

# SUMO-specific protease SUSP4 positively regulates p53 by promoting Mdm2 self-ubiquitination

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**The p53 tumour suppressor has a key role in the control of cell growth and differentiation, and in the maintenance of genome integrity<sup>1,2</sup>. p53 is kept labile under normal conditions, but in response to stresses, such as DNA damage, it accumulates in the nucleus for induction of cell-cycle arrest, DNA repair or apoptosis. Mdm2 is an ubiquitin ligase that promotes p53 ubiquitination and degradation<sup>3–5</sup>. Mdm2 is also self-ubiquitinated and degraded. Here, we identified a novel cascade for the increase in p53 level in response to DNA damage. A new SUMO-specific protease, SUSP4, removed SUMO-1 from Mdm2 and this desumoylation led to promotion of Mdm2 self-ubiquitination, resulting in p53 stabilization. Moreover, SUSP4 competed with p53 for binding to Mdm2, also resulting in p53 stabilization. Overexpression of SUSP4 inhibited cell growth, whereas knockdown of *susp4* by RNA interference (RNAi) promoted of cell growth. UV damage induced SUSP4 expression, leading to an increase in p53 levels in parallel with a decrease in Mdm2 levels. These findings establish a new mechanism for the elevation of cellular p53 levels in response to UV damage.**

Small ubiquitin-related modifier (SUMO) is an ubiquitin-like protein that is conjugated to a variety of cellular proteins<sup>6,7</sup>. Similarly to ubiquitination, sumoylation occurs through three enzymatic steps catalysed by the E1 enzyme Sae1–Sae2, the E2 enzyme Ubc9, and the E3 ligases, including RanBP2 (ref. 8), Pc2 (ref. 9) and PIASs<sup>10,11</sup>. Protein sumoylation participates in the control of diverse cellular processes, including transcriptional regulation, nuclear transport and signal transduction<sup>12–14</sup>. SUMO modification is a reversible process that is catalysed by a family of SUMO-specific proteases. In yeast, two SUMO proteases, Ulp1 and Ulp2, have been identified<sup>15,16</sup>. In humans, several SUMO proteases have been identified, including SENP1, 2, 3, 5 and 6 (refs 12, 17, 18).

p53 is modified by SUMO-1 and overexpression of SUMO-1 or Ubc9 was reported to induce an increase in p53-dependent transcription<sup>19,20</sup>. However, it was also reported that sumoylation of p53 has no effects

on its transcriptional activity and coexpression of PIAS1 and SUMO-1 instead represses p53 activity<sup>21–23</sup>. Mdm2 is also modified by SUMO-1 (refs 24, 25). Interestingly, the nucleoli tumour suppressor p19<sup>ARF</sup> (alternative reading frame), which inhibits Mdm2 and thereby stabilizes p53, promotes sumoylation of Mdm2 (refs 24, 25). However, p19<sup>ARF</sup> recruits Mdm2, Ubc9 and SUMO-1 to the nucleoli<sup>24</sup>. Moreover, the CELO (chicken embryo lethal orphan) adenovirus protein, Gam1 (gallus ante morte 1), which inhibits Sae1–Sae2 (ref. 26) and leads to downregulation of Ubc9, has no overt effect on the ability of p19<sup>ARF</sup> to activate p53 (ref. 27). Thus, the functional relevance of sumoylation of Mdm2, in addition to p53, in the p53–Mdm2 pathway remained unclear.

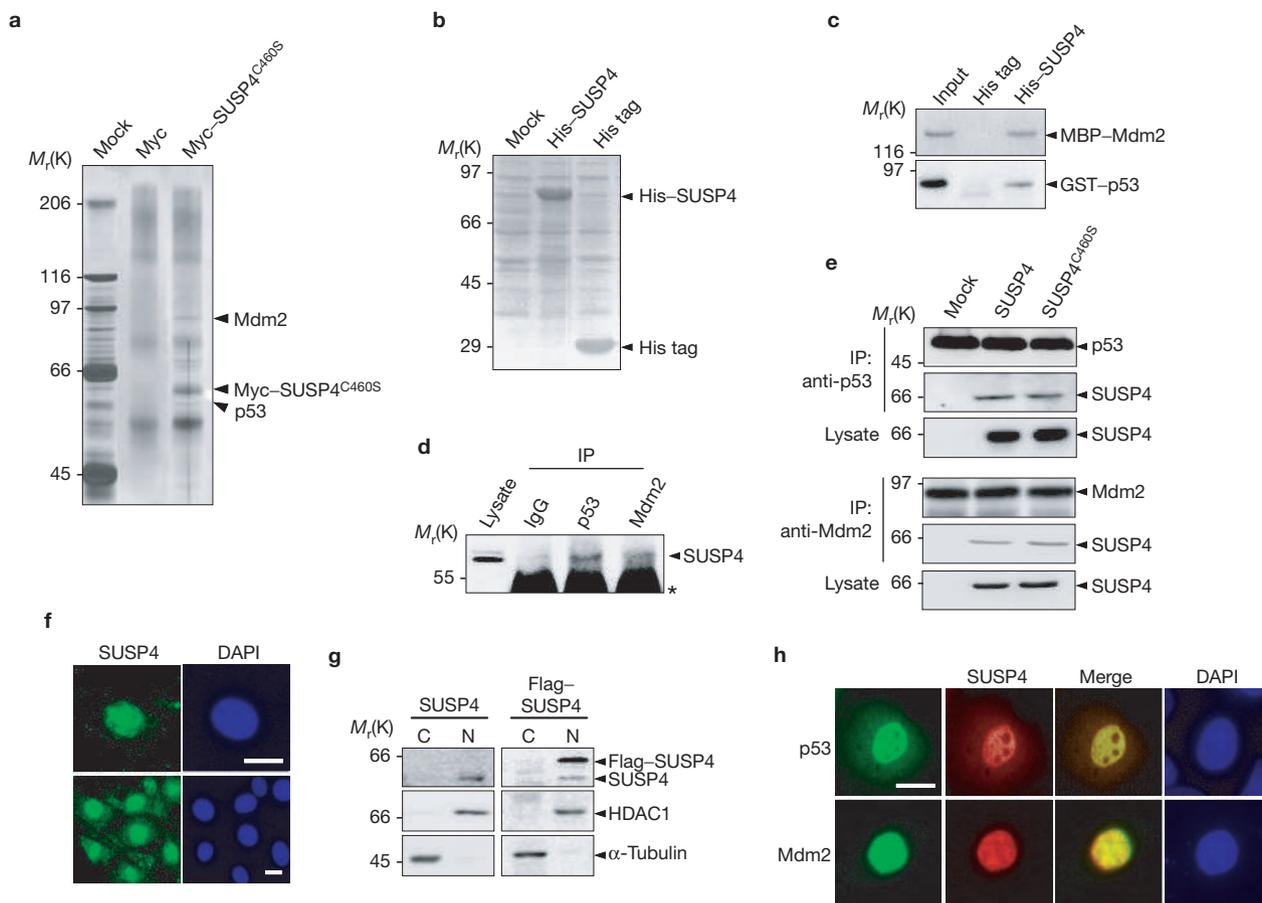
EST database searches for mouse genes identified a partial clone that has a homologous sequence encoding the carboxy-terminal catalytic domains of yeast Ulp1 (ref. 15). Based on the sequence of the clone, we isolated a cDNA for SUMO-1-specific protease from mouse brain mRNAs. The cDNA clone contained a 1,500 base-pair open reading frame encoding a protein of 499 amino acids with a predicted relative molecular mass of 54,890 ( $M_r$ , 54.89K), which we named SUSP4. Similarly to other SUMO proteases, SUSP4 has a conserved His–Asp–Cys catalytic triad (see Supplementary Information, Fig. S1). The C-terminal active-site domain and the remaining amino-terminal region of SUSP4 show approximately 65% and 30% identity with those of human SENP2 and mouse SuPr1, respectively<sup>17,28</sup>, suggesting that SUSP4, like SuPr1, is a mouse homologue of SENP2. SUSP4 was ubiquitously expressed in all tissues and cell lines tested, although the protein levels of SUSP4 varied in different tissues and cell lines (see Supplementary Information, Fig. S2).

p53 and Mdm2 were found to interact with SUSP4 on MALDI-TOF mass spectrometric analysis of the proteins that bound to Myc-SUSP4<sup>C460S</sup> (in which the active site Cys 460 was replaced by Ser), but not to the Myc-tag itself (Fig. 1a). To confirm their interaction, His-tagged SUSP4 and a His tag expressed in *Escherichia coli* (Fig. 1b) were incubated with GST–p53 or MBP–Mdm2. Pulldown assay with NTA resins showed that GST–p53 and MBP–Mdm2 were coprecipitated with His–SUSP4, but not with the His-tag (Fig. 1c), indicating that the proteins directly interact with SUSP4. Immunoprecipitation analysis also revealed that

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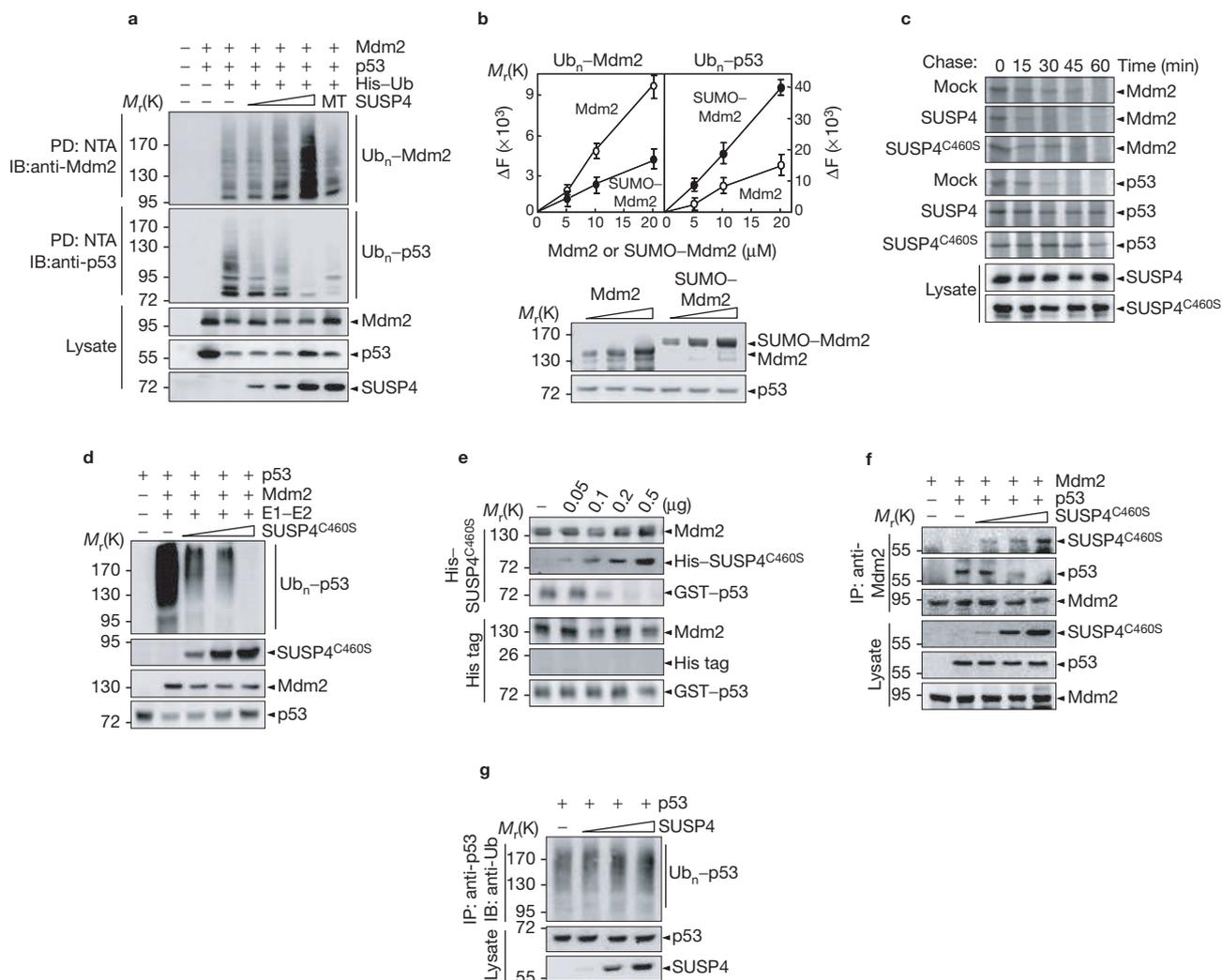
**Figure 1** Interaction of SUSP4 with p53 and Mdm2 and their colocalization. (a) NIH3T3 cells were transfected with pCMV-Myc or pCMV-Myc-SUSP4<sup>C460S</sup>. Cell lysates were incubated with Sepharose beads crosslinked with anti-Myc IgG. After washing, bound proteins were released from the beads by boiling, resolved by SDS-PAGE and silver-stained. Protein bands that appeared only in cells expressing Myc-SUSP4<sup>C460S</sup> were subjected to MALDI-TOF MS analysis. (b) His-SUSP4 or His-tag itself were expressed in *E. coli*. Cell extracts were subjected to SDS-PAGE followed by staining with Coomassie blue R-250. (c) Purified GST-p53 and MBP-Mdm2 were incubated with the *E. coli* extracts obtained in b. After incubation, the samples were treated with NTA resins for 1 h at 4 °C. Precipitates were subjected to SDS-PAGE followed by immunoblot with anti-MBP or anti-GST antibody. Input (20%) indicates the loading controls. (d) NIH3T3 cell lysates were subjected to immunoprecipitation with control IgG or anti-p53 or anti-Mdm2 antibody followed by immunoblot with anti-SUSP4 antibody.

The asterisk indicates the IgG heavy chain. (e) pcDNA-p53 or pcDNA-Mdm2 were transfected into HEK293T cells with pCMV2-Flag-SUSP4, pCMV2-Flag-SUSP4<sup>C460S</sup> or an empty vector (Mock). After incubation for 24 h, cell lysates were subjected to immunoprecipitation with anti-p53 or anti-Mdm2 antibody followed by immunoblot with anti-Flag antibody. Cells lysates were also directly probed with anti-Flag antibody. (f) NIH3T3 cells were stained with purified anti-SUSP4 IgGs. The nuclei were stained with DAPI. The scale bar represents 10  $\mu$ m. (g) Cytosolic (C) and nuclear extracts (N) were prepared from NIH3T3 cells and from the cells that had been transfected with pCMV2-Flag-SUSP4. They were then subjected to immunoblot with anti-SUSP4, anti-HDAC1 or anti- $\alpha$ -tubulin antibodies. (h) pCMV-Myc-SUSP4 was transfected to NIH3T3 cells with pcDNA-p53 or pcDNA-Mdm2. After incubation for 24 h, cells were immunostained for Myc-SUSP4 (red) and p53 or Mdm2 (green). They were then observed using a fluorescent microscope. The scale bar represents 10  $\mu$ m.

endogenous SUSP4 interacts with p53 and Mdm2 (Fig. 1d). Flag-tagged SUSP4 and SUSP4<sup>C460S</sup> also interacted with ectopically expressed p53 or Mdm2. (Fig. 1e). Immunostaining of cells with purified anti-SUSP4 IgGs showed that endogenous SUSP4 localizes predominantly to the nucleus, although a minor fraction was also observed in the cytoplasm (Fig. 1f). To confirm the localization of SUSP4, cytosolic and nuclear extracts were obtained from NIH3T3 cells and immunoblot analysis of these extracts revealed that both endogenous and ectopically expressed SUSP4 localized predominantly to the nuclear fractions (Fig. 1g). As expected, the marker proteins HDAC1 and  $\alpha$ -tubulin were detected in the nuclear and cytosolic fractions, respectively. On overexpression of Myc-SUSP4 with p53 or Mdm2, SUSP4 was found to colocalize with p53 and Mdm2 in the nucleus (Fig. 1h). Collectively, these results demonstrate that SUSP4 interacts with p53 and Mdm2 in the nucleus.

To determine the binding regions of p53 and Mdm2 within SUSP4, His-tagged deletions of SUSP4 were expressed in HEK293T cells with p53 or Mdm2. An SUSP4 truncated mutant containing the N-terminal 200 amino acids (Sd1) interacted with p53, whereas the mutants containing the 201–300 sequence (Sd3 and Sd4) bound to Mdm2 (see Supplementary Information, Fig. S3a). These results indicate that p53 and Mdm2 bind to the N-terminal and middle regions of SUSP4, respectively. To locate the binding regions of SUSP4 within p53 and Mdm2, various deletions of the latter proteins were expressed. The Mdm2 mutants containing the N-terminal 1–158 sequence (Md1 and Md2) interacted with SUSP4 (see Supplementary Information, Fig. S3b), and the p53 mutants carrying the C-terminal 301–393 sequence (Pd3 and Pd4) bound to SUSP4 (see Supplementary Information, Fig. S3c). These results indicate that the SUSP4-binding sites reside within the N- and





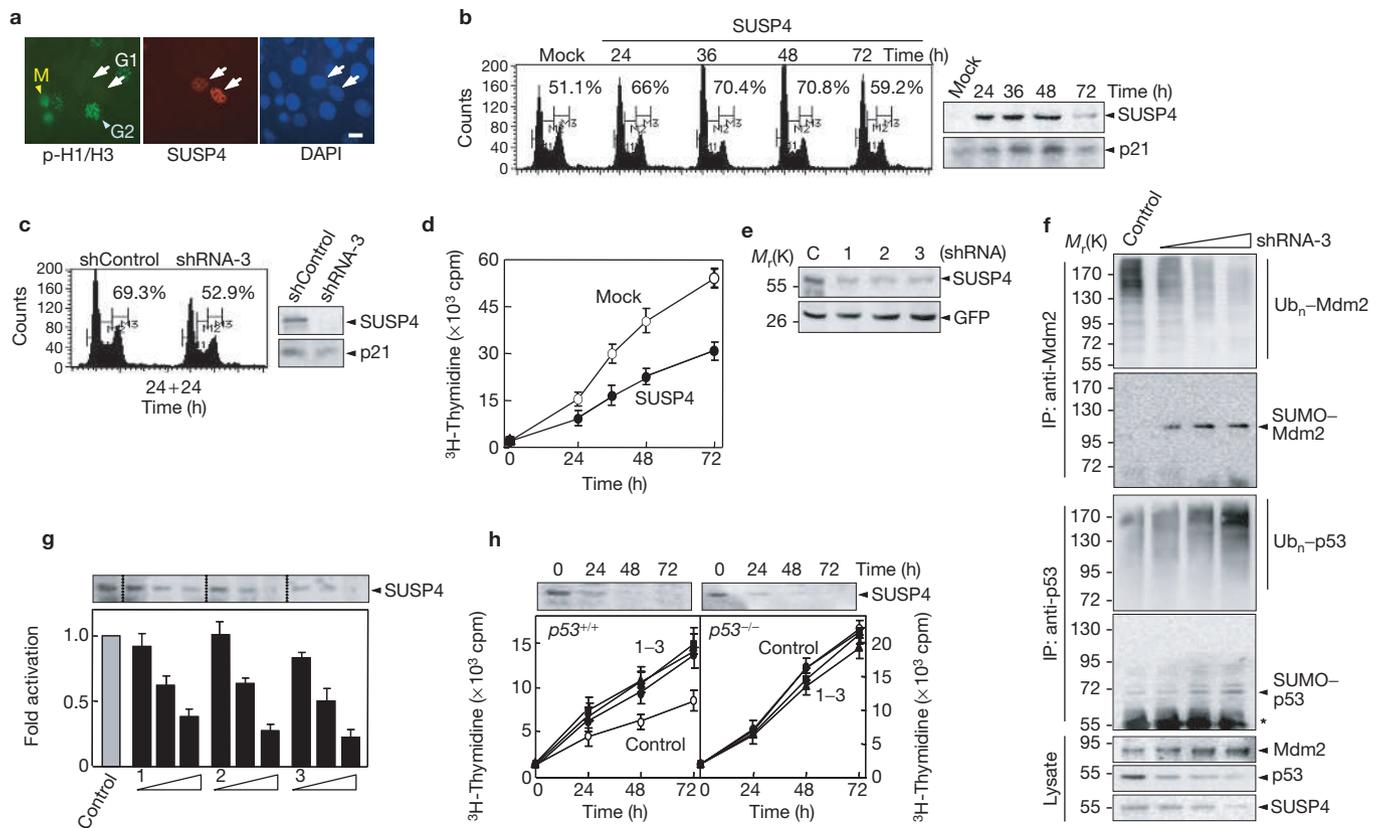
**Figure 3** Effect of SUSP4 on ubiquitination and stability of Mdm2 and p53. (a) pcDNA-Myc-p53, pcDNA-HA-Mdm2 and pcDNA-HisMax-ubiquitin (Ub) were transfected to *p53*<sup>-/-</sup> *mdm2*<sup>-/-</sup> cells with pCMV2-Flag-SUSP4 (2, 4 and 8  $\mu$ g) or pCMV2-Flag-SUSP4<sup>C460S</sup> (8  $\mu$ g). After incubation for 4 h with 10  $\mu$ M MG132, ubiquitinated proteins were pulled down by NTA resins and subjected to immunoblot analysis with anti-Mdm2 or anti-p53 antibodies. Uncropped images of the upper two panels are shown in the Supplementary Information, Fig. S5a. (b) MBP-Mdm2, Sae1-Sae2 and Ubc9 were incubated with or without SUMO-1. Mdm2 and sumoylated Mdm2 were separated using a DEAE-Sepharose column and each was incubated with p53, fluorescein-N-terminal ubiquitin, Uba1, UbcH5 and an ATP-regenerating system for 1 h. The reaction was stopped by adding 150 mM Tris-HCl (pH 7.6) containing 5% SDS and 30% glycerol. The mixtures were immunoprecipitated by anti-Mdm2 or anti-p53 antibodies and the precipitates were quantified using a fluorometer. The same amounts of the input p53, Mdm2 and SUMO-Mdm2 proteins were subjected to SDS-PAGE and Coomassie stained. The data represent the mean  $\pm$  s.d. of three experiments. (c) NIH3T3 cells transfected with an empty vector (Mock), pCMV2-Flag-SUSP4 or

pCMV2-Flag-SUSP4<sup>C460S</sup> were labelled with <sup>35</sup>S-methionine followed by treatment with unlabelled methionine. The labelled proteins were subjected to immunoprecipitation with anti-Mdm2 or anti-p53 antibodies followed by SDS-PAGE, and visualized by autoradiography. (d) Mdm2 (0.1  $\mu$ g) was incubated with SUSP4<sup>C460S</sup> (0.1, 0.2 and 0.5  $\mu$ g) for 30 min at 4  $^{\circ}$ C. The samples were subjected to *in vitro* ubiquitination followed by immunoprecipitation with anti-p53 antibodies. They were then immunoblotted with anti-ubiquitin antibodies. (e) Mdm2 (0.5  $\mu$ g) was incubated with His-SUSP4<sup>C460S</sup> or His-tag itself for 30 min and then with purified GST-p53 (0.2  $\mu$ g) for 1 h. The samples were subjected to immunoprecipitation with anti-Mdm2 antibodies. (f) pcDNA-Mdm2 (1  $\mu$ g) and pcDNA-Myc-p53 (1  $\mu$ g) were transfected to *p53*<sup>-/-</sup> *mdm2*<sup>-/-</sup> cells with pCMV2-Flag-SUSP4<sup>C460S</sup> (0.5, 1 and 2  $\mu$ g). After culturing for 24 h, cell lysates were subjected to immunoprecipitation with anti-Mdm2 antibodies followed by immunoblot with anti-Flag, anti-p53 or anti-Mdm2 antibodies. (g) *p53*<sup>-/-</sup> *mdm2*<sup>-/-</sup> cells were transfected with pcDNA-Myc-p53 and pCMV2-Flag-SUSP4. After incubation as in a, cell lysates were subjected to immunoprecipitation with anti-p53 antibodies and probed with anti-ubiquitin antibodies.

self-ubiquitination and p53 ubiquitination. Furthermore, pulse-chase analysis using <sup>35</sup>S-methionine reveals that SUSP4, but not SUSP4<sup>C460S</sup>, causes a marked decrease in Mdm2 stability (Fig. 3c). SUSP4 also caused p53 stabilization. These results indicate that SUSP4 destabilizes Mdm2 by promoting its self-ubiquitinating activity, thereby leading to p53 stabilization.

Surprisingly, however, expression of SUSP4<sup>C460S</sup>, which cannot desumoylate Mdm2, caused a marked decrease in the level of ubiquitinated p53 (Fig. 3a). Moreover, SUSP4<sup>C460S</sup>, which showed little or no effect

on Mdm2 stability, caused p53 stabilization (Fig. 3c). We showed that SUSP4 binds to the N-terminal region of Mdm2 (see Supplementary Information, Fig. S3). The binding site of p53 has also been identified to reside within the N-terminal region of Mdm2 (ref. 29). Thus, it seemed possible that competition between SUSP4<sup>C460S</sup> and p53 for binding to Mdm2 results in p53 stabilization. To investigate this possibility, we first examined whether SUSP4<sup>C460S</sup> can block p53 ubiquitination by Mdm2 *in vitro*. The level of ubiquitinated p53 was decreased by SUSP4<sup>C460S</sup>



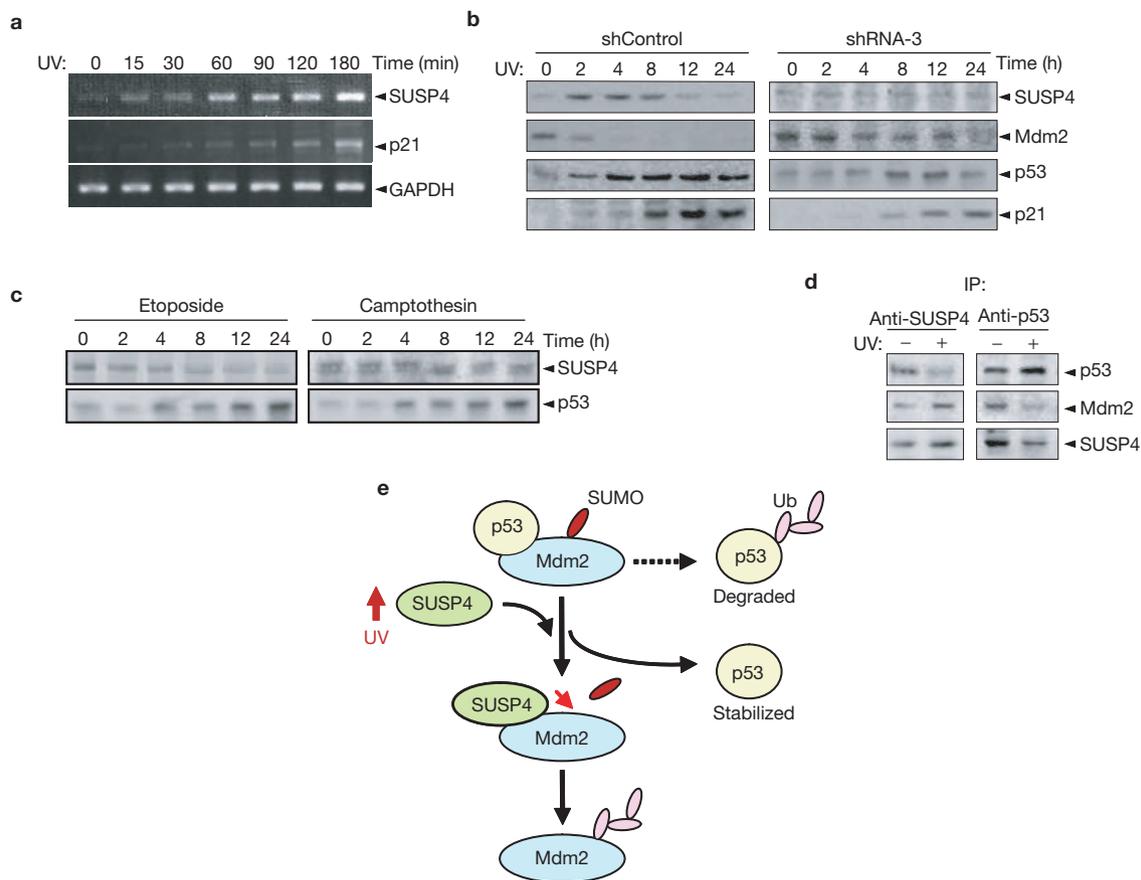
**Figure 4** SUSP4-mediated inhibition of cell growth. **(a)** NIH3T3 cells were transiently transfected with pCMV-Myc-SUSP4. After incubation for 24 h, cells were stained with anti-Myc or a mixture of anti-phospho-histone H1 and H3 antibodies. The scale bar represents 10  $\mu$ m. **(b)** Cells transfected with pCMV2-Flag-SUSP4 or an empty vector (Mock) were cultured for the indicated times and subjected to FACS analysis to determine their DNA content. They were also subjected to immunoblot analysis with anti-SUSP4 or anti-p21 antibodies. The data represents the mean  $\pm$  s.d. of three experiments. **(c)** Cells transfected with pCMV2-Flag-SUSP4 were incubated for 24 h. After incubation, they were transfected with control shRNA (shControl) or shRNA-3 and cultured for a further 24 h. FACS and immunoblot analyses were then performed as in **(b)**. **(d)** Cells cultured as in **(b)** were assayed for  $^3$ H-thymidine incorporation. **(e)** Each of three shRNAs (1–3) or control shRNA (C) was transfected for two rounds into cells with a vector encoding GFP as a transfection control. After incubation for 24 h, cells were subjected to immunoblot analysis with anti-SUSP4

or anti-GFP antibodies. **(f)** Cells transfected with increasing amounts of shRNA-3 were subjected to immunoprecipitation with anti-Mdm2 or anti-p53 antibodies followed by immunoblot with anti-ubiquitin or anti-SUMO-1 antibodies. Uncropped images of the second and fourth panels are shown in the Supplementary Information, Fig. S5b. The asterisk indicates the IgG heavy chain. **(g)** Control shRNA or increasing amounts of shRNAs (1–3) were transfected to cells with pG13-Luc and pcDNA- $\beta$ -Gal. After incubation for 24 h, cells were subjected to luciferase assay. Data represents mean  $\pm$  s.d. of three experiments. Cell lysates were also subjected to immunoblot with anti-SUSP4 antibodies. **(h)**  $p53^{+/+}$  and  $p53^{-/-}$  cells were transfected with control shRNA or shRNAs (1–3). They were then assayed for  $^3$ H-thymidine incorporation. The level of SUSP4 was determined in cells transfected with shRNA-3 by immunoblot with anti-SUSP4 antibodies. The data represents the mean  $\pm$  s.d. of three experiments. Uncropped images of the upper panels are shown in the Supplementary Information, Fig. S5c.

treatment in a dose-dependent manner (Fig. 3d), indicating that SUSP4<sup>C460S</sup> is capable of inhibiting Mdm2-mediated p53 ubiquitination. We then examined whether SUSP4<sup>C460S</sup> actually competes with p53 for binding to Mdm2. Purified Mdm2 was incubated with increasing amounts of His-SUSP4<sup>C460S</sup> and then with p53. Immunoprecipitation analysis using anti-Mdm2 antibodies revealed that the amount of SUSP4<sup>C460S</sup> bound to Mdm2 gradually increased in parallel with a decrease in the amount of p53 bound to Mdm2 (Fig. 3e). *In vivo* competition experiments were also performed. Mdm2 and p53 were expressed in  $p53^{-/-}mdm2^{-/-}$  cells with increasing amounts of SUSP4<sup>C460S</sup>. The amount of SUSP4<sup>C460S</sup> coprecipitated with Mdm2 increased in parallel with a decrease in the amount of p53 coprecipitated with Mdm2 (Fig. 3f). Nearly identical results were obtained with wild-type SUSP4 in both *in vivo* and *in vitro* competition experiments (data not shown). These results indicate that both SUSP4 and SUSP4<sup>C460S</sup> are capable of competing with p53 for binding to Mdm2.

As SUSP4 can also desumoylate p53, we examined the effect of SUSP4 on p53 ubiquitination under conditions where Mdm2 is absent by expressing p53 with increasing amounts of SUSP4 in  $p53^{-/-}mdm2^{-/-}$  cells. In contrast with the results obtained in the presence of Mdm2 (see Fig. 3a), SUSP4 showed little or no effect on p53 ubiquitination in the absence of Mdm2 (Fig. 3g). These results indicate that the effects of SUSP4 on the stability of p53, as well as of Mdm2, are mediated by its action on Mdm2 but not on p53. Taken together, these results suggest that SUSP4 can stabilize p53 in two different modes: by interfering with the interaction between p53 and Mdm2; and by promoting self-ubiquitination and destabilization of Mdm2 through desumoylation of Mdm2.

As SUSP4 increased p53 stability, we examined the effect of SUSP4 on p53 transcriptional activity when NIH3T3 cells were transfected with reporter vectors. SUSP4 caused a marked increase in the activity of both endogenous and ectopically expressed p53 (see Supplementary Information, Fig. S4a). Immunoblot analysis of the same cell lysates again showed that SUSP4



**Figure 5** Induction of SUSP4 expression by UV. (a) NIH3T3 cells were irradiated with UV ( $50 \text{ J m}^{-2}$ ) for 30 s and incubated for the indicated times. Total RNAs were prepared from cells using Triazol reagent. RT-PCR was then carried out using specific primers for *susp4*, *p21* and *GAPDH*. (b) Cells were transfected with control shRNA or shRNA-3 for two rounds. After incubation for 24 h, cells were irradiated with UV. They were then subjected to immunoblot with respective antibodies. (c) Cells were treated with  $20 \mu\text{M}$  etoposide or  $10 \mu\text{g ml}^{-1}$  of camptothecin, and incubated for

the indicated times. They were then subjected to immunoblot with anti-SUSP4 or anti-p53 antibody. (d) Cells that had been treated without (–) or with UV (+) were incubated for 1 h. After incubation, cells were subjected to immunoprecipitation with anti-SUSP4 or anti-p53 antibody followed by immunoblot with respective antibodies. Uncropped images of the left upper two panels and the right lower two panels are shown in the Supplementary Information, Fig. 5Sd. (e) A schematic representation of a model for SUSP4-mediated control of the p53–Mdm2 pathway by SUSP4

stabilizes p53. SUSP4<sup>C460S</sup> also increased the stability and activity of p53. However, SUSP4 had little or no effect on the stability and activity of p50 (NF- $\kappa$ B; see Supplementary Information, Fig. S4b). These results suggest that both SUSP4 and SUSP4<sup>C460S</sup> positively regulate p53 activity by antagonizing endogenous Mdm2. These results also suggest that the SUSP4-mediated increase in p53 activity is independent of p53 sumoylation. To confirm this, p53 or p53<sup>K386R</sup> (in which the SUMO-accepting Lys residue was replaced by Arg) were expressed in cells with SUSP4. SUSP4 caused a marked increase in the stability and activity of both p53 and p53<sup>K386R</sup> (see Supplementary Information, Fig. S4c). Thus, it seems that p53 sumoylation itself has little or no influence on the SUSP4-mediated increase in p53 activity.

We then examined the effect of SUSP4 on the stability and activity of p53 in conditions where Mdm2 was overproduced. As expected, Mdm2 expression resulted in a dramatic reduction in p53 activity by destabilizing it (see Supplementary Information, Fig. S4d). Coexpression of SUSP4 or SUSP4<sup>C460S</sup> reversed the Mdm2-mediated decrease in p53 activity. On the other hand, neither SuPr1 nor SuPr1<sup>C466S</sup> could stabilize p53 or reverse the Mdm2-mediated decrease in p53 activity, indicating that Mdm2 is a specific target of SUSP4. Moreover, Mdm2 expression resulted in a marked reduction in *p21* mRNA levels, but coexpression of SUSP4 or SUSP4<sup>C460S</sup> reversed the inhibitory effect of Mdm2 (see Supplementary Information,

Fig. S4e). Little or no accumulation of *p21* mRNA was observed on expression of NF- $\kappa$ B, regardless of whether SUSP4 or SUSP4<sup>C460S</sup> was coexpressed. These results further demonstrate that SUSP4 positively regulates p53 activity by antagonizing Mdm2 function.

As SUSP4 mediates p53 stabilization, we examined whether SUSP4 is involved in p53-dependent control of cell growth. NIH3T3 cells transfected with pCMV–Myc–SUSP4 were strongly stained by anti-Myc antibodies (Fig. 4a). However, the same cells were barely stained by a mixture of anti-phospho-histone H1 and H3 antibodies, which is a characteristic feature of G1-phase cells. These results suggest that SUSP4 overexpression leads to cell-cycle arrest at the G1 phase. To confirm this, cells transfected with pCMV2–Flag–SUSP4 were cultured for various times and subjected to FACS analysis to determine their DNA contents. The fraction of cells in G1 phase was significantly increased up to 48 h in culture (Fig. 4b). The level of p21 protein was also gradually increased up to 48 h in culture. Both the fraction of G1-phase cells and p21 levels decreased thereafter, most likely due to an increase in the number of cells that were not transfected with pCMV2–Flag–SUSP4, as SUSP4 levels also decreased at 72 h. Furthermore, cotransfection of a *susp4*-specific short hairpin RNA (shRNA-3; see below), but not a negative control vector (shControl), resulted in a decrease in both the fraction of cells in the G1 phase and p21

levels (Fig. 4c), indicating that shRNA-3 can reverse the cell-cycle arrest caused by SUSP4 overexpression. We then examined whether SUSP4-mediated cell-cycle arrest leads to inhibition of cell growth. The rate of <sup>3</sup>H-thymidine incorporation was significantly reduced by transfection of cells with pCMV2-Flag-SUSP4 compared with that of an empty vector (Fig. 4d). Taken together, these results suggest that overproduced SUSP4 can downregulate the growth of cells by stabilizing p53.

To clarify the involvement of SUSP4 in p53-mediated control of cell growth, three *susp4*-specific shRNAs (indicated by 1–3) were transfected into NIH3T3 cells with a vector encoding GFP. All three shRNAs effectively reduced endogenous SUSP4 levels (Fig. 4e). Therefore, we examined the effect of shRNA-3 on ubiquitin and SUMO-1 modification of Mdm2. shRNA-mediated knockdown of *susp4* caused a decrease in the level of ubiquitinated Mdm2 in parallel with an increase in the level of sumoylated Mdm2, leading to stabilization of endogenous Mdm2 and destabilization of endogenous p53 (Fig. 4f). Consistently, all three shRNAs caused a decrease in the transcriptional activity of endogenous p53 in a dose-dependent manner (Fig. 4g). Moreover, the rate of <sup>3</sup>H-thymidine incorporation was significantly increased by transfection of *p53*<sup>+/+</sup> cells with the shRNAs, compared with that observed with the control shRNA (Fig. 4h), indicating that SUSP4 is involved in downregulation of cell growth. In contrast, knockdown of *susp4* had little or no effect on the proliferation of *p53*<sup>-/-</sup> cells (Fig. 4h), demonstrating the link between p53 and SUSP4 in the control of cell growth. Taken together, these results reveal that SUSP4 has an important function in the control of cell growth by p53.

Given that UV damage is one of the major stresses that provokes p53 action, we examined whether UV induces *susp4* gene transcription. *susp4* mRNA levels dramatically increased as early as 15 min after UV irradiation (Fig. 5a). *p21* mRNA level also increased about 1 h after the irradiation. These results suggest that SUSP4 is an UV-inducible protein that stabilizes p53, leading to an increase in *p21* mRNA levels. To determine whether UV-induced SUSP4 influences the endogenous levels of Mdm2 and p53 proteins, NIH3T3 cells transfected with control shRNA or shRNA-3 was irradiated with UV. Concurrent with an increase in SUSP4 levels, Mdm2 levels were sharply declined in cells transfected with control shRNA (Fig. 5b). p53 levels then gradually increased, followed by an increase in endogenous p21 levels. On the other hand, shRNA-mediated knockdown of *susp4* resulted in a marked delay in the accumulation of p53 and p21. These results indicate that SUSP4 has a critical role in the control of the cellular levels of Mdm2 and consequently of p53 and p21, when cells are damaged by UV. However, etoposide and camptothecin, which also cause DNA damage, showed little or no effect on SUSP4 induction (Fig. 5c), suggesting that SUSP4 responds to a limited range of stresses, such as UV.

We then examined whether UV damage causes an alteration in the amounts of p53 and Mdm2 that bind to SUSP4. UV irradiation led to an increase in the amount of Mdm2 bound to SUSP4, with a decrease in the interaction between p53 and SUSP4 (Fig. 5d). UV also caused a decrease in the amounts of SUSP4 and Mdm2 that bind to p53. These results suggest that UV damage leads to an increase in relative abundance of the SUSP4-Mdm2 complex over p53-Mdm2 complex through competition of accumulated SUSP4 with p53 for binding to Mdm2.

Mdm2 targets not only p53, but also itself for ubiquitination, and the fate of p53 is determined accordingly. Recently, Daxx (a multifunctional protein involved in the control of apoptosis and transcription)

was shown to have a key role in switching the Mdm2 activity from p53 to itself in response to DNA damage<sup>30</sup>. Here, we have provided an additional mechanism for the control of Mdm2 function and a model for the involvement of SUSP4 in the control of the p53-Mdm2 pathway, based on the results obtained in this study, is shown in Figure 5d. On UV damage, SUSP4 accumulates and replaces p53 for binding to Mdm2, leading to stabilization of p53 for induction of cell-cycle arrest and cell-growth inhibition. Desumoylation by SUSP4 would then promote self-ubiquitination and degradation of Mdm2 for further stabilization of p53. However, under normal conditions (that is, when SUSP4 levels are low), the SUMO-modification system, including Ubc9, may override SUSP4 and sumoylate Mdm2, leading to promotion of p53 ubiquitination. Thus, SUSP4 seems to have a critical role in switching the Mdm2 activity from p53 to itself in response to UV damage. □

## METHODS

**Plasmids and antibodies.** *susp4* cDNA was isolated from a mouse brain cDNA library and cloned into pcDNA-Myc, pcDNA-HisMax, pCMV-Myc and pCMV2-Flag. It was also cloned into pET-32a for bacterial expression. Therefore, His-SUSP4 and His-tag itself contain an extra-amino acid sequence of approximately 20K corresponding to the multicloning site region of the vector. *susp4*-specific shRNAs were synthesized and cloned into pSilencer 2.0-U6 (Ambion, Austin, TX). Oligonucleotides used were: 1, GATCAGGGATGGTATTTAA; 2, GATGAGGTCATCAATTTCT; 3, GAATGTTACCTGTAAT.

Antibodies against Myc (9E10), SUMO-1 (D-11), p53 (DO-1 and N-19), p50 (H-119), p21 (C-19) and Mdm2 (SMT4) were obtained from SantaCruz (Santa Cruz, CA). Peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse, and donkey anti-goat IgGs, FITC-conjugated goat anti-rabbit IgG, and TRITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-Flag M2, anti-Xpress, anti-Ub (FK1; Affiniti Res, Manhead, UK), and anti-SUMO-2 and -3 (Zymed, San Francisco, CA) antibodies were also used. Rabbit polyclonal anti-SUSP4 antiserum was raised against a recombinant His-tagged N-terminal fragment of SUSP4 (that is, amino acids 1–320). Anti-SUSP4 IgGs were purified by applying the serum onto an affinity column, which had been generated by conjugation of the N-terminal SUSP4 fragments to NHS-activated Sepharose 4 Fast Flow. Anti-SUSP4 IgGs bound to the resins were eluted with 100 mM glycine buffer (pH 3.0).

**Cell culture and transfection.** HEK293T, NIH3T3, MEF cells (*p53*<sup>+/+</sup>, *p53*<sup>-/-</sup>, and *p53*<sup>-/-</sup>*mdm2*<sup>-/-</sup>) were grown at 37 °C in DMEM supplemented with 100 units ml<sup>-1</sup> penicillin, 1 µg ml<sup>-1</sup> streptomycin and 10% FBS. Transfections in HEK293T and NIH3T3 cells were carried out using LipofectaminePlus. MEF cells were transfected by electroporation.

**Immunocytochemistry.** NIH3T3 cells plated on gelatin-coated cover-glasses were fixed with 2% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS. All subsequent dilutions and washes were performed with PBS containing 0.1% Triton X-100 (PBS-T). Nonspecific binding sites were saturated by incubation for 30 min with a blocking solution consisting of 10% goat serum, 1% BSA and 1% gelatin in PBS. Cells were incubated with primary antibody for 1 h and washed with PBS-T four times at 10 min intervals. They were then incubated with FITC- or TRITC-conjugated secondary antibody for 1 h and washed four times. DAPI was used for counterstaining the nuclei. The cover glasses were mounted in Vectashield and cells were visualized under a Zeiss Axioplan II microscope.

**Assays for protein-protein interaction.** Cells were lysed in buffer A consisting of 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 0.5% NP40 and 1× protease inhibitor cocktail. Cell lysates were incubated with appropriate antibodies for 2 h at 4 °C and then with 50 µl of a 50% slurry of protein A-Sepharose for 1 h. The resins were collected by centrifugation and washed five times with buffer B consisting of 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 1 mM EDTA and 0.5% NP40. Bound proteins were eluted by boiling in 0.2% SDS and subjected to SDS-PAGE followed by immunoblot with appropriate antibodies.

To analyse the competition of SUSP4<sup>C460S</sup> with p53 for binding to Mdm2 *in vitro*, Mdm2-affinity resins were prepared by incubation of 0.5 µg of purified Mdm2 for 2 h at 4 °C with protein A-Sepharose that had been treated with anti-Mdm2 antibody. The resins were incubated with increasing amounts of His-SUSP4<sup>C460S</sup> for 30 min at 4 °C and then with 0.2 µg of GST-p53 for the next 1 h. The resins were washed three times with buffer A to remove SUSP4<sup>C460S</sup> and p53 that did not bind to Mdm2. Proteins bound to the resins were then eluted by boiling in 0.2% SDS and subjected to SDS-PAGE.

**Assays for sumoylation and desumoylation.** For *in vitro* assays, *in vitro*-translated Myc-p53, HA-Mdm2 and PML were prepared by using TNT-coupled reticulocyte lysate systems. The resulting proteins were sumoylated by incubation with 10 µg of purified SUMO-1, 1.5 µg of Sae1-Sae2, 2 µg of Ubc9, an ATP-regenerating system (50 mM Tris-HCl at pH 7.5, 5 mM MgCl<sub>2</sub>, 10 mM creatine phosphate, 5 units ml<sup>-1</sup> of phosphocreatine kinase and 5 mM ATP), and 1× protease inhibitor cocktail. After incubation, SUMO-modified proteins were immunoprecipitated with anti-p53, anti-Mdm2 or anti-PML antibody followed by washing with PBS containing 500 units ml<sup>-1</sup> of aprotinase and 1 mM N-ethylmaleimide and then with 2 mM DTT. The substrates were then incubated at 37 °C for 2 h with recombinant His-tagged SUSP4 or SUSP4<sup>C460S</sup>. After incubation, the samples were subjected to SDS-PAGE on 8% gels followed by immunoblot with the same antibodies.

For *in vivo* assays, Myc-p53, Flag-SUMO-1 and Flag-Ubc9 were expressed in HEK293T cells with or without HisMax-SUSP4. HA-Mdm2 or HA-PML were also expressed in cells as above. After culturing for 36 h, cells were lysed by boiling for 10 min in 150 mM Tris-HCl (pH 6.7) buffer containing 5% SDS and 30% glycerol. Cell lysates were diluted twentyfold with buffer A containing 1× protease inhibitor cocktail, incubated with appropriate antibodies for 2 h at 4 °C and treated with protein A-Sepharose<sup>31</sup>. To assay sumoylation by endogenous SUMO-1, NIH3T3 cells that had been transfected with *susp4*-specific shRNAs were cultured for 36 h and then treated as above. The resins were collected by centrifugation and washed five times with buffer B. The samples were then subjected to SDS-PAGE followed by immunoblot analysis.

**<sup>3</sup>H-Thymidine incorporation.** NIH3T3 and MEF cells (2 × 10<sup>4</sup> cells per well) were seeded in 12-well plates and grown for various periods. They were then cultured in serum-free media for 2 h and treated with <sup>3</sup>H-thymidine (1 µCi per well). After incubation for 6 h, cells were subjected to trichloroacetic acid extraction followed by scintillation counting.

**Flow cytometry.** NIH3T3 cells were transfected with an empty vector or pCMV2-Flag-SUSP4 in 60-mm dishes. After incubation, cells were harvested and washed twice with PBS. They were fixed by treatment with 1 ml of 70% ethanol, gently vortexed and kept at 4 °C until used. Fixed cells were washed once with PBS and resuspended in a propidium iodide solution (10 µg ml<sup>-1</sup>) containing 4% NP40 and RNase A (250 µg ml<sup>-1</sup>). Propidium iodide-stained cells were then analysed for their DNA contents by using a FACS instrument (BD Biosciences, Franklin Lakes, CA).

**Pulse-chase analysis.** NIH3T3 cells that had been transfected with appropriate vectors were cultured for 18 h. Cells were divided into four fractions and further cultured on 60-mm dishes for 12 h. They were then incubated with 100 µCi ml<sup>-1</sup> of <sup>35</sup>S-methionine in methionine-free DMEM for 30 min at 37 °C. After incubation, cells were washed twice with PBS and further incubated in DMEM supplemented with 10% FBS and 2 mM unlabelled methionine for various periods. Cell lysates prepared in buffer A were subjected to immunoprecipitation with anti-p53 or anti-Mdm2 antibodies, followed by SDS-PAGE. Gels were then fixed, dried and visualized by autoradiography.

**Accession numbers** The GenBank accession number for *susp4* cDNA is AF366264.

Note: Supplementary Information is available on the Nature Cell Biology website.

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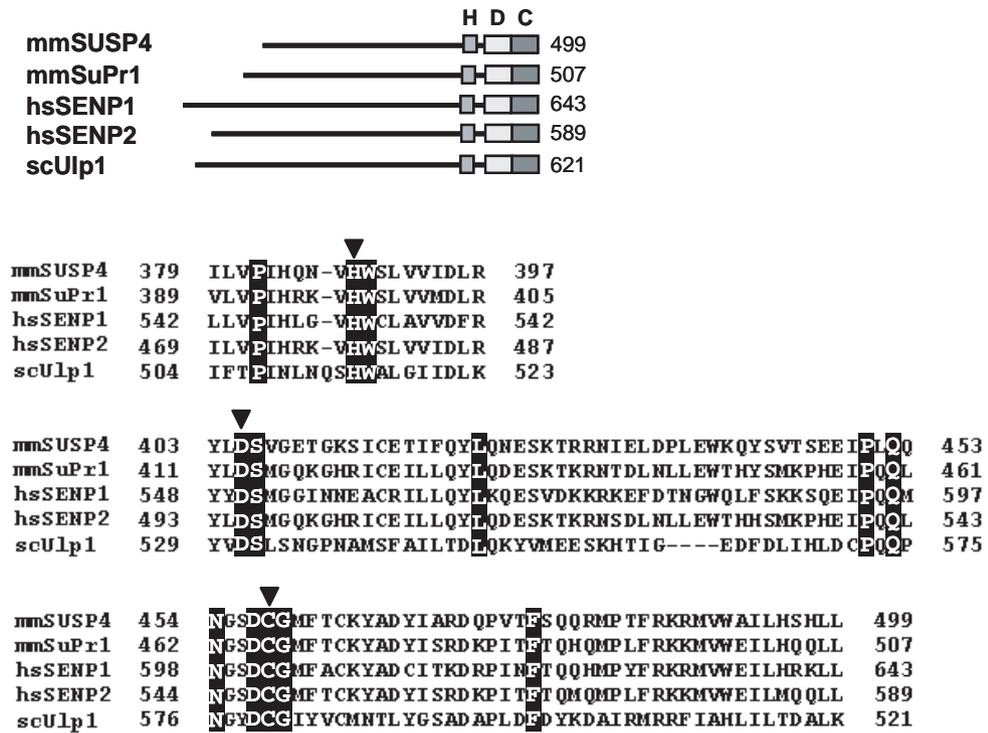
#### COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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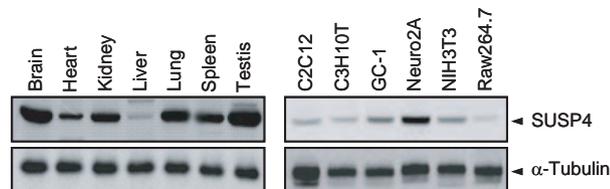
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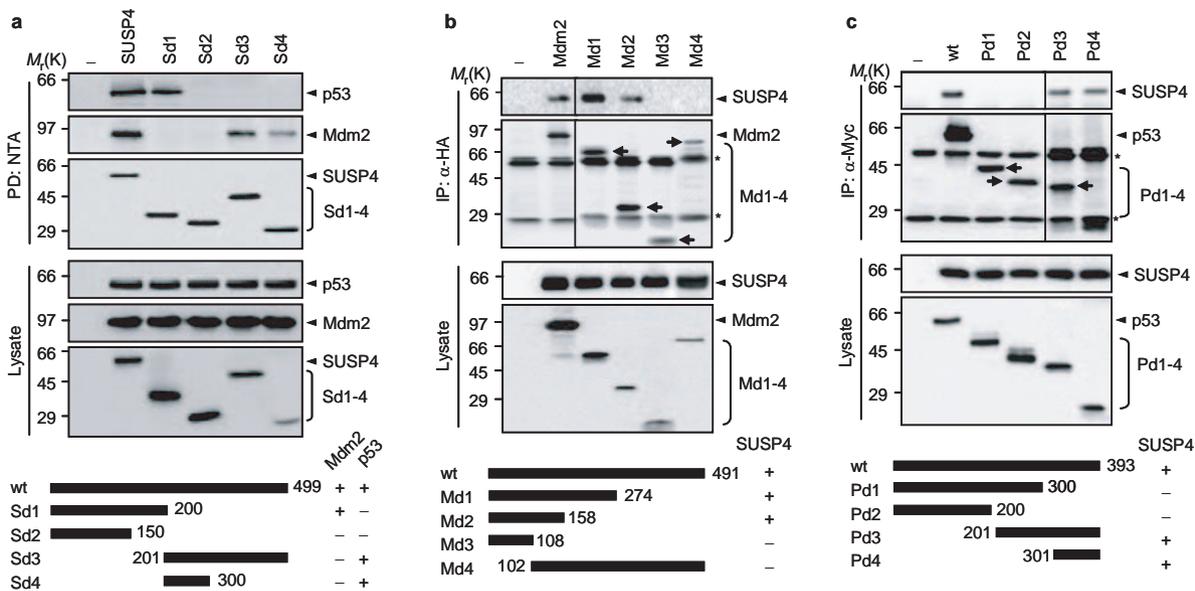
**Figure S1** Sequence of SUSP4. Schematic diagrams compares the primary structure of mouse SUSP4 with that of other SUMO-specific proteases. The boxes denote the conserved domains carrying the His-Asp-Cys catalytic triad

(upper). The sequences of conserved active site domains of SUSP4 were compared with those of other SUMO proteases. The catalytic residues were indicated by the triangles, and the invariant residues were shaded (lower).



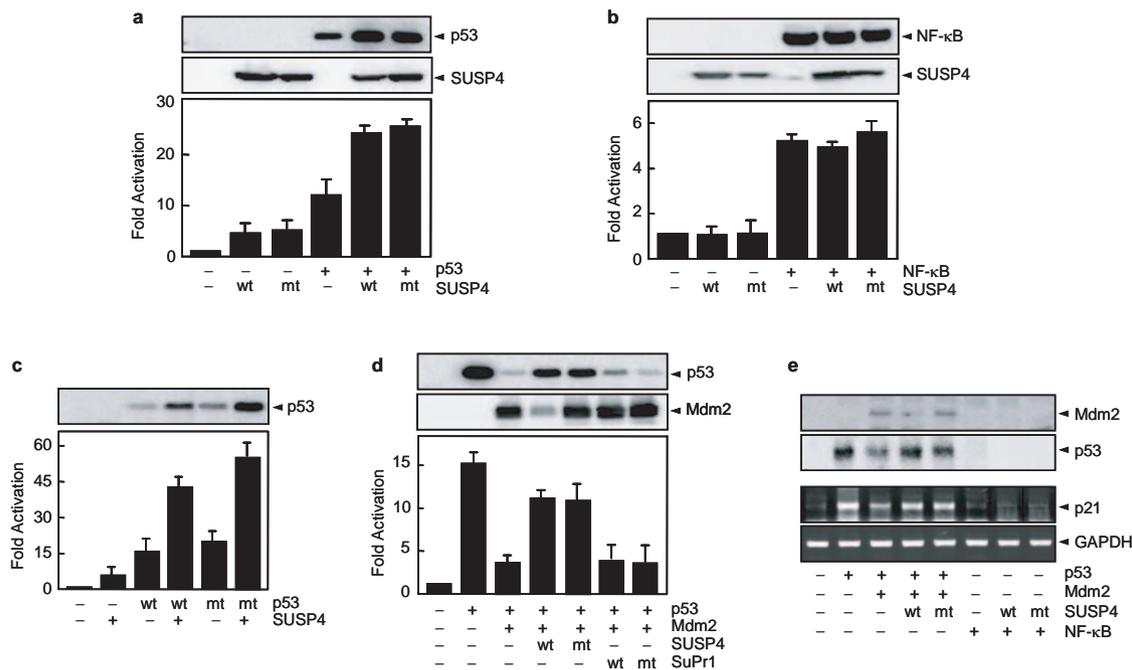
**Figure S2** Expression of SUSP4 in various tissues and cell lines. Extracts were prepared from various mouse tissues and cell lines. Aliquots of them

(50 µg) were then subjected to SDS-PAGE followed by immunoblot with anti-SUSP4 or anti- $\alpha$ -tubulin antibody.



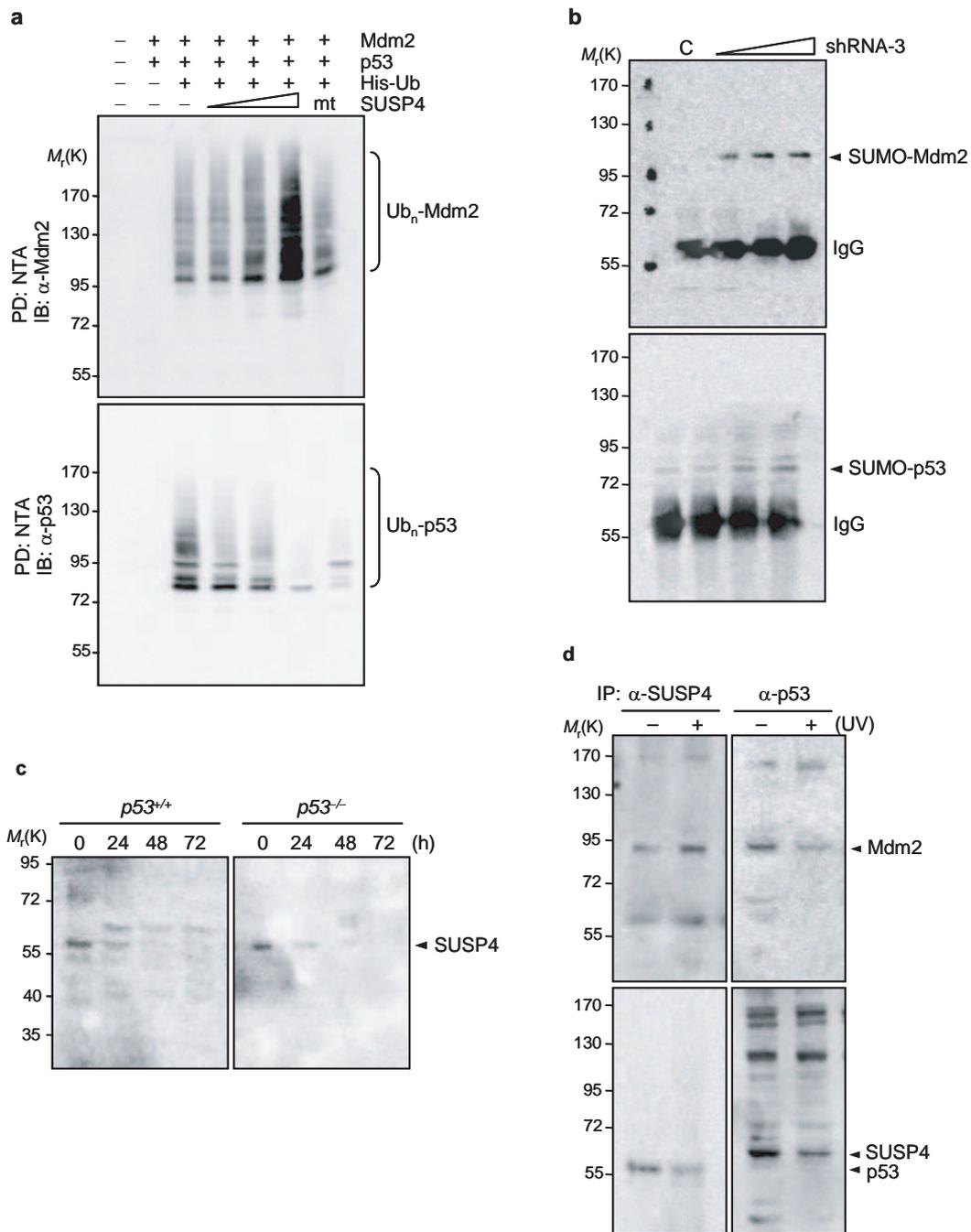
**Figure S3** Mapping of binding regions within SUSP4, p53, and Mdm2. (a) pcDNA-p53 and pcDNA-Mdm2 were co-transfected to HEK293T cells with pcDNA-HisMax-SUSP4 or vectors expressing SUSP4 deletion mutants (Sd1-Sd4). His-tagged proteins were pulled down (PD) by treatment with NTA resins and subjected to immunoblot with anti-p53, anti-Mdm2, or anti-Xpress antibody. The abilities of SUSP4 and its deletions to interact with p53 and Mdm2 were shown as “+” or “-”. (b) pCMV2-Flag-SUSP4 was transfected to cells with pcDNA-HA-Mdm2 or vectors expressing Mdm2 deletions (Md1-Md4). Cell lysates were subjected to immunoprecipitation

with anti-HA antibody followed by immunoblot with anti-Flag or anti-HA antibody. The asterisks indicate the IgG heavy and light chains, and the arrows show the deletion mutants. The abilities of Mdm2 and its deletions to interact with SUSP4 were shown as “+” or “-”. (c) pCMV2-Flag-SUSP4 was transfected to cells with pcDNA-Myc-p53 or the vectors expressing p53 deletions (Pd1-Pd4). Cell lysates were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblot with anti-Flag or anti-Myc antibody. The abilities of p53 and its deletions to interact with SUSP4 were shown as “+” or “-”.



**Figure S4** SUSP4-mediated promotion of p53 transcriptional activity. (a) pG13-Luc and pcDNA-β-Gal reporter vectors were transfected to NIH3T3 cells with indicated combinations of pCMV2-Flag-SUSP4 (wt), pCMV2-Flag-SUSP4-C460S (mt), and pcDNA-Myc-p53. Cell lysates were subjected to immunoblot analysis and luciferase assay. The luciferase activities seen with transfection of the indicated vectors were expressed as relative folds to the activity seen with transfection of empty vectors after normalization with β-galactosidase activity. Total amounts of transfected DNA were kept constant by supplementing proper amounts of empty vectors. Data represents mean (SD of triplicates). (b-d) Experiments were performed as in a, but using

different combinations of the vectors. In b, pGL2-Luc and pcDNA-p50 were used in place of pG13-Luc and pcDNA-Myc-p53. In c, both pcDNA-Myc-p53 (wt) and pcDNA-Myc-p53-K386R (mt) were used. In d, pcDNA-Mdm2, pCMV2-Flag-SuPr1 (wt), and pCMV2-Flag-SuPr1-C466S (mt) were also used. (e) NIH3T3 cells were transfected with the indicated combinations of pcDNA-Myc-p53, pcDNA-Mdm2, pcDNA-p50, and pCMV2-Flag-SUSP4 (wt) or pCMV2-Flag-SUSP4-C460S (mt). Cell lysates were immunoblotted with anti-p53 or anti-Mdm2 antibody (upper). Total RNAs were extracted from the cells using Trizol reagent (Invitrogen), and subjected to real-time RT-PCR using specific primers for p21 and GAPDH (lower).



**Figure S5** (a) Full scans of the upper two panels of Fig. 3a. (b) Full scans of the second and fourth panels of Fig. 4f. (c) Full scans of the upper panels

of Fig. 4h. (d) Full scans of the left upper two panels and the right lower two panels in Fig. 5d.