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The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2

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Mdm2 is an E3 ubiquitin ligase that promotes its own ubiquitination and also ubiquitination of the p53 tumour suppressor. In a bacterial two-hybrid screen, using Mdm2 as bait, we identified an Mdm2-interacting peptide that bears sequence similarity to the deubiquitinating enzyme USP2a. We have established that full-length USP2a associates with Mdm2 in cells where it can deubiquitinate Mdm2 while demonstrating no deubiquitinating activity towards p53. Ectopic expression of USP2a causes accumulation of Mdm2 in a dose-dependent manner and consequently promotes Mdm2-mediated p53 degradation. This differs from the behaviour of HAUSP, which deubiquitinates p53 in addition to Mdm2 and thus protects p53 from Mdm2-mediated degradation. We further demonstrate that suppression of endogenous USP2a destabilises Mdm2 and causes accumulation of p53 protein and activation of p53. Our data identify the deubiquitinating enzyme USP2a as a novel regulator of the p53 pathway that acts through its ability to selectively target Mdm2.

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Introduction

p53 plays a critical role in preserving the integrity of the genome. Diverse cellular stresses result in p53 stabilisation and activation, which leads to the expression of genes involved in cell-cycle arrest and apoptosis (Vogelstein *et al*, 2000; Michael and Oren, 2003). Inhibition of p53 activity either through mutation or other mechanisms, including overexpression of Mdm2 and loss of p14ARF, is frequently required for tumour progression. Restoration of the endogenous p53 pathway is an attractive therapeutic approach for tumours expressing wild-type p53 (Lain and Lane, 2003; Woods and Lane, 2003; Vousden and Prives, 2005).

Under normal conditions, p53 is tightly regulated. Mdm2 binds the N-terminal domain of p53, inhibiting its transcriptional activity directly (Momand *et al*, 1992; Oliner *et al*, 1993), and its E3 ligase activity promotes p53 ubiquitination and proteasomal degradation (Haupt *et al*, 1997; Honda *et al*, 1997; Kubbutat *et al*, 1997). Mdm2 can also inhibit the transcriptional activity of p53 by promoting its conjugation to the ubiquitin-like molecule, NEDD8 (Xirodimas *et al*, 2004). As Mdm2 is itself a transcriptional target of p53 (Barak *et al*, 1993; Wu *et al*, 1993), the relationship between Mdm2 and p53 defines a negative feedback loop (Picksley and Lane, 1993; Prives, 1998; Oren *et al*, 2002). Mdm2 has also been reported to have a number of p53-independent functions (Ganguli and Wasylyk, 2003) and is overexpressed in a number of tumours, where it has been proposed to promote tumour survival through both repression of p53 and p53-independent mechanisms (Wang *et al*, 2001).

Deubiquitinating enzymes (DUBs) represent one of the largest families of proteins involved in the ubiquitin–proteasome system (Nijman *et al*, 2005). The abundance of family members presumably allows for diversity and specificity of DUB activity, although relatively little is known about the physiological substrates of individual members of the family. DUBs have several functions, which include processing and recycling of ubiquitin (Wing, 2003) and reversal of ubiquitination of specific substrate proteins, analogous to the reversal of protein phosphorylation by phosphatases (Li *et al*, 2002; Brummelkamp *et al*, 2003; Kovalenko *et al*, 2003; Graner *et al*, 2004). There is precedent for a role of DUB activity within the p53–Mdm2 pathway, as demonstrated by the activity of HAUSP, which deubiquitinates p53, Mdm2 and Mdmx. The effects of endogenous HAUSP on the p53 pathway are concentration-dependent. Partial knockdown of HAUSP causes destabilisation of p53, whereas more complete suppression can cause destabilisation of Mdm2 and Mdmx and accumulation of p53 (Li *et al*, 2002, 2004; Cummins *et al*, 2004; Meulmeester *et al*, 2005).

In this study we show that the deubiquitinating enzyme USP2a associates with Mdm2 *in vivo*. USP2a can deubiquitinate Mdm2, without reversing Mdm2-mediated ubiquitination of p53. Ectopic expression of USP2a therefore causes an increase in the levels of Mdm2 and promotes p53 degradation. Transfection of tumour-derived cell lines with siRNA-targeting USP2a results in increased p53 protein expression and upregulation of p53 target genes. We thus identify USP2a as a regulator of the Mdm2/p53 pathway that makes a significant contribution to repression of p53 activity *in vivo*. As such, the Mdm2-selective deubiquitinating enzyme USP2a represents a potential target for therapeutic interventions aimed at reactivating wild-type p53 in tumours.

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Results

A bacterial two-hybrid screen identified Mdm2-interacting peptides

In order to identify Mdm2 binding partners that might not be identified by more conventional means, we undertook a bacterial two-hybrid screen, using full-length Mdm2 as bait against a human breast cDNA target library (Phizicky and Fields, 1995; Toby and Golemis, 2001). Three million Mdm2 bait and cDNA library target cotransformants were plated, and within the first 250 000 clones screened, we identified seven positive clones. Interestingly, all of these clones expressed peptides and not full-length proteins. Five of the clones were derived from the 5′ untranslated region (5′UTR) of ubiquitin B. These clones expressed 20–21-mer peptides encoded by the normally untranslated sequence that is immediately upstream of the initiating ATG for ubiquitin B. Examination of this sequence, herein referred to as clone 6245, revealed that it possessed the key residues with the requisite spacing necessary for binding to Mdm2 via the p53-binding pocket (Bottger *et al*, 1996) (Figure 1A). Clone 6012, derived from the 3′ UTR region of ubiquilin 4, expressed a 16-mer peptide with similar properties. The fact that this screen identified clones that encoded peptides with p53-like characteristics validated the methodology, and indicated that we were accurately identifying bona fide Mdm2-interacting peptides. The remaining clone, designated 5251, expressed a 91-mer protein sequence that was derived from the coding region of NPC2, but which was the result of an out-of-frame fusion. No region of this 91-mer peptide bore any resemblance to p53. However, a 10-amino acid stretch of clone 5251 showed near identity with sequence in the N terminus (residues 72–81) of the deubiquitinating enzyme, USP2a (Figure 1B). To further characterise the interaction of the various peptides with Mdm2, an ELISA was used to detect interaction of purified Mdm2 with the peptides. Biotinylated peptides that spanned the relevant regions of clones 6245, and 5251, as well as the region of human USP2a with sequence similarity to clone 5251 were synthesised. Peptides were bound to streptavidin-coated 96-well plates and incubated with purified Mdm2. Clone 5251 and the homologous peptide derived from USP2a bound to Mdm2 (Figure 1C). In order to determine whether 5251 and USP2a-derived peptides could bind to Mdm2 *in vivo*, the peptides were expressed as C-terminal fusions with EGFP and transfected into U-2 OS cells. Cell lysates were immunoprecipitated with anti-Mdm2 antibody and blotted for EGFP. EGFP was undetectable in immunoprecipitates from cells transfected with the control construct, which expressed EGFP fused to an unrelated peptide (Figure 1D). The positive control peptide, EGFP-12.1, which is a p53-like sequence optimised for Mdm2 binding (Bottger *et al*, 1996), and EGFP fusions expressing either 5251 or USP2a-derived peptides were detected in Mdm2 immunoprecipitates.

As USP2a-derived peptides could interact with Mdm2 both *in vitro* and *in vivo*, we then examined whether full-length USP2a interacts with Mdm2 in cells. U-2 OS cells were transfected with USP2a and cell lysates were immunoprecipitated with anti-Mdm2 antibody, 4B2, or control IgG. Full-length ectopically expressed USP2a specifically co-immunoprecipitated with endogenous Mdm2 (Figure 1E). To determine whether Mdm2 associates with endogenous USP2a,

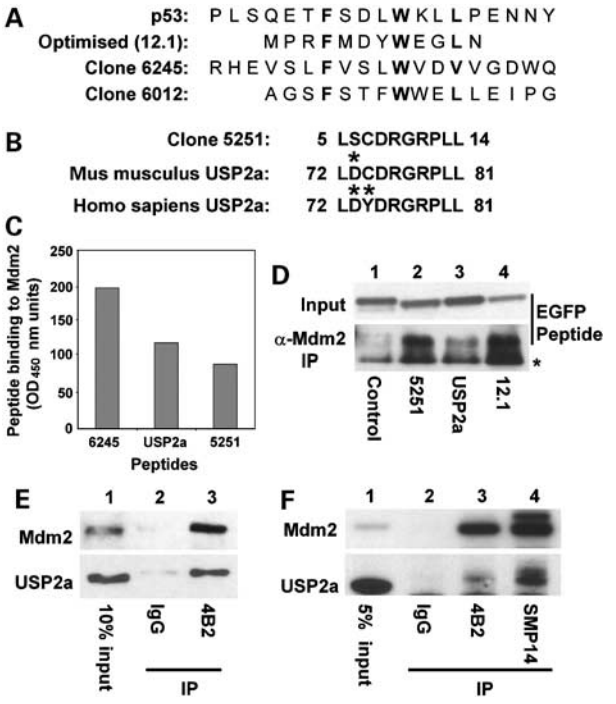

Figure 1 USP2a interacts with Mdm2. (**A**) Mdm2-interacting peptides were identified in a bacterial two-hybrid screen. p53-like sequences, from clones identified in the bacterial two-hybrid screen, aligned with the relevant portion of wild-type p53 and a p53-derived sequence optimised for binding to Mdm2. (**B**) Clone 5251 has homology with USP2a. Alignment of the relevant portion of clone 5251 sequence with mouse and human USP2a. (**C**) Peptides identified by two-hybrid screening interact with Mdm2. 16-mer biotinylated peptides derived from USP2a, clone 5251 and clone 6245 were used to capture purified Mdm2 in an ELISA. (**D**) EGFP-peptide fusion constructs were expressed in U-2 OS cells. Cell lysates were immunoprecipitated with α-Mdm2 antibody 4B2 and blotted for EGFP. Control (lane 1) expresses EGFP fused to an unrelated peptide. Top panel shows an EGFP blot from 7% of the IP input. *Denotes IgG light chain. (**E**) USP2a forms complexes with Mdm2 *in vivo*. USP2a was expressed in U-2 OS cells. Cell lysates were immunoprecipitated with α-Mdm2 antibody or control IgG and Mdm2 and USP2a detected by Western blotting. (**F**) Immunoprecipitations were performed from lysates of untransfected NTERA-2 cell with an irrelevant IgG or with anti-Mdm2 antibodies 4B2 or SMP14. Immunoprecipitates were analysed by Western blotting for Mdm2 and USP2a.

Mdm2 was immunoprecipitated with 4B2 and SMP14 from lysates of NTERA-2 testicular embryonal carcinoma cells. USP2a co-precipitated with Mdm2, indicating that endogenous USP2a forms complexes with Mdm2 *in vivo* (Figure 1F).

We have shown that the peptide corresponding to residues 72–81 of USP2a can bind to Mdm2 both *in vitro* and *in vivo*. To further map the site(s) required for Mdm2 binding to USP2a, bacterially expressed GST-Mdm2 was incubated with ^{35}S-labelled full-length USP2a and with N- and C-terminal deletions of USP2a (Figure 2A). GST-Mdm2 but not GST interacted with full-length USP2a. USP2a 200–605, which lacks the USP2a peptide 72–81, and USP2a 1–403 also bound to Mdm2 (Figure 2B). Loss of binding to Mdm2 required deletion of both the N- and C-termini of USP2a. This indicates that there are at least two binding sites for Mdm2 in USP2a: one at the N terminus, as discovered in the two-hybrid screen, and another at the C-terminus. To map binding sites for USP2a in Mdm2, ^{35}S-labelled full-length

USP2a was incubated with a series of GST-tagged Mdm2 deletions (Figure 2C). GST-Mdm2 1–110 and 276–491 both bound to USP2a (Figure 2D). Thus, consistent with the presence of two Mdm2 binding sites in USP2a, there are also at least two USP2a binding sites in Mdm2.

USP2a causes accumulation of Mdm2 and leads to an Mdm2-dependent decrease in p53 levels

Mdm2 is an E3 ligase that promotes the ubiquitination of p53 (Honda *et al*, 1997; Fang *et al*, 2000; Honda and Yasuda, 2000). Mdm2 also 'auto-ubiquitinates' and is itself targeted for degradation by the proteasome. The expression of Mdm2 and p53 can be regulated by HAUSP as a consequence of its ability to deubiquitinate both proteins (Li *et al*, 2004). We investigated whether expression of the deubiquitinating enzyme, USP2a, would affect Mdm2 and p53 protein levels. To address effects on Mdm2 independently of p53, H1299 cells (p53 null) were cotransfected with Mdm2 and increasing amounts of USP2a. Expression of USP2a led to a dose-dependent increase in Mdm2 levels that was similar in magnitude to the increase in Mdm2 resulting from the over-expression of HAUSP (Figure 3A). Mutation of the active site His residue in the conserved His box of DUBs has been shown to render them catalytically inactive (Yoo *et al*, 2005). Cotransfection of a His-box catalytic mutant of USP2a (H549A) resulted in only a modest accumulation of Mdm2

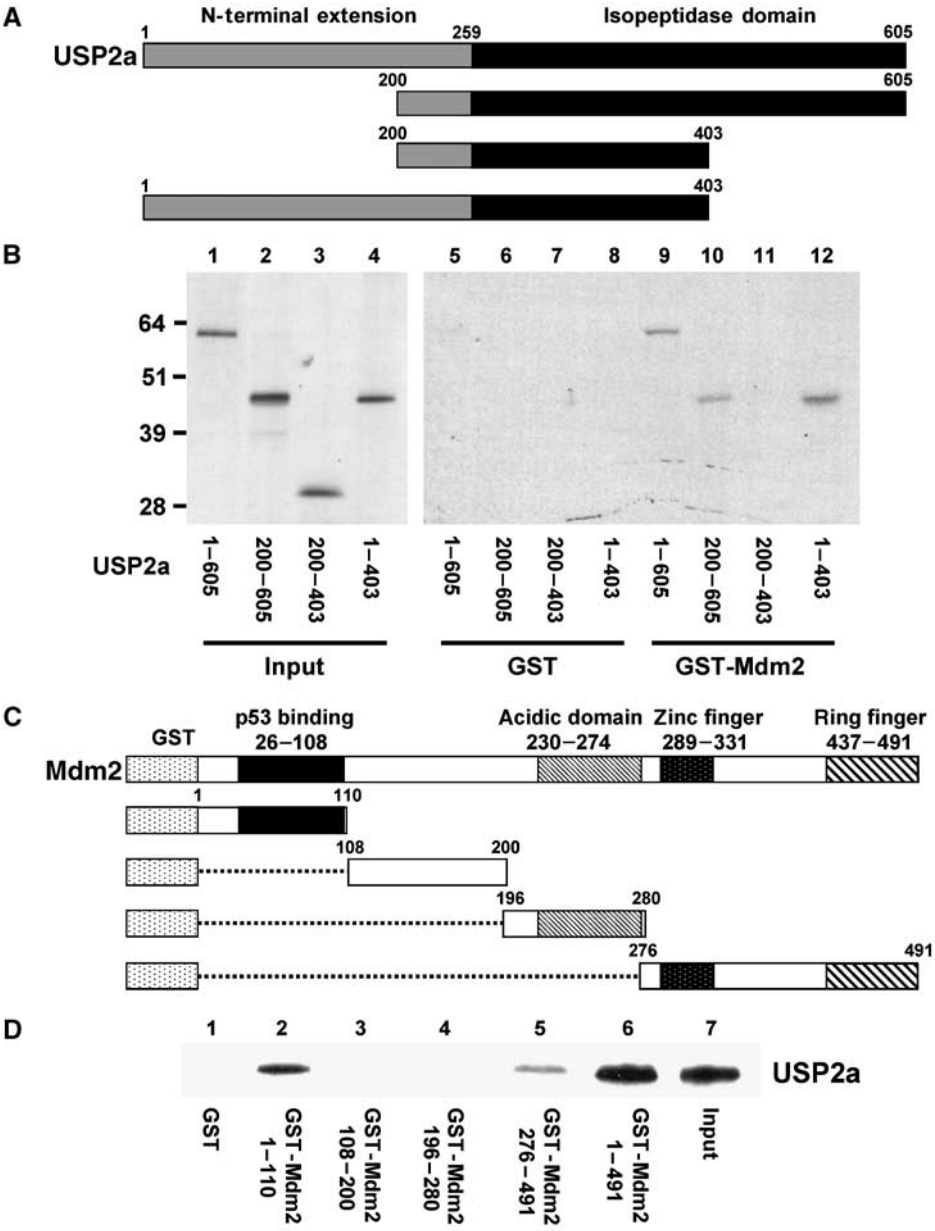

Figure 2 Mapping of the sites of interaction. (**A**) Schematic representation of wild-type USP2a and its deletions. (**B**) Mapping of Mdm2 binding sites in USP2a. ^{35}S-labelled full-length USP2a (1–605) and the indicated N- and C-terminal deletions were prepared by IVT. The labelled proteins were incubated with GST or GST-Mdm2 and bound proteins were detected by autoradiography. (**C**) Schematic representation of wild-type Mdm2 and its deletions. (**D**) Mapping of USP2a binding sites in Mdm2. Full-length ^{35}S-labelled USP2a (1–605) prepared by IVT was incubated with GST, GST-full-length Mdm2 (1–491) or the indicated GST-Mdm2 deletion mutant. Bound proteins were detected by autoradiography.

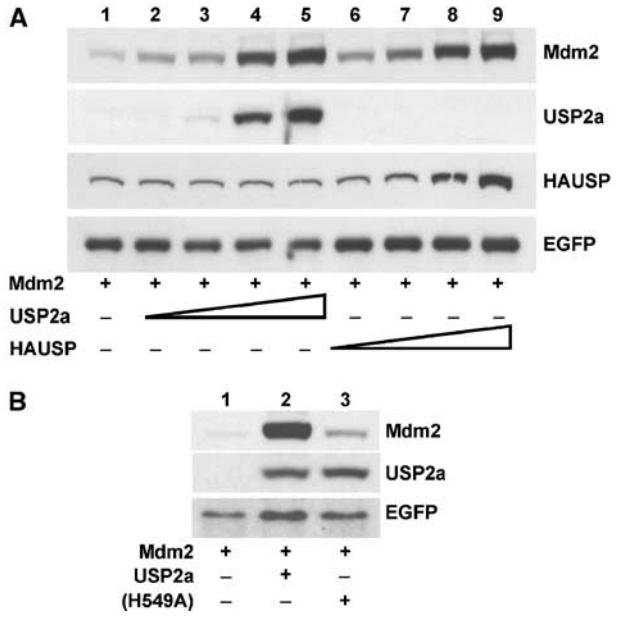

Figure 3 Exogenous wild-type USP2a causes accumulation of Mdm2. (**A**) H1299 cells were transfected with a constant amount of Mdm2 and increasing amounts (1, 3, 10 and 15 μg) of USP2a (lanes 2–5) or HAUSP (lanes 6–9). Levels of protein expression were determined by Western blotting. (**B**) Mdm2 accumulation is dependent on the deubiquitinating activity of USP2a. H1299 cells were transfected with Mdm2 and wild-type USP2a or a catalytic mutant of USP2a (H549A). Protein expression was analysed by Western blotting.

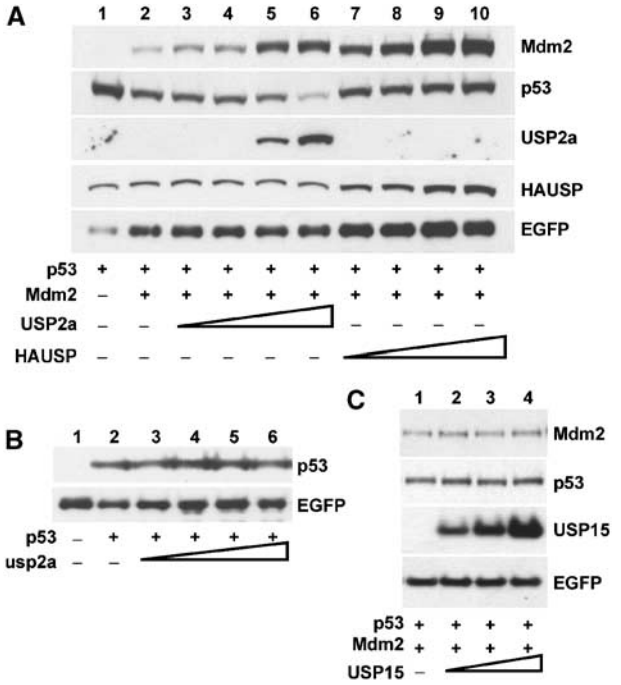

Figure 4 USP2a promotes Mdm2-mediated degradation of p53. (**A**) H1299 cells transfected with p53 (lanes 1–10) and Mdm2 where indicated (lanes 2–10) in the presence of increasing amounts (1, 3, 10 and 15 μg) of USP2a (lanes 3–6) or HAUSP (lanes 7–10) were lysed and blotted for the indicated proteins. (**B**) The effect of USP2a on p53 levels is dependent on the ectopic expression of Mdm2. H1299 cells were transfected with p53 and increasing amounts (1, 3, 10 and 15 μg) of USP2a. p53 and EGFP protein expression was analysed by Western blotting. (**C**) Mdm2 and p53 levels are not regulated by USP15. H1299 cells were transfected with Mdm2 and p53 alone (lane 1) or in the presence of increasing amounts (2, 5 and 15 μg) of USP15 (lanes 2–4). Protein expression was determined by Western blotting.

(Figure 3B), indicating that the deubiquitinating activity of USP2a is required for its effects on Mdm2 expression.

To determine the effect of USP2a on Mdm2-mediated degradation of p53 H1299, cells were transfected with Mdm2 and p53 and increasing amounts of USP2a. Overexpression of USP2a resulted in a dose-dependent increase in Mdm2 levels, with a concomitant decrease in p53 levels (Figure 4A). In contrast, in the absence of exogenous Mdm2, USP2a had no direct effect on cotransfected p53 levels in H1299 cells (Figure 4B). For comparison, we also examined the effects of HAUSP in these assays. As previously observed (Li *et al*, 2004; Meulmeester *et al*, 2005), expression of HAUSP protected p53 from Mdm2-mediated degradation despite high levels of Mdm2 (Figure 4A). In contrast, even at the highest dose of USP2a, no rescue of p53 degradation was observed. Overexpression of another DUB, USP15, which has been shown to have deubiquitinating activity (Hetfeld *et al*, 2005), had no effect on Mdm2 or p53 protein levels (Figure 4C), indicating, as previously observed (Li *et al*, 2004; Meulmeester *et al*, 2005), that overexpression of a deubiquitinating enzyme *per se* does not stabilise Mdm2/p53.

USP2a deubiquitinates Mdm2 in cells

To investigate whether Mdm2 is a substrate for the deubiquitinating activity of USP2a, the effect of USP2a cotransfection on Mdm2 ubiquitination was examined. In order to inhibit degradation of ubiquitinated proteins, cells were treated with the proteasome inhibitor MG132 for 6 h before lysis in SDS–urea sample buffer. High-molecular-weight Mdm2 and p53 conjugates corresponding to proteins modified with endogenous ubiquitin or ubiquitin-like molecules were detected in extended exposures of Western blots of total cell lysates (Figure 5A). Cotransfected HAUSP reduced the level of both Mdm2 and p53 conjugates. Wild-type USP2a but not the catalytic site mutant reduced the relative level of Mdm2 conjugates. In contrast, the levels of conjugated p53 were not affected by USP2a. To confirm that the high-molecular-weight conjugates observed in total cell lysates represent ubiquitinated species, H1299 cells were cotransfected with His_6-ubiquitin and treated with MG132 before harvesting. Ubiquitinated proteins were purified from transfected cell lysates using Ni^{2+}-agarose beads and the purified lysates were blotted for p53 and Mdm2. Control transfections, lacking the His_6-ubiquitin construct, were performed in parallel to demonstrate that the purified species detected were ubiquitin conjugates (Figure 5B, lanes 1 and 5). HAUSP was able to deubiquitinate both Mdm2 and p53. Expression of USP2a, however, led to a dramatic decrease in the ubiquitinated species of Mdm2 (Figure 5B, upper panel, compare lanes 2 and 4), whereas p53 ubiquitination was increased (compare lanes 6 and 8). This indicates that USP2a deubiquitinates Mdm2, but is ineffective in deubiquitinating p53 *in vivo*. To further investigate the specificity of both USP2a and HAUSP, we blotted Ni^{2+}-purified cell lysates for ubiquitin in order to assess any effects on the total cellular population of ubiquitinated species. Neither USP2a nor HAUSP had any global effects on the total ubiquitinated species in the lysates under

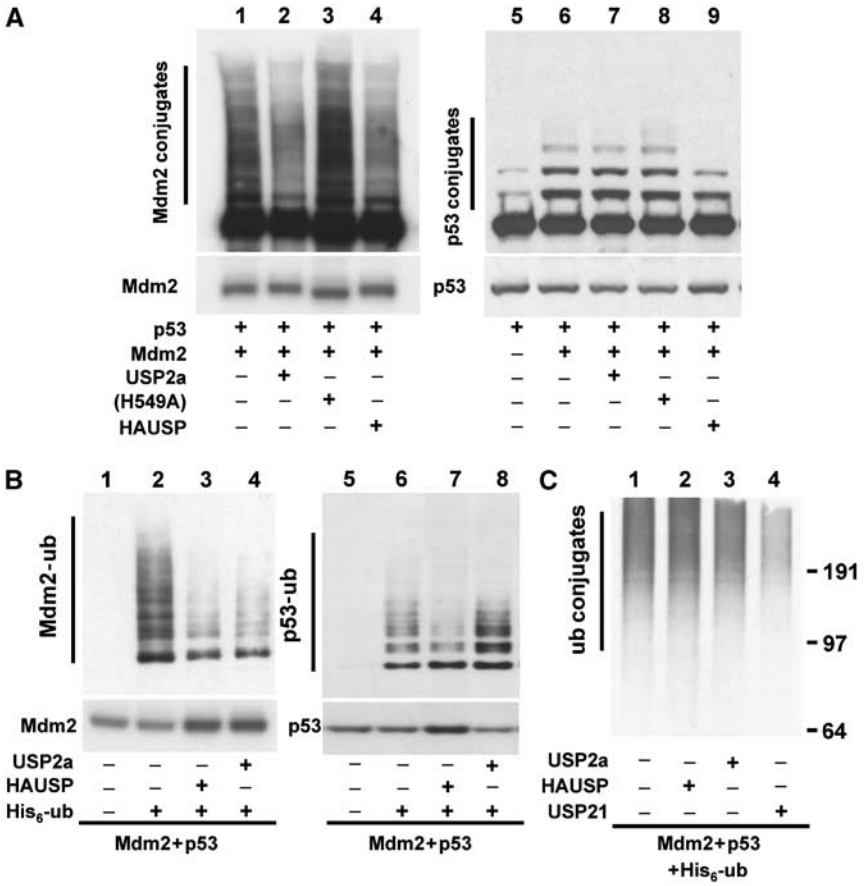

Figure 5 USP2a selectively deubiquitinates Mdm2 *in vivo*. (**A**) Western blots of total cell lysates. H1299 cells were transfected with p53 (lanes 1–9) and Mdm2 (lanes 1–4 and 6–9) in the presence of wild-type USP2a, a catalytic mutant of USP2a (H549A) or HAUSP. MG132 was added to the cells 6 h before harvesting in SDS–urea sample buffer. Samples were analysed by Western blotting for Mdm2 and p53. The lower panels are short exposures showing the level of unmodified protein. The upper panels are extended exposures of the same blots showing protein conjugates. To allow a direct comparison of the effects of DUB expression on the stoichiometry of Mdm2 conjugates, the amount of extracts loaded onto the SDS–PAGE gels was adjusted so that each sample contained matched amounts of unmodified Mdm2. (**B**) Ni^{2+}-agarose purification of ubiquitinated proteins. H1299 cells were transfected with Mdm2 and p53 alone (lanes 1 and 5) or in the presence of His_6-ubiquitin (His_6-ub) (lanes 2–4 and 6–8) and the indicated DUBs. Cells were treated with MG132 for 4 h before lysis. Lysates were blotted for Mdm2 and p53 (bottom panels). Ubiquitinated proteins were purified using Ni^{2+}-agarose and blotted for Mdm2 or p53 (upper panels). (**C**) USP2a expression does not alter the general pattern of ubiquitin conjugates. H1299 cells were transfected with Mdm2, p53, His_6-ub and the indicated DUBS and were treated with MG132 for 4 h before lysis. Ubiquitinated proteins were purified using Ni^{2+}-agarose and analysed by Western blotting for ubiquitin.

conditions where USP21, as reported previously (Gong *et al*, 2000), was able to reverse global ubiquitination (Figure 5C, lane 4).

Knockdown of USP2a increases p53 protein levels and transcriptional activity

We next sought to determine the contribution of endogenous USP2a to the regulation of the p53 pathway. NTERA-2 testicular embryonal carcinoma cells, which express wild-type p53 and Mdm2, were transfected with a control siRNA, or siRNAs targeting either USP2a or Mdm2. siRNA-mediated knockdown of Mdm2 resulted in increased expression of p53, confirming that levels of p53 are regulated by endogenous Mdm2 in these cells (Figure 6A). USP2a knockdown also caused accumulation of p53. This USP2a siRNA, which targets the 5′UTR of USP2a, has been characterised previously and shown to effectively reduce USP2a protein levels (Graner *et al*, 2004). A decrease in Mdm2 protein expression was frequently observed following transfection with USP2a siRNA, as shown in Figure 6A; however in some experiments, no change or a modest increase was observed. As a feedback loop exists between p53 and Mdm2, activation of p53 and increases in Mdm2 mRNA would tend to counteract decreases in Mdm2 protein expression. To determine the effect of USP2a knockdown on Mdm2 and p53 protein stability, NTERA-2 cells were transfected with control or USP2a siRNA, and 48 h later, cycloheximide was added to inhibit protein synthesis. Suppression of USP2a destabilised Mdm2, whereas the stability of p53 was increased (Figure 6C). These observations are consistent with suppression of endogenous USP2a causing a reduction in deubiquitination of Mdm2, which then promotes Mdm2 degradation and leads to attenuation of Mdm2-mediated degradation of p53.

USP2a is overexpressed in prostate cancer and has been shown to regulate the stability of fatty acid synthase in LNCaP prostate cancer-derived cells (Graner *et al*, 2004). We investigated whether USP2a also regulates the p53 pathway in these cells, which express wild-type p53, by transfecting them with control, USP2a or Mdm2 siRNAs. USP2a knockdown resulted in a decrease in Mdm2 protein

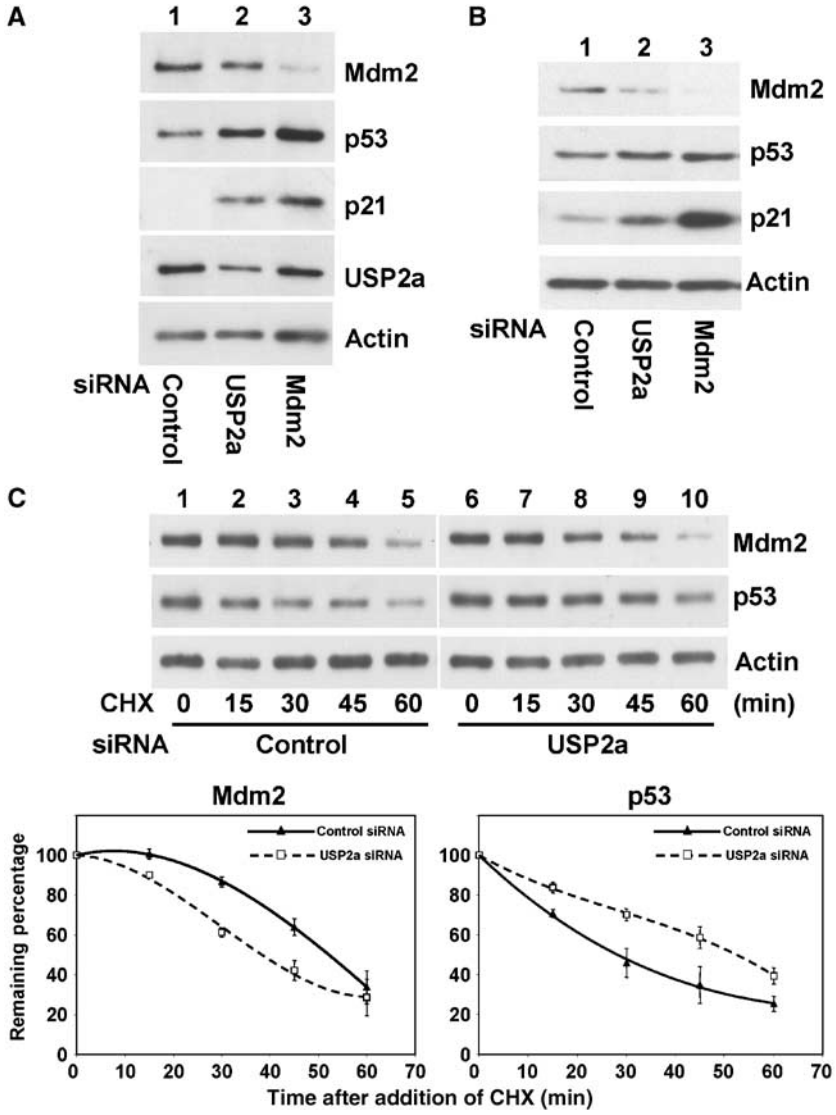

Figure 6 Suppression of endogenous USP2a causes accumulation of p53. (**A**) NTERA-2 cells transfected with the indicated siRNAs were lysed after 70 h and protein expression analysed by Western blotting. (**B**) LNCaP prostate cancer cells transfected with the indicated siRNAs were lysed after 48 h and protein expression was determined by Western blotting. (**C**) Knockdown of USP2a destabilises Mdm2 and stabilises p53. Forty-eight hours after transfection with control or USP2a synthetic siRNA duplexes, NTERA-2 cells were incubated with cycloheximide (20 μg/ml) for the indicated times. Mdm2 and p53 protein expression was determined by Western blotting. Because USP2a knockdown increases expression of p53, the Western blot shown in the left panel (control siRNA) was exposed for longer than that in the right panel (USP2a siRNA) to allow comparison of similar intensities of p53 signal. The lower panel shows quantification of the levels of Mdm2 and p53. The results are expressed as a percentage of expression in the absence of cycloheximide and are the average ± s.e.m. of three experiments.

expression and an increase in p53 protein levels (Figure 6B), indicating that endogenous USP2a also suppresses p53 in this cell line.

Knockdown of USP2a in both NTERA-2 and LNCaP cells resulted in increased protein expression of the p53 target gene *p21* (Figure 6A and B) We examined the effects of USP2a suppression on mRNA levels of p53-responsive genes. To determine the dependence on endogenous p53, experiments were carried out in an NTERA-2-derived cell line overexpressing a dominant-negative p53 mini-protein (DDp53) (Ostermeyer *et al*, 1996; Smart *et al*, 1999) and a matched control cell line stably transfected with empty vector (CMVNeo). The DDp53 mini-protein has been well characterised and consists of the first 14 amino acids of p53 fused to the oligomerisation domain of p53 (Shaulian *et al*, 1992). DDp53 interacts with the oligomerisation domain of endogenous p53, blocking its oligomerisation and consequently its transcriptional activity. USP2a knockdown in control CMVNeo NTERA-2 cells caused an increase in the levels of p21 and Bax mRNA (Figure 7A). DDp53 inhibited induction of p21 and BAX mRNAs following USP2a knockdown. The *Mdm2* gene has a promoter that is p53 independent (P1) and a second promoter that is p53 responsive (P2). Consistent with the operation of the feedback loop involving p53 activation, USP2a knockdown resulted in a p53-dependent increase in the levels of Mdm2 mRNA synthesised from the P2 promoter, while having no effect on Mdm2 mRNA expression from the P1 promoter. These data indicate that suppression of USP2a results in transcriptional activation of p53.

To determine the effect of USP2a suppression on cell-cycle progression, NTERA-2 cells were transfected with control,

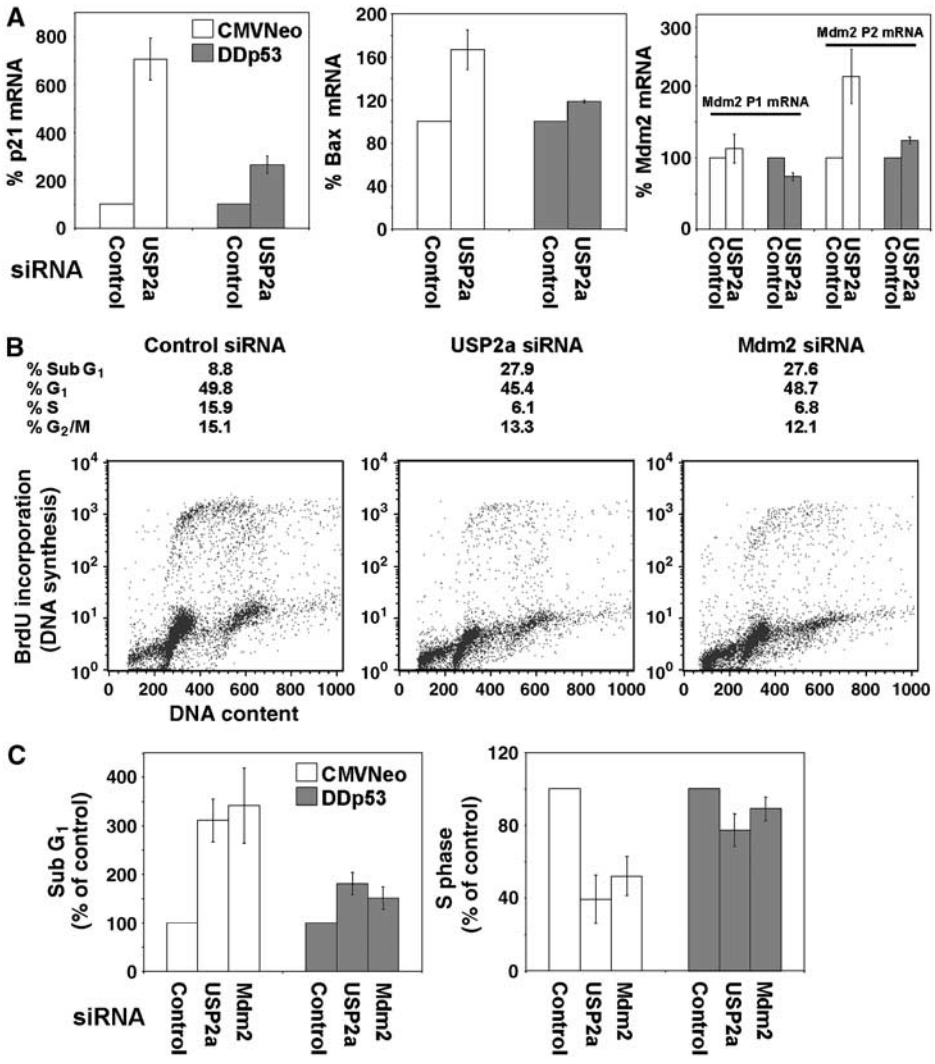

Figure 7 Knockdown of USP2a activates p53 in NTERA-2 cells. (**A**) USP2a suppression causes a p53-dependent increase in p53 target gene expression. Control (CMVNeo) or NTERA-2 cells expressing dominant-negative p53 (DDp53) were transfected with control or USP2a siRNA, total RNA was isolated and p53-target gene mRNA levels were quantitated by real-time PCR. mRNA levels are normalised to actin and expressed as a percentage of control (control siRNA transfection in the particular cell line). Values are means ± s.d. of four experiments. (**B**) Suppression of USP2a causes cell death. NTERA-2 cells were transfected with the indicated siRNA. Seventy-two hours after transfection, cells were pulsed with BrdU and harvested for flow cytometry. The percentage cell-cycle distribution and the profile of a representative experiment are shown. (**C**) The effects of USP2a knockdown on cell-cycle progression are p53 dependent. Control (CMVNeo) or NTERA-2 cells expressing dominant-negative p53 (DDp53) were transfected with the indicated siRNAs, pulse-labelled with BrdU and harvested for FACS analysis. The results are expressed as a percentage of control (control siRNA transfection in the particular cell line) and are the average ± s.d. of four experiments.

USP2a or Mdm2 siRNAs, pulse-labelled with BrdU to measure DNA synthesis and analysed by FACS. Knockdown of USP2a, like knockdown of Mdm2, was associated with a decrease in DNA synthesis and an increase in the proportion of cells in the sub-G_1 population (Figure 7B). Therefore, knockdown of USP2a, like suppression of Mdm2, results in increased cell death. DDp53 attenuated the decrease in DNA synthesis and the increase in cell death observed following USP2a or Mdm2 knockdown, indicating that these cell-cycle effects are dependent on the activity of endogenous p53 (Figure 7C).

Discussion

Despite the critical role of the ubiquitination pathway in the regulation of cellular processes, surprisingly little is known about the substrate specificity of individual deubiquitinating enzymes. In this study we have identified Mdm2 as a substrate for the deubiquitinating enzyme USP2a. USP2a acts selectively on the p53 pathway in that it can deubiquitinate Mdm2 without reducing p53 ubiquitination. Suppression of USP2a results in the destabilisation of Mdm2 and activation of p53 *in vivo*. The identification of USP2a as a regulator of Mdm2 will further our understanding of the mechanism of control of p53.

The role of USP2a in normal tissues has not been defined previously. It has been suggested that USP2a may play a part in normal spermatogenesis (Lin *et al*, 2000), a process in which p53 is also thought to be involved (Rotter *et al*, 1993; Schwartz *et al*, 1999). In mice and rats, USP2a mRNA levels are highest in testes, but mRNA is also expressed in many other tissues (Lin *et al*, 2001; Park *et al*, 2002; Gousseva and Baker, 2003). We have confirmed these results with a panel

of human RNA (data not shown). This suggests that USP2a could play a role in multiple normal tissues.

USP2a associates with Mdm2 *in vivo*. Our two-hybrid screen identified sequences within the N-terminus of USP2a that are sufficient for binding to Mdm2. However, deletion of both the N- and C-termini of USP2a was required to prevent complex formation with Mdm2. This indicates that there is an additional Mdm2 binding site in the C-terminus of USP2a. The USP2 gene encodes a second deubiquitinating enzyme, USP2b, as a result of alternative splicing of 5′ exons. USP2a and USP2b have different N-terminal extensions, but share an identical C-terminal catalytic core (Gousseva and Baker, 2003; Graner *et al*, 2004), which contains the canonical isopeptidase Cys and His boxes which include the residues critical for catalysis. USP2b may consequently interact with Mdm2 through its common C-terminus. Further studies will be required to investigate the role of USP2b in the regulation of Mdm2. Consistent with the presence of two binding sites in USP2a, two regions of Mdm2 were able to associate with USP2a. Residues 1–110 at the N-terminus of Mdm2 were able to bind USP2a. In addition a second USP2a binding site mapped to residues 276–491. This latter region contains both the zinc finger and ring finger of Mdm2. Multiple contact points in USP2a could aid in properly orientating the DUB to mediate deubiquitination of its substrate. HAUSP has been shown to interact with residues 147–159 (Sheng *et al*, 2006) and 223–232 of Mdm2 (Hu *et al*, 2006), indicating that Mdm2 also has multiple binding sites for HAUSP, which are distinct from the USP2a binding sites.

We show that USP2a deubiquitinates Mdm2 *in vivo*. The behaviour of USP2a displays specificity with respect to the components of the p53 pathway. Overexpression of USP2a consequently results in elevated levels of Mdm2 and enhanced Mdm2-mediated degradation of p53. We compared the effects of USP2a overexpression with those elicited by HAUSP (Cummins *et al*, 2004; Li *et al*, 2004; Meulmeester *et al*, 2005). In marked contrast to USP2a, HAUSP protected p53 from Mdm2-mediated degradation, despite the presence of high levels of Mdm2. This correlated with the ability of HAUSP to deubiquitinate both Mdm2 and p53. USP2a has previously been shown to stabilise fatty acid synthase (Graner *et al*, 2004), and it is probable that additional substrates also exist. We have demonstrated that a peptide from the N-terminal extension of USP2a binds Mdm2. Such extensions of deubiquitinating enzymes are proposed to be involved in mediating their interaction with specific substrates (D'Andrea and Pellman, 1998). Indeed, the N-terminus of HAUSP has been shown to interact with both p53 and Mdm2 (Hu *et al*, 2006).

We investigated the role of endogenous USP2a in regulation of the p53 pathway. siRNA-mediated knockdown of USP2a in the NTERA-2 testicular embryonal carcinoma cell line resulted in destabilisation of Mdm2, stabilisation of p53 and an increase in p53 protein expression. Suppression of USP2a caused a p53-dependent increase in the mRNA levels of p53-responsive genes involved in both cell-cycle arrest (*p21*) and apoptosis (*Bax*) and of *Mdm2* itself. This indicates that the accumulated p53 is transcriptionally active. Knockdown of USP2a, like siRNA-mediated suppression of Mdm2, resulted in a p53-dependent decrease in DNA synthesis and an increase in cell death. This demonstrates that regulation of the p53 pathway by USP2a is of physiological significance. We propose that in this tumour cell line USP2a, contributes to the maintenance of Mdm2 stability and thus promotes p53 degradation and limits p53 activity. When USP2a is inhibited, Mdm2 is destabilised, allowing accumulation and transcriptional activation of p53.

In the majority of cases, attenuation of p53 function occurs during tumour progression to facilitate escape from p53 surveillance. In approximately half of tumours p53 is inactivated by mutation, whereas in tumours expressing wild-type p53 other mechanisms are involved in its suppression. USP2a could act as an oncogene when overexpressed by inhibiting wild-type p53 function through stabilisation of Mdm2. Prostate cancers frequently express wild-type p53, and overexpression of Mdm2 without gene amplification has been linked with poor prognosis (Osman *et al*, 1999). Furthermore, Priolo *et al* (2006) show that USP2a is oncogenic, and that it is overexpressed in approximately half of prostate tumours. They also demonstrate that high levels of USP2a in prostate cancers correlate with changes in gene expression patterns that are consistent with the repression of p53 transcriptional activity. These observations suggest that overexpression of USP2a could cause suppression of wild-type p53 during the development of prostate cancer. Consistent with this hypothesis, we observed that knockdown of USP2a causes accumulation of p53 and p21 protein in the LNCaP prostate cancer-derived cell line. Intriguingly, USP2a is upregulated in response to androgens (Graner *et al*, 2004). Androgens promote the survival of prostate cancers, and there is evidence that this involves androgen-mediated repression of p53 function (Sengupta and Wasylyk, 2004). Further work will be required to determine whether USP2a participates in androgen regulation of p53.

We observed that USP2a knockdown results in the p53-dependent death of NTERA-2 cells. This result is noteworthy as there is considerable interest in the development of non-genotoxic therapies aimed at activating wild-type p53 in tumours (Lain and Lane, 2003; Woods and Lane, 2003). Additionally, Mdm2 has tumour-promoting activity in the absence of normally functioning p53 (Wang *et al*, 2001; Ganguli and Wasylyk, 2003). The selective destabilisation of Mdm2 would make USP2a a useful target for therapeutic intervention in p53-positive tumours, as well as in p53-negative backgrounds, in which Mdm2 is required for tumour progression. USP2a-directed therapies could take advantage of two approaches. In addition to therapeutic agents that inhibit USP2a directly, agents could also be developed that block the interaction of USP2a with Mdm2. Our screen identified a peptide with similarity to USP2a that binds to Mdm2. The development of optimised peptides that prevent binding of USP2a to Mdm2 would allow validation of the therapeutic potential of specifically disrupting the interaction of Mdm2 with USP2a and could contribute to the discovery of small molecules that disrupt this interaction (Bottger *et al*, 1997; Vassilev *et al*, 2004).

It will be of interest to determine whether USP2a and HAUSP function simultaneously in regulating p53 or whether one of them might predominate depending upon the cellular conditions or cell type. Cell type differences in dependency on particular DUBs could contribute towards the development of tumour-selective therapies for p53 activation. Indeed, the overexpression of USP2a in prostate cancer, a tissue in which it is not normally highly expressed (Graner *et al*, 2004), suggests that USP2a might be a suitable therapeutic target

in cancers of specific tissues. Additional studies will be required to further elucidate the degree of importance of USP2a in controlling Mdm2 stability and activity in normal development and cancer and of USP2a regulation in the signaling pathways controlling p53. It is becoming increasingly obvious, however, that numerous feedback loops are involved in the regulation of the p53–Mdm2 pathway, and that deubiquitinating enzymes play a significant role within these feedback loops.

Materials and methods

Bacterial two-hybrid plasmids, libraries and screening

The BacterioMatch™ Two-Hybrid System was purchased from Stratagene. The pBT bait plasmid was modified to include a short flexible linker sequence to create the modified plasmid, pBT-Link. Full-length human Mdm2 was cloned into pBT-Link to prepare the bait. For screening purposes, a human breast cDNA library in pTRG was purchased from Stratagene. Screening was performed according to the manufacturer's instructions, with cotransformants being plated onto CTCK (carbenicillin, tetracycline, chloramphenicol and kanamycin) plates containing 375 μg/ml carbenicillin.

Expression constructs

EGFP-peptide fusions were generated by annealing oligonucleotides encoding the appropriate peptide sequence and ligation into pEGFP-C1 (Clontech). Peptide sequences were as follows: EGFP-control: SGLRSRAQASNSAVDGTAGPGSTGSR; EGFP-5251: APTGLSCDRGR PLLLSR; EGFP-USP2: PSSLLDYDRGRPLLRPD; EGFP-12.1: PLSMP RFMDYWEGLNEN. Full-length human HA-tagged USP2a was cloned by PCR from IMAGE clone #3635143, utilising primers that added the HA epitope at the 5′ end. Human Myc-USP15 and USP21 were cloned by PCR using primers that incorporated the myc epitope at the 5′ end. All PCR products were ligated into pcDNA3. His_6-ubiquitin was cloned by PCR of a single copy of the ubiquitin sequence into pcDNA3-His_6 (Invitrogen). Plasmids used to express human Mdm2 and p53 have been described previously (Xirodimas *et al*, 2001). Plasmids for bacterial expression of GST-Mdm2 deletions were obtained from Dr David Meek.

ELISA

Ninety-six-well ELISA plates were coated with 5 μg/ml streptavidin and washed three times with 0.1% NP-40/PBS and once with PBS. Wells were blocked with 3% BSA in PBS overnight at 4°C. Plates were washed and incubated for 1 h at room temperature with biotinylated peptides in 0.1% BSA/PBS and were washed five times with 0.1% NP-40/PBS and twice with PBS. 20 ng of bacterially expressed human Mdm2 protein (a gift from Dr David Meek) was added to each well and incubated for 1 h at RT. Bound Mdm2 was detected by incubation with α-Mdm2 antibody 4B2, followed by peroxidase-conjugated anti-mouse secondary antibody (Jackson Laboratories) at a dilution of 1:20 000 in 0.1% BSA/PBS. Plates were washed and TMB substrate (ImmunoPure® TMB Substrate kit, Pierce) was added and allowed to develop at RT (5–20 min). The reaction was stopped with 2 M H_2SO_4 and OD_{450} was read in an MRX Microplate Reader (Dynatech Laboratories). Biotinylated peptide (Mimotopes Pty Ltd) sequences were as follows: 6245: SGSGRHEVSLFVSLWVDVVGDWQ; 6012: SGSGAGSFSTFWWEL-LEIPG; 5251: SGSGAPTGLSCDRGRPLLLS; USP2: SGSGPSSLLDY DRGRPLLRP.

Transfections

H1299 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and gentamycin. 5×10^5 cells were seeded per 10-cm tissue culture plate and transfected using the calcium phosphate method. At least 2 h before transfection, the cells were transferred into DMEM and 10% FBS. Cells were transfected with the following plasmids, which express human proteins: 3 μg CMV-Mdm2, 0.3 μg pcDNA3-p53 and the indicated amount of pcDNA3-DUB. Eighteen hours after transfection, cells were washed with PBS and the medium was changed back to RPMI 10% FBS. Cells were harvested 36–48 h after transfection. The NTERA-2-DDp53 cell line was derived by transfecting NTERA-2 testicular embryonal carcinoma cells with plasmid pCMVNeop53DD, which expresses a truncated mouse p53 consisting of residues 1–14 and 302–390 (Ostermeyer *et al*, 1996; Smart *et al*, 1999). The control cell line NTERA-2-CMVNeo was derived by transfecting NTERA-2 cells with pCMVNeo. Stable cell lines were selected at 0.3 mg/ml G418 and surviving clones were pooled.

Western blot analysis

The primary antibodies used were 4B2 for Mdm2, DO-1 for p53 and L523 for USP2 (Abgent). An anti-USP2a polyclonal antibody was a gift from Dr S Wing, anti-EGFP antibody was obtained from Roche and anti-ubiquitin antibody was purchased from Novus. Cells were washed twice with PBS at 4°C. Cell extracts were prepared by direct lysis in SDS–urea sample buffer: two times Laemmli sample buffer supplemented with 8 M urea. DTT or 2-mercaptoethanol was added to a final concentration of 100 or 10 mM, respectively. Proteins were resolved by SDS–PAGE. Gels were transferred overnight to nitrocellulose membranes and processed as described (Saville *et al*, 2004). Peroxidase-conjugated secondary antibodies were supplied by Jackson Laboratories. Bound antibody was detected by ECL (Amersham) or SuperSignal® West Dura Extended Duration Substrate (Pierce).

Immunoprecipitation

U-2 OS cells were cultured in DMEM supplemented with 10% FBS and gentamycin. 1.5×10^6 cells were seeded on 10-cm dishes and transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Twenty hours after transfection, the proteasome inhibitor, MG132 (Calbiochem), was added to a final concentration of 10 μM and incubated for an additional 4 h at 37°C. For EGFP-peptide co-immunoprecipitation, cells were transfected with 2 μg of the appropriate EGFP-peptide construct and lysed in NP-40 lysis buffer: 0.5% NP-40, 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM EDTA and 1 mM DTT, supplemented with complete protease inhibitor mixture (Roche Applied Science). Anti-Mdm2 antibody, 4B2, coupled to protein G Sepharose was added to each lysate and rotated at 4°C for 1 h. Immunoprecipitates were washed four times with NP-40 lysis buffer and eluted in Laemmli sample buffer, 100 mM DTT for 20 min at RT and analysed by Western blotting. For full-length USP2a co-immunoprecipitations, cells were transfected with 20 μg of pcDNA3 HA-USP2a. Cells were lysed in NP-40 lysis buffer, protein G Sepharose-coupled 4B2 or mouse IgG was added, and samples were rotated for 1–2 h at 4°C. To investigate complex formation between endogenous proteins, 2×10^6 NTERA-2 cells were seeded on 15-cm dishes and immunoprecipitation carried out with the indicated antibodies as described above.

In vitro interaction assays

In vitro translation reactions (IVT) were carried out using the TNT-quick-coupled rabbit reticulocyte transcription/translation system according to the manufacturer's instructions (Promega). ^{35}S-labelled protein was mixed with 3 μg of bacterially expressed GST, or GST-Mdm2 and incubated for 2 h at 4°C in 250 μl of binding buffer: 20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% Ipegal, 2 mM DTT, 0.05% BSA, 5% glycerol and 5 mM benzamidine. Complexes were washed once in binding buffer containing 500 mM NaCl and three times in binding buffer containing 200 mM NaCl. Complexes were resolved by SDS–PAGE and bound ^{35}S-labelled proteins detected by autoradiography.

In vivo ubiquitination assay

H1299 cells were cultured and transfected as described above. Cells were transfected with combinations of the following as indicated: 3 μg CMV-Mdm2, 0.3 μg pcDNA3-p53, 5 μg pcDNA3-His_6-ubiquitin and 15 μg of the appropriate DUB expression vector. Forty-four hours after transfection, MG132 was added to each plate at a final concentration of 10 μM and incubated for an additional 4 h at 37°C. Cells were washed twice with PBS and lysed in 1 ml of 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4 and 0.01 M Tris–HCl pH 8.0. BCA protein assays (Pierce) were performed and lysates were adjusted. Lysates were incubated with Ni^{2+}-NTA-agarose beads (Qiagen) overnight at 4°C with rotation. Purification of His_6-ubiquitinated conjugates was performed as described (Xirodimas *et al*, 2001).

siRNAs and knockdown of USP2a

Nonspecific control duplex #I and Mdm2 SMART pool (M-003279–01) were purchased from Dharmacon. The USP2a siRNA duplex is identical to that characterised by Graner *et al* (2004). NTERA-2 cells were maintained in DMEM, 10% FBS and gentamycin (50 μg/ml).

For transfections, NTERA-2 cells were plated at 1×10^5 cells/35-mm dish. Transfection with single siRNA synthetic duplexes or SMART pools (30–100 nM) was carried out using Oligofectamine (Invitrogen) according to the manufacturer's instructions.

RNA preparation and real-time PCR

Total RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's instructions, including an on-column DNase treatment step. Mdm2 P1 and P2 primers and p21 and Bax primers and 6-FAM/TAMRA-labelled probes were as described (Saville *et al*, 2004; Phelps *et al*, 2005).

Flow cytometry

Flow cytometry analysis of cells was performed as described previously (Smart *et al*, 1999). Seventy-two hours after transfection, cells were labelled with 30 μM BrdU for 15 min, detached by trypsinisation and fixed at 4°C in ethanol. BrdU incorporation was detected using an anti-BrdU antibody from Becton Dickinson and cells were stained in PBS containing 25 μg/ml propidium iodide. Analysis of BrdU incorporation and propidium iodide staining was carried out using a Becton-Dickinson FACScan.

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References

Barak Y, Juven T, Haffner R, Oren M (1993) mdm2 expression is induced by wild type p53 activity. *EMBO J* **12:** 461–468

Bottger A, Bottger V, Sparks A, Liu WL, Howard SF, Lane DP (1997) Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo. *Curr Biol* **7:** 860–869

Bottger V, Bottger A, Howard SF, Picksley SM, Chene P, Garcia-Echeverria C, Hochkeppel HK, Lane DP (1996) Identification of novel mdm2 binding peptides by phage display. *Oncogene* **13:** 2141–2147

Brummelkamp TR, Nijman SM, Dirac AM, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature* **424:** 797–801

Cummins JM, Rago C, Kohli M, Kinzler KW, Lengauer C, Vogelstein B (2004) Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* **428**, 1 p following 486

D'Andrea A, Pellman D (1998) Deubiquitinating enzymes: a new class of biological regulators. *Crit Rev Biochem Mol Biol* **33:** 337–352

Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* **275:** 8945–8951

Ganguli G, Wasylyk B (2003) p53-independent functions of MDM2. *Mol Cancer Res* **1:** 1027–1035

Gong L, Kamitani T, Millas S, Yeh ET (2000) Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. *J Biol Chem* **275:** 14212–14216

Gousseva N, Baker RT (2003) Gene structure, alternate splicing, tissue distribution, cellular localization, and developmental expression pattern of mouse deubiquitinating enzyme isoforms Usp2–45 and Usp2–69. *Gene Expression* **11:** 163–179

Graner E, Tang D, Rossi S, Baron A, Migita T, Weinstein LJ, Lechpammer M, Huesken D, Zimmermann J, Signoretti S, Loda M (2004) The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer Cell* **5:** 253–261

Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387:** 296–299

Hetfeld BK, Helfrich A, Kapelari B, Scheel H, Hofmann K, Guterman A, Glickman M, Schade R, Kloetzel PM, Dubiel W (2005) The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1. *Curr Biol* **15:** 1217–1221

Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420:** 25–27

Honda R, Yasuda H (2000) Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. *Oncogene* **19:** 1473–1476

Hu M, Gu L, Li M, Jeffrey PD, Gu W, Shi Y (2006) Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53–MDM2 pathway. *PLoS Biol* **4:** e27

Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature* **424:** 801–805

Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. *Nature* **387:** 299–303

Lain S, Lane D (2003) Improving cancer therapy by non-genotoxic activation of p53. *Eur J Cancer* **39:** 1053–1060

Li M, Brooks CL, Kon N, Gu W (2004) A dynamic role of HAUSP in the p53–Mdm2 pathway. *Mol Cell* **13:** 879–886

Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416:** 648–653

Lin H, Keriel A, Morales CR, Bedard N, Zhao Q, Hingamp P, Lefrancois S, Combaret L, Wing SS (2000) Divergent N-terminal sequences target an inducible testis deubiquitinating enzyme to distinct subcellular structures. *Mol Cell Biol* **20:** 6568–6578

Lin H, Yin L, Reid J, Wilkinson KD, Wing SS (2001) Divergent N-terminal sequences of a deubiquitinating enzyme modulate substrate specificity. *J Biol Chem* **276:** 20357–20363

Meulmeester E, Maurice MM, Boutell C, Teunisse AF, Ovaa H, Abraham TE, Dirks RW, Jochemsen AG (2005) Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol Cell* **18:** 565–576

Michael D, Oren M (2003) The p53–Mdm2 module and the ubiquitin system. *Semin Cancer Biol* **13:** 49–58

Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69:** 1237–1245

Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123:** 773–786

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* **362:** 857–860

Oren M, Damalas A, Gottlieb T, Michael D, Taplick J, Leal JF, Maya R, Moas M, Seger R, Taya Y, Ben-Ze'Ev A (2002) Regulation of p53: intricate loops and delicate balances. *Ann NY Acad Sci* **973:** 374–383

Osman I, Drobnjak M, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C (1999) Inactivation of the p53 pathway in prostate cancer: impact on tumor progression. *Clin Cancer Res* **5:** 2082–2088

Ostermeyer AG, Runko E, Winkfield B, Ahn B, Moll UM (1996) Cytoplasmically sequestered wild-type p53 protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide. *Proc Natl Acad Sci USA* **93:** 15190–15194

Park KC, Kim JH, Choi EJ, Min SW, Rhee S, Baek SH, Chung SS, Bang O, Park D, Chiba T, Tanaka K, Chung CH (2002) Antagonistic regulation of myogenesis by two deubiquitinating enzymes, UBP45 and UBP69. *Proc Natl Acad Sci USA* **99:** 9733–9738

Phelps M, Phillips A, Darley M, Blaydes JP (2005) MEK-ERK signaling controls Hdm2 oncoprotein expression by regulating hdm2 mRNA export to the cytoplasm. *J Biol Chem* **280:** 16651–16658

Phizicky EM, Fields S (1995) Protein–protein interactions: methods for detection and analysis. *Microbiol Rev* **59:** 94–123

Picksley SM, Lane DP (1993) The p53–mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? *Bioessays* **15:** 689–690

Priolo C, Tang D, Brahamandan M, Benassi B, Sicinska E, Ogino S, Farsetti A, Porrello A, Finn S, Zimmermann J, Febbo P, Loda M

(2006) The isopeptidase USP2a protects human prostate cancer from apoptosis. *Cancer Res* **66:** 8625–8632

Prives C (1998) Signaling to p53: breaking the MDM2–p53 circuit. *Cell* **95:** 5–8

Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA, Levine AJ (1993) Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. *Proc Natl Acad Sci USA* **90:** 9075–9079

Saville MK, Sparks A, Xirodimas DP, Wardrop J, Stevenson LF, Bourdon JC, Woods YL, Lane DP (2004) Regulation of p53 by the ubiquitin-conjugating enzymes UbcH5B/C *in vivo*. *J Biol Chem* **279:** 42169–42181

Schwartz D, Goldfinger N, Kam Z, Rotter V (1999) p53 controls low DNA damage-dependent premeiotic checkpoint and facilitates DNA repair during spermatogenesis. *Cell Growth Differ* **10:** 665–675

Sengupta S, Wasylyk B (2004) Physiological and pathological consequences of the interactions of the p53 tumor suppressor with the glucocorticoid, androgen, and estrogen receptors. *Ann NY Acad Sci* **1024:** 54–71

Shaulian E, Zauberman A, Ginsberg D, Oren M (1992) Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol Cell Biol* **12:** 5581–5592

Sheng Y, Saridakis V, Sarkari F, Duan S, Wu T, Arrowsmith CH, Frappier L (2006) Molecular recognition of p53 and MDM2 by USP7/HAUSP. *Nat Struct Mol Biol* **13:** 285–291

Smart P, Lane EB, Lane DP, Midgley C, Vojtesek B, Lain S (1999) Effects on normal fibroblasts and neuroblastoma cells of the activation of the p53 response by the nuclear export inhibitor leptomycin B. *Oncogene* **18:** 7378–7386

Toby GG, Golemis EA (2001) Using the yeast interaction trap and other two-hybrid-based approaches to study protein–protein interactions. *Methods* **24:** 201–217

Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303:** 844–848

Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. *Nature* **408:** 307–310

Vousden KH, Prives C (2005) P53 and prognosis: new insights and further complexity. *Cell* **120:** 7–10

Wang H, Nan L, Yu D, Agrawal S, Zhang R (2001) Antisense anti-MDM2 oligonucleotides as a novel therapeutic approach to human breast cancer: *in vitro* and *in vivo* activities and mechanisms. *Clin Cancer Res* **7:** 3613–3624

Wing SS (2003) Deubiquitinating enzymes—the importance of driving in reverse along the ubiquitin–proteasome pathway. *Int J Biochem Cell Biol* **35:** 590–605

Woods YL, Lane DP (2003) Exploiting the p53 pathway for cancer diagnosis and therapy. *Hematol J* **4:** 233–247

Wu X, Bayle JH, Olson D, Levine AJ (1993) The p53–mdm-2 autoregulatory feedback loop. *Genes Dev* **7:** 1126–1132

Xirodimas D, Saville MK, Edling C, Lane DP, Lain S (2001) Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 *in vivo*. *Oncogene* **20:** 4972–4983

Xirodimas DP, Saville MK, Bourdon JC, Hay RT, Lane DP (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118:** 83–97

Yoo KJ, Lee HJ, Lee H, Lee KY, Lee SH, Chung HM, Baek KH (2005) Expression and functional analyses of mHAUSP regulating apoptosis of cervical adenocarcinoma cells. *Int J Oncol* **27:** 97–104