Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization

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The p53 tumour suppressor is a short-lived protein that is maintained at low levels in normal cells by Mdm2-mediated ubiquitination and subsequent proteolysis1–3. Stabilization of p53 is crucial for its tumour suppressor function1–5. However, the precise mechanism by which ubiquitinated p53 levels are regulated in vivo is not completely understood. By mass spectrometry of affinity-purified p53-associated factors, we have identified herpesvirus-associated ubiquitin-specific protease (HAUSP) as a novel p53-interacting protein. HAUSP strongly stabilizes p53.


even in the presence of excess Mdm2, and also induces p53-dependent cell growth repression and apoptosis. Significantly, HAUSP has an intrinsic enzymatic activity that specifically deubiquitinates p53 both in vitro and in vivo. In contrast, expression of a catalytically inactive point mutant of HAUSP in cells increases the levels of p53 ubiquitination and destabilizes p53. These findings reveal an important mechanism by which p53 can be stabilized by direct deubiquitination and also imply that HAUSP might function as a tumour suppressor in vivo through the stabilization of p53.

By serving as a signal for specific cellular protein degradation, ubiquitination is crucial in the physiological regulation of many cellular processes\(^1\)–\(^4\). The ubiquitination of p53 was first discovered in papilloma-virus-infected cells through the functions mediated by the viral E6 protein\(^5\); however, in normal cells, Mdm2 functions as a ubiquitin ligase (E3) that directly mediates the ubiquitination and subsequent degradation of p53 (refs 11–13). Numerous studies imply the existence of multiple pathways involved in p53 stabilization\(^14\)–\(^19\). In response to DNA damage, p53 is phosphorylated at multiple sites, and these phosphorylation events promote p53 stabilization by preventing the binding with Mdm2 and rendering p53 more resistant to Mdm2-mediated degradation\(^14\)–\(^15\). Furthermore, through inhibiting Mdm2-mediated ubiquitin ligase activity, the p14\(^{ARF}\) tumour suppressor can stabilize p53 in vivo in response to oncogene activation\(^20\). Overall, regulation of the p53 ubiquitination levels is of intense interest but remains less well understood.

Using a biochemical purification method with glutathione S-transferase (GST)–p53 affinity chromatography (see Methods and refs 21,22), we have identified a novel p53-binding protein from nuclear extracts of human lung carcinoma cells (H1299). As indicated in Fig. 1a, there are several proteins present in the fractions eluted from the GST–p53 affinity column as well as other columns. Strikingly, only one protein, p135 (relative molecular mass \(\sim 135,000\) (Mr, 135K)) was specifically present in the associated factors obtained from the GST–p53 column but not from either the GST column or the control column (Fig. 1a, compare lane 3 with lanes 1 and 2). After a large preparation, purification of HAUSP, and interactions between p53 and HAUSP. Silver staining analysis of an SDS–PAGE gel containing the eluates from the indicated columns. Peptide sequences derived from the p135 protein band were obtained by mass spectrometry. b, p53 interacts with HAUSP in cells. Western blot analysis of the whole cell extract (WCE) or immunoprecipitates (IP/M2) from the transfected cells by anti-p53 monoclonal antibody (DO-1). c, Interaction between endogenous p53 and HAUSP proteins. The top panel shows a western blot analysis of control immunoprecipitates with the pre-immune serum (lane 1) or immunoprecipitates with the anti-HAUSP antibody (IP/HAUSP) from H460 cells that were either untreated (lane 3) or treated with a DNA damage reagent (etoposide) (lane 2) or a proteasome inhibitor (LNL) (lane 4). The middle and bottom panels show similar analyses of nuclear extracts by anti-p53 (middle) or anti-actin monoclonal antibody (bottom).

Figure 2 HAUSP interacts with and stabilizes p53 in vivo. a, b, Enhancement of the steady-state levels of p53 (a), but not p27 (b), by HAUSP. Western blot analysis of cell extracts from the H1299 cells transfected with p53 alone (lanes 1 and 3), or with p53 and HAUSP together (lanes 2 and 4), with anti-p53 monoclonal antibody (DO-1) (a). Western blot analysis of cell extracts from the H1299 cells transfected with p27 alone (lanes 1 and 3), or with p27 and HAUSP together (lanes 2 and 4), with anti-p27 monoclonal antibody (b). c, Protection of p53 from Mdm2-mediated degradation by HAUSP. Western blot analysis of extracts from the cells transfected with p53 (lane 1), with p53 and HAUSP together (lane 2) or with p53 and Mdm2 together (lane 3), or in combination with different amounts of HAUSP (lanes 4–6), with anti-p53 monoclonal antibody (DO-1). d, Regulation of the expression levels of endogenous proteins by HAUSP. Cell extracts from both mock-infected and pBabe-HAUSP–infected IMR-90 cells were analysed for expression levels of each protein by western blot analysis. e, Regulation of the half-life of endogenous p53 by HAUSP. Cell extracts from both mock-infected and pBabe-HAUSP–infected IMR-90 cells, harvested at different time points as indicated after pretreatment with cycloheximide were analysed for p53 protein levels by western blotting with anti-p53 monoclonal antibody (DO-1). f, Regulation of the ubiquitination levels of endogenous p53 by HAUSP. Cell extracts from both mock-infected cells and pBabe-HAUSP–infected IMR-90 cells pretreated with LNL for 4 h were first immunoprecipitated with anti-p53 polyclonal antibody, then analysed for ubiquitination levels by western blotting with anti-p53 monoclonal antibody (DO-1).
enough material of the p135 band was obtained for mass spectrometry; five peptide sequences were obtained, all of which were derived from the herpesvirus protein Vmw110-associated cellular factor known as HAUSP (also known as human USP7 (ref. 6)). HAUSP belongs to the ubiquitin-specific processing protease (UBP) family of deubiquitination enzymes (DUBs) and contains the characteristic Cys and His motifs at the core enzymatic domain. Interestingly, the amino-terminal and carboxy-terminal extensions of HAUSP with no significant homology to other members of the UBP family, which are thought to be critical for the substrate specificity, bind directly to p53 in vitro (see Supplementary Information). To evaluate interactions in vivo by immunoprecipitation analysis, p53-null cells (H1299) were transfected with p53 and a Flag-tagged HAUSP expression vector. As shown in Fig. 1b, p53 was readily immunoprecipitated from the cells transfected with both Flag-HAUSP and p53 (lane 4) but not from cells transfected with p53 alone (lane 2). By using the HAUSP-specific antibody, we also examined the interaction between the endogenous p53 and HAUSP proteins. Western blot analysis showed that p53 was present in the anti-HAUSP immunoprecipitates from cell extracts of human lung carcinoma cells (H460), but not in the control immunoprecipitates obtained with the preimmune serum (Fig. 1c). This interaction was strongly detected in cells subjected to genotoxic stress (Fig. 1c, compare lanes 2 and 3), whereas only a slight enhancement was detected in the cells treated with a proteasome inhibitor LLNL (lane 4). These results indicate that p53 interacts with HAUSP in vivo and that the possible regulation of p53 by HAUSP might be still effective in the cells during the DNA damage response.

To determine the functional consequence of the p53–HAUSP interaction, we tested whether HAUSP affects stabilization of p53. As indicated in Fig. 2a, HAUSP expression significantly increased the steady-state cellular levels of p53. In contrast, HAUSP had no obvious effect on the levels of p27 (Fig. 2b), another short-lived tumour suppressor protein whose stability is also regulated by the ubiquitination pathway. Moreover, as shown in Fig. 2c, HAUSP effectively rescues p53 from Mdm2-mediated degradation. Thus, although overexpression of Mdm2 significantly induced p53 degradation (compare lane 3 with lane 1), degradation of p53 was inhibited in a dose-dependent manner upon expression of HAUSP (lanes 4–6). Furthermore, we examined the effect of HAUSP expression on stabilization of endogenous p53. Normal human fibroblast IMR-90 cells were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing HAUSP. We first examined the protein levels of endogenous p53 by western

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**Figure 3** Effects of HAUSP on p53-mediated cell growth repression (a) and apoptosis (b). **a**. A pair of human lung carcinoma cells (H1299 (p53+/+) and H460 (p53+/−)) and a pair of mouse embryo fibroblasts (MEF (p53+/+) and MEF (p53−/−)) were infected with either pBabe-vector or pBabe-HAUSP. At 24 h after infection, cells were split and kept in the medium with puromycin, and surviving colonies were counted after 2 weeks. **b**. H1299 cells were transfected with p53 alone, with HAUSP alone, with p53 and Mdm2 together or with p53, Mdm2 and HAUSP together, as indicated. After transfection the cells were fixed, stained for p53 with fluorescein-isothiocyanate-conjugated anti-p53 antibody and analysed for apoptotic cells (sub-G1) according to DNA content (propidium iodide staining).

**Figure 4** Deubiquitination of p53 by HAUSP both in vivo and in vitro. **a**. Regulation of p53 ubiquitination levels in vivo. Western blot analysis of immunoprecipitates with the M2/Flag antibody from the cells transfected with Flag-p53 (lane 1), with Flag-p53 and Mdm2 together (lane 2), or in combination with different expression vectors as indicated (lanes 3–5), with anti-p53 monoclonal antibody (DO-1). **b**. Regulation of p53 stability by the HAUSP mutant. Western blot analysis of H1299 cell extracts from the cells transfected with p53 (lane 1), with p53 and Mdm2 together (lane 2), or in combination with different expression vectors as indicated (lanes 3–5), with anti-p53 monoclonal antibody (DO-1). The CMV–GFP expression vector was included in each transfection as a transfection efficiency control, and levels of GFP were detected with anti-GFP monoclonal antibody (JL-8; Clontech). **c**. Deubiquitination of p53 in vitro by HAUSP. The purified ubiquitinated p53 protein (lane 1) was incubated with the purified recombinant proteins of either HAUSP (lane 2) or HAUSP−cs (lane 3).
null H1299 cells (Fig. 3a). Similar cell growth repression by HAUSP encoding HAUSP, and cultured for 2 weeks under pharmacological empty pBabe-puro control retrovirus or a pBabe-puro retrovirus effect on cell growth in a colony formation assay. A pair of human in vivo specifically stabilizes p53 cells (Fig. 2f). These data therefore demonstrate that HAUSP ubiquitination levels of p53 in the pBabe-HAUSP-infected cells were also reduced in comparison with the levels in the mock-infected cells (Fig. 2f). These data therefore demonstrate that HAUSP specifically stabilizes p53 in vivo.

To investigate the biological role of HAUSP, we examined its effect on cell growth in a colony formation assay. A pair of human lung carcinoma cells (H1299 and H460) were infected with either an empty pBabe-puro control retrovirus or a pBabe-puro retrovirus encoding HAUSP, and cultured for 2 weeks under pharmacological selection. Strikingly, HAUSP strongly inhibited the growth of H460 cells expressing wild-type p53 but had no significant effect on p53-null H1299 cells (Fig. 3a). Similar cell growth repression by HAUSP was also observed in MEF p53+/+ cells but not MEF p53−/− cells (Fig. 3a), indicating that the cell growth repression by HAUSP is dependent on p53. We also tested whether HAUSP directly affects p53-dependent apoptosis. H1299 cells were transfected with p53 alone, with p53 and Mdm2, or with p53, Mdm2 and HAUSP. After transfection the cells were fixed, stained for p53, and analysed for apoptotic cells (sub-G1) according to DNA content32. As indicated in Fig. 3b, although overexpression of p53 alone induced significant apoptosis (31.0%), Mdm2 strongly reduced the level of p53-dependent apoptosis (11.2%). However, expression of HAUSP effectively attenuated the inhibitory effect of Mdm2 on p53-mediated apoptosis (28.5% versus 11.2%; Fig. 3b). These data demonstrate that HAUSP is crucially involved in both the regulation of p53-dependent apoptosis and the inhibition of cell growth.

To elucidate the molecular mechanism by which HAUSP stabilizes p53, we tested whether HAUSP directly controls the levels of p53 ubiquitination in vivo. As indicated in Fig. 4a, a high level of ubiquitinated p53 was found in cells transfected with p53 and Mdm2 (lane 2); however, p53 ubiquitination was significantly abrogated by HAUSP expression (compare lanes 3 and 2). In contrast, HAUSP had no effect on the levels of ubiquitinated p27 (see Supplementary Information). Notably, an unrelated human UBP family member (human USP11) (refs 23–25) that is defective in p53 binding (see Supplementary Information) had no obvious effect on the levels of p53 ubiquitination (Fig. 4a, lane 5) or stabilizing p53 (Fig. 4b, lane 5). Significantly, a HAUSP mutant with a short deletion at the core domain lost the ability both to stabilize p53 (Fig. 4b, lane 4) and to reduce the cellular levels of p53 ubiquitination (Fig. 4a, lane 4), indicating that stabilization of p53 by HAUSP requires its deubiquitinating enzymatic activity. To confirm the specific deubiquitination activity of HAUSP on p53, we examined whether HAUSP can directly deubiquitinate p53 in a purified system. The HAUSP protein was expressed in bacteria and purified to near homogeneity. The ubiquitinated form of p53 was purified on an M2 affinity column under conditions of high stringency from cells transfected with a Flag-tagged p53 expression vector. The highly purified in vitro system was used in this assay to avoid possible contamination by either inhibitory factors (namely, p14ARF) or any enzymes involving the ubiquitination of p53. As shown in Fig. 4c, p53 was efficiently deubiquitinated upon incubation with purified recombinant HAUSP (lane 2). These results therefore demonstrate that HAUSP can specifically deubiquitinate p53 both in vitro and in vivo.

Interestingly, HAUSP-cs, a point mutant of HAUSP in which a highly conserved Cys residue at the core domain was replaced by Ser, retained its strong binding with p53 (see Supplementary Information); however, it was functionally defective in deubiquitinating p53 in vivo (Fig. 4c, lane 3). Significantly, in contrast to the effect of wild-type HAUSP, the expression of HAUSP-cs in the cells increased the level of p53 ubiquitination (Fig. 5a, compare lanes 4 and 2), indicating that HAUSP-cs might function as a dominant-negative mutant through interfering with endogenous HAUSP-mediated deubiquitination of p53. To corroborate these results, we also tested whether HAUSP-cs expression affects the levels of p53 proteins in cells. As shown in Fig. 5b, expression of HAUSP-cs together with p53 slightly, but significantly, decreases the levels of p53 proteins (compare lanes 1 and 3 with lanes 2 and 4). To demonstrate further that HAUSP regulates endogenous p53, we introduced HAUSP-cs into normal human cells: IMR-90 cells were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing HAUSP-cs. As indicated in Fig. 5c, in the mock-infected cells the level of p53 proteins was markedly increased by DNA damage (lanes 1, 3 and 5); however, HAUSP-cs expression led to a significant attenuation of p53 stabilization under both normal and DNA-damage conditions (lanes 2, 4 and 6). Taken together, these results indicate that HAUSP is crucially involved in both the deubiquitination and the stabilization of p53 under physiological conditions.

**Figure 5** The dominant-negative effects of HAUSP-cs in human cells. **a**, Western blot analysis of immunoprecipitates with the M2/Flag antibody from the cells transfected with Flag-p53 (lane 1), with Flag-p53 and Mdm2 together (lane 2), or in combination with HAUSP and HAUSP-cs as indicated (lanes 3 and 4), with anti-p53 monoclonal antibody (DO-1). All cells were treated with 50 μM LNL for 4 h before being harvested. **b**, Western blot analysis of immunoprecipitates with M2/Flag antibody from human SJSA cells either transfected with expression vectors of Flag-p53 alone (lanes 2 and 4) or with expression vectors of Flag-p53 and HAUSP-cs together (lanes 1 and 3), with anti-p53 monoclonal antibody (DO-1) The CMV–GFP expression vector was included in each transfection as a transfection efficiency control, and levels of GFP were detected with anti-GFP monoclonal antibody (DO-1). **c**, Western blot analysis of the cell extracts from both mock-infected and pBabe-HAUSP-cs-infected IMR-90 cells with anti-p53 monoclonal antibody (DO-1). Cells were either not treated (lanes 1 and 2) or treated with 20 μM etoposide (lanes 3–6) for either 1 or 2 h as indicated. **d**, A model for the regulation of p53 stability by Mdm2, HAUSP and ARF: p53 is ubiquitinated (ub) by Mdm2 and subsequently degraded by the 26S proteasome, whereas the ARF tumour suppressor induces p53 stabilization through inhibiting Mdm2-mediated ubiquitin ligase activity. HAUSP can deubiquitinate p53 directly and rescue the ubiquitinated p53 from degradation.
**letters to nature**

Our data suggest that HAUSP-mediated stabilization of p53 acts through its intrinsic deubiquitinating enzymatic activity. Deubi-
quination, which removes the ubiquitin moiety from ubiquitin-
modified proteins, is now recognized as an important regulatory
step23–25. The large number of UBP homologues that have been
identified in mammalian cells23–25, none has yet been impli-
cated in stabilizing specific substrates in vivo. HAUSP might
represent the first example of a mammalian protein that can directly
debiquitinate and stabilize a specific cellular factor (p53).

Our findings have significant implications for the potential tumour
suppression function of HAUSP and also predict that many UBP
family proteins, like HAUSP, might interact with different substrates
in vivo for deubiquitination as well as subsequent protein
stabilization.

Previous studies have indicated that HAUSP interacts with the
herpesvirus protein Vmw110 and that a subset of the HAUSP
proteins is localized with the promyelocytic leukemia (PML)
nuclear body4. There is also now increasing evidence showing
that ARF is dispensable for p53 activation induced by some types of
oncogenic stress27–29. These studies, together with our findings,
therefore further indicate potential regulation of the p53–HAUSP
interaction in viral infection, DNA damage response and other types of
stress response. Stabilization of p53 is crucial for its effects on cell
growth repression and apoptosis. Many studies have proposed that
stabilization of p53 in response to various types of stress can be
achieved through inhibition of the Mdm2–p53 interaction and/or
Mdm2-mediated ubiquitin ligase1–12. Our findings reveal that
ubiquitination of p53 is a dynamic process in vivo and that
ubiquitinated p53 can be rescued from degradation by HAUSP
through direct deubiquitination (Fig. 5d). It is very likely that
changing the balance between the Mdm2-mediated ubiquitination
and HAUSP-mediated deubiquitination of p53 is the key for p53
stabilization in vivo.

### Methods

**Plasmas and antibodies**

To construct the HAUSP and USP11 expression vectors, the DNA sequences corresponding to the full-length proteins15,16 were amplified by polymerase chain reaction (PCR) from Marathon-Ready Hela cDNA (Clontech) and subcloned either into a pDNA3-Topo vector (Invitrogen) or with a Flag-tag either in a PET-11 vector for expression in bacteria or a PCIN4 vector and pBabe vector for expression in mammalian (for example, 200 mM NaCl for binding, 500 mM NaCl for elution)

**Identification of HAUSP as a p53-binding protein**

Elminating non-specific protein binding is critical for successfully identifying a genuine binding partner for p53 in the affinity chromatography assay11,12. We modified the salt
concentration range between the binding and elution conditions compared with the previous method (for example, 200 mM NaCl for binding, 500 mM NaCl for elution)

To limit the number of proteins eluted. Furthermore, nuclear extracts were extensively precleared with the GST column before being loaded on the GST–p53 affinity column. The mock purification was performed simultaneously on both the GST–p53 and Mdm2 expression vectors. After treatment as described above, the ubiquitinated p53 was purified from the cell extracts on the M2-affinity column with the

**Stabilization of p53 and detection of ubiquitination levels of p53 in vivo and in vitro**

The p53-null H1299 cells were transfected with 0.1–2 μg cytomegalovirus (CMV)–Flag–p53, 2.5 μg CMV–Mdm2, 2 μg CMV–green fluorescent protein (CMV–GFP), and 5–16 μg either CMV–HAUSP or the same quantities of expression vectors for the indicated HAUSP, mutants or other proteins. At 24 h after transfection, cells were lysed in a Flp-lbuc buffer

(600 mM Tris-HCl pH 7.8, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 1 mM DTT, 10% glycerol and fresh proteinase inhibitors) for western blot

Electrophoresis. Western analysis. The levels of GFP were detected with the anti-GFP monoclonal antibody (1:8; Clontech) as a subtraction efficiency control. The ubiquitination levels of p53 were detected essentially as described previously30. The cells were treated for 4 h with a proteasome inhibitor, LLLN (Sigma) (50 μM) before being harvested, and were then lysed in Flp-lbuc buffer with mild sonication. The cell extracts were immunoprecipitated with

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Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays

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Small molecules that alter protein function provide a means to modulate biological networks with temporal resolution. Here we demonstrate a potentially general and scalable method of identifying such molecules by application to a particular protein, Ure2p, which represses the transcription factors Gln3p and Nil1p. By probing a high-density microarray of small molecules generated by diversity-oriented synthesis with fluorescently labelled Ure2p, we performed 3,780 protein-binding assays in parallel and identified several compounds that bind Ure2p. One compound, which we call uretupamine, specifically activates a glucose-sensitive transcriptional pathway downstream of Ure2p. Whole-genome transcription profiling and chemical epistasis demonstrate the remarkable Ure2p specificity of uretupamine and its ability to modulate the glucose-sensitive subset of genes downstream of Ure2p. These results demonstrate that diversity-oriented synthesis and small-molecule microarrays can be used to identify small molecules that bind to a protein of interest, and that these small molecules can regulate specific functions of the protein.

The progress in identifying and expressing all human proteins present an opportunity to develop a small-molecule modulator for every protein function. Small-molecule approaches to study protein function have illuminated diverse fields of biology. Examples include tetrodotoxin, which enabled the dissection of the action potential, and agonists of peroxisome-proliferator-activated receptor-γ such as rosiglitazone, which illuminated the regulation of adipogenesis. However, in most cases no small molecule that can modulate the function of a protein of interest is known, and there is currently no efficient method of identifying these biological probes. Using the example of the yeast protein Ure2p, we demonstrate a general two-step method that does not require a high-resolution structure or a previously characterized small molecule known to bind the protein. First, diversity-oriented synthesis is used to produce structurally complex and diverse small molecules efficiently. Second, the resulting compounds are screened for their ability to bind a protein of interest by using small-molecule microarrays, a technique for extremely high-throughput parallel-binding assays. Cell-based studies can subsequently determine which functions of the protein are modulated by each small molecule.

The yeast protein Ure2p has been widely studied in several different contexts. Ure2p is the central repressor of genes involved in glucose metabolism.

Figure 1 The library synthesis and identification of uretupamine. a, Outline of the diversity-oriented synthesis leading to uretupamine and other library members. b, An expanded view of 64 compound spots on the 3,780-member small-molecule microarray (~800 spots cm-2). Cy5-labelled Ure2p was passed over a microarray of the 1,3-dioxane small-molecule library, and the resulting slide was washed three times and scanned for fluorescence. The spot corresponding to uretupamine A is shown.