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; Voudsen 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; Meultmeester et al., 2005).

In this study we show that the deubiquitinating enzyme USP2a associates with Mdm2 in vivo. USP2a can deubiquiti- nate 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 deubiquiti- nating enzyme USP2a represents a potential target for therapeutic interventions aimed at reactivating wild-type p53 in tumours.
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 ubiquitin 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, 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 35S-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, 35S-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 overexpression 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. 35S-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 35S-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 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 His6-ubiquitin and treated with MG132 before harvesting. Ubiquitinated proteins were purified from transfected cell lysates using Ni²⁺-agarose beads and the purified lysates were blotted for p53 and Mdm2. Control transfections, lacking the His6-ubiquitin construct, were performed in parallel to demonstrate that the purified species detected were ubiquitin conjugates (Figure 5B, see lanes 5 and 7). 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 Ni²⁺-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
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 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) Ni²⁺-agarose purification of ubiquitinated proteins. H1299 cells were transfected with Mdm2 and p53 alone (lanes 1 and 5) or in the presence of His₆-ubiquitin (His₆-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 Ni²⁺-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, His₆-ub and the indicated DUBs and were treated with MG132 for 4 h before lysis. Ubiquitinated proteins were purified using Ni²⁺-agarose and analysed by Western blotting for ubiquitin.

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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,
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-G1 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 normal tissues.
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 (Goussev 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 deubiquitates 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 (Bortger 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 pB7T bait plasmid was modified to include a short flexible linker sequence to create the modified plasmid, pBFT-Link. Full-length human Mdm2 was cloned into pBFT-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 cotransfomers being plated onto CTCK (carbenicillin, tetracycline, chloramphenicol and kanamycin) plates containing 375 µg/ml carbenicillin.

Expression constructs

EGF-peptide fusions were generated by annealing oligonucleotides encoding the appropriate peptide sequence and ligation into pEGFP-C1 (Clontech). Peptide sequences were as follows: EGF-control: SGGLRSQAQNSAVDQGCTGQSRR; EGF-P2: APTGLSCDRG; PLLLR; EGF-4SP: PSSLLYDDRGRPLLPNR; EGF-12: PLSMPRFDYWEGLNEN. Full-length human HA-tagged USP2a was cloned by PCR from IMAGE clone #3635143, utilizing primers that added the HA epitope at the 5’ end. Human Myc-USP15 and USP21 were expressed from pcDNA3-His6 (Invitrogen). Plasmids used to express full-length human Mdm2 were transferred into DMEM and 10% FBS. Cells were transfected with 20 µg of the appropriate DUB expression vector. Forty-four surviving clones were pooled. The USP2a siRNA duplex is identical to that characterised by Graner et al (2004). Peroxidase-conjugated secondary antibodies were supplied by Jackson Laboratories. Blurred 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 × 10⁶ 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 EGF-peptide co-immunoprecipitation, cells were transfected with 2 µg of the appropriate EGF-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 Laemmlli sample buffer, 100 mM DTT for 20 min at RT and analysed by Western blotting. For full-length USP2a co-immunoprecipitations, cell 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 × 10⁶ 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). 35S-labelled protein was mixed with 3 µg of bacterially expressed GST, or GST-Mdm2 and incubated for 2 h at 37°C. To investigate complex formation between endogenous proteins, 2 × 10⁶ NTERA-2 cells were seeded in 15-cm dishes and immunoprecipitation carried out with the indicated antibodies as described above.

In vivo ubiquitination assay

H1299 cells were cultured and transfected as described above. Cells were transfected with combinations of the following plasmids: 3 µg CMV-Mdm2, 0.3 µg pcDNA3-p53, 5 µg pcDNA3-His6-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 NaHPO4/Na2HPO4 and 0.01 M Tris–HCl pH 8.0. BCA protein assay (Pierce) were performed and lysates were adjusted. Lysates were incubated with Ni2+–NTA-agarose beads (Qiagen) overnight at 4°C with rotation. Purification of His6-ubiquitinated conjugates was performed as described (Xirodimas et al, 2001).

siRNAs and knockdown of USP2a

Non-specific control duplex #1 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 RNAspray (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 (Saville 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**


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