub1 suppresses p53 ubiquitination as efficiently as USP7 in cells (Supplementary Figure S3C). In addition, we observed that Otub1 also suppressed p53 ubiquitination mediated by ARF-BP1 (Supplementary Figure S3D). Together, these results indicate that Otub1 either deubiquitinates p53 or suppresses p53 ubiquitination in cells.

Otub1 regulates p53 in cells independent of its catalytic residue Cys91

Previous structure studies have revealed the putative catalytic triad, Cys 91, His 265, and Asp 267, in the Otub1 (Edelmann *et al*, 2009). In agreement with these studies, we found that mutation of Cys 91 to Ser (C91S) abolished the activity of Otub1 to cleave K48-linked polyUb chains (Supplementary Figure S5A). Thus, C91 is essential for catalytic activity of Otub1. To test whether the catalytic activity is required for

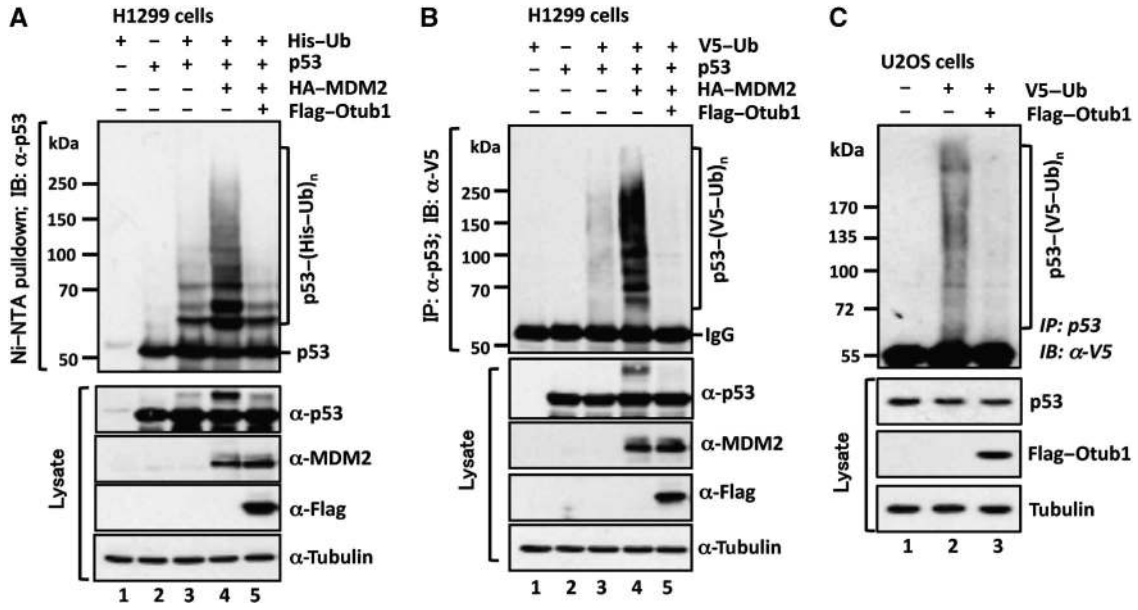


Figure 4 Otub1 suppresses p53 ubiquitination in cells. (**A**, **B**) H1299 cells were transfected with different combinations of plasmids encoding p53, HA–MDM2, Flag-Otub1 with His–Ub (**A**) or V5–Ub (**B**). The cells were subjected to pulldown using Ni^{2+}-NTA bead under denaturing conditions (**A**) or co-IP with anti-p53 antibodies (**B**), followed by IB. The ubiquitinated species of p53 are indicated. (**C**) Otub1 suppresses ubiquitination of endogenous p53. U2OS cells were transfected with or without V5–Ub and Flag-Otub1 plasmids for 48 h and treated with MG132 for 6 h. The cell lysates were immunoprecipitated with anti-p53 (DO-1) antibodies followed by IB using anti-V5 antibodies.

Otub1 to regulate p53 in cells, we examined whether mutating C91 either to Ala (C91A) or to Ser (C91S) would affect the activity of Otub1 to deubiquitinate, stabilize, and activate p53. Surprisingly, this was not the case (Figure 5), as neither the C91A nor the C91S mutants abolished the ability of Otub1 to induce endogenous p53 and p21 (compare lanes 3 and 4 with lane 2, Figure 5A), block MDM2-mediated p53 degradation (compare lanes 4 and 5 with lane 3, Figure 5B), or reverse MDM2-mediated p53 ubiquitination (compare lanes 6 and 7 with lane 5, Figure 5C). Similar to wt Otub1, the C91S mutant also completely suppressed cell proliferation determined by colony formation assays (Figure 6E). Therefore, the catalytic C91 is not required for p53 stabilization and activation by Otub1 in cells.

Asp88 is critical for Otub1 to regulate p53 ubiquitination in cells

To elucidate how Otub1 regulates p53 in cells, we then examined whether deletion of residues 88–91(DGNC) at the OTU domain would affect the ability of Otub1 to regulate p53. These four residues are highly conserved in the Otub1 protein across species and among other OTU family DUBs as well (Balakirev *et al*, 2003; Komander and Barford, 2008). Interestingly, deletion of these four residues (Flag–Otub1$^{\Delta 4}$) completely abolished the ability of Otub1 to induce p53, p21, and MDM2 in U2OS cells (Supplementary Figure S4A) and to suppress p53 ubiquitination in H1299 cells (data not shown). Knowing that C91 was not required, we focused on D88, as cysteine proteases often require Asp (D) or Asn (N) to assist Cys in catalysis (Komander *et al*, 2009). Further, it was initially predicted that D88 is critical for Otub1 DUB activity (Balakirev *et al*, 2003). Indeed, we found that mutation of D88 to A completely abolished the ability of Otub1 to cleave K48-linked polyUb chain (lane 4, Supplementary Figure S5A). Similar to Otub1$^{\Delta 4}$, mutation of Asp88 to Ala (Otub1^{D88A}) completely abolished the induction of p53, p21, and MDM2 proteins (Figure 6A) as well as their mRNA (Figure 6B) in U2OS cells. Unlike wt Otub1, expression of Otub1^{D88A} was unable to abrogate MDM2-mediated p53 degradation (Figure 6C) or reverse MDM2-mediated p53 polyubiquitination (Figure 6D). It also failed to suppress colony formation in U2OS cells (Figure 6E). These results indicate that D88 is required for the activity of Otub1 towards p53 in cells. Intriguingly, Otub1^{D88A} interacted with p53 stronger than wt Otub1 (Figure 6F), which could again be explained by the 'substrate-trapping' mechanism. Together, these results reveal that D88 is critical for Otub1 to suppress p53 ubiquitination in cells. Of note, expression of Flag–Otub1^{D88A} decreased the levels of p53, p21, and MDM2 protein (Figure 6A) and mRNA (Figure 6B) compared with vector control. Knockdown of endogenous Otub1 does not further decrease Otub1^{D88A}-mediated p53 reduction (Supplementary Figure S4B), suggesting that Otub1^{D88A} may act as a dominant-negative mutant of Otub1.

Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, suppresses MDM2-mediated p53 ubiquitination in vitro

To examine whether Otub1 deubiquitinates p53 *in vitro*, we expressed and purified recombinant wt Otub1 with a N-terminal His tag (His–Otub1) and its C91S and D88A mutants from bacteria and generated ubiquitinated p53 from cells transfected with Flag–p53, MDM2, and His–Ub using an anti-Flag affinity purification method (Li *et al*, 2002; Yuan *et al*, 2010). Purified wt His–Otub1, but not the C91S and D88A mutants, efficiently cleaved K48-linked polyUb chains *in vitro* (Supplementary Figure S5A), demonstrating its deubiquitinating enzyme activity. Interestingly, wt Otub1, but not the Otub1^{C91S} and Otub1^{D88A} mutants, slightly re-

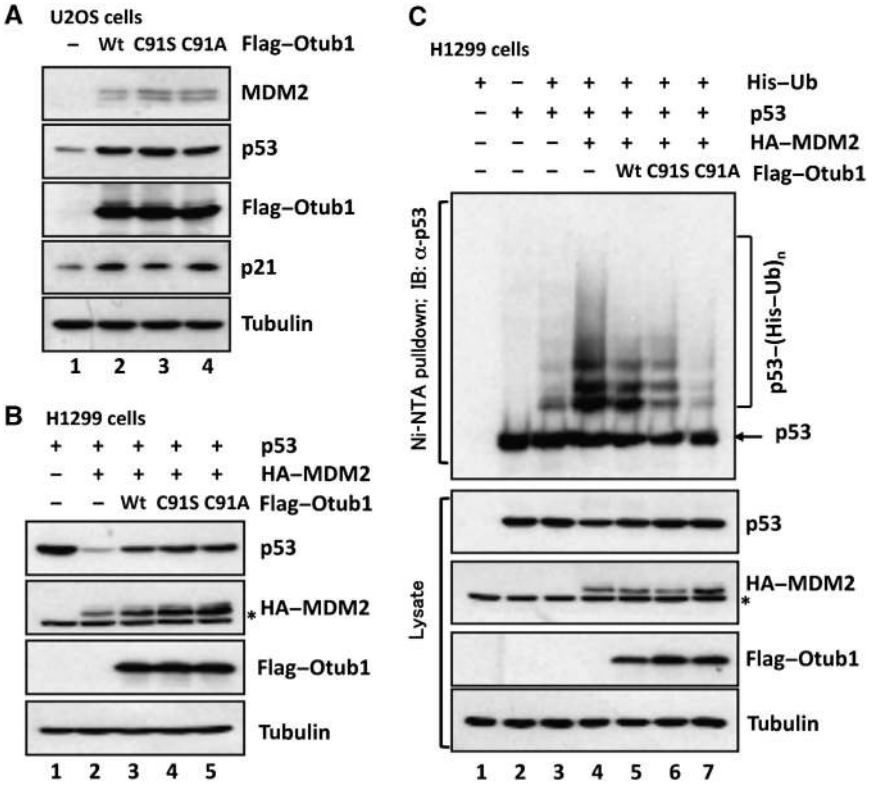


Figure 5 Otub1 regulates p53 independently of its catalytic Cys 91 in cells. (**A**) Mutation of residue C91 does not abolish the activity of Otub1 to induce p53. U2OS cells transfected with Flag–Otub1 or its point mutant C91S or C91A were examined for the expression of indicated proteins using IB. (**B**) Mutation of residue C91 does not abolish the activity of Otub1 to inhibit MDM2-mediated p53 degradation. H1299 cells were transfected with different combinations of plasmids as indicated followed by IB using indicated antibodies. The asterisk indicates a nonspecific antibody-reacting band. (**C**) Mutation of residue C91 does not abolish the activity of Otub1 to suppress MDM2-mediated p53 ubiquitination. H1299 cells were transfected with different combinations of indicated plasmids. *In vivo* ubiquitination assay was conducted using Ni^{2+}-NTA pulldown under denaturing conditions followed by IB. The ubiquitinated species of p53 are indicated. The asterisk indicates a nonspecific antibody-reacting band.

duced polyubiquitinated p53, resulting in slightly increase of monoubiquitinated p53 (Supplementary Figure S5B). This suggests that Otub1 possesses weak DUB activity towards polyubiquitinated p53. To further understand how $Otub1^{C91S}$ stabilizes p53 in cells, we examined whether Otub1 also directly suppresses p53 ubiquitination. To this end, *in vitro* ubiquitination reactions were assembled using recombinant Ub-activating enzyme (UbE1), UbcH5 (E2), MDM2, p53, Ub in the absence or presence of His-tagged $Otub1^{wt}$, $Otub1^{C91S}$, or $Otub1^{D88A}$. As shown in Figure 7A, wt Otub1 and $Otub1^{C91S}$, but not $Otub1^{D88A}$, drastically suppressed MDM2-mediated p53 ubiquitination *in vitro*. This effect was dose-dependent, as increasing amount of Otub1 resulted in decreasing amounts of ubiquitinated species of p53 and increasing amounts of non-ubiquitinated p53 (Figure 7B). These results indicate that Otub1 directly suppresses p53 ubiquitination independent of its DUB activity and imply Otub1 may inhibit the activity of MDM2 (E3) and/or UbcH5 (E2). We then examined the physical association between Otub1 and MDM2. We found that Otub1 directly interacts with MDM2 in cells (Supplementary Figure S6A) and *in vitro* (Supplementary Figure S6B and C). We also showed that Otub1 co-immunoprecipitated with both p53 and MDM2, indicating that the three proteins may form a complex in cells (Supplementary Figure S6D). Furthermore, Otub1 also suppresses MDM2 autoubiquitination (Supplementary Figure S6E) and stabilizes MDM2 (Supplementary Figures S1E, F and S6F) independent of its catalytic activity.

$Otub1^{wt}$ and $Otub1^{C91S}$, but not $Otub1^{D88A}$, bind to UbcH5 and suppress UbcH5-dependent Ub chain formation

It has recently been shown that Otub1 inhibits DNA-damage-dependent chromatin ubiquitination via suppressing UBC13, a cognate E2 enzyme for RNF168 E3 ligase (Nakada *et al*, 2010). This study also showed that Otub1 suppresses the activity of UbcH5 in the presence of the TRAF6 (E3) enzyme. To understand whether Otub1 suppresses p53 ubiquitination by inhibiting UbcH5, the MDM2 cognate E2, *in vitro* reactions were assembled using recombinant E1, UbcH5c, MDM2, and Ub in the absence or presence of His-tagged $Otub1^{wt}$, $Otub1^{C91S}$, or $Otub1^{D88A}$, followed by IB with anti-conjugated Ub antibody (FK2). Similar to the case of Ubc13/RNF168 (Nakada *et al*, 2010), MDM2 greatly stimulated the Ub-conjugating activity of UbcH5 to form polyUb chains (compare lane 3 with lane 2, Figure 7C). Interestingly, $Otub1^{wt}$ and $Otub1^{C91S}$, but not $Otub1^{D88A}$, drastically suppressed this activity (lanes 4 and 5 with lane 6, Figure 7C), suggesting that Otub1 suppresses the activity of UbcH5/MDM2. To further examine whether Otub1 directly suppresses UbcH5, we performed similar reactions as above in the absence of MDM2. As shown in Figure 7D, $Otub1^{wt}$ and $Otub1^{C91S}$, but

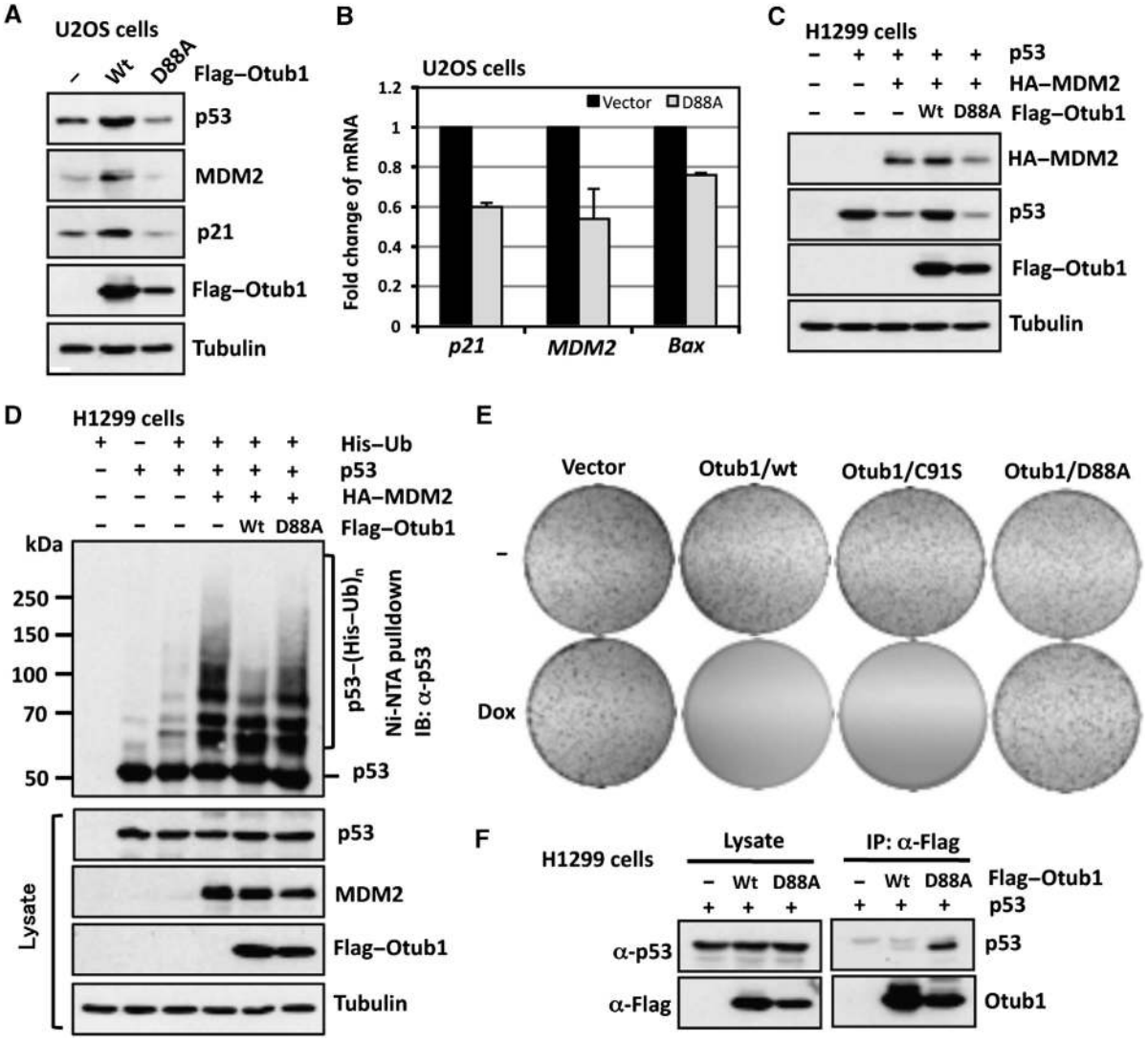


Figure 6 Mutation of D88 to A (Otub1^{D88A}) abolishes the effect of Otub1 on p53. (**A**) Otub1^{D88A} reduces p53 levels. U2OS cells transfected with wt or D88A mutant of Otub1 or control vector were assayed for the expression of indicated proteins by IB. (**B**) Otub1^{D88A} inhibits p53 activity. U2OS cells transfected with control or the D88A mutant of Otub1 were subjected to RT–qPCR analysis to detect the mRNA expression of *p21*, *mdm2*, and *bax* genes. (**C**) Otub1^{D88A} does not block MDM2-mediated p53 degradation. H1299 cells transfected with the indicated plasmids were subjected to IB analysis to detect the expression of the indicated proteins. (**D**) Otub1^{D88A} does not suppress MDM2-mediated p53 ubiquitination in cells. H1299 cells transfected with different combinations of plasmids as indicated were subjected to Ni^{2+}-NTA pulldown followed by IB using anti-p53 antibodies. The protein expression is shown in the bottom panels. (**E**) Otub1^{D88A} does not inhibit cell proliferation. Colony formation assays were performed in T-Rex-U2OS-Flag, T-Rex-U2OS-Flag-Otub1, T-Rex-U2OS-Flag-Otub1^{C91S}, and T-Rex-U2OS-Flag-Otub1^{D88A} cells in the absence or presence of 2 μg/ml Dox for up to 3 weeks. The colonies were visualized by staining with crystal violet blue. (**F**). Mutation of D88 to A increases the binding of Otub1 to p53. H1299 cells were transfected with p53 together with wt or the D88A mutant of Flag–Otub1. Co-IP assays were then performed using anti-Flag antibodies followed by IB assay.

not Otub1^{D88A}, markedly suppressed UbcH5c-mediated polyUb chain formation. This effect was dose-dependent as shown in Supplementary Figure S7A. These results indicate that Otub1 directly suppresses UbcH5, although we cannot exclude the possibility that Otub1 also suppresses MDM2 (Figure 7C). Interestingly, it seems that Otub1^{C91S} has stronger E2 suppressing activity (Figure 7D and Supplementary Figure S7A) and stronger effect in suppressing p53 ubiquitination (Figures 5C and 7A) compared with Otub1^{wt}. Consistent with the previous study (Nakada *et al*, 2010), we showed that ectopic wt Otub1 and Otub1^{C91S}, but not Otub1^{D88A}, co-immunoprecipitated with all three forms of V5-UbcH5 (5a, 5b, 5c; Figure 7E and supplementary Figure S7B–D) and endogenous UbcH5 (Figure 7F) in cells. Interestingly, Otub1^{C91S} showed stronger interaction with UbcH5 compared with Outb1^{wt}. Thus, Otub1 suppresses UbcH5 activity by physically associating with UbcH5, and the stronger effect for Otub1^{C91S} is associated with its stronger binding to UbcH5.

Expression of Otub1^{D88A} attenuates p53 induction in response to DNA damage

Next, we explored the physiological role of Otub1 in the regulation of p53 signalling. As Otub1^{D88A} may act as a dominant-negative mutant of Otub1 (Figure 6), we asked whether this mutant would affect p53 signalling in response

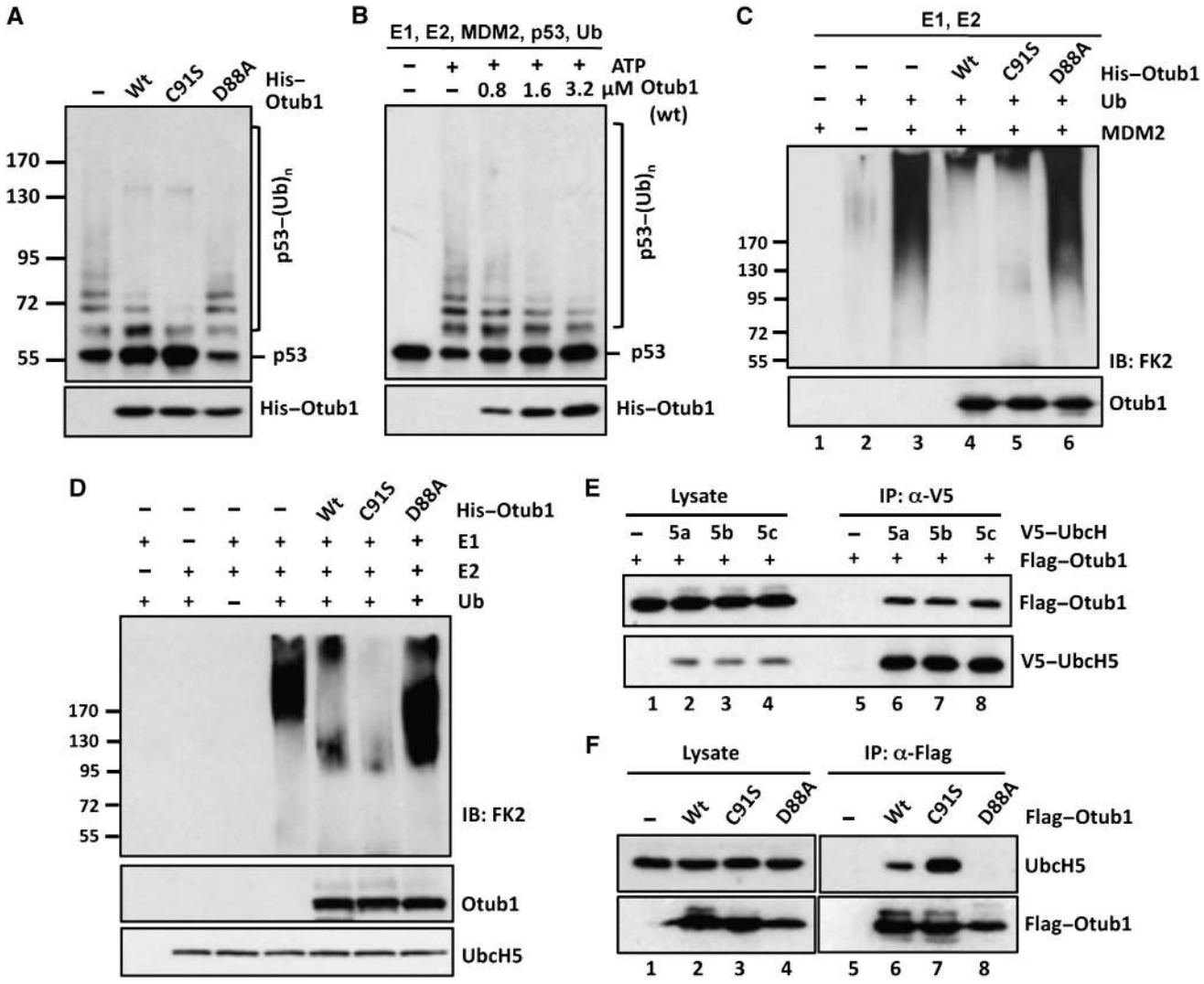


Figure 7 Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, inhibit MDM2-mediated p53 ubiquitination and suppress UbcH5 activity *in vitro*. (**A**) Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, suppress MDM2-mediated p53 ubiquitination *in vitro*. The *in vitro* ubiquitination reactions were conducted as described in Materials and Methods, and assayed by IB with anti-p53 (DO-1) and anti-Otub1 antibodies. The unmodified p53 and ubiquitinated p53 are indicated (top panel). (**B**) Dose-dependent suppression of MDM2-mediated p53 ubiquitination by Otub1 *in vitro*. The *in vitro* ubiquitination assay was conducted as above in the absence or presence of indicated amounts of wt His–Otub1, followed by anti-p53 (top panel) and anti-Otub1 (bottom panel) antibodies. (**C**) Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, suppress UbcH5-dependent ubiquitin chain formation *in vitro*. The *in vitro* ubiquitination reactions were conducted as described in Materials and Methods in the presence of E1, UbcH5c (E2), ATP, and combinations of Ub, MDM2, His-tagged Otub1^{wt}, Otub1^{C91S}, or Otub1^{D88A} as indicated. The reactions were assayed by IB with anti-conjugated Ub antibody (Clone FK2; top panel). The Otub1 protein is shown in the bottom panel. (**D**) Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, suppress UbcH5-dependent ubiquitin chain formation *in vitro* in the absence of MDM2. The *in vitro* ubiquitination assay was conducted as above in the presence of combinations of E1, E2, Ub, His-tagged Otub1^{wt}, Otub1^{C91S}, or Otub1^{D88A} as indicated. The reactions were assayed by IB with anti-conjugated Ub antibody (Clone FK2; top panel). The Otub1 and UbcH5 proteins are shown in the bottom panels. (**E**) Otub1 interacts with UbcH5 in cells. H1299 cells transfected with Flag–Otub1 alone or together with V5-tagged UbcH5s (5a, 5b, and 5c) were subjected to co-IP with anti-V5 antibodies followed by IB. (**F**) Otub1^{wt} and Otub1^{C91S}, but not Otub1^{D88A}, interact with endogenous UbcH5. H1299 cells transfected with indicated plasmid were immunoprecipitated with anti-Flag antibodies followed by IB with anti-UbcH5 antibodies.

to DNA damage. To this end, we transfected U2OS cells with control or Otub1^{D88A} plasmid and then treated them with different genotoxic agents, including neocarzinostatin (NCS, mimicking γ-irradiation and causing DNA double-strand break, Figure 8A) and etoposide (Eto, Figure 8B) for different lengths of time. IB analysis showed that expression of Otub1^{D88A} significantly abolished induction of p53 by both agents. Additionally, the induction of p21 and MDM2 by both agents at 5 h was also significantly reduced by expression of Otub1^{D88A}. MDM2 was initially reduced by both treatments (2 h), which is consistent with other reports (Meulmeester *et al*, 2005; Lee *et al*, 2006). Similarly, expression of Otub1^{D88A} significantly attenuated p53 activation following UVC irradiation (Figure 8C) and treatment with 5-fluorouracil (5-FU; Supplementary Figure S4C). The effect of Otub1^{D88A} on suppressing p53 induction in response to DNA damage was specific, as overexpression of wt Otub1 did not result in an inhibition of p53 induction upon treatment with NCS (Figure 8D). Further, the induction of p21 and mdm2 mRNA following treatment with NCS,

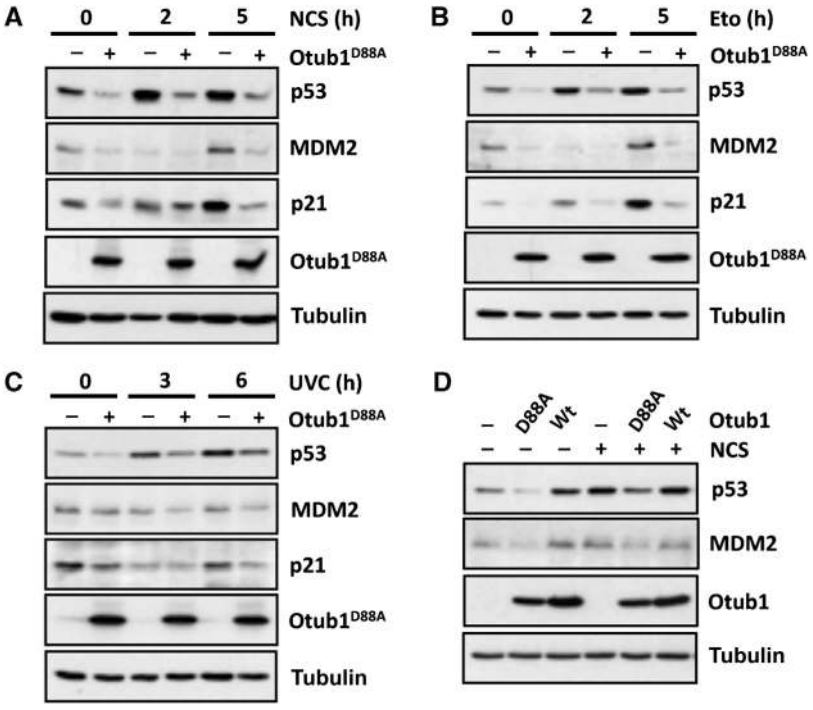


Figure 8 Overexpression of Otub1^{D88A} suppresses p53 induction in response to DNA damage. (**A–C**) U2OS cells were transfected with control vector or Flag–Otub1^{D88A} vector for 36 h followed by treatment with NCS (0.5 μg/ml, **A**), Eto (20 μM, **B**), or UVC (40 J/m^2, **C**) for indicated times. The cells were assayed for expression of indicated proteins by IB. (**D**) Otub1^{D88A}, but not wild-type Otub1, suppresses p53 induction by NCS treatment. U2OS cells transfected with control, wt Otub1, or Otub1^{D88A} were treated with NCS (0.5 μg/ml) for 5 h. The cells were assayed for expression of indicated proteins by IB.

Eto, or 5-FU was significantly inhibited by expression of Otub1^{D88A} (Supplementary Figure S4D). These results indicate that Otub1^{D88A} attenuated p53 activation in cells in response to DNA damage possibly by interfering with endogenous Otub1.

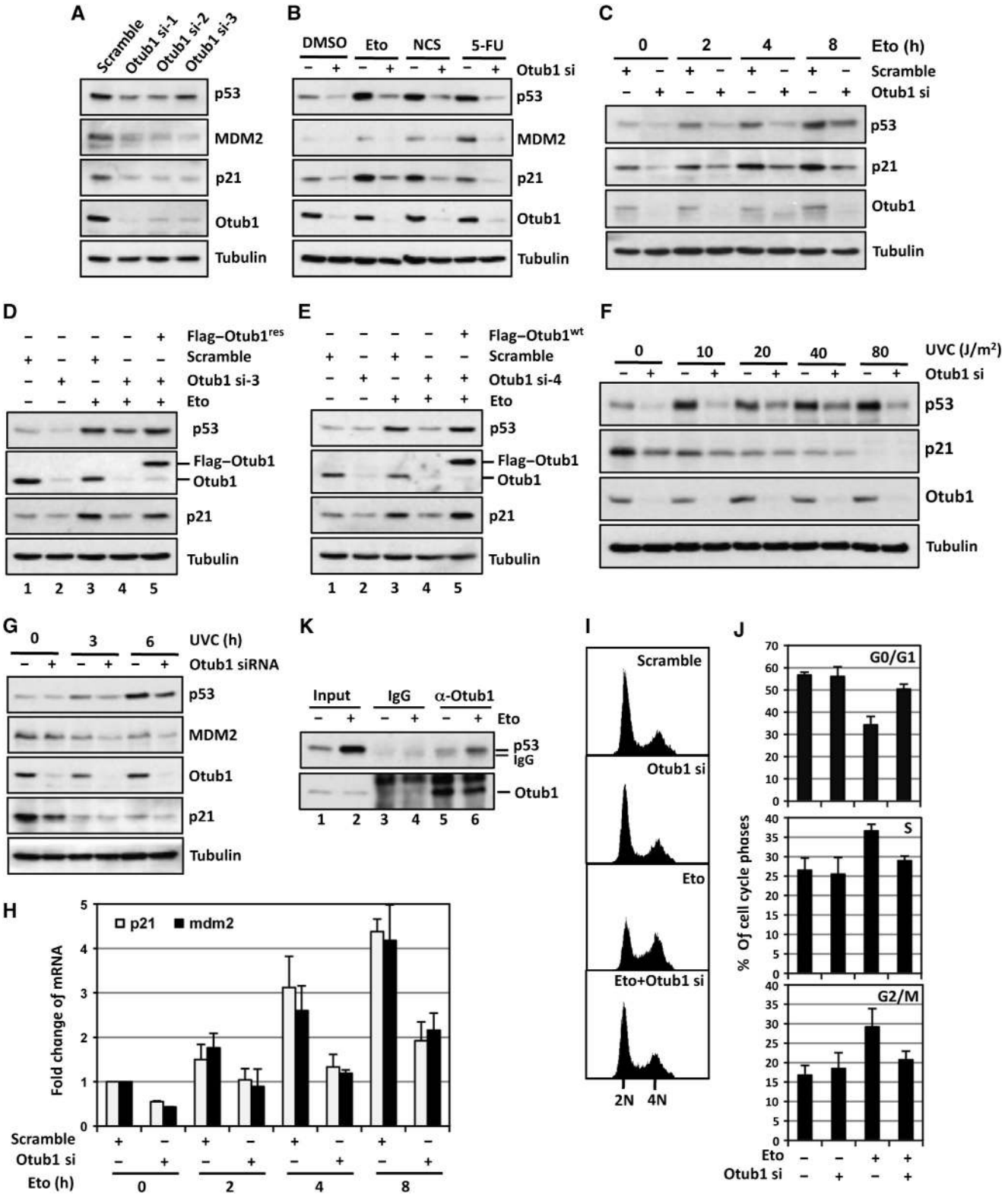
Knockdown of endogenous Otub1 also inhibits p53 activation in response to DNA damage

To further investigate the physiological significance of Otub1 in cells, siRNA-mediated knockdown of endogenous Otub1 was conducted. As shown in Figure 9A, knockdown of Otub1 slightly but consistently reduced the steady-state level of p53 in U2OS cells. This is not an off-target effect, as knockdown of Otub1 by three different siRNAs against different sequences in the *Otub1* gene, all slightly, reduced p53 levels. The levels of p21 and MDM2 protein were also reduced (Figure 9A), suggesting that p53 activity is reduced upon Otub1 knockdown. Strikingly, knockdown of endogenous Otub1 drastically suppressed the p53 induction by DNA damage, including treatment of cells with Eto, NCS, and 5-FU (Figure 9B). The levels of p21 and MDM2 were also drastically reduced by knockdown of Otub1 in response to the above treatments. The effect of Otub1 in attenuating p53 response was also confirmed by a time-course study showing that knockdown of Otub1 attenuated p53 induction throughout the treatment of cells with Eto (Figure 9C). To confirm the role of Otub1 in p53 stabilization in response to DNA damage, we performed siRNA knockdown and rescue experiments. As shown in Figure 9D, transfection of the Flag–Otub1 plasmid, which is resistant to siRNA-3 (Flag–Otub1^{res}), completely rescued the p53 response to the treatment with Eto in Otub1 siRNA-3-transfected cells (compare lane 5 with lane 4). Similarly, transfection with wt Flag–Otub1 also rescued the p53 induction following treatment with Eto in cells transfected with Otub1 siRNA-4, which targets the 3′-untranslated region (3′-UTR) of the *Otub1* mRNA that is not present in the wt Flag–Otub1 plasmid (Figure 9E, compare lane 5 with lane 4). Also, time-course (Figure 9G) and dose–response (Figure 9F) studies showed that knockdown of Otub1 attenuated p53 induction by UV irradiation. RT–qPCR assays clearly showed that knockdown of Otub1 greatly suppressed the expression of p53 target genes *p21* and *mdm2* in time-dependent manner (Figure 9H). To examine the physiological function of Otub1 in p53 response to DNA damage, we performed cell cycle analysis. Treatment of U2OS cells with Eto results in G2/M- and S-phase arrest, which was abolished by knocking down Otub1 (Figure 9I and J), suggesting that Otub1 plays a critical role in the G2/M- and S-phase checkpoints in response to DNA damage. Finally, treatment with Eto drastically increased the interaction of Otub1 with p53 (Figure 9K and Supplementary Figure S8A and C) and MDM2 (Supplementary Figure S6G) in cells. Together, these results indicate that Otub1 plays a critical role in p53 stabilization and activation in cells in response to DNA damage.

Otub1 is a cytoplasmic DUB

To test where Otub1 regulates p53, we examined the cellular localization of endogenous Otub1 using both immunofluorescence (IF) staining and cell fractionation assays. As shown in Figure 10A and B, endogenous Otub1 is predominantly localized in the cytoplasm in U2OS cells. Neither treatment

with NCS nor treatment with Eto significantly changed the cytoplasmic localization of Otub1, whereas p53 was accumulated in the nucleus in response to both treatments (Figure 10A and B). The cytoplasmic localization of Otub1 was further verified by IF (Figure 10C) and cell fractionation assays (Figure 10D) in WI38 cells as well as in RKO cells (Supplementary Figure S8B). Again, treatment of WI38 cells with Eto did not result in a significant change in the cytoplasmic localization of Otub1 (Figure 10C and D). Finally, Otub1 did not suppress MDM2-mediated ubiquitination of a nuclear export signal (NES)-mutated p53, $p53^{L348A/L350A}$, which is retained in the nucleus (Stommel *et al*, 1999)



(compare lane 6 with lane 5, Figure 10E). Thus, Otub1 regulates p53 in the cytoplasm.

Discussion

Otub1 is a member of OTU domain-containing cysteine protease and has been shown to catalyse the cleavage of K48-linked poly-Ub *in vitro* (Balakirev *et al*, 2003; Soares *et al*, 2004; Edelmann *et al*, 2009; also see Supplementary Figure S5A). Soares *et al* (2004) first reported that Otub1 regulates T-cell anergy by enhancing the degradation of a key Ub E3 ligase called GRAIL (gene related to anergy in lymphocytes) independently of its deubiquitinase activity (Soares *et al*, 2004). Recently, it has been shown that Otub1 deubiquitinates tumour necrosis factor receptor-associated factor (TRAF)-3 and TRAF6, leading to the inhibition of viral-induced INFβ production (Li *et al*, 2010). Otub1 is ubiquitously expressed in most tissues (Soares *et al*, 2004), suggesting that it might have a broader function. Indeed, it has been shown that Otub1 deubiquitinates oestrogen receptor-α and regulates its level and activity (Stanisic *et al*, 2009), deubiquitinates RhoA small GTPase to facilitate the cellular uptake of *Yersinia* bacteria (Edelmann *et al*, 2010), and suppresses DNA-damage-dependent chromatin ubiquitination (Nakada *et al*, 2010). In this study, we found that Otub1 directly suppresses MDM2-mediated p53 ubiquitination in cells and *in vitro*, leading to stabilization and activation of p53. Overexpression of Otub1 induces p53-dependent apoptosis and drastic cell growth inhibition. Thus, we have identified Otub1 as a novel p53 regulator.

Otub1 contains a catalytic triad consisting of C91, H265, and D268 (Edelmann *et al*, 2009) and possesses *in vitro* DUB enzymatic activity towards polyUb chains (Supplementary Figure S5A). However, mutation of the catalytic C91 did not abolish the ability of Otub1 to inhibit MDM2-mediated p53 ubiquitination in cells (Figure 5) and *in vitro* (Figure 7). Point mutations at H265, D268, or all three of the catalytic residues were unable to abolish Otub1 function (data not shown). Our data further revealed that Otub1 and its C91S mutant bind to the UbcH5 and significantly inhibit its Ub-conjugating activity, regardless of the presence or absence of MDM2 (Figure 7C and D). Thus, it appears that Otub1 suppresses p53 ubiquitination in cells primarily via non-canonical inhibition of UbcH5, although we cannot exclude the possibility that Otub1 also directly inhibits MDM2 E3 activity. This observation is consistent with a non-canonical mechanism by which Otub1 suppresses DNA-damage-induced chromatin ubiquitination (Nakada *et al*, 2010). On the other hand, Otub1 possesses weak *in vitro* DUB enzymatic activity towards polyubiquitinated p53 (Supplementary Figure S5B). This weak activity might be due to the Otub1 conformation that is incompatible with typical functional catalytic cores—the imidazole ring of H265 is positioned far from catalytic C91 and sandwiched between a Pro and a Glu (Edelmann *et al*, 2009). Whether and to what extend this weak DUB activity contributes to the function of Otub1 in regulating p53 in cells is not clear. However, the direct association of Otub1 with p53 (Figure 3C) and MDM2 (Supplementary Figure S6) suggest that Otub1 may bind to the p53–MDM2–UbcH5 complex, wherein it suppresses the activity of E2/E3 while it may also cleave ployUb chains attached to p53 (Figure 10F). Interestingly, compared with wt Otub1, the Otub1^{C91S} mutant possesses stronger suppressing effects on MDM2-mediated p53 ubiquitination in cells (Figure 5C) and *in vitro* (Figure 7A) and on the Ub-conjugating activity of UbcH5 in the absence of MDM2 (Figure 7D). Also, Otub1^{C91S} binds to UbcH5 stronger (Figure 7F and Supplementary Figure S7C and D) than wt Otub1. Thus, it is likely that mutating this residue converts Otub1 to a conformation that favours its tight association with UbcH5 and stronger suppression of its activity.

On the other hand, mutation of the D88 residue abolished the activity of Otub1 to suppress p53 ubiquitination in cells (Figure 6) and *in vitro* (Figure 7). Consistently, Otub1^{D88A} failed to bind to and suppress UbcH5 (Figure 7). D88 was initially predicted to be a catalytic residue that is conserved across species and among other OTU family DUBs (Balakirev *et al*, 2003), and the D88A mutant failed to cleave polyUb chain *in vitro* (Supplementary Figure S5A). Thus D88 is essential for Otub1's DUB activity, albeit it is not among the catalytic triad (Edelmann *et al*, 2009). D88 is located in a loop closely preceding the C91-containing active-site helix within the OTU domain (Nanao *et al*, 2004; Edelmann *et al*, 2009). C91 may contact D88 to form a novel oxyanion hole (Nanao *et al*, 2004; Edelmann *et al*, 2009) essential for stabilization of the oxyanion reaction intermediate in the enzymatic reaction

Figure 9 Otub1 is required for p53 induction in response to DNA damage. (**A**) Knockdown of Otub1 by siRNA reduced the level of endogenous p53. U2OS cells were transfected with scrambled or one of the three siRNAs against the *Otub1* gene. The cells were then assayed for the expression of indicated proteins using IB. (**B**, **C**). Knockdown of Otub1 by siRNA attenuated p53 induction in response to DNA damaging agents. U2OS cells were transfected with scrambled or Otub1 siRNA mixture (equal molecular ratio of the three Otub1 siRNAs shown in **A**) for 48 h and then treated with Eto (20 μM), NCS (0.5 μg/ml) and 5-FU (50 μg/ml) for 5 h (**B**) or with Eto (20 μM) and harvested at different time points as indicated (**C**). The cells were then assayed for the expression of the indicated proteins using IB. (**D**) Introduction of siRNA-resistant Otub1 (Otub1^{res}) rescues the p53 induction following DNA damage in Otub1 knockdown cells. U2OS cells transfected with scrambled or Otub1 siRNA-3 with control or Flag-Otub1^{res} plasmid as indicated for 48 h, followed by treatment with Eto (20 μM) for 5 h. The cells were assayed for the expression of the indicated proteins using IB. (**E**) Re-introduction of wild-type Otub1 rescues the p53 response following DNA damage in Otub1 knockdown cells. U2OS cells were transfected with scrambled or Otub1 siRNA-4 (targeting the 3′-UTR of the *Otub1* mRNA) with control or wild-type Flag–Otub1 plasmid as indicated for 48 h, followed by treatment with Eto (20 μM) for 5 h. The cells were assayed for the expression of the indicated proteins using IB. (**F**, **G**). Knockdown of Otub1 by siRNA attenuated p53 induction in response to UVC treatment. U2OS cells were transfected with scrambled or Otub1 siRNA mixture as in **B** for 48 h and then treated with different doses of UVC for 6 h (**F**) or 40 J/m^2 for different time points (**G**). The cells were then assayed for the expression of the indicated proteins using IB. (**H**) Knockdown of Otub1 attenuates p53 transactivation activity upon DNA damage. U2OS cells were transfected with scrambled or Otub1 siRNA and then treated with Eto (20 μM). The cells were harvested at different time points and assayed for the mRNA expression of *p21* and *mdm2* genes by RT–qPCR. (**I**, **J**) Knockdown of Otub1 attenuates DNA damage induced by G2/M- and S-phase checkpoints. U2OS cells transfected with scrambled or Otub1 siRNA were treated with Eto (20 μM). The cells were harvested at 24 h after treatment and assayed for cell cycle profile. Representative histograms for cell cycle profile are shown in **I**, and the percentages of cells in different cell cycle phase are summarized from three independent experiments in (**J**). (**K**) DNA damage increases the p53–Otub1 interaction in cells. U2OS cells treated with dimethylsulphoxide (DMSO) or Eto (20 μM) for 5 h were subjected to co-IP with polyclonal anti-Otub1 antibodies or pre-immune IgG followed by IB.

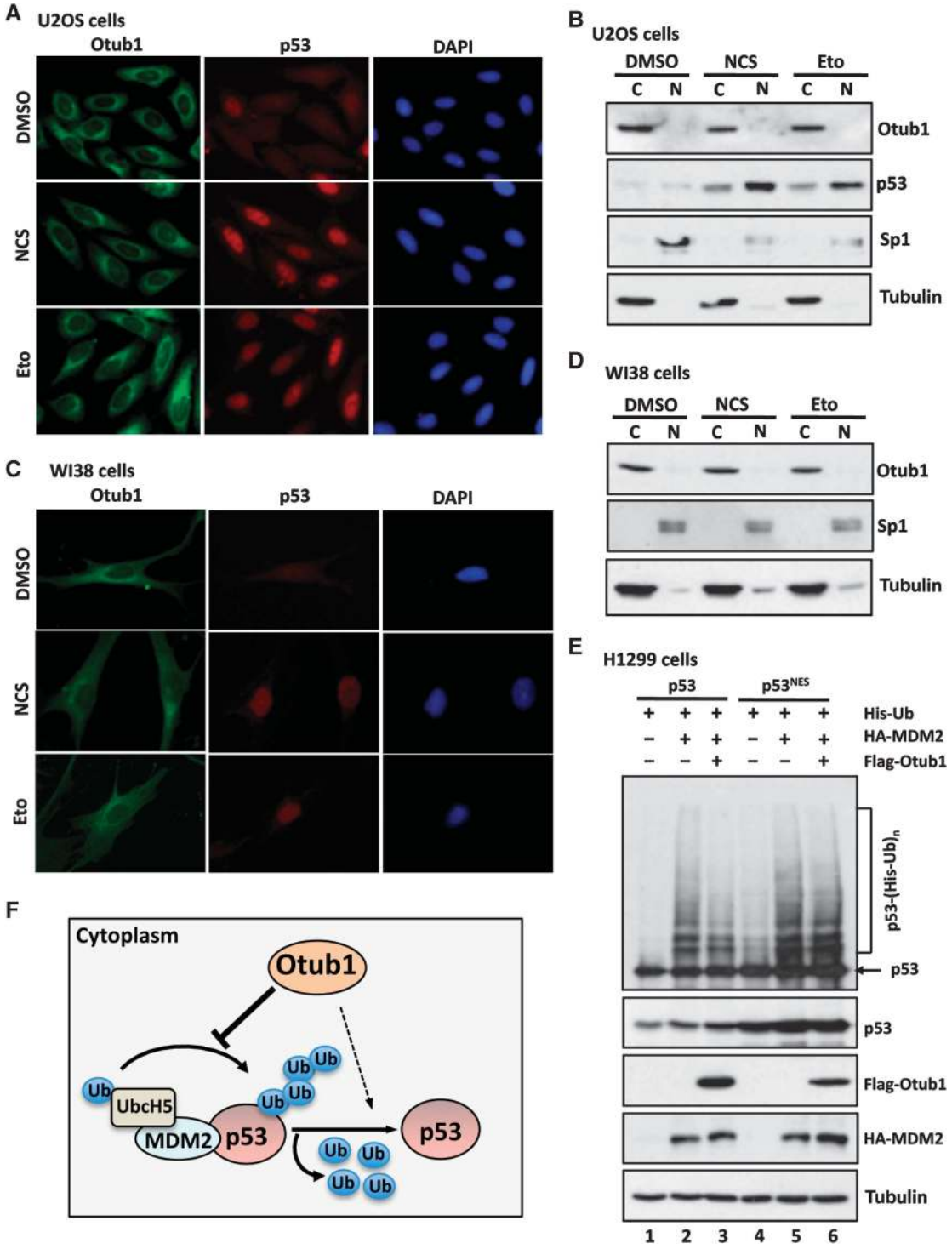


Figure 10 Otub1 is a cytoplasmic DUB. (**A**–**D**) DNA damage does not change the cytoplasmic localization of Otub1. U2OS (**A**, **B**) and WI38 (**C**, **D**) cells treated with NCS (0.5 μg/ml), Eto (20 μM), or dimethylsulphoxide (DMSO) for 5 h were immunostained with monoclonal anti-Otub1 (green) and polyclonal anti-p53 (red) antibodies together with DPAI (blue) (**A**, **C**). The cells were also fractionated to the cytoplasmic (C) and nuclear (N) fractions followed by IB detection of the indicated proteins. Sp1 is a nuclear protein, whereas tubulin is used as cytoplasmic marker (**B**, **D**). (**E**) Otub1 does not suppress MDM2-mediated ubiquitination of nuclear retained p53. H1299 cells were transfected with wt p53 (lanes 1–3) or nuclear export signal (NES)-mutated p53 ($p53^{L348A/L350A}$; lanes 4–6) together with other plasmids as indicated. *In vivo* ubiquitination assays were conducted using Ni^{2+}-NTA pulldown under denaturing conditions followed by IB. The ubiquitinated species of p53 are indicated. (**F**) A schematic diagram for Otub1 regulation of p53 ubiquitination in the cytoplasm. Otub1 suppresses p53 ubiquitination in the cytoplasm primarily by non-canonical inhibition of UbcH5/MDM2 activity, while it may also possess weak canonical DUB catalytic activity to deubiquitinate p53. Arrow indicates activation, whereas bar indicates inhibition.

(Nijman *et al*, 2005). Thus, mutating D88 may disrupt the structure of the OTU domain. Supporting this notion, neither the Otub1 N-terminal fragment lacking the OTU domain nor the C-terminal fragment, which disrupts the OTU domain, can induce p53 when overexpressed even though both bind to p53 (Supplementary Figure S3B). Also, the Otub1 mutant with D88A, C91S, and H265A mutations failed to suppress DNA-damage-induced chromatin ubiquitination (Nakada *et al*, 2010). These results indicate that Otub1 regulation of p53 ubiquitination and its binding to UbcH5 might require the integrity of the OTU domain.

Functionally, overexpression of Otub1^{D88A} significantly reduced the levels and activity of p53 and suppressed p53 activation in response to DNA damage (Figure 8), suggesting that this mutant may act as a dominant-negative mutant of Otub1, considering that its strong binding to p53 (Figure 6F) may interfere with the binding of endogenous Otub1 to p53. Knockdown of endogenous Otub1 drastically attenuated the p53 stabilization and activation in response to DNA damage (Figure 9). DNA damaging treatments markedly increased the interaction between p53 and Otub1 (Figure 9K and Supplementary Figure S8A). Importantly, Otub1 is essential for the G2/M- and S-phase checkpoints in response to DNA damage (Figure 9I and J). These results indicate that Otub1 plays a critical role in p53 signalling in response to DNA damage.

Interestingly, Otub1 has been suggested to act as an inhibitor of DNA repair by suppressing Ubc13/RNF168 activity. Under physiological condition, Otub1 may set a threshold for RNF168 activity to dynamically control chromatin ubiquitination (Nakada *et al*, 2010). It is likely that upon DNA damage, Otub1 transiently dissociates from Ubc13–RNF168 complex allowing RNF168 to catalyse ubiquitination of chromatin at DNA damage sites (Nakada *et al*, 2010), whereas it associates with UbcH5–MDM2 complex to suppress MDM2-mediated p53 ubiquitination, leading to p53 stabilization and activation. This dual action would likely work in concert to promote DNA damage response by initiating cell cycle arrest and subsequent DNA repair. This dual action might also play a role in lethal DNA damage response. In this condition, Otub1 may cause sustained p53 activation and apoptosis by suppressing UbcH5–MDM2 and permanently dissociating from the Ubc13/RNF168. Nevertheless, as knockdown of Otub1 reduces the levels of p53 and impairs p53 induction in response to DNA damage, our results indicate that Otub1 plays a crucial role in maintaining homeostatic levels of p53 under physiological conditions, via stoichiometric interplay with the MDM2–UbcH5 complex. It may also play a role in determining the functional outcome of the p53 activation.

Our results indicate that Otub1 regulates p53 in the cytoplasm and remains in the cytoplasm upon DNA damage (Figure 10). In contrast, USP7 is a nuclear p53 deubiquitinase and USP10 is primarily a cytoplasmic p53 deubiquitinase but a fraction of it translocates into the nucleus following DNA damage (Yuan *et al*, 2010). Therefore, these three DUBs regulate p53 function in different compartments, ensuring fine control of p53 stability and activity in response to stress. Future studies would examine how Otub1 regulates p53 signalling and whether it is subjected to posttranslational modifications in response to DNA damage. Altogether, our study places Otub1 as one of the central molecules that modulate the p53 stability and activity and suggests that Otub1 might have tumour suppressor function. It will be interesting to test whether expression of Otub1 is altered in human cancers and whether Otub1 suppresses tumourigenesis *in vivo*.

Materials and methods

Cell culture, plasmids, antibodies, and reagents

Human p53-null lung non-small-cell carcinoma H1299 cells, human p53-proficient osteosarcoma U2OS cells, human p53-null osteosarcoma Saos2 cells, and human fibroblast WI38 cells were cultured as described (Sun *et al*, 2010; Challagundla *et al*, 2011). Full-length human Otub1 and Otub2 cDNAs were amplified from HeLa cell mRNA by RT–PCR and cloned into pcDNA3–Flag vector to generate Flag–Otub1 and Flag–Otub2 vectors, respectively. The primers were 5′-CGCGGATCCGCGGCGGAGGAACCTCAGCAG-3′ and 5′-CGCTCTAGACTATTTGTAGAGGATATCGTAGTGTCC-3′ for Otub1 and 5′-CGCGGATCCAGTGAAACATCTTTCAACCTAATATCAG-3′ and 5′-CCGGAATTCTCAATGTTTATCGGCTGCATAAAGG-3′ for Otub2. Human Otub1 was also cloned into pcDNA3-V5 vector to generate the pcDNA3-V5–Otub1 vector. The Flag–p53 and Flag–HAUSP plasmids were obtained from Addgene. All Flag-tagged Otub1 deletion mutants and Flag-tagged p53 deletion mutants were generated by PCR and cloned into pcDNA3–Flag vector. All Otub1 plasmids with point mutations and the mutant p53 with residues Leu 348 and Leu 350 converted to Ala (p53$^{L348A/L350A}$) were constructed using site-directed mutagenesis (Stratagene). GFP–Otub1 was constructed by inserting an Otub1 cDNA into the pEGFP-C1 vector (Clontech). The plasmids encoding p53, HA–MDM2, His$_6$-tagged Ub (His–Ub), and V5–Ub have been described (Dai and Lu, 2004; Sun *et al*, 2011). For generation of tet-inducible expression system, the Flag–Otub1, its point mutants, and Flag–Otub2 cDNAs were subcloned into pcDNA4-TO (Invitrogen) vector to generate pcDNA4-TO-Flag-Otub1, its mutants, and pcDNA4-TO-Flag-Otub2 plasmids. N-terminal His-tagged Otub1 (His–Otub1) bacterial expression vector and its mutants (C91S and D88A) were constructed by PCR into pPROEX-HT vector (Invitrogen).

Anti-Flag (M2, Sigma), anti-p21 (Ab-11, NeoMarkers), monoclonal anti-p53 (DO-1, Santa Cruz), polyclonal anti-p53 (FL393, Santa Cruz), anti-MDM2 (SMP14, Santa Cruz), anti-Ub (Santa Cruz), anti-V5 (Invitrogen), anti-cleaved PARP (Asp214; Cell Signaling), monoclonal anti-Otub1 (Abcam) antibodies were purchased. To generate rabbit polyclonal anti-Otub1 antibodies, recombinant His–Otub1 protein was expressed in *E. coli* containing pet24a–His–Otub1 vector and purified using Ni^{2+}-NTA purification method. The purified protein was used as an antigen to raise the polyclonal anti-Otub1 antibodies. Other reagents, including MG-132 (Peptide Inc.), cycloheximide (Calbiochem), etoposide (Sigma), NCS (Sigma), and 5-fluorouracil (Sigma), were purchased.

Establishment of Otub1 and Otub2 expression cell lines

To generate tet-inducible expression of Otub1 or Otub2 in U2OS cells, T-Rex-U2OS cells (Invitrogen) were transfected with pcDNA4-TO, pcDNA4-TO-Flag-Otub1 or its mutants (C91S and D88A), or pcDNA4-TO-Flag-Otub2. The cells were then split into selection medium containing 50 μg/ml of hygromycin and 100 μg/ml of Zeocin, and selection was continued for 2 weeks. Single colonies were isolated, expanded, and screened by IB analysis for Dox-induced expression using anti-Flag antibodies. Similar procedures were used to establish tet-inducible expression of Otub1 in Saos2, H1299 cells, HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cells that have been stably transfected with pcDNA6-TR plasmid (Invitrogen). The cells were selected in selection medium containing 10 μg/ml of Blasticidin (Invitrogen) and 100 μg/ml of Zeocin. All the cells were cultured in DMEM medium supplemented with 10% tetracycline system-approved FBS (Clontech) in the presence of proper selective antibiotics. Doxycycline (Dox, 2 μg/ml; Sigma) was used to induce tet-controlled gene expression.

Lentiviruses

To generate lentiviral expression of Otub1, Flag-Otub1 cDNA with in-frame stop codon was amplified by PCR and inserted into the pENTR™/D-TOPO® vector using pENTR Directional TOPO Cloning Kits (Invitrogen). The cDNA was then cloned into pLenti4/V5-DEST

using Gateway LR recombination reaction following the manufacturer's protocol (Invitrogen). The resulting vector pLenti4/Flag-Otub1 was then transfected with VSVG, pLP1, pLP2 plasmids into 293FT cells using Calcium Chloride (Promega). The viruses were then used to infect cells in the presence of polybrene (6 μg/ml). The cells were harvested at 48-h posttransduction for IB analysis.

Transfection, IB and co-IP analyses
Cell transfection, cell lysate preparation, IB, and co-IP assays were performed as previously described (Dai *et al*, 2008). See Supplementary data for details.

RT–qPCR analysis
Total RNA preparation, reverse transcriptions, and qPCR were performed as described by Sun *et al* (2010). See Supplementary data for details.

GST-fusion protein association assays
See Supplementary data for details.

Flow cytometry
Cells were fixed in ethanol and stained in 500 μl of PI (Sigma) stain buffer (50 μg/ml PI, 200 μg/ml RNase A, and 0.1% Triton X-100) at 37 °C for 30 min. The cells were analysed for DNA content using a Becton Dickinson FACScan flow cytometer. For cells expressing GFP or GFP–Otub1, GFP-positive cells were gated and analysed for DNA content. Cells were also stained with FITC-conjugated anti-Annexin V antibody following the manufacturer's protocol (PharMingen). Data were collected using CellQuest and the ModFit programs.

Cell fractionation. Cell fractionation assays were performed as described by Challagundla *et al* (2011). See Supplementary data for details.

Immunofluorescence staining
U2OS cells treated with or without DNA damaging agents were fixed and stained with monoclonal anti-Otub1 and polyclonal anti-p53 antibodies followed by staining with Alexa Fluor 488 (green) goat anti-mouse antibody and Alexa Fluor 546 (red) goat anti-rabbit antibody (Molecular Probes, OR) as well as DAPI for DNA staining. Stained cells were analysed under a Leica inverted fluorescence microscope.

In vivo ubiquitination assay
In vivo ubiquitination assay was conducted as previously described (Dai *et al*, 2004; Sun *et al*, 2011). See Supplementary data for details.

In vitro deubiquitination assay
The His–Otub1 and its mutant proteins were purified from bacteria using Ni^{2+}-NTA purification method. For *in vitro* deubiquitination of K48-linked polyUb chain, the K48-Ub_{3-7} (200 ng, Boston Biochem) was incubated with 1 μM (final concentration) of purified His–Otub1 (wt, C91S, or D88A) in 20-μl reaction buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM DTT at 37 °C for 4 h. The reactions were stopped by adding SDS sample buffer and assayed by IB. For *in vitro* deubiquitination of p53, ubiquitinated p53 was generated from H1299 cells transfected with Flag–p53, HA–MDM2, and His–Ub using anti-Flag affinity purification (Li *et al*, 2002). The ubiquitinated p53 was then incubated with 3.2 μM (final concentration) of purified His–Otub1 (wt, C91S, or D88A) in deubiquitination buffer consisting of 50 mM Tris–HCl (pH 7.5), 10 mM DTT at 37 °C for 8 h. The reactions were resolved in SDS–PAGE gel followed by IB.

In vitro ubiquitination assay
The *in vitro* p53 ubiquitination assays were conducted in a total of 20-μl reaction buffer containing recombinant p53 (20 ng), MDM2 (100 ng), UbE1 (0.025 μM, Boston Biochem), UbcH5 (0.4 μM, Biomol), Ub (40 μM, Boston Biochem), 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT in the absence or presence of 1.6 μM or indicated concentrations of His–Otub1 (wt, C91S, and D88A) at 37 °C for 2 h. The reactions were stopped by adding SDS sample buffer followed by IB with anti-p53 antibodies. For detection of Ub-conjugating activity of UbcH5, reactions were assembled in a total of 20-μl reaction buffer containing recombinant UbE1 (0.025 μM), UbcH5 (0.4 μM), MDM2 (100 ng), Ub (40 μM), 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM ATP, and 1 mM DTT, in the absence or presence of 3.2 μM or indicated concentrations of His–Otub1 (wt, C91S, and D88A) at 37 °C for 16 h. The reaction was stopped and assayed by gradient SDS–PAGE gel in non-denaturing conditions followed by IB with anti-conjugated Ub (clone FK2, Millipore; Nakada *et al*, 2010).

RNA interference
The 21-nucleotide siRNA duplexes with a 3′ dTdT overhang were synthesized by Dharmacon Inc. (Lafayette, CO). The target sequences for Otub1 are 5′-GACCAGGCCTGACGGCAAC-3′ (siRNA-1), 5′-GCAGACCTCTGTCGCCGAC-3′ (siRNA-2), 5′-CCGACTACCTTGTGG TCTA-3′ (siRNA-3), and 5′-GTGGTTGTAAATGGTCCTA-3′ (siRNA-4). siRNA-1, -2, and -3 target the coding region, whereas siRNA-4 targets the 3′-UTR of the *Otub1* mRNA. The control scramble II RNA sequence is 5′-GCGCGCTTTGTAGGATTCG-3′. For rescue experiments using siRNA-3, the targeting sequence in pcDNA3-Flag–Otub1 vector was mutated to CGGATTATCTCGTCGTGTA by mutagenesis (mutated nucleotides are underlined) to generate Otub1 expression vector resistant to siRNA-3 (Flag–$Otub1^{res}$). These siRNA duplexes (100 nM) were introduced into cells using SilentFect Lipid Reagent (Bio-Rad) following the manufacturer's protocol. The cells were analysed 48 h after transfection.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: M-SD and X-XS conceived and designed experiments; X-XS, KBC, and M-SD performed the experiments and analysed the results. M-SD and X-XS wrote and edited the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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