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USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1

Xinna Zhang[1], Franklin G Berger[2], Jianhua Yang[3] and Xiongbin Lu[1,*]

[1]Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA, [2]Department of Biological Sciences, Center for Colon Cancer Research, University of South Carolina, Columbia, SC, USA and [3]Department of Pediatrics, Texas Children's Cancer Center, Baylor College of Medicine, Houston, TX, USA

Tumour suppressor p53 levels in the cell are tightly regulated by controlled degradation through ubiquitin ligases including Mdm2, COP1, Pirh2, and ARF-BP1. The ubiquitination process is reversible via deubiquitinating enzymes, such as ubiquitin-specific peptidases (USPs). In this study, we identified ubiquitin-specific peptidase 4 (USP4) as an important regulator of p53. USP4 interacts directly with and deubiquitinates ARF-BP1, leading to the stabilization of ARF-BP1 and subsequent reduction of p53 levels. *Usp4* knockout mice are viable and developmentally normal, but showed enhanced apoptosis in thymus and spleen in response to ionizing radiation. Compared with wild-type mouse embryonic fibroblasts (MEFs), *Usp4*−/− MEFs exhibited retarded growth, premature cellular senescence, resistance to oncogenic transformation, and hyperactive DNA damage checkpoints, consistent with upregulated levels and activity of p53 in the absence of USP4. Finally, we showed that USP4 is overexpressed in several types of human cancer, suggesting that USP4 is a potential oncogene.

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Introduction

p53 (encoded by the *TP53* gene) is a transcription factor and serves as a pivotal tumour suppressor in animals (Harris and Levine, 2005). Mutations in the p53 tumour suppressor protein are the most commonly observed genetic alterations in human cancer. About half of all human cancers contain inactivating p53 mutations, and many other cancers exhibit genetic alterations in p53 regulatory genes (e.g. MDM2, ARF) that result in functional suppression of p53 activity (Martin *et al*, 2002; Toledo and Wahl, 2006). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours (Donehower *et al*, 1992; Jacks *et al*, 1994). P53 becomes activated in response to a variety of stressors and directs a transcriptional programme that prevents the proliferation of genetically unstable cells, or initiates apoptosis if severe damage occurs in the cell (Toledo and Wahl, 2006).

Given the important role of p53 in cell activities, an exquisite control mechanism has evolved to prevent its errant activation and enable rapid stress responses when necessary. Central to this regulation are the p53 inhibitors: Mdm2, Mdm4, COP1, ARF-BP1, and Pirh2 in the E3 ubiquitin ligase family (Kruse and Gu, 2009). Mdm2 was the first identified E3 ubiquitin ligase that polyubiquitylates p53 and itself for subsequent proteasomal degradation (Haupt *et al*, 1997). Interestingly, one transcriptional target of p53 is the *Mdm2* gene. Induced Mdm2 in turn destabilizes p53 as part of an oscillating negative feedback regulatory loop (Barak *et al*, 1993). ARF-BP1 (ARF-binding protein 1, also known as HUWE1) was recently identified as another critical E3 ubiquitin ligase in regulating p53 levels (Chen *et al*, 2005). ARF-BP1 is a HECT domain-containing E3 ubiquitin ligase, which interacts directly with the p53 protein and induces p53 ubiquitination. Binding to ARF-BP1, ARF strongly represses ARF-BP1-mediated p53 ubiquitination. Inactivation of ARF-BP1 stabilizes p53 and induces apoptosis. ARF-BP1 also ubiquitinates Myc through a lysine 63-linked polyubiquitin chain (Adhikary *et al*, 2005). This ubiquitination does not cause Myc degradation but significantly alters transcription properties of Myc. TopBP1 was recently identified as a target of ARF-BP1. Herold *et al* (2008) reported that TopBP1 is degraded by ARF-BP1 if it is not bound to chromatin. Expression of Myc leads to dissociation of TopBP1 from chromatin, reduces the amount of total TopBP1 and attenuates DNA damage response.

Ubiquitination is a key regulatory event in the p53 pathway, which has been the focus of many studies. Deubiquitinating enzymes (DUBs), which mediate the removal and processing of ubiquitin, comprise another facet of the story. They may be functionally as important as E3 ubiquitin ligases, but are less well understood. DUBs are divided into four subclasses based on their Ub-protease domains: ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase, Otubain protease, and Machado-Joseph disease protease (Nijman *et al*, 2005). USP7 (also named HAUSP) was the first identified USP that binds to and stabilizes p53. In the presence of USP7 overexpression, p53 levels were sufficiently stabilized to induce cell growth arrest and apoptosis (Li *et al*, 2002). However, it was later found that USP7 also interacted with Mdm2 and exhibited strong deubiquitinase activity and stabilization of the protein (Cummins and Vogelstein, 2004; Li *et al*, 2004). These data suggest that USP7-mediated deubiquitination of Mdm2 is required to maintain a sufficient level of the Mdm2 protein to act as an E3 ligase for p53 (Hu *et al*, 2006). Another deubiquitinating enzyme USP2a was identified as an Mdm2-interacting protein, which can only deubiquitinate Mdm2 while demonstrating no deubiquitinase activity towards p53 (Stevenson *et al*, 2007). A latest report

*Corresponding author. Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.
Tel.: +1 713 745 6247; Fax: +1 713 792 8747;
E-mail: xlu2@mdanderson.org

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by the Lou group showed that USP10 deubiquitinates p53 in the cytoplasm and this deubiquitination reverses Mdm2-induced p53 nuclear export and degradation (Yuan *et al*, 2010).

In the present study, a cDNA expression library consisting of 41 USPs was employed to screen novel regulators in the p53 signalling pathway. Several ubiquitin-specific peptidases (USPs) were identified that modulated p53 activation after DNA damage, one of which was USP4. We report that USP4 binds directly with and stabilizes ARF-BP1 via deubiquitination, promoting ARF-BP1-dependent ubiquitination and degradation of p53. Further, knockout of USP4 in *Usp4−/−* mouse embryonic fibroblasts (MEFs) leads to the activation of p53, upregulation of apoptosis, premature cell senescence, and reduced oncogene-associated transformation. Finally, consistent with its potential oncogenic functions, USP4 was shown to be significantly overexpressed in several types of human cancer.

Results

USP4 downregulates p53 levels and activity

Given the complexity of the regulatory network for p53, we postulated that more USPs may be involved in the regulation of p53. To this end, we generated a library of expression vectors encoding 41 human USPs. Using this library, we screened for USPs that increased or suppressed the activity of p53 in human osteosarcoma U2OS cells that express wild-type p53. The cells were transfected with vectors expressing each individual USP, p21 promoter-driven reporter luciferase and control Renilla luciferase. Cells were then treated with a radiomimetic DNA damaging agent, neocarzinostatin (NCS) to stimulate the p53-associated signalling pathway (Zhang *et al*, 2009). Controlled by the p53-targeting p21 promoter, expression levels of the reporter luciferase were measured to assess the p53 activity. Compared with the control, 4 out of 41 USPs were shown to increase p53 activity over 50%, including USP15, USP19, USP30, and USP36 (Figure 1A). USP10 was shown to increase p53 activity by 45%, consistent with the recent report showing that USP10 stabilizes p53 through direct deubiquitination (Yuan *et al*, 2010). In contrast, as many as 15 USPs had profound negative effects on p53 activity (more than two-fold), indicating that USPs probably modulate p53 activity more frequently through promoting p53 inhibitors, such as E3 ubiquitin ligases (Figure 1A). One of the negative p53 regulators is USP4, which reduced the DNA damage-induced p53 activity by three-fold. To determine if USP4 inhibits p53 activity through transcriptional regulation, we quantified p53 mRNA levels in cells with overexpressed or silenced USP4. Altered levels of USP4 had no notable effects on p53 mRNA levels, excluding the possibility that the transcription of p53 was influenced (Figure 1B, upper panel). However, overexpression of USP4 dramatically reduced the transcriptional activity of p53, shown by the reduced reporter activities controlled by the promoters of two p53 transcriptional targets, p21 and Mdm2 (Figure 1B, bottom panel). USP4-CA mutant, in which the critical cysteine 311 residue in the enzymatic domain of USP4 was mutated to inactivate USP4 (Supplementary Figure S1), failed to inhibit p53 activity, suggesting that the effects of USP4 on p53 are dependent on its deubiquitinase activity. In contrast, silencing USP4 by two individual short hairpin RNAs (shRNAs) remarkably increased the p53 activity (Figure 1B; Supplementary Figure S2A).

To further examine how p53 is regulated by USP4, we assessed the steady-state levels of p53 in U2OS cells after NCS treatment (Figure 1C). Levels of p53 increased within 2 h and further increased to the peak 8 h after treatment. Overexpression of USP4 in U2OS cells significantly reduced p53 levels compared with the control cells transfected with empty expression vector (Figure 1C). In contrast, inhibiting USP4 by its specific small interfering RNAs (siRNAs) resulted in higher levels of p53. The results suggested that USP4 inhibited the stabilization of p53 (Figure 1C; Supplementary Figure S3). One possibility for the observed USP4 effects is that USP4 deubiquitinates and stabilizes p53-targeting E3 ubiquitin ligases including Mdm2, COP1, Pirh2, or ARF-BP1. We employed a pair of isogenic HCT116 cell lines that are *p53+/+* or *p53−/−*. In the HCT116p53+/+ cells, we showed that wild-type, but not the ubiquitinase-dead mutant form of USP4 (USP4-C311A) negatively regulated p53 levels, similar to the results obtained from U2OS cells (Figure 1D; Supplementary Figure S2B). Considering the complexity of the feedback loops between p53 and p53-targeting E3 ligases, we examined the effects of USP4 on E3 ligases in the p53-null cells to eliminate the transcriptional regulation from p53. Altered USP4 levels appeared to have no effects on Mdm2, COP1, and Pirh2, while ARF-BP1 was positively regulated by wild-type but not the ubiquitinase-dead mutant USP4, indicating that ARF-BP1 is probably a deubiquitination substrate of USP4 (Figure 1D). Another known target of ARF-BP1, TopBP1 was also promoted by USP4 (Figure 1D) (Herold *et al*, 2008).

USP4 interacts with ARF-BP1

We next examined whether USP4 directly binds to ARF-BP1. Western blot analyses of U2OS cell lysates immunoprecipitated with USP4 antibodies resulted in positive ARF-BP1 bands (Figure 2A, left panel). Reciprocal immunoprecipitation-western blot analyses in which U2OS cell lysates were immunoprecipitated with ARF-BP1 antibodies and probed with USP4 antibodies confirmed the USP4–ARF-BP1 interaction (Figure 2A, right panel), indicating endogenous USP4 binds to endogenous ARF-BP1. Subsequent experiments to determine whether the enzymatic activity of USP4 is essential for the interaction showed that both wild-type and ubiquitinase-dead mutant USP4 bound to ARF-BP1 at similar levels, suggesting the enzymatic activity of USP4 is not a prerequisite for its ARF-BP1-binding activity (Figure 2B).

We also generated three deletion constructs of USP4 and performed similar binding experiments to identify the binding requirements between USP4 and ARF-BP1 (Figure 2C). Two domains are found on USP4: catalytic domain (residue 302 to C-terminus, or 302-CT) and a N-terminal DUSP (domain present in ubiquitin-specific proteases, residues 1–187) that usually surrounds catalytic domain without any identified functions. The results from the binding experiments showed that neither N-terminus nor C-terminus of USP4 is solely sufficient for the binding of USP4 with ARF-BP1. USP4 (188-CT) retains the binding capacity of USP4, suggesting that the catalytic domain and a part of N-terminal sequence (188–301) are required for USP4 to bind with ARF-BP1.

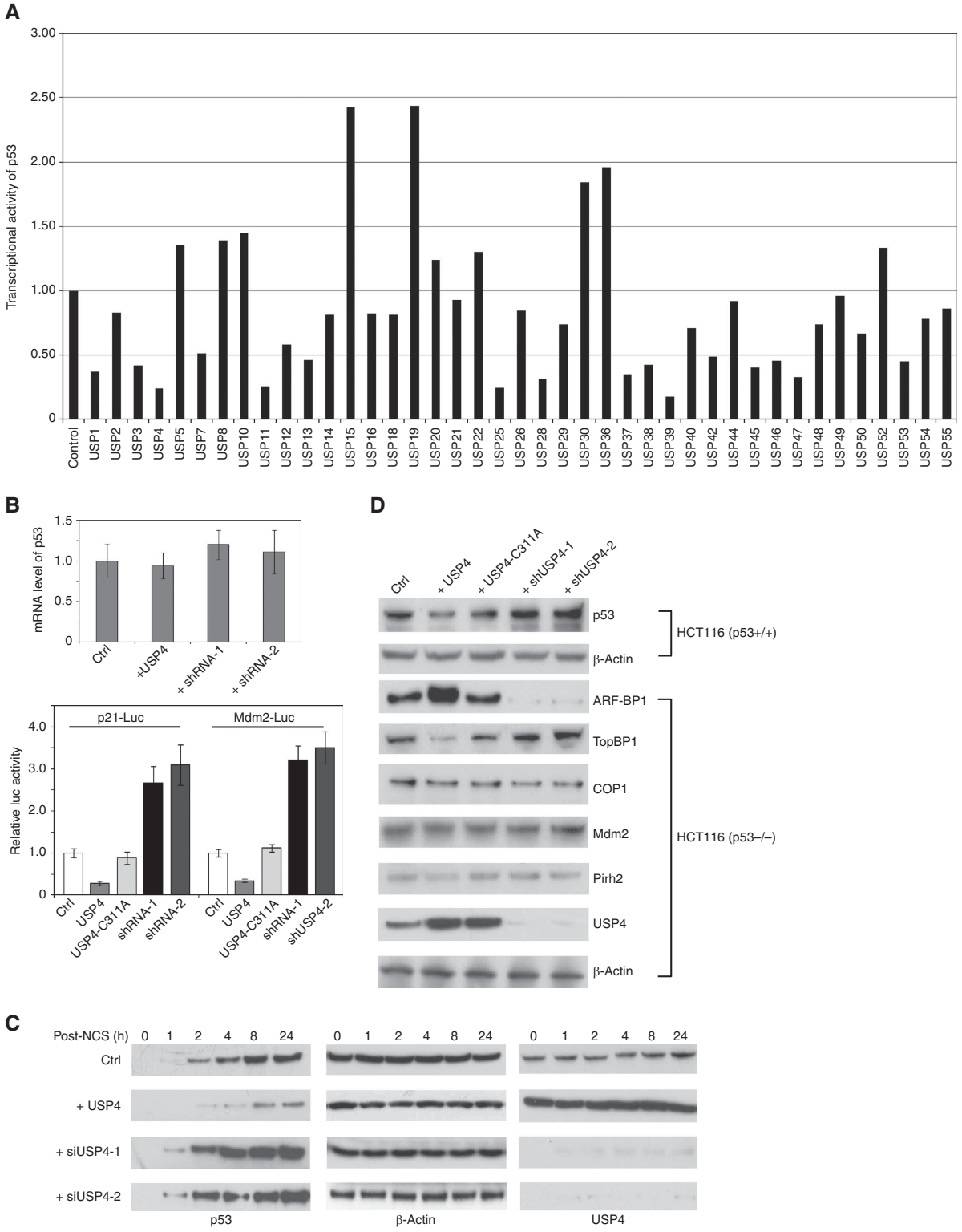

Figure 1 USP4 inhibits p53 induction. (**A**) The effects of overexpressed ubiquitin-specific peptidases (USPs) on the activity of p53. U2OS cells were transfected with control or USP expression vector, p21 promoter-driven firefly luciferase expression vector, and Renilla luciferase expression vector. At 24 h after transfection, cells were treated with NCS (500 ng/ml) and then harvested 16 h after treatment. Firefly luciferase activity was measured and normalized to the activity of Renilla luciferase. (**B**) USP4 affects p53 activity, but not the transcription of p53. U2OS cells were transfected with control vector or vector expressing wild-type human USP4 or its shRNA. P53 mRNA levels were determined by quantitative RT–PCR (upper panel). At the bottom panel, U2OS cells were transfected with vectors expressing p21 or Mdm2 promoter-driven firefly luciferase, together with vector expressing wild-type USP4, ubiquitinase-dead mutant USP4-C311A or USP4 shRNA. Cells were treated with NCS (500 ng/ml). (**C**) USP4 inhibits p53 induction in the DNA damage response. U2OS cells were transfected with USP4 siRNA or USP4 expression vector. Cells were treated by NCS (500 ng/ml) and then harvested at indicated time points. Protein levels were assessed by immunoblotting. (**D**) USP4 upregulates ARF-BP1, but not other p53-targeting E3 ubiquitin ligases. HCT116p53 + / + and HCT116p53 − / − cells were transfected with control vector or vector expressing wild-type USP4, USP4-C311A, or USP4 shRNA. Cell lysates were applied to the analyses of protein levels by immunoblotting.

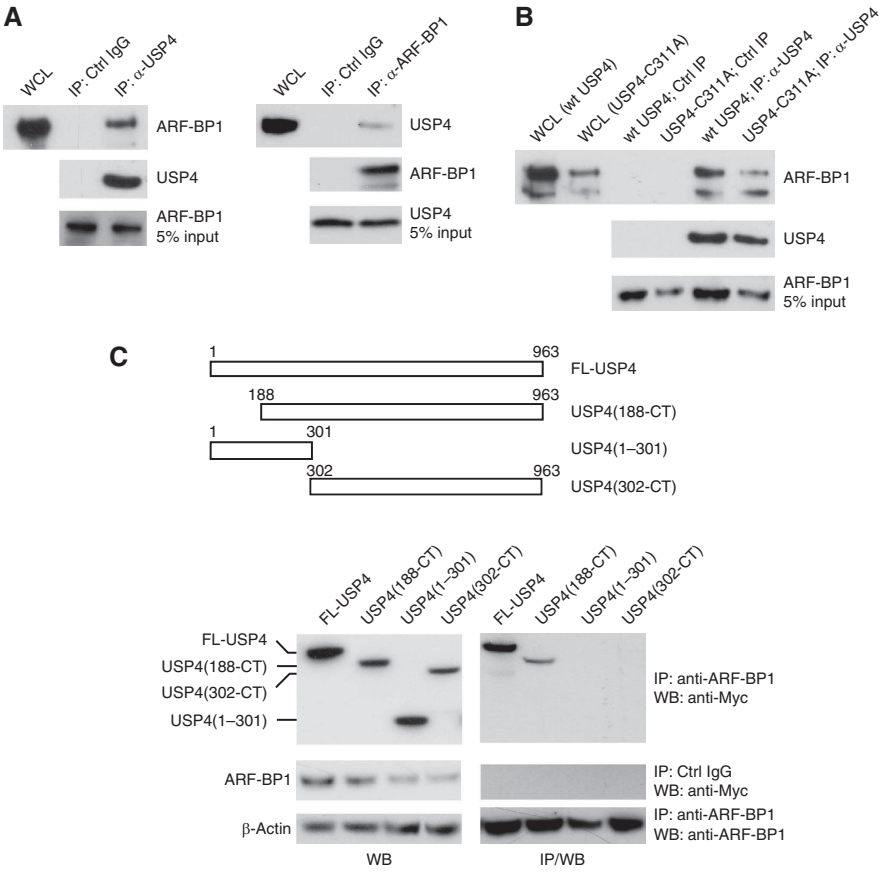

Figure 2 USP4 interacts with ARF-BP1. (**A**) Endogenous USP4 binds to endogenous ARF-BP1. Immunoprecipitation of U2OS cell lysates with anti-USP4 or control antibody followed by immunoblotting with anti-ARF-BP1 antibody showed that endogenous ARF-BP1was detected in the USP4-containing immunoprecipitates (left panel). A reciprocal experiment utilizing immunoprecipitation with anti-ARF-BP1 antibody followed by immunoblotting with anti-USP4 antibody confirmed their interaction (right panel). (**B**) USP4–ARF-BP1 interaction is not dependent on the activity of USP4. U2OS cells were transfected with vector DNA expressing wild-type or mutant USP4. Immunoprecipitation of lysates with anti-USP4 or control antibody followed by immunoblotting with anti-ARF-BP1 antibody showed that ARF-BP1 was detected in the wild or mutant USP4-containing immunoprecipitates. (**C**) Binding requirements between USP4 and ARF-BP1. Full-length (FL-) and three deletion constructs of USP4 (Myc-tagged) were individually transfected to U2OS cells. A schematic representation of USP4 mutants is shown at the top. USP4 was detected by western blotting using anti-Myc antibodies in the ARF-BP1 immunoprecipitates.

USP4 deubiquitinates and stabilizes ARF-BP1

ARF-BP1 was previously reported to be self-ubiquitinated (Chen *et al*, 2006). The interaction between USP4 and ARF-BP1 suggested that ARF-BP1 might be a substrate of USP4. We assessed the ubiquitination of ARF-BP1 in U2OS cells transfected with control vector or vectors expressing wild-type USP4, USP4-C311A, or USP4 shRNAs. Cells were treated with proteasome inhibitors MG101 and MG132 that prevented degradation of ubiquitinated proteins (Zhang *et al*, 2009). Altered levels of USP4 appeared to have no notable effects on global ubiquitination of proteins (Figure 3A). However, overexpression of wild-type USP4 reduced the ubiquitination of ARF-BP1, while knockdown of USP4 markedly increased the levels of ubiquitinated ARF-BP1. The effects of USP4 were dependent on its activity as USP4-C311A failed to inhibit the ubiquitination of ARF-BP1 (Figure 3A).

Since ARF-BP1 is a critical E3 ubiquitin ligase for p53, it was expected that p53 was inhibited by USP4 through ARF-BP1. Thus, we further examined whether USP4 affected the ubiquitination of p53 in U2OS cells treated with or without NCS. Overall, ubiquitination of p53 was inhibited after DNA damage, consistent with previous observations on the DNA damage-induced stabilization of p53 (Lu *et al*, 2007). Overexpression of USP4 dramatically promoted the ubiquitination of p53, while knockdown of USP4 by siRNA abolished the ubiquitination of p53 (Figure 3B). To exclude the possibility that USP4 indirectly influences the ubiquitination of ARF-BP1 in cells, we assessed the *in vitro* biochemical activity of USP4 using self-ubiquitinated ARF-BP1 as substrates. Immunopurified ARF-BP1 was first incubated with ubiquitin, E1 and E2 ubiquitin enzymes to produce self-ubiquitinated ARF-BP1 (Figure 3C, lane 2). We performed a titration experiment to incubate ubiquitinated ARF-BP1 with varying amounts of bacterial purified USP4 proteins. Increasing amounts of USP4 proteins resulted in decreasing ubiquitination levels of ARF-BP1 (Figure 3C), while USP4 (C311A) and negative control USP14 showed no activity on the ubiquitinated ARF-BP1 (Supplementary Figure S4). These results provided strong evidence that USP4 possesses specific and intrinsic deubiquitination activity towards ARF-BP1.

Polyubiquitination of proteins is the triggering signal that leads to protein degradation in the proteasome (Harper and Schulman, 2006). Thus, USP4-mediated deubiquitination may lead to the stabilization of ARF-BP1. We examined whether and to what extent USP4 augments the stability of ARF-BP1. Stability of ARF-BP1 was assessed in

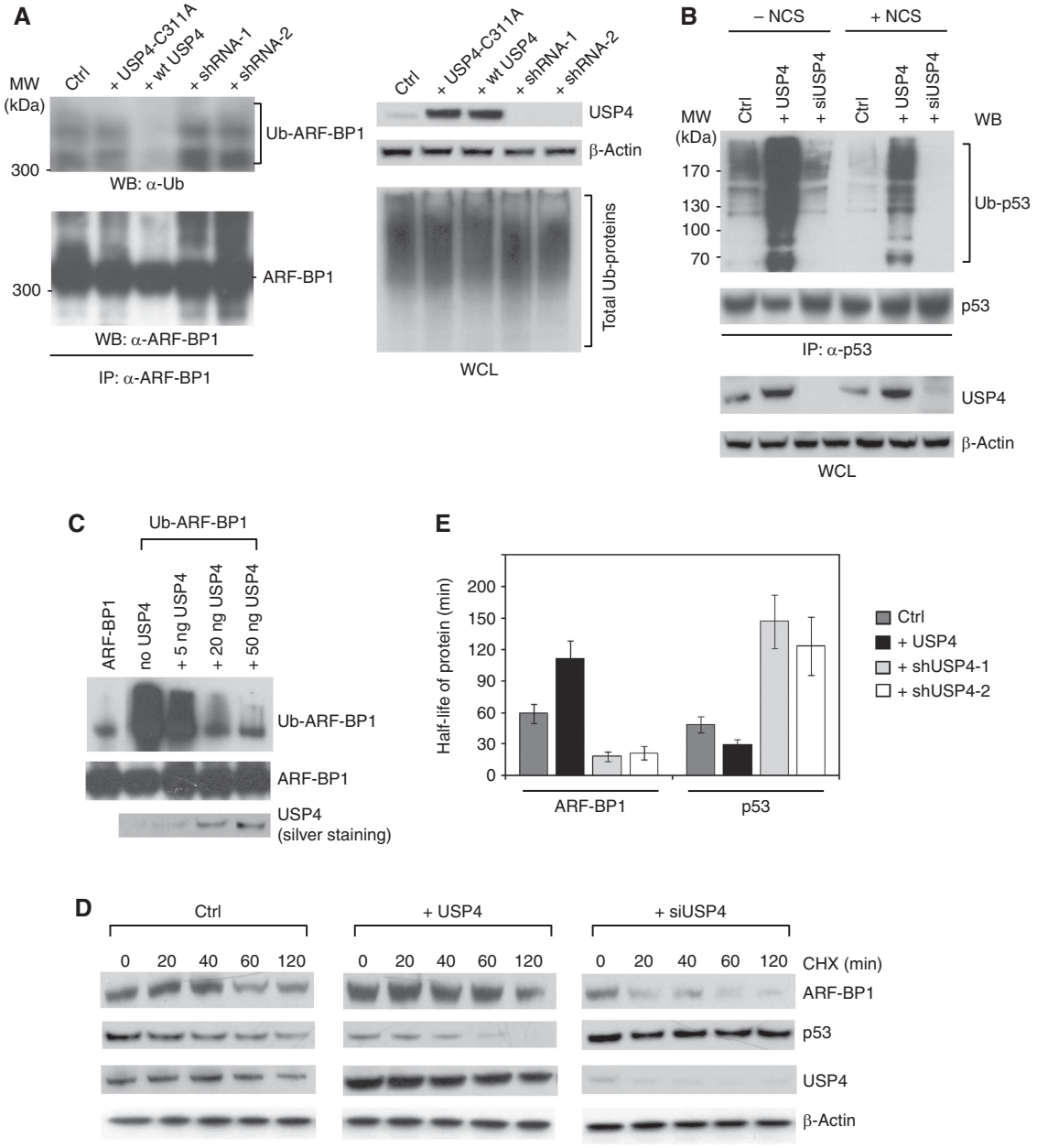

Figure 3 USP4 deubiquitinates ARF-BP1 *in vivo* and *in vitro*. (**A**) USP4 inhibits polyubiquitination of ARF-BP1 *in vivo*. U2OS cells were transfected with FLAG tagged wild-type or mutant USP4, USP4 shRNA, or control expression vector. Transfected cells were treated with protease inhibitors MG132 and MG101, and harvested 4 h after treatment. Cell lysates were analysed by immunoblotting for protein levels, or immunoprecipitated with anti-FLAG antibody and immunoblotted by anti-ubiquitin antibody. (**B**) USP4 enhances polyubiquitination of p53 *in vivo*. U2OS cells were transfected with USP4 siRNA, control vector, or USP4 expression vector. Transfected cells were treated with or without NCS (200 ng/ml) and with protease inhibitors MG132 and MG101, and harvested 4 h after NCS treatment. Cell lysates were precipitated with anti-p53 antibody and immunoblotted by anti-ubiquitin antibody. (**C**) USP4 deubiquitinates ARF-BP1 *in vitro*. Immunopurified ARF-BP1 was incubated with ubiquitin, E1 and E2 ubiquitin enzymes, and varying amounts of bacterial purified USP4 proteins. Reaction products were analysed by immunoblotting using anti-ubiquitin antibody. Varying amounts of USP4 proteins used in the assays were shown as silver staining. (**D**) USP4 stabilizes ARF-BP1, but destabilizes p53. U2OS cells were transfected with USP4 shRNA, control, or USP4 expression vector. Transfected cells were treated with 100 μg/ml of cyclohexamide (CHX) to block protein synthesis. Cells were harvested at various times after CHX treatment and protein levels were analysed by immunoblotting. (**E**) USP4 extends the half-life of ARF-BP1. U2OS cells stably expressing USP4 or its shRNAs were treated with CHX as described above. Half-lives of ARF-BP1 and p53 were calculated from three separate experiments for the graph.

cells transfected with vector expressing USP4 or USP4 shRNAs. Cells were treated with cycloheximide (CHX) to block protein synthesis. ARF-BP1 appeared to be a relatively short-lived protein with a half-life about 60 min (Figure 3D). Overexpression of USP4 clearly stabilized ARF-BP1, while depletion of USP4 by shRNAs destabilized ARF-BP1. Whereas USP4 promoted the stability of ARF-BP1, it consequently inhibited the stability of p53, a bona fide ubiquitination substrate of ARF-BP1 (Figure 3D). To better assess the effects of USP4 on the half-lives of ARF-BP1 and p53, we generated U2OS cells stably expressing USP4 or its shRNAs. Half-lives of proteins were measured in similar CHX blocking assays.

Overexpressed USP4 extended the half-life of ARF-BP1 from 59.8 to 116.2 min, while two specific USP4 shRNAs reduced the half-life of ARF-BP1 to 14.9 and 16.4 min, respectively (Figure 3E). In contrast, overexpressed USP4 reduced the half-life of p53 in U2OS cells from 50.3 to 28.2 min, and silencing USP4 augmented the stability of p53 with a half-life increased to 122–147 min, suggesting that USP4 is an important player regulating cellular p53 levels (Figure 3E).

USP4 sensitizes cells to DNA damaging agents in a P53-dependent manner

P53 is one of the important mediators of cell-cycle checkpoints and apoptosis in the DNA damage response (Toledo and Wahl, 2006). If USP4 modulates p53 levels by deubiquitination, it was expected that USP4 might impair the activation of DNA damage checkpoints and apoptosis. We treated HCT116p53+/+ and HCT116p53−/− cells with two DNA damaging agents, doxorubicin and etoposide that were widely used as cancer chemotherapy drugs. Cell viability was measured 2 days after treatment. Overexpression of ecotopic USP4 decreased the sensitivity of HCT116 cells to the treatment with doxorubicin when p53 was expressed, while silencing USP4 resulted in an increase in the sensitivity (IC50: Ctrl-0.15 μM, +USP4-0.58 μM, and −USP4-0.064 μM) (Figure 4A; Supplementary Figure S5A). However, the sensitivity of p53-null HCT116 cells to doxorubicin was not significantly influenced by varying levels of USP4. Similar results were obtained from cells treated with Nutlin-3 that activates p53 by inhibiting p53–Mdm2 interaction (Supplementary Figure S6) (Vassilev *et al*, 2004).

To examine whether USP4 inhibits DNA damage-induced cell-cycle checkpoints, we performed flow cytometry-based cell-cycle profiling analyses in HCT116p53+/+ and HCT116p53−/− cells transfected with control or USP4 expression vector (Figure 4B; Supplementary Figure S5B). Cells were treated with NCS to activate DNA damage checkpoints. In untreated HCT116p53+/+ cells, overexpression of USP4 appeared to promote DNA synthesis and cell proliferation evidenced by the higher percentage of S-phase cells (26.3% in USP4-overexpressing cells versus 17.0% in control cells) (Figure 4B). G1/S and G2/M checkpoints were triggered 12 h after DNA damage and a majority of cells were arrested in G1 and G2/M phases 24 h after damage. Only 13.8% of cells arrested at G1 phase and 9.6% of cells stayed in S phase in the USP4-overexpressing cells, whereas 27.7% of cells were in G1 phase and almost no cells were in S phase in control cells. These results suggested that USP4 overexpression led to a defective G1 cell-cycle arrest. The absence of p53 in HCT116p53−/− cells resulted in defective G1/S checkpoints, as shown by the accumulation of cells in S phase 12 h after DNA damage and a smaller percentage of cells (16.7% in HCT116p53−/− cells versus 27.7% in HCT115p53+/+ cells) arrested in G1 phase at 24 h. USP4 overexpression exhibited only mild effects in the cell-cycle profiling in the HCT116p53−/− cells (Figure 4B). These results suggested that USP4 is involved in the regulation of G1/S cell-cycle checkpoint partly in a p53-dependent manner.

Generation and characterization of USP4 knockout mice

To understand the biological functions of USP4 *in vivo*, we collaborated with Deltagen to generate *Usp4* knockout mice with germ line transmission via homologous recombination in 129Sv mouse embryonic stem (ES) cells injected into C57BL/6 strain blastocysts. To inactivate the *Usp4* gene, a targeting construct was designed in which a fusion gene expression cassette (β-galactosidase LacZ gene and neomycin-resistance gene) replaced genomic *Usp4* sequences containing exons 7 and 8. These two exons contain highly conserved sequences, and the expected targeting event results in an allele expressing a severely truncated mRNA with no *Usp4* coding sequences beyond exon 7 and no deubiquitinase domain (Figure 5A). Introduction of the targeting construct into ES cells were selected and validated by Southern blotting analyses. Amplified positive ES clones were used to reconstitute mice with a germ line mutation in the *Usp4* allele that were backcrossed to C57BL/6 mice to ensure 99% C57BL/6 genetic background. *Usp4* heterozygotes were crossed with each other to determine the effects of *Usp4* nullizygosity on embryonic development. Primers were designed for PCR genotyping as shown in Figure 5B. *Usp4*−/− mice were viable at birth, developed normally, and showed no observable phenotypic defects up to 6 months of age. To confirm the absence of intact USP4 expression in the null mice, we performed western blot analysis on tissue lysates harvested from *Usp4*+/+ and *Usp4*−/− mice. *Usp4* was expressed in all mouse tissues from the wild-type mice, but was highly expressed in the skeletal muscle and heart. As expected, no protein products of the *Usp4* gene were observed in *Usp4*-null mice, confirming the authentic knockout of the *Usp4* gene (Figure 5C). We further examined the radiosensitivity of wild-type and knockout mice to ionizing radiation (IR). *Usp4*+/+ and *Usp4*−/− mice were treated or mock treated with 5 Gy IR. Thymi and spleens were harvested 4 h after treatment and cells were isolated and prepared for apoptosis analyses by Annexin V and propidium iodide staining and flow cytometry. Absence of *Usp4* enhanced the sensitivity of mice to IR treatment, leading to significantly higher levels of apoptosis in both thymi and spleens, consistent with the higher levels of p53 in the *Usp4*−/− cells (Figure 5D and E).

Usp4-null MEFs exhibited retarded growth, elevated apoptosis, premature senescence, and resistance against oncogenic transformation

To assess whether the absence of *Usp4* had any effect on MEF cellular phenotypes, we isolated MEFs from littermate *Usp4*+/+ and *Usp4*−/− embryos and cultured them under standard conditions. It was observed that *Usp4*−/− MEFs grew more slowly than their *Usp4*+/+ counterparts (Figure 6A). Growth curve analyses confirmed that the proliferation rates of early passage *Usp4*−/− MEFs were substantially retarded compared with *Usp4*+/+ MEFs. Four days after plating 2.0×10^4 *Usp4*−/− MEFs, they grew to a mean of 4.9×10^4 cells per plate, versus a mean of 7.0×10^4 cells per plate for *Usp4*+/+ cells (Figure 6A). We further examined cell apoptosis induced by DNA damage stress. *Usp4*+/+ and *Usp4*−/− MEFs were treated with or without IR (5 Gy) or ultraviolet radiation (UV, 30 J/m^2). Apoptotic cells were quantified and analysed by Annexin V-PI staining and flow cytometry 24 h post-radiation. Untreated *Usp4*+/+ and *Usp4*−/− MEFs had low apoptosis rates, a characteristic of the early passage of MEFs. *Usp4*−/− cells demonstrated elevated apoptosis and sensitivity to IR and UV treatments as compared with wild-type MEFs. In the IR- and UV- treated

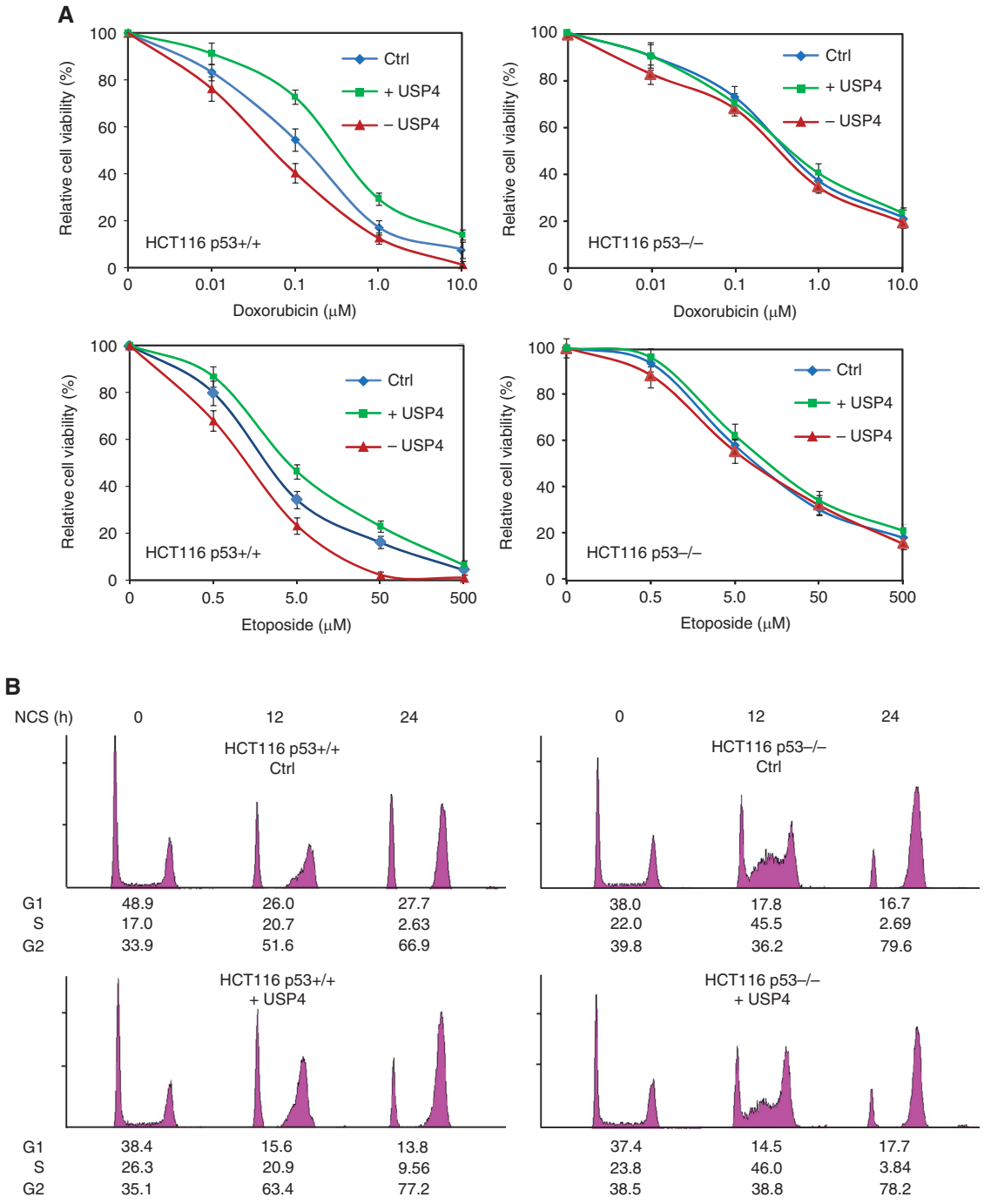

Figure 4 USP4 regulates cell viability and cell-cycle checkpoints in a p53-dependent manner. (**A**) USP4 desensitizes cells to DNA damaging agents in a p53-dependent manner. HCT116p53+/+ and HCT116p53−/− cells were transfected with control vector or vectors expressing USP4 or its shRNA. Transfected cells were treated with doxorubicin or etoposide at indicated concentrations for 1 day and cell viability was measured 2 days after treatment. (**B**) USP4 inhibits G1/S cell-cycle checkpoints. HCT116p53+/+ and HCT116p53−/− cells were transfected with control or USP4 expression vector. Transfected cells were treated with 200 ng/ml of NCS to induce cell-cycle checkpoints. Cells were harvested at 0, 12, or 24 h after treatment, fixed and stained with propidium iodide, and analysed by flow cytometry.

Usp4−/− cells, the percentages of apoptotic cells were increased to 66.0 and 62.4% compared with 42.1 and 38.4% in the treated *Usp4*+/+ cells, respectively (Figure 6B).

P53 is considered to be a master player in cellular senescence (Campisi, 2005). If USP4 functions as an inhibitor for p53, we expected that removal of *Usp4* would result in enhanced premature cellular senescence. We assessed the cellular senescence in two pairs of littermate MEFs by senescence-associated β-galactosidase staining at pH 6, a known characteristic of senescent cells not found in presenescent (Reznikoff *et al*, 1996; Serrano *et al*, 1997), quiescent or immortal cells. At passage 4, *Usp4*+/+ MEFs exhibit low levels of senescence (3–5% of total cells), while depletion of *Usp4* in the *Usp4*−/− counterparts dramatically promoted the senescence to much higher levels (32–35% of total cells) (Figure 6C). Senescence marker, p16INK4a was also significantly

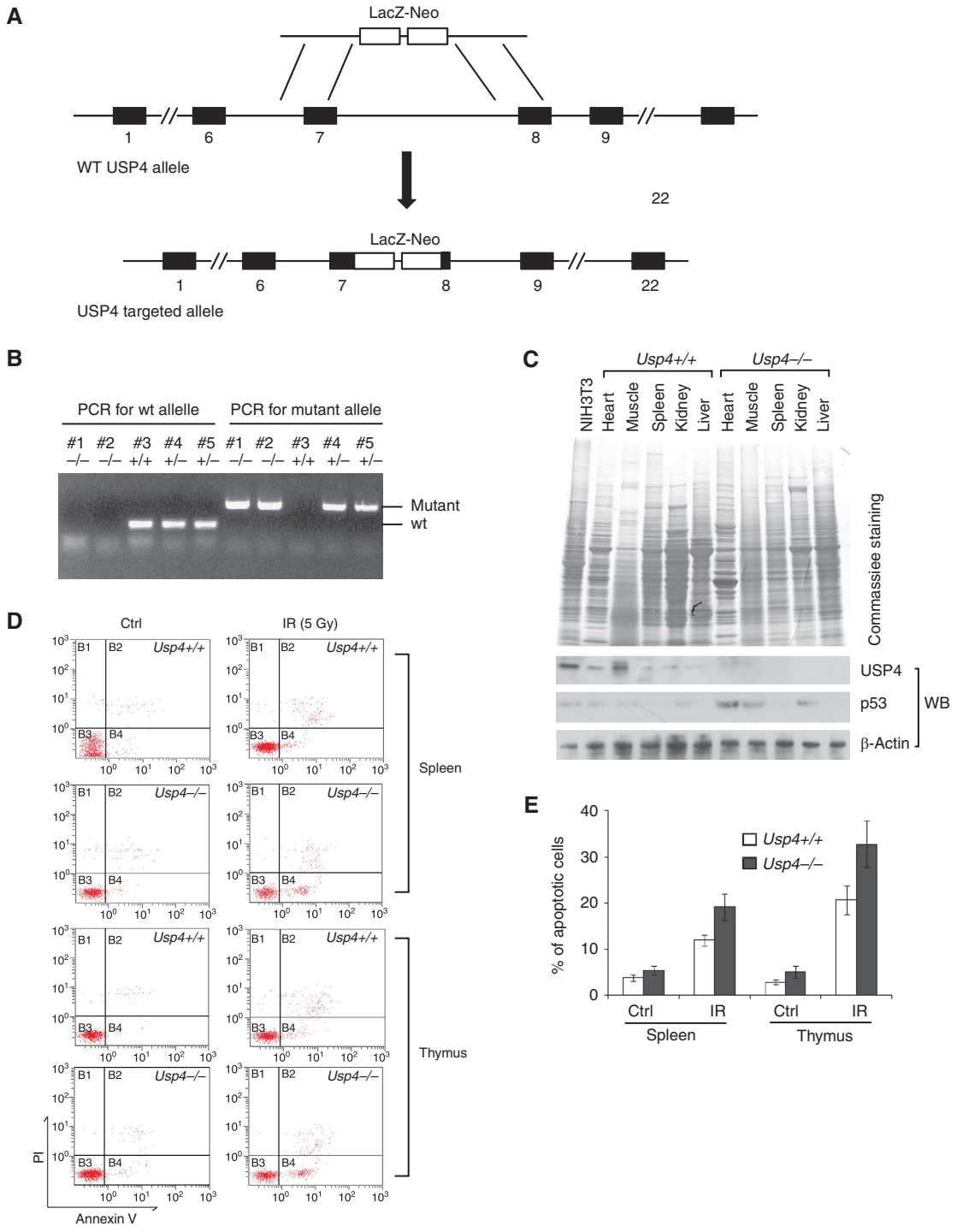

Figure 5 Generation and characterization of USP4 knockout mice. (**A**) Gene-targeting strategy for *Usp4* inactivation. Targeting deletes highly conserved domains in exons 7 and 8 and replaces them with a LacZ-Neo marker cassette. (**B**) PCR genotyping of wild-type and mutant mice. (**C**) USP4 proteins were not detected in *Usp4*−/− mice. Protein lysates of various tissues from *Usp4*+/+ and *Usp4*−/− mice were analysed by Coommassie blue staining and immunoblotting. (**D**) Loss of USP4 dampens IR-induced apoptosis. *Usp4*+/+ and *Usp4*−/− mice were treated with 5 Gy of ionizing radiation. Thymi and spleens were harvested 4 h after treatment and cells were isolated and prepared for apoptosis analyses by Annexin V and propidium iodide staining and flow cytometry. (**E**) Percentages of apoptotic cells were quantified and graphed from the above experiments in Figure 5D.

upregulated in the *Usp4*−/− cells. The evidence for augmented p53 activity in the *Usp4*−/− cells could account for their premature senescence phenotype (Figure 6C; Supplementary Figure S7). We next examined the expression of proteins in the p53 and apoptotic signalling pathways in *Usp4*+/+ and *Usp4*−/− MEFs treated with or without IR (Figure 6D). As expected, USP4 was not detectable in the *Usp4*−/− MEFs, and levels of ARF-BP1 were much lower than in the *Usp4*+/+ MEFs (Figure 6D). As a result of reduced ARF-BP1 levels, p53 and its transcriptional target p21 were robustly induced after

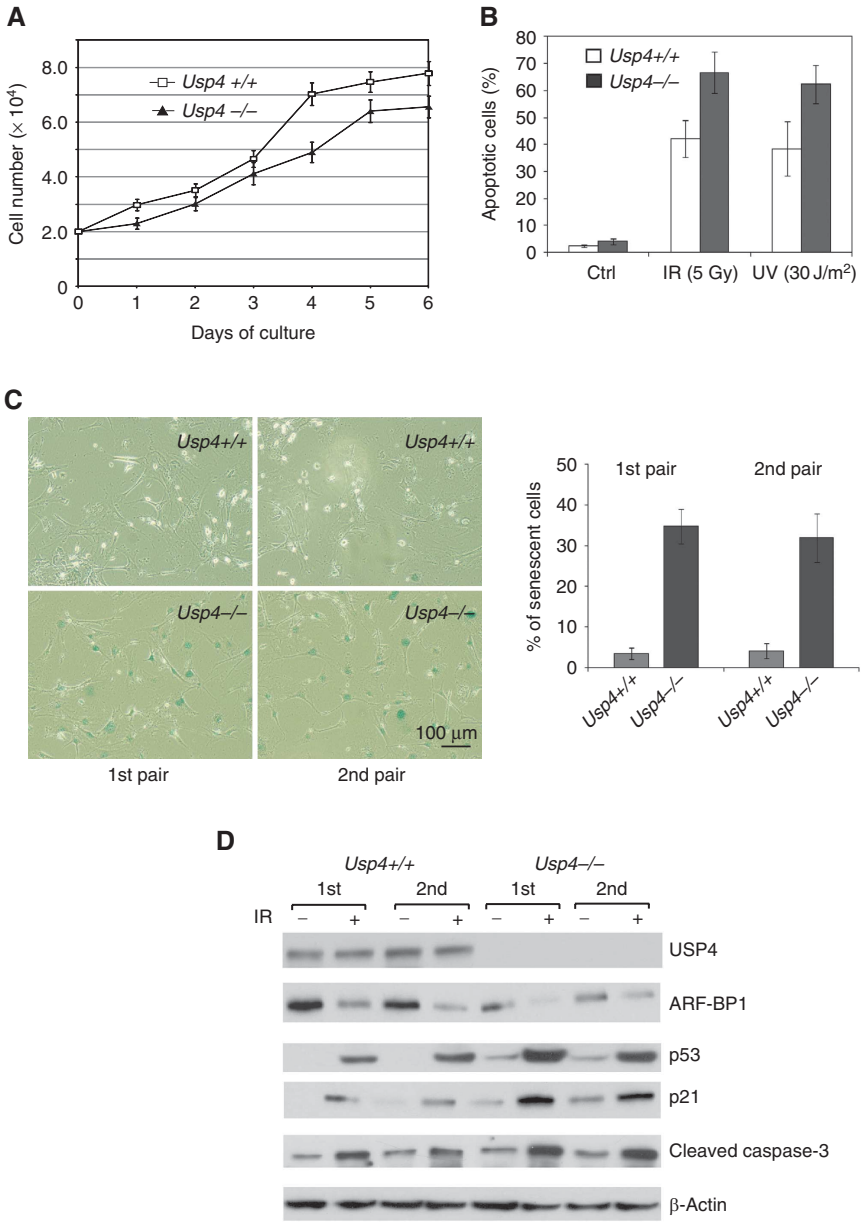

Figure 6 Characterization of *Usp4*+/+ and *Usp4*−/− MEFs. (**A**) *Usp4*−/− MEFs have retarded cell proliferation. *Usp4*+/+ and *Usp4*−/− MEFs were seeded at 20 000 cells/well and cultured up to 6 days. Cells were counted at indicated time points. (**B**) Absence of USP4 sensitizes cells to DNA damaging treatments. *Usp4*+/+ and *Usp4*−/− MEFs were treated with or without UV (30 J/m^2) or IR (5 Gy). Apoptosis cells were analysed by Annexin V-PI staining and flow cytometry. (**C**) *Usp4*−/− MEFs exhibit premature cell senescence. Two pairs of littermate MEFs were assessed at passage 4 for cell senescence by β-galactosidase staining at pH 6. Blue-stained cells are senescence cells. Percentages of senescent cells are shown in the graph to the right. (**D**) ARF-BP1 is downregulated in *Usp4*−/− MEFs. Levels of various proteins in two pairs of littermate MEFs were analysed by immunoblotting.

IR treatment at higher levels in the *Usp4*−/− cells. An apoptosis indicator, cleaved caspase-3 was increased to higher levels in the *Usp4*−/− cells as compared with the *Usp4*+/+ cells, further confirming that loss of *Usp4* activated p53-associated apoptosis (Figure 6D).

USP4 is a potential oncogene overexpressed in several types of human cancer

Previous studies have shown that USP4 may be an oncogene that is overexpressed in small cell tumours and adenocarcinomas of the lung (Gray *et al*, 1995). To investigate whether USP4 deficiency interfered with oncogene-induced transformation of primary MEFs, we transformed *Usp4*+/+ and *Usp4*−/− MEFs with the oncogenes *H-ras* and *Myc* and assessed colony formation in soft agar. While either H-ras or Ras overexpression alone did not transform MEFs, co-overexpression of the two genes resulted in enhanced transformation efficiency, consistent with the previous reports showing the cooperation between these two genes in oncogenic transformation of mouse primary cells (Figure 7A) (Land *et al*, 1983, 1986). Absence of USP4 conferred transformation resistance on the *Usp4*−/− MEFs, suggesting elevated p53 levels in these cells may contribute to the suppression of oncogenic transformation.

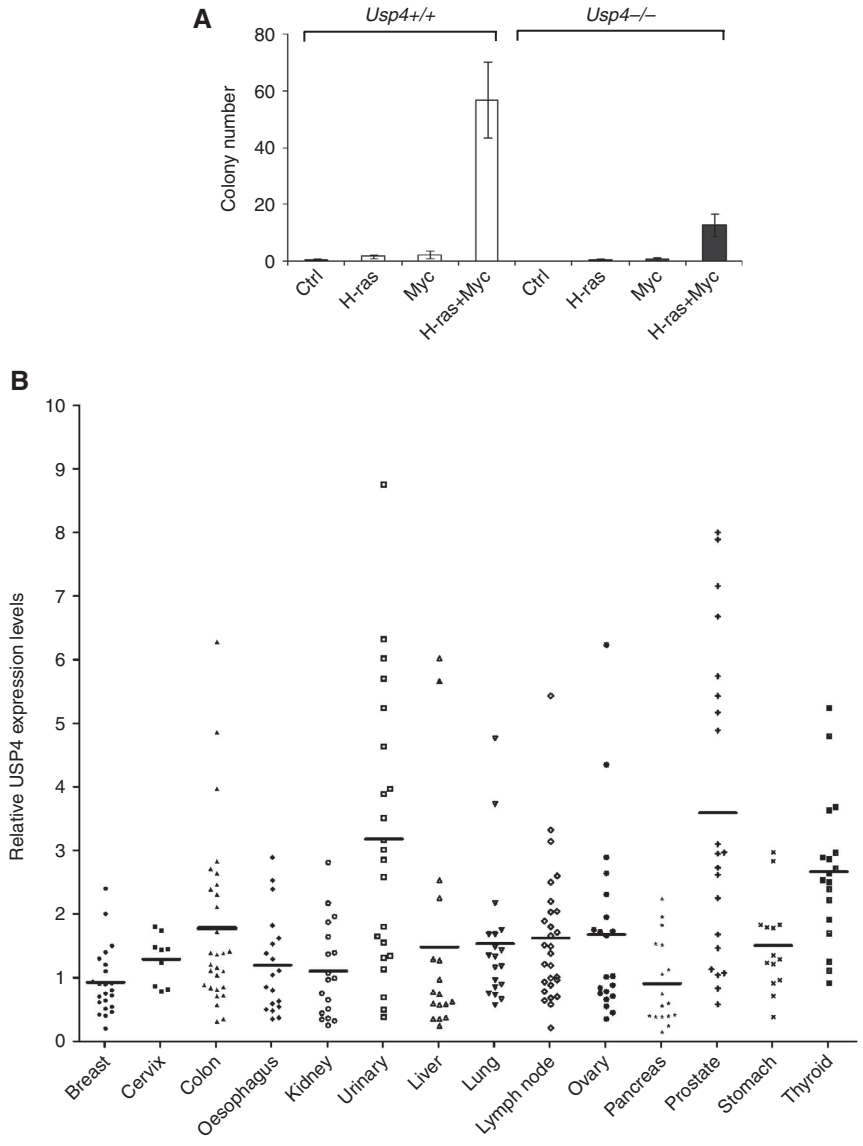

Figure 7 USP4 is a potential oncogene. (**A**) Absence of USP4 confers transformation resistance in MEFs. *Usp4*+/+ and *Usp4*−/− MEFs were infected by retroviruses expressing oncogenes H-ras or Myc. Transformation was determined by soft-agar colony-formation assays. (**B**) USP4 is overexpressed in several types of human cancer. Cancer tissue arrays for quantitative PCR were used to quantify the mRNA levels of USP4 that were normalized to the levels of β-actin. Bars in the graph show mean expression levels of USP4 in each type of cancer.

To better understand the potential roles of USP4 in tumourigenesis, we examined the expression levels of USP4 in 14 types of human cancer samples and normal tissue samples (Figure 7B). Cancer tissue arrays for quantitative PCR were used to quantify the mRNA levels of USP4 that were normalized to the levels of β-actin. In general, USP4 levels were elevated in a majority of cancer types except for human breast cancer and pancreatic cancer. Of all cancer samples tested, urinary bladder and prostate cancer samples displayed significantly higher USP4 levels (3.3-fold and 3.9-fold, respectively) than control normal tissues, suggesting that USP4 may contribute to the initiation and growth of some human tumours (Figure 7B).

Discussion

In unstressed cells, p53 is maintained at low physiological levels to allow cells to proliferate. A major player in controlling p53 levels is Mdm2, which is supported by evidence from animal studies showing that embryonic lethality of Mdm2−/− mice was rescued by nullizygosity of p53. This simplified regulatory model becomes far more complicated when p53 needs to be induced to respond to external and internal stresses (Batchelor *et al*, 2008). Post-translational modifications, including phosphorylation, acetylation, methylation, sumoylation, and ubiquitination orchestrate to manipulate the functionality and stability of p53 during stress response (Toledo and Wahl, 2006). For instance, phosphorylation of p53 at multiple sites has long been considered to stabilize p53 by preventing Mdm2 from degrading p53 (Olsson *et al*, 2007). Acetylation on lysine residues of p53 circumvents the ubiquitination on these residues (Lee and Gu, 2010). Therefore, the p53 regulatory loop is not a simply linear loop but part of a complex and multifaceted regulatory network that involves a number of regulators and effectors.

Although Mdm2 is thought to be one of the primary E3 ubiquitin ligases that target p53 for ubiquitination and degradation, a few more E3 ligases have been reported to have similar activities in regulating p53 levels. COP1, Pirh2, and ARF-BP1 directly interact with p53 and target p53 for proteasome-mediated degradation (Leng *et al*, 2003; Dornan *et al*, 2004; Chen *et al*, 2005). The Gu group tested the differential effects of these known E3 ligases on p53 stabilization by RNAi knockdown. As expected, silencing Mdm2 significantly stabilized p53, but depletion of COP1 or Pirh2 only had a modest effect. Surprisingly, inactivation of ARF-BP1 induced the highest levels of p53 activity compared with the other E3 ligases including Mdm2, suggesting that ARF-BP1 is likely to be a key modulator for p53 (Chen *et al*, 2005). Identified as a stable interactor of ARF, ARF-BP1 turned out to be a bona fide E3 ubiquitin ligase that ubiquitinates p53 and itself, reminiscent of Mdm2 in substrate specificity. It remains unclear whether ARF-BP1 is ubiquitinated by other E3 ligases. However, the interplay between Mdm2, COP1, and Pirh2 has begun to be studied, showing their mutual regulation (Wang *et al*, 2011).

Since ubiquitination is a reversible process, deubiquitinases were assumed to have important roles in regulating p53. Several USPs, including USP7, USP2a, and USP10, have been reported to regulate p53 and/or Mdm2 (Li *et al*, 2002, 2004; Stevenson *et al*, 2007; Yuan *et al*, 2010). In the present study, we attempted to identify novel USPs that regulate p53 activity and levels. From our screens, a number of USPs turned out to be negative regulators for p53, suggesting that they did not directly deubiquitinate p53 but possibly promoted the activity or levels of the E3 ubiquitin ligases in the p53 regulatory loop. ARF-BP1, but not other E3 ligases, was shown to specifically bind to and deubiquitinate USP4. Altered levels of USP4 influenced the stability and levels of ARF-BP1 *in vitro* and *in vivo*.

Depletion of USP4 promoted p53 levels via destabilization of ARF-BP1, rendering cells sensitive to DNA damaging agents. Elevated activity and levels of p53 were observed in *Usp4−/−* MEFs, leading to premature senescence and increased cell apoptosis. The findings reported here clarified a mechanism by which USP4 regulates p53 signalling through ARF-BP1. While ARF-BP1 is certainly an important mediator of USP4 functions, it remains uncertain whether USP4 is integrated into the p53 regulatory network from other nodes. Previous studies have shown that USP4 physically or functionally interacts with other proteins that function in the p53 pathway. For example, one report showed that USP4 bound with the retinoblastoma protein, an important tumour suppressor that intersects with the p53 pathways (Blanchette *et al*, 2001). In addition to the p53 pathways, USP4 appears to function in other key regulatory pathways. A recent study reported that knockdown of USP4 activated β-catenin-associated transcription (Zhao *et al*, 2009a). By interacting with two known Wnt signalling components: Nemo-like kinase and T-cell factor 4, USP4 may integrate into canonical Wnt signalling in a variety of physiological and pathological conditions.

While our data suggested a primary role of USP4 in the p53 pathways, it is also likely that other targets of ARF-BP1 or USP4 contribute to the regulation of DNA damage response and p53 activity. For example, TopBP1 inhibits p53 activity and appears to be negatively regulated by ARF-BP1 (Herold *et al*, 2008; Liu *et al*, 2009). The regulation is mediated by an interaction between two BRCT domains of TopBP1 and the DNA-binding domain of p53, leading to inhibition of p53 promoter binding activity. Because USP4 stabilizes ARF-BP1, it should have activated p53 through the ARF-BP1-TopBP1-p53 pathway. However, our results showed that USP4 inhibits p53 levels and activity. We examined the effects of USP4 on TopBP1. In Figure 1D, it shows that TopBP1 is negatively regulated by USP4. As a key player in the DNA damage response, TopBP1 has p53-independent functions. Stabilization of ARF-BP1 by USP4 would affect both p53-dependent and -independent pathways. We performed the same assays shown in Figure 4 on the HCT116p53+/+ and HCT116p53−/− cells with altered levels of TopBP1 and USP4 (Supplementary Figure S8). The results demonstrated that (1) in the presence or absence of p53, knocking down TopBP1 significantly sensitized the cells to DNA damaging treatment, suggesting a p53-independent role of TopBP1 in the DNA damage response and (2) modulating USP4 levels had only mild effects on cell viability when TopBP1 was knocked down in the cells, indicating that TopBP1 is an indirect but important downstream target of USP4 in the DNA damage response. We also showed that the effects of USP4 on G2/M checkpoint and p53 levels are not completely independent on TopBP1 (Supplementary Figure S9). Therefore, we predict that multiple targets of ARF-BP1 may contribute to the *in vitro* and *in vivo* functions of USP4, of which p53 is one of the most important.

In vivo functions of ARF-BP1 have not been well characterized so far. From published studies, ARF-BP1 appears to be multifunctional and involved in neural development, spermatogenesis, mental retardation, and tumourigenesis (Hall *et al*, 2007; Liu *et al*, 2007; Parsons *et al*, 2009; Zhao *et al*, 2009b). In cells, ARF-BP1 has important roles in DNA damage repair, cell-cycle checkpoints, and apoptosis. USP4 knockout mice used in our laboratory showed reduced levels of ARF-BP1 and developmental normality, suggesting that a low level of ARF-BP1 may be sufficient for embryonic and tissue development. Another intriguing observation is that the USP4 knockout mice are viable and develop normally despite their increased levels of transcriptionally active p53. Deletion of Mdm2, an important p53 E3 ligase, causes embryonic lethality due to p53 stabilization and activation (Jones *et al*, 1995). p53 activity and level are regulated by a complex network of regulators (Toledo and Wahl, 2006). While ARF-BP1 appears to be an important player in regulating p53 level, Mdm2 may be a primary regulator to maintain the physiological levels of p53. In addition, other USP4 targets may also contribute to the regulation of p53 activity.

Our current studies suggest that USP4 may be an oncogene because (1) USP4 inhibits p53 and p53-associated apoptosis and cell-cycle checkpoints, (2) depletion of USP4 promotes cell senescence, (3) loss of USP4 inhibits oncogene-induced primary cell transformation, and (4) USP4 is overexpressed in a subset of human cancers. More evidence should be provided from *in vivo* studies. It will be of great interest to further examine whether loss of USP4 confers a tumour-resistance phenotype in tumour-prone animal models, or whether USP4 overexpression promotes tumourigenesis *in vivo*.

Materials and methods

Cell lines and tissue culture

The U2OS cell line is a human osteosarcoma line that was obtained from the American Type Culture Collection. HCT116p53+/+ and HCT116p53−/− cell lines were obtained from the Vogelstein laboratory at Johns Hopkins University. Cells were cultured and stored according to the supplier's instructions. Once resuscitated, cell lines are routinely authenticated through cell morphology monitoring, growth curve analysis, species verification by isoenzymology and karyotyping, identity verification using short tandem repeat profiling analysis, and contamination checks. Expression of p53 in the HCT116 cell lines was confirmed by immunoblotting before they were used in the experiments. Primary *Usp4*+/+ and *Usp4*−/− MEFs were harvested and cultured as previously described (Lu *et al*, 2007).

Western blot analysis, antibodies, and purified proteins

Immunoprecipitations, western blot analysis, and immunoprecipitation-western blot analyses were performed by standard methods described previously (Zhang *et al*, 2009). To separate polyubiquitinated ARF-BP1 from its non-ubiquitinated form, cell lysates were run in 4–15% gradient SDS gel at constant 20 V for 24–36 h. Anti-actin (#1616), anti-p53 (#126), HRP-anti-goat IgG (#2020), HRP-anti-rabbit IgG (#2302), HRP-anti-mouse IgG (#2302), anti-ubiquitin (#8017), and anti-USP4 (#79322) were purchased from Santa Cruz; anti-ARF-BP1 (#IHC-00439), anti-COP1 (#A300-894A), anti-Pirh2 (#A300-357) antibodies were purchased from Bethyl Laboratories. Anti-Mdm2 (#M7815 and #M8558), anti-FLAG (F2922) antibodies and anti-FLAG beads (A2220) were obtained from Sigma-Aldrich. Bacterial GST-tagged USP4 proteins (#H00007375-P01) were obtained from Abnova.

In vitro *ubiquitination and deubiquitination assays*

The *in vitro* ubiquitination assay was performed as described previously with modifications (Chen *et al*, 2005). For the self-ubiquitination assay, immunopurified ARF-BP1 proteins were mixed with other components, including E1 (10 ng), E2 (His-UbcH5a, 100 ng), and 5 μg of His-ubiquitin in 20 μl reaction buffer with the addition of 2 μl of Mg-ATP solution (1 mM final). The reaction was stopped after 60 min at 37°C by adding 2 μl of 10 × stop buffer. All reagents were purchased from Boston Biochem. In deubiquitination assays, self-ubiquitinated ARF-BP1 proteins were incubated with varying amount of bacterial USP4 proteins for 1 h at 37°C in the ubiquitination buffer. Deubiquitinated products were analysed by western blotting.

Plasmid constructs and cell transfection

The human USP4 cDNA clone (#MMM1013-65091) was purchased from Open Biosystems and verified by DNA sequencing. The DNA fragment of USP4 was amplified by PCR and subcloned to pcDNA3.1 vector with an N-terminal FLAG or Myc tag. The enzymatically dead mutant of USP4 (C311A) was generated using site-directed mutagenesis Quikchange kit from Stratagene. Cell transfections were performed by using Fugene HD transfection reagents from Roche following the manufacturer's instructions.

Luciferase assays

U2OS cells were transfected with either a p21 or an Mdm2 promoter-driven luciferase construct, and a control Renilla luciferase construct using Fugene HD transfection reagent (#11633400, Roche). Cells were incubated for 24 h and then treated with 500 ng/ml of NCS. Luciferase activity was determined 16 h after treatment with the Dual Luciferase System (Promega) following the manufacturer's instructions.

shRNA-mediated knockdown of USP4

To stably knockdown USP4, oligonucleotides targeting human USP4 (shRNA-1: 5′AACATGTCCGAGTTTGTCTGT3′, shRNA-2: 5′AACTGTA AGAAGCATCAACAG3′) were cloned into pSUPER-retro vector (Oligoengine). U2OS cells were transfected by the shRNA expression vector DNA using Fugene HD transfection reagent (#11633400, Roche). Transfected cells were kept under selection in medium containing 500 μg/ml G418 (#345810, Calbiochem). Individual colonies were propagated and validated.

Protein stability measurement, cell proliferation, and viability assays

In protein half-life assays, 100 μg/ml CHX (#C4859, Sigma-Aldrich) was added to cell culture to block protein synthesis. Cells were collected at indicated time points and protein levels were measured and quantified by western blotting and phosphoimager. The half-life of proteins was calculated from two independent experiments. In cell proliferation assays, cells were seeded at a concentration of 1000 cells/well in flat bottom 96-well microplates. Cell proliferation was measured at the indicated time using reagent WST-1 (#05015944001, Roche), according to the manufacturer's instructions. In cell viability assays, cells were seeded into 96-well plates at 3000 cells/well and incubated for 24 h. Each drug was then added at various concentrations to quadruplicate wells in a final volume of 100 μl of medium. After 24 h of incubation, cells were cultured for 2 days and cell viability was measured using MTS tetrazolium reagent (CellTiter 96 One Solution, Promega) following the manufacturer's instruction.

Apoptosis assays and soft agar colony-formation assays

In apoptosis assays, harvested cells were suspended in PBS buffer containing 0.1% BSA, and fixed with cold ethanol. Cells were then washed, stained with propidium iodide (50 μg/ml) and Annexin V-FITC, and analysed by flow cytometry. The soft agar colony-formation assay was performed as previously described. Cells were infected with retroviruses expressing H-ras and/or Myc, and plated in 0.3% top agarose in six-well dishes and cultured for 2 weeks. Colonies were counted under a light microscope.

Supplementary data

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

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Author contributions: XZ, FGB, and XL designed and carried out the experiments; JY provided reagents for deubiquitination assays; and XZ and XL wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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