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Article

HLA-B-associated transcript 3 (Bat3) stabilizes and activates p53 in a HAUSP-dependent manner

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The p53 pathway is a highly complex signaling network including several key regulators. HAUSP is a critical component of the p53 pathway acting as a deubiquitinase for both p53 and its key repressor Mdm2. Here, we identified a novel HAUSP-interacting protein, HLA-B-associated transcript 3 (Bat3) and found it to be capable of inducing p53 stabilization and activation via a HAUSP-dependent mechanism, resulting in cell growth inhibition. Surprisingly, the deubiquitylating enzymatic activity of HAUSP was not required for this phenomenon. Co-immunoprecipitation showed that p53 coexisted in a complex with Bat3 and HAUSP *in vivo*, and HAUSP may serve as a binding mediator to enhance the interaction between p53 and Bat3. Further studies revealed that formation of this three-protein complex interfered with the binding of p53 to its proteasome receptor S5a and promoted the accumulation of p53 in nucleus. Notably, Mdm2 protein abundance is also regulated by Bat3 in the presence of HAUSP. Overexpression of Bat3 and HAUSP increases Mdm2 protein levels without influencing the p53–Mdm2 interaction and Mdm2-mediated p53 ubiquitination, indicating that Bat3–HAUSP-mediated protein stabilization is not specific to p53 and different mechanisms may be involved in Bat3-mediated regulation of p53–Mdm2 pathway. Together, our study unravels a novel mechanism by which p53 is stabilized and activated by HAUSP-mediated interaction with Bat3 and implies that Bat3 might function as a tumor suppressor through the stabilization of p53.

Keywords: Bat3, p53 stabilization, HAUSP, S5a, proteasome recognition

Introduction

p53, as a major tumor suppressor protein, plays a fundamental and important role in the control of cell cycle arrest, apoptosis, and senescence. The p53 signaling pathway, involving multicomponents, is a highly complex signaling network. During normal homeostasis, p53 is a short-lived protein and maintained at low protein levels predominantly through Mdm2-mediated ubiquitylation and subsequent degradation (Michael and Oren, 2003; Yang et al., 2004; Liu et al., 2019). Mdm2 is an E3 ubiquitin ligase, which can not only promote p53 ubiquitylation (Honda et al., 1997) and negatively regulate p53-mediated transcriptional activity (Honda and Yasuda, 2000) but also target Mdm2 itself for auto-ubiquitylation and proteasome degradation (Fang et al., 2000; Honda and Yasuda, 2000). In addition, the Mdm2 gene can be transcriptionally activated by p53, which leads to repression of p53 activity and forms an auto-regulatory feedback loop in the p53 regulation network (Wu et al., 1993).

The herpes virus-associated ubiquitin-specific protease HAUSP is a critical component of the p53–Mdm2 pathway. HAUSP can specifically deubiquitinate both p53 and Mdm2 and has a dynamic role in regulating p53 stabilization and degradation (Li et al., 2002,2004; Tavana and Gu, 2017). p53 and Mdm2 bind to the N-terminal TRAF-like domain of HAUSP vgin a mutually exclusive manner, and the balance between the Mdm2-mediated ubiquitination and HAUSP-mediated deubiquitination is considered as the key for p53 homeostasis and activation (Hu et al., 2006). Meanwhile, Brooks et al. (2007) found that the direct interaction betweenx HAUSP and

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p53 was not completely required for it to efficiently antagonize Mdm2-mediated ubiquitination, despite mutually exclusive binding, and that the existence of p53–Mdm2–HAUSP complex allowed HAUSP to enzymatically function *in trans* on p53 through indirect binding with Mdm2. In addition, several proteins have been shown to interact with HAUSP for targeting p53–Mdm2 pathways, such as TSPYL5 (Epping et al., 2011), DAXX (Ronai, 2006), and ICP0 (Antrobus and Boutell, 2008). Therefore, the identification and investigation of proteins interacting with HAUSP is of significance for deeply understanding p53 signal pathways.

HLA-B-associated transcript 3 (Bat3) also known as BAG6/Scythe, was originally identified as a gene within the human major histocompatibility complex that encodes the anti-apoptotic ubiquitin-like protein (Banerji et al., 1990). Bat3 acts as a specific negative regulator for mitochondria-mediated apoptosis (Thress et al., 1998) and may protect cells from apoptosis by promoting the degradation of certain apoptotic factors (Colon-Ramos et al., 2003; Minami et al., 2007). Subsequent studies found that Bat3 functions as a proteasomal substrate-associated protein and provides a transient platform linking the 26S proteasome and various defective substrates for efficient proteasome targeting and ubiquitin degradation (Minami et al., 2010; Claessen and Ploegh, 2011; Xu et al., 2012; Payapilly and High, 2014; Tanaka et al., 2016). Bat3 also acts as a chaperoning protein and directly interacts with nascent tail-anchored proteins through their C-terminal transmembrane domain, shielding the exposed hydrophobic segments and preventing the ubiquitin-positive cytoplasmic aggregation or inappropriate interactions (Mariappan et al., 2010; Mock et al., 2015). Despite being a quality control and anti-apoptotic regulator under normal condition, Bat3 can also contribute to apoptosis when the cell death program has been initiated under certain stress conditions. Desmots and colleagues found that Bat3 regulates the stability of the apoptosis-inducing factor during ER stress (Desmots et al., 2008). Sasaki et al., (2007) and Sebti et al., (2014a, b) reported that Bat3 acts as a positive regulator of p53 transactivation by stabilizing its interaction with the acetyltransferase p300 and enhancing its acetylation during apoptosis and autophagy. Therefore, Bat3 may play multiple and/or dynamic roles during different conditions; however, the precise mechanisms are not completely understood.

In this study, we first identified Bat3 as a novel HAUSP-interacting protein. Further investigation showed that Bat3 is capable of stabilizing and activating p53 depending on the presence of HAUSP but, surprisingly, independent of its deubiquitylating enzymatic activity. Notably, we revealed that a p53–HAUSP–Bat3 trimeric protein complex could exist *in vivo* with HAUSP serving as a binding mediator for enhanced interaction between p53 and Bat3, which antagonizes the interaction of p53 with its proteasome receptor S5a and promotes the accumulation of p53 in the nucleus. These findings support the notion that Bat3 is an essential regulator of p53 and reveal a novel mechanism by which p53 is stabilized and activated by HAUSP-dependent interaction with Bat3. It also implies the potential role of Bat3 in tumor suppression through p53 modulation.

Results

HAUSP interacts with Bat3

To screen for the proteins that interact with HAUSP, we detected the affinity-purified HAUSP-associated proteins in the fractions eluted with Flag peptide using mass spectrometric analysis and identified Bat3 as a novel HAUSP-interacting protein. To confirm this result, we examined the interaction of exogenous HAUSP with Bat3 in an overexpression condition in 293T cells and found that HAUSP readily pulled down Bat3 in the immunoprecipitation assay (Figure 1A). Furthermore, this interaction was also confirmed at endogenous protein levels (Figure 1B). Since the non-specific protein–protein binding was remarkably detected in the whole-cell extracts, we used a small-scale biochemical fractionation scheme described previously (Wysocka et al., 2001) and found the specific interaction of endogenous HAUSP with Bat3 in the soluble nuclear (SN) fraction (Figure 1B) in HCT116 cells. In addition, immunofluorescence analysis in both HCT116 and HeLa cells revealed the co-localization of HAUSP and Bat3 mainly in the nucleus (Figure 1C).

Bat3 stabilizes and activates p53 in a HAUSP-dependent manner

To determine the functional consequence of the HAUSP–Bat3 interaction in the p53 pathway, we then tested whether Bat3 affects stabilization and activation of p53. Flag-p53 was co-transfected into HCT116 cells with Ha-HAUSP or/and Ha-Bat3. As indicated in Figure 2A, the overexpression of HAUSP, as expected, effectively inhibited the degradation of p53 in HCT116 cells (lane 2 vs. lane 1) and, notably, Bat3 expression enhanced the steady-state cellular levels of p53 induced by HAUSP in a dose-dependent manner (lanes 3 and 4 vs. lane 2). However, the overexpression of Bat3 alone, without HAUSP, had no obvious effect on the levels of exogenous p53 (lanes 5 and 6 vs. lane 1). Meanwhile, as shown in Figure 2B, the endogenous p53 protein levels following Bat3 overexpression was relatively stable with an increased half-life in comparison with the p53 levels following vector control. The depletion of Bat3 mediated by small interfering RNA (siRNA) could significantly reduce the half-life of p53 but did not affect the stability of HAUSP protein in IMR90 cells (Figure 2C). To further confirm that the observed reduction of p53 half-life was indeed caused by specific depletion of Bat3 and not by off-target effects, we tested whether the phenotype could be rescued by a Bat3 mutant containing two silent point mutations within the siBat3 recognition site (ΔRNAi-Bat3). The half-life of p53 protein was detected in IMR90 cells transfected with siBat3 plus either wild-type Bat3 (WT-Bat3) or ΔRNAi-Bat3. WT-Bat3 could be depleted by siBat3 and not rescue the half-life of p53 (Figure 2D, lanes 1–4). In contrast, ΔRNAi-Bat3 was resistant to siBat3 and restored the protein levels of p53 after cycloheximide (CHX) treatment

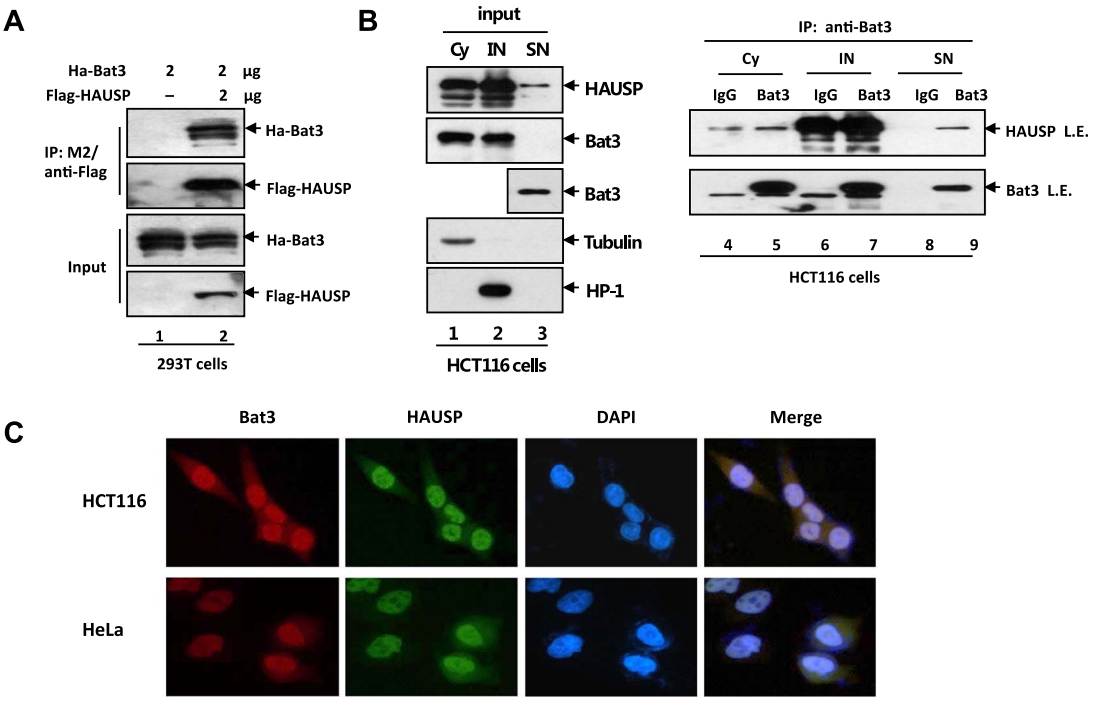

Figure 1 HAUSP interacts with Bat3. (**A**) Exogenous HAUSP interacts with exogenous Bat3. 293T cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. (**B**) Endogenous HAUSP interacts with endogenous Bat3. HCT116 cell extracts were collected and separated through small-scale biochemical fractionation. Each separated fractionation was immunoprecipitated with either Bat3 antibody or control IgG antibody, followed by western blot analysis. Cy, soluble cytosolic fraction; SN, soluble nuclear fraction; IN, insoluble pellet fraction containing chromatin and nuclear matrix components. (**C**) Endogenous HAUSP and Bat3 co-localize in HCT116 and HeLa cells. HAUSP and Bat3 were visualized by immunofluorescence with specific antisera in both HCT116 and HeLa cells. The nucleus was stained with DAPI. Scale bar, 10 μm.

(Figure 2D, lanes 5–8). Next, we examined the half-life of p53 in cells knocking down both Bat3 and HAUSP in order to evaluate the role of HAUSP in Bat3-induced p53 stabilization. As expected and reported previously (Li et al., 2004), the p53 protein was stabilized upon siRNA-mediated depletion of HAUSP because the self-ubiquitinated Mdm2 was extremely unstable upon the silence of HAUSP, leading to indirect p53 stabilization (Figure 2E, lanes 10–12), strikingly, the half-life of endogenous p53 also became long in cells depleting both Bat3 and HAUSP, just like that in cells silencing HAUSP (Figure 2E, lanes 7–9 vs. lanes 10–12), suggesting that Bat3 stabilizes p53 in a HAUSP-dependent manner.

Next, we examined the transcription of p53 target genes using quantitative RT-PCR. We found that levels of p21 and Puma transcripts were markedly increased in cells co-transfected with Bat3 and HAUSP compared with the cells transfected with HAUSP alone (Figure 2F), but both transcripts were not activated by the ectopic expression of Bat3 without HAUSP, suggesting that Bat3 also activates p53-dependent transcriptional activation depending on the presence of HAUSP.

To investigate the biological role of Bat3, we examined its effect on cell proliferation. We found that, in comparison with the cells transfected with HAUSP, overexpression of both Bat3 and HAUSP had a stronger inhibitory effect on the growth of HCT116 cells (Figure 2G). In contrast, the depletion of Bat3 in IMR90 cells promoted the cell growth in the HAUSP-wild-type cells but not in the HAUSP-silent cells (Figure 2H). Meanwhile, the protein levels of p53 and p21 were correspondingly increased in Bat3–HAUSP overexpressing cells (Figure 2G) and decreased in Bat3-depleting cells (Figure 2H). In corroboration of these data, we found that Bat3 stabilized p53 and activated p53-dependent transcriptional activation and cell growth repression depending on the presence of HAUSP.

Bat3-induced p53 stabilization is independent of the deubiquitination of p53 by HAUSP

HAUSP has an intrinsic enzymatic activity that specifically deubiquitinates p53 and strongly stabilizes p53 even in the presence of excess Mdm2 (Li et al., 2002; Brooks et al., 2007). To unravel the mechanism underlying the HAUSP-dependent p53 stabilization induced by Bat3, we next tested whether Bat3 directly influenced the deubiquitination of p53 by HAUSP *in vivo*. We firstly examined the ubiquitination levels of p53 in HCT116 cells by immunoprecipitation of p53 and western blotting with anti-Ha antibody. The result showed that the ubiquitination level of p53, as expected, was abrogated by HAUSP (Figure 3A, lane 1 vs. lane 2), but unexpectedly, the expression of Bat3 increased

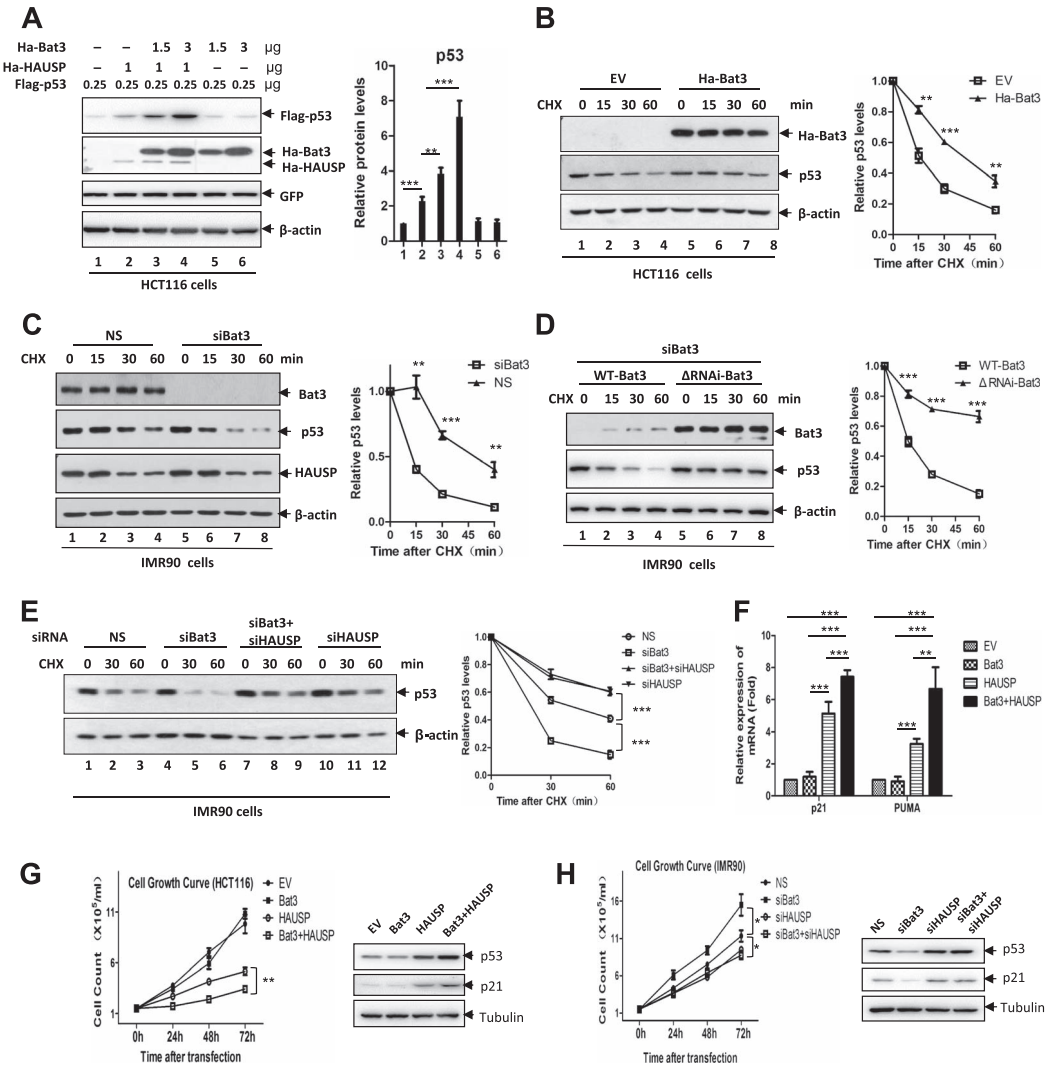

Figure 2 Bat3 stabilizes and activates p53 in a HAUSP-dependent manner. (**A**) Overexpression of Bat3 upregulates p53 in a HAUSP-dependent manner. HCT116 cells transfected with the indicated plasmids were analyzed by western blotting (left panel). Plasmid expressing GFP (0.75 μg) was used as an internal control for transfection efficiency. The total amount of DNA in each transfection was made up to 5 μg with empty vector (EV). p53 protein levels were quantified by densitometry and normalized to the levels of β-actin (right panel). The value of normalized p53 at lane 1 was set at 1.0. Data are mean ± SD from three independent experiments. (**B**) HCT116 cells transfected with EV or Ha-tagged Bat3 were treated with 50 μg/ml CHX for the indicated time periods and then analyzed by western blotting (left panel). p53 protein levels were quantified by densitometry and normalized to the levels of β-actin (right panel). The value of normalized p53 at time 0 was set at 1.0. Data are mean ± SD from three independent experiments. (**C**) Knockdown of Bat3 shortens the half-life of p53. IMR90 cells transfected with non-silencing (NS) or Bat3 siRNA (siBat3) were treated with 50 μg/ml CHX for the indicated time periods. (**D**) Rescue of the half-life of p53 protein by introducing a Bat3 mutant resistant to RNAi (ΔRNAi-Bat3). IMR90 cells subjected to Bat3 KD with siBat3 were transfected with wild-type Bat3 (WT-Bat3) or ΔRNAi-Bat3. The cells were then treated with CHX (50 μg/ml) for the indicated times. (**E**) Knockdown of HAUSP inhibits the shortening of p53 half-life induced by Bat3 silence. IMR90 cells transfected with NS, siBat3, HAUSP siRNA (siHAUSP), or Bat3 plus HAUSP siRNA (siBat3 + siHAUSP) were treated with 50 μg/ml CHX for the indicated time periods. The protein levels of p53 in **C**, **D**, and **E** were determined and quantified as described in **B**. (**F**) Quantitative real-time PCR analysis of p21 and PUMA mRNA in IMR90 cells transfected with the indicated plasmids. Data shown are the relative amounts of specific mRNA normalized to GAPDH. Data are mean ± SD from three independent experiments. (**G**) p53-mediated cell growth repression in HCT116 cells transfected with EV, Bat3, HAUSP, or Bat3 plus HAUSP. The ratio for the transfected Bat3 and HAUSP plasmid is 2:1, and the total amount of DNA in each transfection was made up to 2 μg with EV. (**H**) IMR90 cells transfected with NS, siBat3, siHAUSP, or siBat3 + siHAUSP. Data shown in **G** and **H** are the cell numbers counted at different time points after transfection. The protein levels of p53 and its target gene p21 at 24 h after transfection were detected by western blotting. Statistical analyses were performed using GraphPad Prism software with *t*-test or ANOVA test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

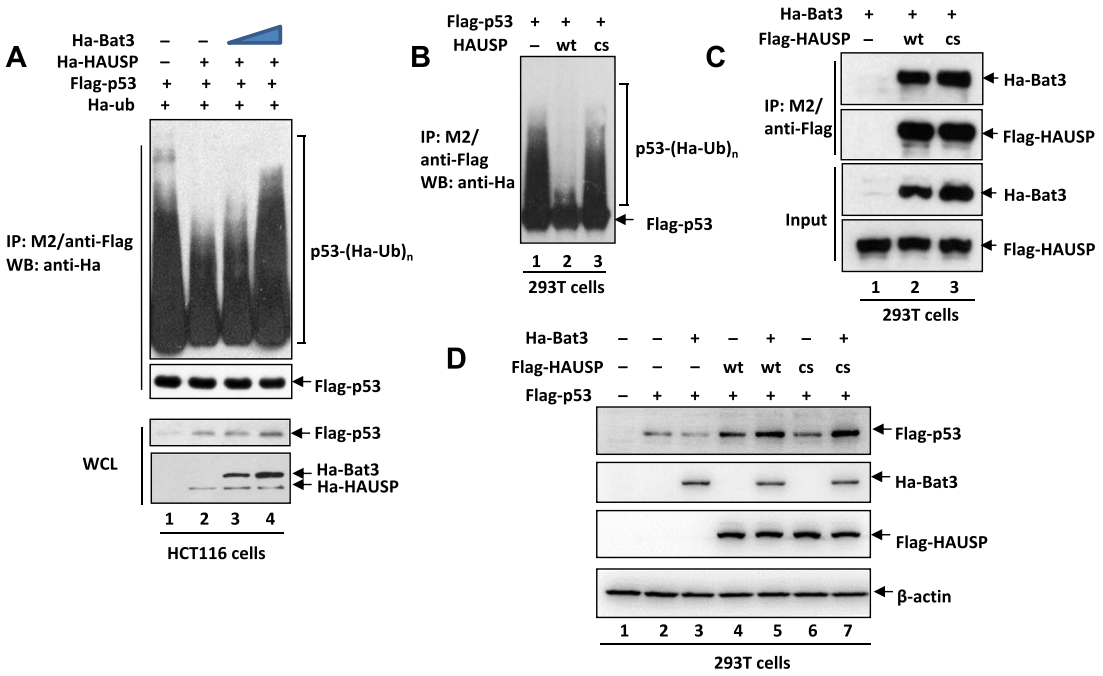

Figure 3 Bat3-induced p53 stabilization is independent of the deubiquitination of p53 by HAUSP. (**A**) The effect of Bat3 on HAUSP-mediated deubiquitination of p53 *in vivo*. HCT116 cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis using anti-Ha monoclonal antibody. All cells were treated with 20 μM MG132 for 4 h before being harvested. WCL, whole-cell lysates. (**B**) A point mutant of HAUSP (HAUSP-cs) was functionally defective in deubiquitinating p53 compared with the wild-type HAUSP (HAUSP-wt) in 293T cells transfected with HAUSP-wt or HAUSP-cs plasmid followed by p53 ubiquitination analysis. (**C**) The enzymatic site mutant of HAUSP (HAUSP-cs) does not affect its interaction with Bat3. 293T cells transfected with either HAUSP-wt or HAUSP-cs, together with Ha-Bat3, were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. (**D**) Overexpression of Bat3 and HAUSP stabilizes p53 independent of the enzymatic activity of HAUSP. 293T cells transfected with the indicated plasmids were analyzed by western blotting.

the levels of p53 ubiquitination in a dose-dependent manner (Figure 3A, lanes 3 and 4 vs. lane 2), suggesting that Bat3 might not stabilize p53 through enhancing the deubiquitinating ability of HAUSP but function as a negative regulator interfering with HAUSP-mediated deubiquitination of p53. To further confirm this result, a HAUSP mutant, HAUSP-cs (Li et al., 2002), which was functionally defective in deubiquitinating (Figure 3B, lane 3) and stabilizing p53 (Figure 3D, lane 6), was constructed by replacing a highly conserved Cys residue at the core domain with the Ser residue. Notably, HAUSP-cs still kept the ability both to interact with Bat3 (Figure 3C, lane 3) and to assist Bat3 for p53 stabilization (Figure 3D, lane 7), indicating that Bat3-induced p53 stabilization does not require the deubiquitinating enzymatic activity of HAUSP.

Bat3 induces HAUSP-mediated Mdm2 stabilization without influencing the p53–Mdm2 interaction and Mdm2-mediated p53 ubiquitination

We then examined whether Mdm2 was also directly affected by the presence of excess Bat3 and HAUSP, as it is a key repressor of p53 and represents a physiological substrate for HAUSP-mediated deubiquitylating activity. Western blot analysis showed that, just like p53, the increased expression of Bat3 in p53-deficient H1299 cells enhanced the Mdm2 stabilization only in the presence of HAUSP expression (Figure 4A). Moreover, the ubiquitination of Mdm2 abrogated by HAUSP was also restored by Bat3 expression in the p53-deficient cell line H1299 (Figure 4B). Also, we examined the half-life of Mdm2 in cells depleting Bat3 and/or HAUSP and found that the endogenous Mdm2 became slightly unstable upon the silence of Bat3 (Figure 4C, lanes 5–8 vs. lanes 1–4), but Bat3 knockdown could not affect the half-life of Mdm2 in the HAUSP-depleted cells (Figure 4C, lanes 13–16 vs. lanes 9–12) due to the extreme instability of Mdm2 in the absence of HAUSP. These data indicated that Bat3 could directly regulate Mdm2 stability and that Bat3–HAUSP-mediated protein stabilization may not be specific to p53.

Since the effect of Bat3 on Mdm2 would likely indirectly affect p53 ubiquitination and degradation in p53 wild-type cells, we next examined the protein levels of Mdm2 and p53 simultaneously in HCT116 cells transfected with Flag-p53, Flag-Mdm2, Ha-HAUSP, or/and Ha-Bat3. As shown in Figure 4C, overexpression of Bat3 effectively enhanced HAUSP-mediated protein stabilization of both p53 and Mdm2 (Figure 4C, lane 5 vs. lane 4), which is consistent with the results in Figures 2A and 4A. However, the protein level of p53 was not

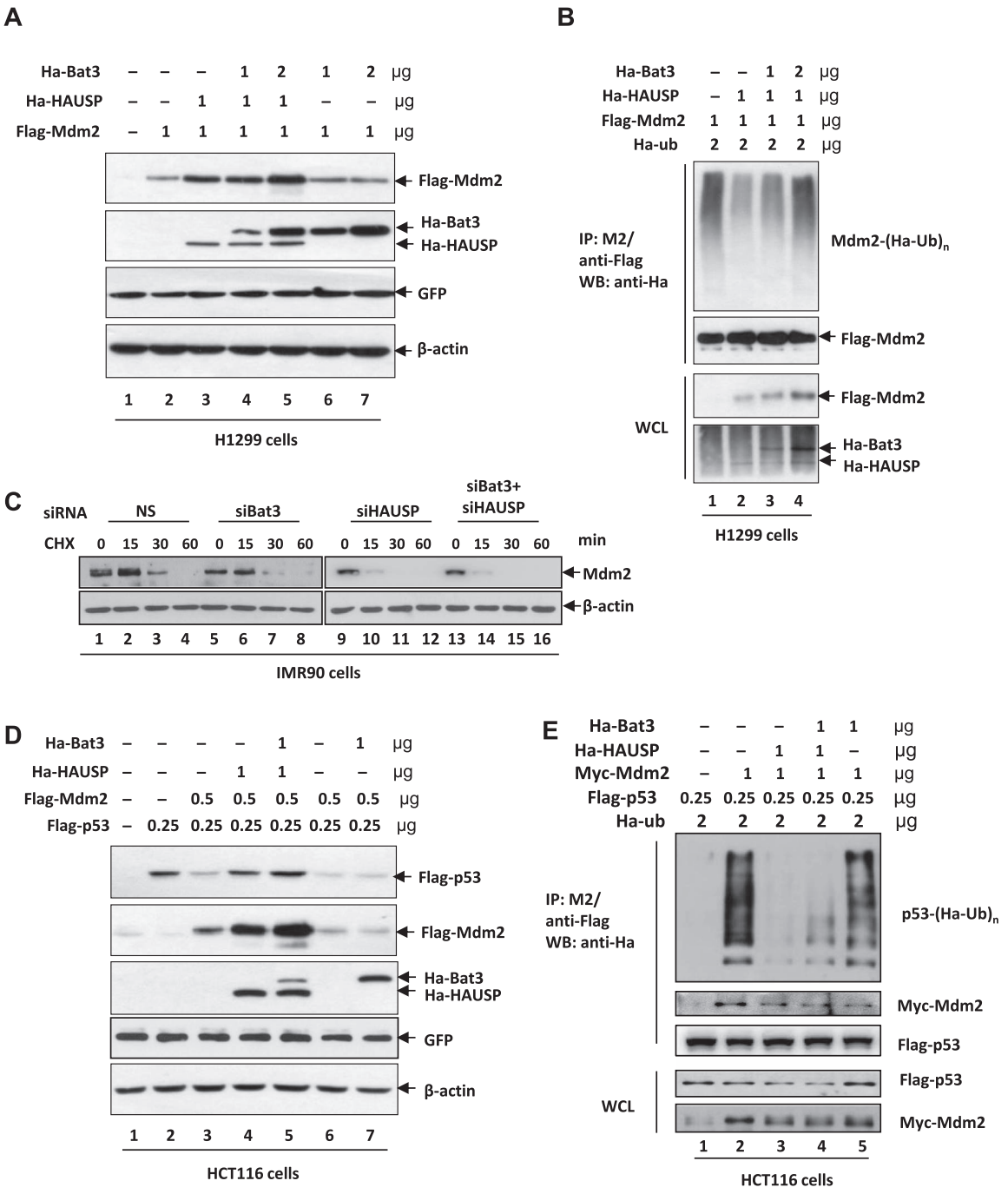

Figure 4 Bat3 induces HAUSP-mediated Mdm2 stabilization without influencing the p53–Mdm2 interaction and Mdm2-mediated p53 ubiquitination. (**A**) Overexpression of Bat3 upregulates Mdm2 in a HAUSP-dependent manner in the p53-deficient cell line. H1299 cells transfected with the indicated plasmids were analyzed by western blotting. (**B**) The effect of Bat3 on HAUSP-mediated deubiquitination of Mdm2 *in vivo*. H1299 cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis using anti-Ha monoclonal antibody. All cells were treated with 20 μM MG132 for 4 h. (**C**) Depletion of Bat3 cannot affect the half-life of Mdm2 in the HAUSP-depleted cells. HCT116 cells transfected with non-silencing siRNA (NS), Bat3 siRNA (siBat3), HAUSP siRNA (siHAUSP), or Bat3 plus HAUSP siRNA (siBat3 + siHAUSP) were treated with 50 μg/ml CHX for the indicated time periods. (**D**) Overexpression of Bat3 upregulates both Mdm2 and p53 proteins depending on the presence of HAUSP in HCT116 cells. HCT116 cells transfected with the indicated plasmids were analyzed by western blotting. (**E**) Bat3 does not influence the p53–Mdm2 interaction and Mdm2-mediated p53 ubiquitination. HCT116 cells were transfected with the indicated plasmids. The interaction of Mdm2 with p53 and the ubiquitination of p53 mediated by Mdm2 were detected by immunoprecipitation and western blotting. All cells were treated with 20 μM MG132 for 4 h.

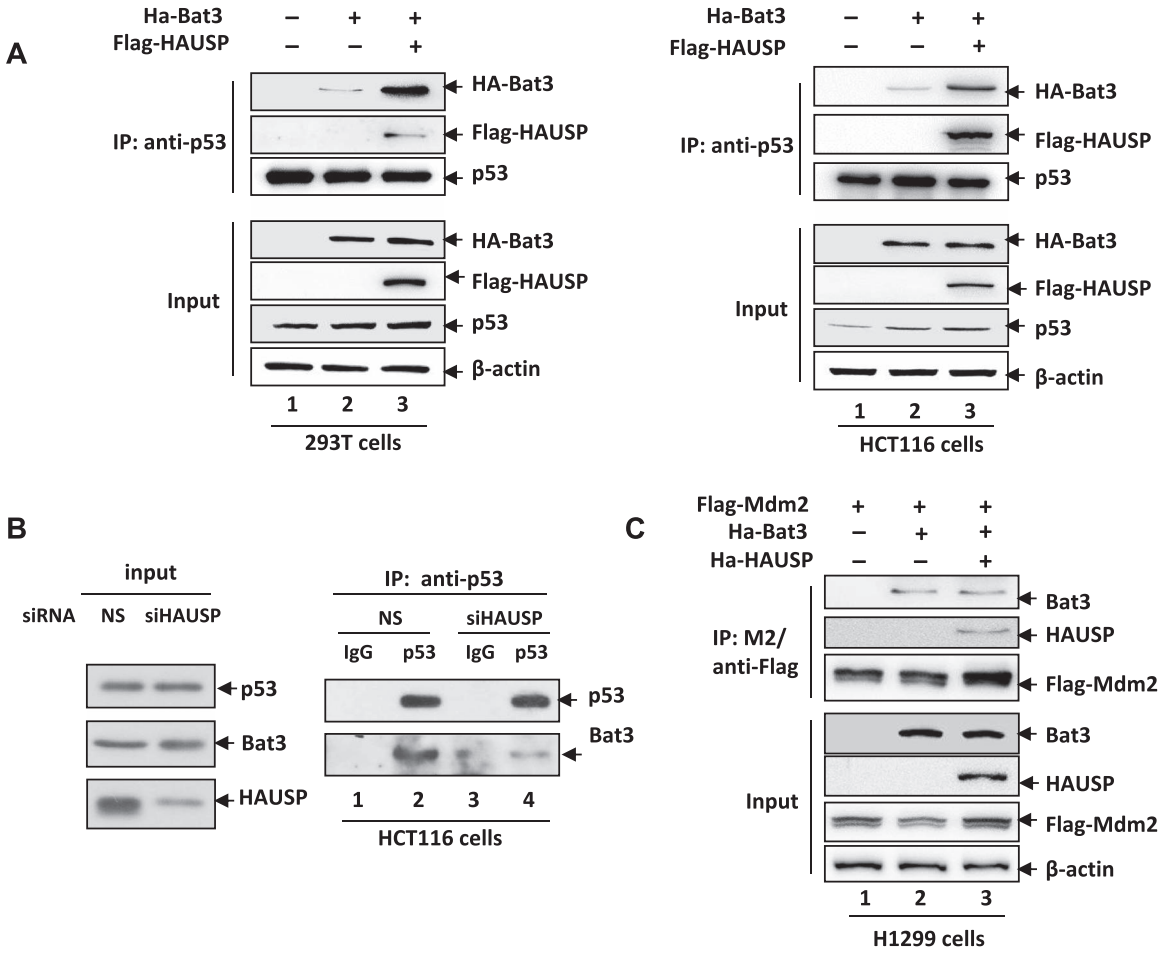

Figure 5 HAUSP enhances the association of p53 with Bat3 without effect on the interaction between Mdm2 and Bat3. (**A**) Overexpression of HAUSP enhances the interaction of p53 with Bat3 *in vivo*. 293T cells (left panel) and HCT116 cells (right panel) were transfected with the indicated plasmids for 24 h and treated with MG132 for 4 h. The whole-cell lysates were immunoprecipitated with p53 monoclonal antibody followed by western blot analysis. (**B**) Knockdown of HAUSP attenuates the interaction of p53 with Bat3 *in vivo*. HCT116 cells were transfected with non-silencing siRNA (NS) or HAUSP siRNA (siHAUSP) for 24 h and treated with MG132 for 4 h. The whole-cell lysates were subjected to immunoprecipitation and western blot analysis. (**C**) Overexpression of HAUSP does not affect the interaction of Mdm2 with Bat3. H1299 cells were transfected with the indicated plasmids for 24 h and treated with MG132 for 4 h. The whole-cell lysates were immunoprecipitated with M2/Flag antibody followed by western blot analysis.

decreased with the increase in Mdm2 protein. Moreover, p53 proteins purified from HCT116 cells transfected with or without Bat3 were readily captured and ubiquitinated by recombinant Mdm2 (Figure 4D, lane 5 vs. lane 2) and deubiquitinated by HAUSP (lane 3 vs. lane 2), but the HAUSP-mediated deubiquitination of p53, to some extent, was inhibited by Bat3 (lane 4 vs. lane 3), which was also in agreement with the data in Figure 3A. Since many E3 ubiquitin ligases, including Mdm2, undergo self-ubiquitination as a means of regulating their own stabilities, the increased amount of Mdm2 levels in Bat3-overexpressing cells may contribute to the increased ubiquitination of both p53 and its own. We speculate that the obstruction of proteasome recognition by Bat3–HAUSP may lead to the accumulation of ubiquitinated p53 and Mdm2 in transfected conditions. Taken together, these data demonstrated that Bat3 induced HAUSP-mediated Mdm2 stabilization without influencing the p53–Mdm2 interaction and Mdm2-mediated p53 ubiquitination.

HAUSP selectively enhances the association of p53 with Bat3 without affecting the interaction between Mdm2 and Bat3

The mechanism for HAUSP stabilizing p53 is attributed to its interaction with the C-terminal region of p53 (Brooks et al., 2007). We thereby examined whether Bat3 interacts with p53. Ha-Bat3 was transfected into 293T cells (Figure 5A, left panel) or HCT116 cells (Figure 5A, right panel) with or without Flag-HAUSP for 24 h followed by the treatment with MG132 for 4 h. Immunoprecipitation of protein extracts with anti-p53 antibody and subsequent immunoblot analysis revealed a trimeric protein interaction between p53, Bat3 and HAUSP

as both HA-Bat3 and Flag-HAUSP were detected in the p53-immunoprecipitated complex (Figure 5A). Surprisingly, the addition of HAUSP led to an enhanced interaction between p53 and Bat3 in both cell lines (Figure 5A, lane 3 vs. lane 2). To confirm that the observed increase in the interaction between p53 and Bat3 is indeed mediated by HAUSP, we knocked down HAUSP using siRNA in HCT116 cells. As a result, co-immunoprecipitation analysis showed that the amount of endogenous Bat3 associated with p53 decreased with HAUSP knockdown (Figure 5B, lane 4 vs. lane 2), indicating that HAUSP may play a role in mediating the association of p53 with Bat3.

We next wished to deduce whether Mdm2, Bat3 and HAUSP could also form a three-protein complex. To avoid that Bat3 and HAUSP may associate with Mdm2 through indirect binding with p53, we performed immunoprecipitation assay in p53-deficient H1299 cells co-transfected with Mdm2, Bat3, and HAUSP. The protein complex of co-immunoprecipitated Flag-Mdm2 contained Bat3 as well as HAUSP (Figure 5C, lane 3); however, HAUSP did not affect the amount of Bat3 pulled down by Flag-Mdm2 (Figure 5C, lane 3 vs. lane 2), indicating that HAUSP selectively enhanced the interaction between Bat3 and p53 but not the interaction between Bat3 and Mdm2.

HAUSP serves as a binding mediator through its interactions with p53 and Bat3

We next asked how HAUSP enhances the interaction of p53 with Bat3 and whether it serves as a binding mediator between the two proteins. As the p53-binding element of HAUSP has been previously mapped to its N-terminal residues 53–208 (Hu et al., 2002; Holowaty et al., 2003), we first sought to identify the functional domain in HAUSP that is responsible for binding to Bat3, and the results are summarized in Figure 6A. The C-terminus of HAUSP was found to form a stable complex with Bat3 in 293T cells co-transfected with Ha-Bat3 and Flag-tagged HAUSP deletion mutants (Figure 6B, lane 5). Then, we examined whether both p53- and Bat3-binding regions of HAUSP were required for its functions in increasing the interaction of p53 with Bat3 and in mediating the Bat3-induced p53 stabilization. The immunoprecipitation assay showed that the deletion mutants of HAUSP lacing either the p53-binding region or Bat3-binding region lost its ability to enhance the association of p53 with Bat3 (Figure 6C, lanes 4–6 vs. lane 3). To confirm this phenomenon, we examined the direct interactions between p53 and Bat3 by mixing bacterially purified His-Bat3 and GST-p53 with Flag antibody-purified HAUSP or its deletion mutants followed by GST pull-down assay *in vitro*. Western blot analysis showed that the increased amount of Bat3 could be pulled down specifically by GST–p53 only in the presence of full-length HAUSP, whereas not in other deletion mutants (Figure 6D). Consistent with this result, the western blot analysis also demonstrated that Bat3 stabilized p53 protein only in the presence of full-length of HAUSP instead of in the presence of any deletion mutants of HAUSP (Figure 6E, lanes 4-6 vs. lane 3). These results suggest that HAUSP may serve as a binding mediator of p53 and Bat3, resulting in the increased interaction between the two proteins and subsequent p53 stabilization induced by Bat3.

Amino acids 1–255 in the N-terminal region of Bat3 are required for its co-mediated p53 stabilization with HAUSP

To further determine which region/domain of Bat3 is involved in the p53–HAUSP–Bat3 complex formation, we generated a series of deletion mutants (Figure 7A) and measured their interaction with either HAUSP or p53 using co-immunoprecipitation assay. The N-terminal region of Bat3 containing an ubiquitin-linked domain was found to have capacity to form a stable complex with both HAUSP (Figure 7B, lane 3) and p53 (Figure 7C, lane 3), but other deletions, which are lacking the N-terminus, completely abolish the ability to form complexes (Figure 7B, lanes 4 and 5; Figure 7C, lanes 5 and 6). Accordingly, the 1–255 and 1–753 deletion mutants of Bat3 retained the ability to stabilize p53 by addition of HAUSP (Figure 7D, lanes 5 and 6), indicating that amino acids 1–255 in the N-terminal region of Bat3 can effectively induce p53 stabilization mediated by HAUSP, probably by forming a three-protein complex with these two proteins.

p53–HAUSP–Bat3 complex prevents p53 from binding to proteasome receptor S5a and promotes the accumulation of p53 in nucleus

The stabilization and degradation of p53 are regulated by the ubiquitin–proteasome system, which has the ability to capture substrates by recognizing their covalently linked polyUbs. S5a, also known as Rpn10/PSMD4, has been revealed to be an intrinsic subunit of 26S proteasome that plays a key role in the recognition and recruitment of ubiquitinated p53 (Elangovan et al., 2009, 2010; Sparks et al., 2014). To investigate whether Bat3–HAUSP induces p53 stabilization through interfering with its recognition to S5a, we examined the interaction of S5a with p53 in HCT116 cells transfected with/without Bat3 and HAUSP by immunoprecipitation assay. Surprisingly, Flag-tagged S5a readily interacted with p53 in the absence of the exogenous HA-Bat3 and HA-HAUSP (Figure 8A, lanes 1) and in the presence of either HA-Bat3 or HA-HAUSP (Figure 8A, lanes 2 and 3), but failed to bind to p53 when both proteins were co-overexpressed in HCT116 cells (Figure 8A, lane 4). This disrupted interaction between p53 and S5a was also confirmed at endogenous protein levels (Figure 8B), in which the interactions of endogenous p53 and S5a were monitored in the S5a-immunoprecipitated complex pulled down from HCT116 cells transfected with HA-Bat3 and/or HA-HAUSP. In addition, just like the phenomenon observed in cells knocking down S5a (Sparks et al., 2014), the ectopic expression of Bat3 and HAUSP resulted in the accumulation of p53 in the nucleus (Figure 8C). To further confirm this phenomenon, we then tested the localization of p53–HAUSP–Bat3 complex in HCT116 cells transfected with HA-Bat3 and Flag-HAUSP using cellular fractionation followed by immunoprecipitation and western blotting. As seen in Figure 8D, the majority of p53–HAUSP–Bat3 complex existed in the nucleus

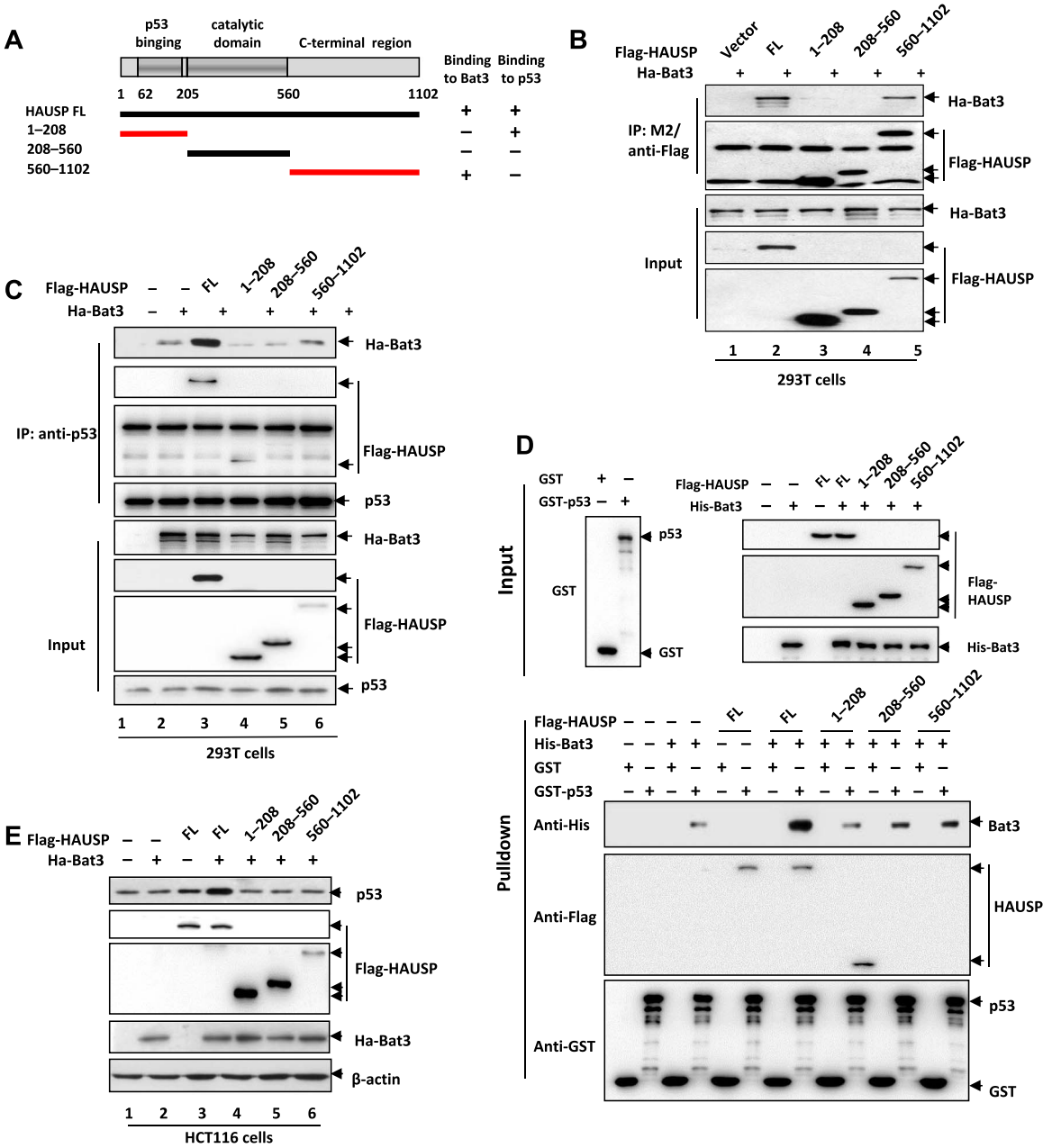

Figure 6 HAUSP serves as a binding mediator through its interactions with p53 and Bat3. (**A**) Schematic diagram illustrating the full-length (FL) and truncated mutants of HAUSP. The binding ability of each truncated mutant to Bat3 and p53 is indicated. (**B**) Mapping the domain of HAUSP binding to Bat3. 293T cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. (**C**) Overexpression of the HAUSP truncated mutants fails to enhance the interaction of p53 with Bat3. 293T cells transfected with either FL or truncated Flag-tagged HAUSP, together with Ha-Bat3, were immunoprecipitated with p53 antibody, followed by western blot analysis. (**D**) HAUSP-truncated proteins fail to enhance the interaction of p53 with Bat3 *in vitro*. Purified His-Bat3 protein, Flag-tagged HAUSP-truncated proteins, and GST-tagged p53 protein or GST control protein were incubated in PBS buffer with glutathione agarose for 2 h at 4°C. The incubated proteins were then immunoblotted using anti-His, Flag, or GST antibodies. (**E**) Overexpression of Bat3- and HAUSP-truncated mutants fails to stabilize p53. HCT116 cells transfected with either FL or truncated Flag-tagged HAUSP, together with Ha-Bat3, were analyzed by western blotting.

of HCT116 cells (lower panel, lane 4), and the percentage of p53 proteins in nucleus increased accordingly in whole-cell lysates (upper panel, lanes 4 and 8). These results suggest that the formation of p53–HAUSP–Bat3 complex may prevent p53 from being recognized and binding to its proteasome receptor S5a, subsequently leading to the accumulation and stabilization of p53 in nucleus (Figure 8E) though we still do not know whether there is conformational change in Bat3 and p53 at present.

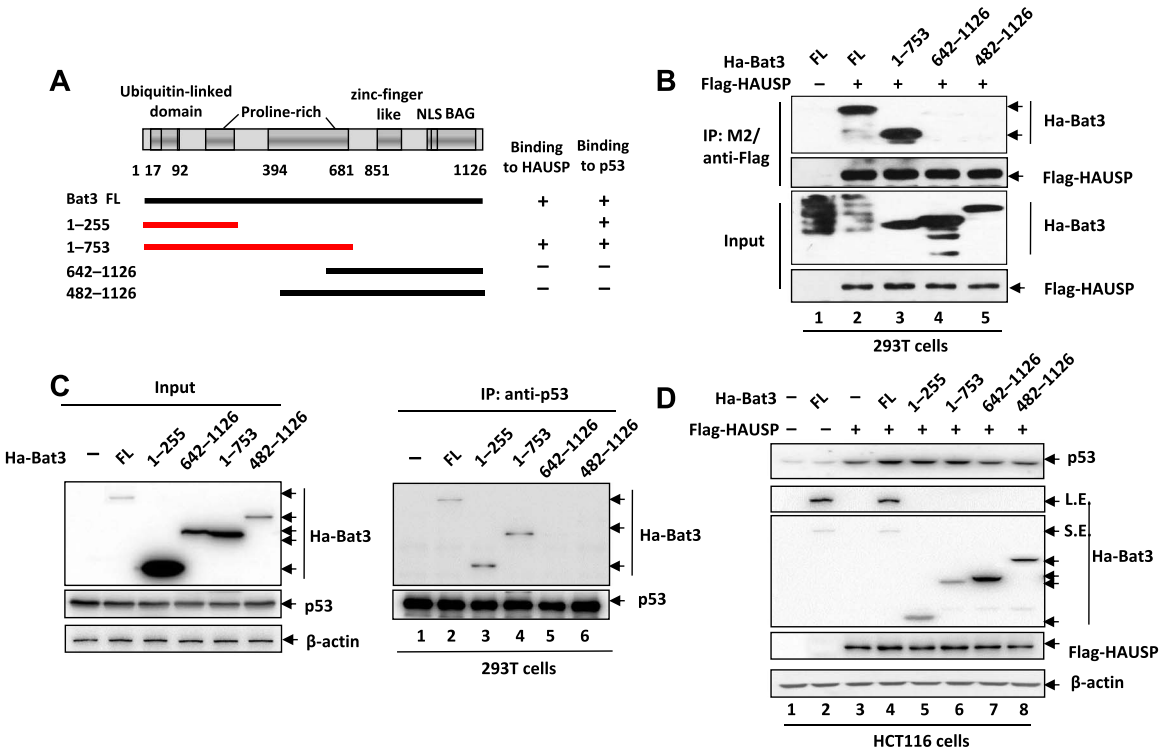

Figure 7 Amino acids 1–255 in the N-terminal region of Bat3 are required for its co-mediated p53 stabilization with HAUSP. (**A**) Schematic diagram illustrating the full-length (FL) and truncated mutants of Bat3. The binding ability of each truncated mutant to HAUSP and p53 is indicated. (**B**) Mapping the domain of Bat3 binding to HAUSP. 293T cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. (**C**) Mapping the domain of Bat3 binding to p53. 293T were transfected with the indicated plasmids for 24 h and treated with MG132 for 4 h. The whole-cell lysates were immunoprecipitated with p53 monoclonal antibody followed by western blot analysis. (**D**) HCT116 cells transfected with either FL or truncated Flag-tagged Bat3, together with Flag-HAUSP, were analyzed by western blotting. L.E., long time exposure; S.E., short time exposure.

Discussion

In this study, we found that the role of Bat3 in stabilization and activation of p53 depended on the presence of HAUSP and started to unravel the molecular mechanism underlying this phenomenon. We first showed that Bat3 extended the half-life of p53 and activated p53-dependent transcription and cell growth repression by forming a complex with HAUSP and p53 (Figures 2, 5, and 6). It has been reported that Bat3 acts as a cofactor to enhance p53 transactivation through a Bat3–p300 connection and subsequent p53 acetylation in response to DNA damage (Sasaki et al., 2007). Our findings support the role of Bat3 in modulating the activity of p53 but provides the important evidences for a novel mechanism by which HAUSP serves as a binding mediator of Bat3 and p53 leading to the enhancement of Bat3–p53 association and the increment of p53 nuclear accumulation.

HAUSP is known as a major regulator of Mdm2 and, consequently, p53 levels. Under normal conditions, HAUSP preferably binds to and deubiquitilates Mdm2, which leads to p53 ubiquitination and downregulation. Upon DNA damage, the binding affinity of HAUSP to Mdm2 is greatly reduced due to ATM-mediated Mdm2 phosphorylation allowing for HAUSP to preferably stabilize p53 (Meulmeester et al., 2005; Pereg et al., 2005). The Bat3-mediated function described here provides an interesting example that adds a layer of feedback regulation to balance the HAUSP–p53–Mdm2 network. In transfected conditions, HAUSP selectively enhanced the interaction of Bat3 and p53 but not Mdm2 (Figure 5), leading to the failure of p53 to bind to proteasomal receptor and suffer from degradation. We hypothesized that, under the normal condition, Bat3 mainly targeted and stabilized p53 through indirectly interacting with HAUSP, consequently upregulating the Mdm2 level, because the net outcome of Bat3 overexpression is stabilization and functional activation of p53 (Figure 2). However, under the DNA damage condition, in agreement with previous reports (Sasaki et al., 2007), Bat3 did not affect the stability of p53 protein when it had already been upregulated (Supplementary Figure S1). This suggests that Bat3 plays a role in maintaining the steady-state cellular levels of p53.

Bat3 is a cytoplasmic-nucleo shuttling protein containing a nuclear localization signal (NLS). In normal cells, endogenous Bat3 localizes in both cytoplasm and nucleus (Wang et al., 2011) and functions as a quality control and anti-apoptotic regulator. Under some stress conditions, the nuclear Bat3 levels were observed to increase (Manchen and Hubberstey, 2001; Tsukahara et al., 2009), and Bat3 is capable of modulating transactivation of methylase SET1A (Nguyen et al., 2008) and

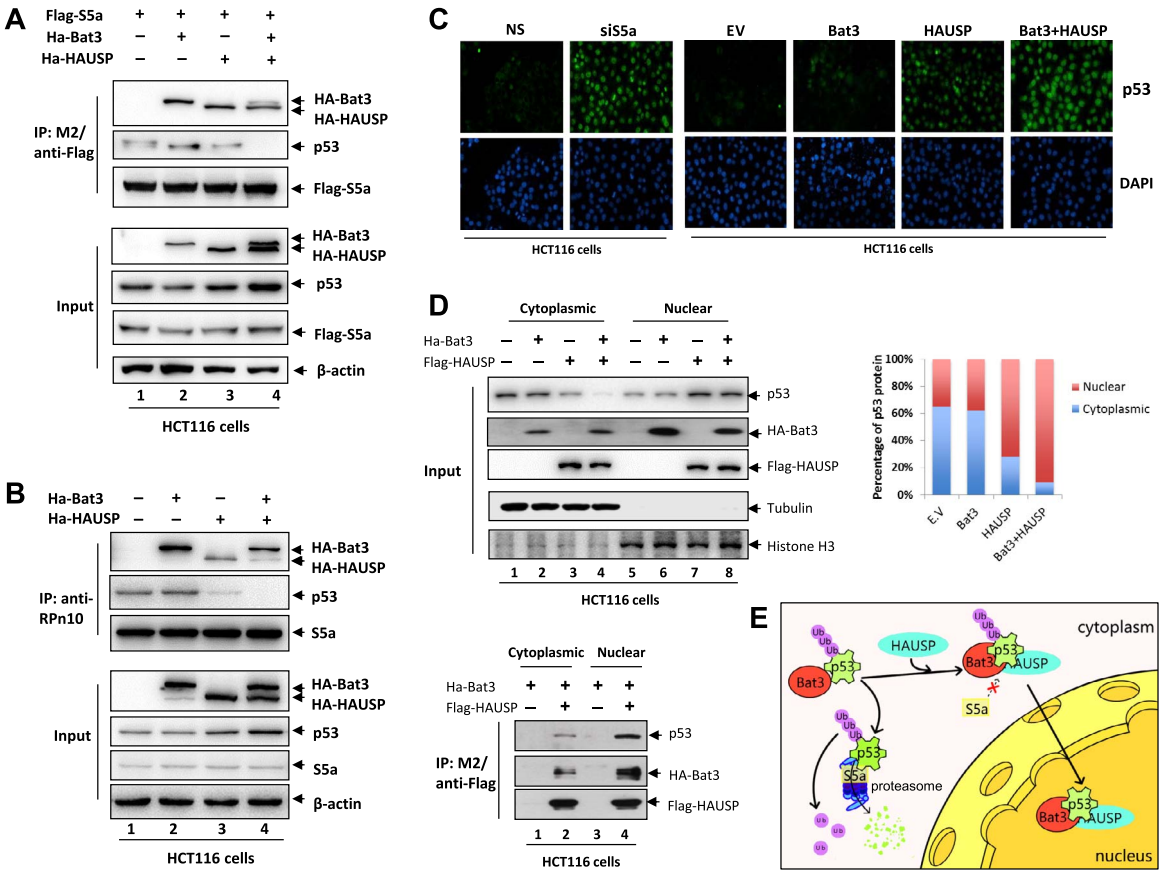

Figure 8 p53–HAUSP–Bat3 complex prevents p53 from binding to proteasome receptor S5a and promotes the accumulation of p53 in the nucleus. (**A**) Overexpression of Bat3 and HAUSP inhibits the interaction of exogenous S5a with p53. HCT116 cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. (**B**) Overexpression of Bat3 and HAUSP inhibits the interaction of endogenous S5a with p53. HCT116 cells transfected with the indicated plasmids were immunoprecipitated with PRn10 antibody, followed by western blot analysis. (**C**) Overexpression of Bat3 and HAUSP causes the accumulation of p53 in the nucleus. HCT116 cells transfected with either EV or the indicated plasmids were analyzed by immunofluorescence to detect endogenous p53 (shown in green) and DNA was stained with DAPI (shown in blue). (**D**) p53–HAUSP–Bat3 complex is mainly localized in the nucleus and promotes the nuclear accumulation of p53. The nuclear and cytoplasmic extracts from HCT116 cells transfected with the indicated plasmids were immunoprecipitated with M2/Flag antibody, followed by western blot analysis. All cells were treated with 20 μM MG132 for 4 h. The percentage of p53 protein levels in nuclear and cytoplasmic fractionations of whole-cell lysates was quantified by densitometry and normalized to the levels of tubulin or histone H3 (right panel). (**E**) Schematic diagram of a model for the proposed mechanism by which Bat3 stabilizes and activates p53 in a HAUSP-dependent manner. A p53–HAUSP–Bat3 trimeric protein complex exists *in vivo*. HAUSP may serve as a binding mediator through its interactions with p53 and Bat3, which enhances the association of p53 and Bat3 and prevents p53 from binding to the proteasome receptor S5a.

DOT1L (Wakeman et al., 2012) and tumor suppressor p53 (Sasaki et al., 2007; Sebti et al., 2014a, b). We found that Bat3 specifically interacted with HAUSP in the SN fraction and targeted p53 for nuclear accumulation. Meanwhile, it also had a capacity to interfere with HAUSP-mediated deubiquitination of p53 in the cytoplasm. These data suggest that Bat3 has both nuclear and cytosolic functions in tumor cells. Currently, we do not exactly know how Bat3 balances the stabilization and degradation of p53 under different conditions, but the region that is required for stabilizing p53 and associating with HAUSP superficially overlaps with the ubiquitin-linked domain of Bat3 (Figure 7). The N terminus of Bat3 containing the evolutionarily conserved ubiquitin-linked domain has been reported to play a key role in the recognition of polyubiquitinated polypeptides as well as potential defective proteins with hydrophobicity stretches (Minami et al., 2010; Tanaka et al., 2016), which improves their assembly, solubilization, and/or degradation efficiency (Wang et al., 2011; Xu et al., 2012). It is likely that, through the conserved N-terminal domain, Bat3, acting as a multifunctional cofactor, can directly or indirectly mediate interactions of p53 with several different proteins that function to mediate stabilization (such as HAUSP), transactivation (such as CBP/p300), and/or ubiquitinational degradation (such as Mdm2) under different conditions.

Although HAUSP has been an attractive regulator for p53 and Mdm2 stability, its ability to function in this capacity, and the underlying mechanism, was still not fully understood. Several proteins have been shown to interact with HAUSP for targeting p53–Mdm2 pathways. For instance, the breast cancer marker protein TSPYL5 has capacity to interact with HAUSP and reduce its deubiquitination activity towards p53, resulting in increased p53 ubiquitination. (Epping et al., 2011). Tang et al. found that Daxx directed HAUSP toward its associated protein Mdm2 and MdmX, which leads to increasing Mdm2 ligase activity towards p53 (Tang et al., 2006). Here, we identified Bat3 as a novel HAUSP-interacting protein and found it to be capable of inducing p53 stabilization and activation via a HAUSP-dependent mechanism. However, unlike other proteins associated with HAUSP, the regulation of Bat3 on p53 did not rely on the deubiquitylating enzymatic activity of HAUSP. We found that the ubiquitin-like domain of Bat3 was required for simultaneous association with both p53 and HAUSP, thus enabling the formation of a triple complex. The formation of this complex enhanced the interaction of p53 and Bat3, thereby preventing p53 from recognizing its proteasome receptor S5a and subsequent degradation. This model provides molecular insights into the function of HAUSP independent of the enzymatic activity by focusing Bat3 as an example, which may be represent a novel mechanism mediated by HAUSP to target the p53–Mdm2 pathway.

It has been reported that proteasomal recognition of p53 and Mdm2 was mediated by different pathways. S5a selectively participates in the recognition of ubiquitinated p53 by the proteasome but not in the recognition of Mdm2 (Mayor et al., 2007; Sparks et al., 2014). In support of previous studies, our model stipulates that the HAUSP association selectively enhances the binding of Bat3 to p53, which prevented p53 from the degradation mediated by the S5a receptor pathway, but not Mdm2, suggesting that Bat3 may, cooperating with HAUSP, affect p53 and its major E3 ligase Mdm2 through different pathways. This stabilization mechanism mediated by Bat3 selectively targeting S5a may result in more precise and efficient modulation of p53. How the association of HAUSP stabilizes the Bat3–p53 complex and prevents its binding to S5a and how it directs Bat3 towards p53 but not Mdm2 are questions that will be answered by future structural and biochemical studies.

In conclusion, we provided evidence to suggest that Bat3 stabilizes and activates p53 in a HAUSP-dependent mechanism. Since p53 plays a fundamental role in tumor control, and the Bat3 gene has been found to have biallelic inactivating mutations in several colon cancer cell lines (Ivanov et al., 2007), it will be important to investigate the potential role of Bat3 in tumorigenesis and human diseases.

Materials and methods

Cell culture and plasmids

Human 293T, HCT116, H1299, and IMR90 cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and penicillin (100 U/ml)–streptomycin (100 µg/ml) at 37°C in 5% CO_2. Plasmids expressing Flag-p53, Flag-Mdm2, Ha-HAUSP, Flag-HAUSPwt, Flag-HAUSPcs, Ha-ub, and all truncation mutants of Flag-HAUSP were described previously (Li et al., 2002; Tang et al., 2006). Ha-Bat3 was from Dr Tomohide Tsukahara (Sapporo Medical University). Plasmids expressing Flag-tagged S5a and all truncation mutants of Ha-Bat3 were generated by PCR and cloned into the pRK5 vector. Primer sequences are in the Supplemental Material. The Bat3 mutant resistant to siBat3 (ΔRNAi-Bat3) was generated by introducing two silent point mutations into the siBat3 recognition site with the oligonucleotide 5′-GGACAAATTTGGAATTTCTCCAA-3′ and was verified by sequencing.

Antibodies and drugs

The following antibodies were used for immunoprecipitation and western blotting: p53 (DO-1, FL-393) and HA (Y-11) from Santa Cruz Biotechnology; p21 from BD Biosciences; β-actin (1–19) and S5a from Cell Signaling Technology; human Mdm2 (Ab-1) from Calbiochem; Flag (M2) and tubulin from Sigma; Bat3 (B01P) from Abnova; HAUSP (BL851) from Bethyl Laboratories; CHX from Amresco; and MG132 from Sigma.

RNA interference and transfection

The siRNA target sequences for endogenous Bat3 were 5′-GGACAAACCUGGAAUUUCUUU-3′; endogenous HAUSP were 5′-CCCAAATTATTCCGCGGCAAA-3′. The non-silencing sequences were sense 5′-UUCUCCGAACGUGUCACGU-3′ and antisense 5′-ACGUGACACGUUCGGAGAA-3′. siRNA was transfected using Lipofectamine RNAiMAX for two or three rounds in each experiment, and plasmids were transfected using Lipofectamine 3000 (Life Technologies) or jetPRIME *in vitro* DNA and siRNA transfection reagent (Polyplus-transfection) according to the manufacturer's instruction. Plasmid expressing GFP was used as an internal control to monitor the transfection efficiency. The total amount of DNA in each transfection was kept constant using empty vector.

Immunofluorescence microscopy

The cells cultured on glass coverslips were transfected with siRNA or plasmids. At 24 h post transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 on ice, blocked with 1% bovine serum albumin, and incubated with antibody against p53 (FL-393), followed by a FITC-conjugated IgG antibody. Nuclei were stained with DAPI. Images were captured using a Nikon Eclipse Ni-E microscope.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting were performed as described previously (Tang et al., 2006; Cui et al., 2014). Briefly, 24–48 h after transfection with siRNA or plasmids, cells were lysed in lysis buffer (50 mM Tris–Cl at pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, protease inhibitor cocktail and 10% glycerol) and pre-cleared with protein G Sepharose beads (GE Healthcare) for 2 h at 4°C. The lysates were then immunoprecipitated with indicated antibodies plus protein G Sepharose

overnight or M2 beads (Sigma) for 4 h at 4°C. Beads were washed and boiled. Protein samples were analyz by western blotting.

For western blotting, cells were prepared using lysis buffer (50 mM Tris–Cl at pH 8.0, 150 mM NaCl, 1.0% NP-40, 10% glycerol, 20 mM NaF, 1 mM DTT) supplemented with protease inhibitors. The protein samples were resolved by SDS–PAGE and transferred onto nitrocellulose membranes, which were blocked in 5% skim milk in PBST (PBS containing 0.5% of Tween-20) and probed with the indicated antibodies.

Ubiquitination assay

Ubiquitination assay *in vivo* was conducted as described previously (Tang et al., 2006; Cui et al., 2014). In brief, cells were transfected with the indicated plasmids for 20 h followed by treatment with MG132 for 4 h, then lysed in 1% SDS. After boiling for 10 min, cell lysates were diluted 10 times with cold lysis buffer supplemented with a protease inhibitor cocktail and 10 mM N-ethylmaleimide (Sigma) and were immunoprecipitated with M2 beads (Sigma) for 4 h at 4°C. The levels of ubiquitination were detected by western blot analysis probed with anti-Ha antibody.

GST pulldown assay

Purified His-Bat3 protein and Flag-HAUSP-truncated proteins were incubated with GST-tagged p53 protein or GST control protein in PBS buffer with glutathione agarose (GE Healthcare) for 2 h at 4°C. The incubated proteins were then washed and immunoblotted using anti-His, anti-Flag, or anti-GST antibodies.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNAs from cells were extracted with TRIzol (Invitrogen) following the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the QuantiTect Reverse Transcription kit (TaKaRa). Quantitative RT-PCR (qPCR) reaction was performed by using a Fast SYBR Mixture (TaKaRa) and in a ViiATM 7 Real-Time PCR System (Applied Biosystems). Each reaction was performed in triplicate, and the results were normalized to the expression of the housekeeping gene GAPDH. Primer sequences are in the Supplementary material.

Cell growth and proliferation assay

HCT116 cells transfected with the indicated plasmids were seeded at 10^5 cells per well and grown in 6-well plates. IMR90 cells transfected with the indicated siRNAs were seeded at 5×10^4 cells per well and grown in 12-well plates. At 0, 24, 48, or 72 h post transfection, cells were washed with PBS twice and the cell numbers were counted using a cell counting instrument (Countstar, Zhuoyuelianhe Biotech). All the cells transfected with the indicated plasmids or siRNA for 24 h were lysed for western blot analysis.

Small-scale biochemical fractionation

Small-scale biochemical fractionation was performed as described by Wysocka et al. (2001), and the details are in the Supplemental Material.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software with Student's *t*-test or ANOVA test on least three independent replicates. A *P*-value of less than 0.05 was considered to be statistically significant for each test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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Conflict of interest: none declared.

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