

# Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2

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**In response to environmental stress, the p53 phosphoprotein is stabilized and activated to inhibit cell growth. p53 stability and activity are negatively regulated by the murine double minute (Mdm2) oncoprotein in an autoregulatory feedback loop. The inhibitory effect of Mdm2 on p53 has to be tightly regulated for proper p53 activity. Phosphorylation is an important level of p53 regulation. In response to DNA damage, p53 is phosphorylated at several N-terminal serines. In this study we examined the role of Ser20, a potential phosphorylation site in human p53, in the regulation of p53 stability and function. Substitution of Ser20 by Ala (p53-Ala20) significantly increases the susceptibility of human p53 to negative regulation by Mdm2 *in vivo*, as measured by apoptosis and transcription activation assays. Mutation of Ser20 to Ala renders p53 less stable and more prone to Mdm2-mediated degradation. While the *in vitro* binding of p53 to Mdm2 is not increased by the Ala20 mutation, the same mutation results in a markedly enhanced binding *in vivo*. This is consistent with the conclusion that phosphorylation of Ser20 *in vivo* attenuates the binding of wild-type p53 to Mdm2. Peptides bearing non-phosphorylated Ser20 or Ala20 compete with p53 for Mdm2 binding, while a similar peptide with phosphorylated Ser20 does not. This implies a critical role for Ser20 in modulating the negative regulation of p53 by Mdm2, probably through phosphorylation-dependent inhibition of p53–Mdm2 interaction.**

**Keywords:** apoptosis/degradation/Mdm2/p53/phosphorylation

## Introduction

The p53 phosphoprotein plays a key role in tumor suppression and in the cellular response to stress. The p53 protein is normally expressed at low levels in a latent inactive form. However, upon exposure to stress conditions such as hypoxia, DNA damage or reduction in ribonucleotide

triphosphate levels, p53 is modified post-translationally with subsequent accumulation of active protein (Gottlieb and Oren, 1996; Levine, 1997; Agarwal *et al.*, 1998; Giaccia and Kastan, 1998; Prives, 1998). Activation of p53 by stress leads to growth inhibition. This includes the induction of growth arrest, presumably to allow sufficient time for DNA repair, or apoptosis which eliminates abnormal cells. These activities are abrogated by mutations in p53, which constitute the most common type of genetic alterations in many different human malignancies (Velculescu and El-Deiry, 1996). Most of the mutations are localized to the central DNA-binding domain of p53, and these mutations prevent p53 from binding to its target DNA sequences (e.g. Unger *et al.*, 1993).

The biological effects of p53 are largely due to sequence-specific binding to DNA and transcriptional activation of target genes. The transactivation domain of p53, located at the N-terminus of the protein (Unger *et al.*, 1992), interacts with components of the basal transcription machinery (Lu and Levine, 1995) and promotes the transcription of genes containing p53-binding sequences. Different sets of p53 responsive genes mediate different functions of p53, including growth arrest by *p21<sup>waf1/cip1</sup>* and *14-3-3 $\sigma$*  (El-Deiry *et al.*, 1993; Hermeking *et al.*, 1997) and apoptosis by *bax*, *CD95/Fas*, *IGF-BP3* and *PIG3* (Buckbinder *et al.*, 1995; Miyashita and Reed, 1995; Owen-Schaub *et al.*, 1995; Polyak *et al.*, 1997).

The protein stability and biochemical activities of p53 are tightly regulated by a variety of mechanisms. One of the mechanisms most likely to mediate p53 regulation is phosphorylation. The p53 protein is phosphorylated on several serine residues within the N- and C-terminal regions by several cellular kinases (reviewed in Steegenga *et al.*, 1996; Milczarek *et al.*, 1997; Fuchs *et al.*, 1998a; Giaccia and Kastan, 1998; Meek, 1998; Prives, 1998). There are at least three phosphorylation sites near the C-terminus of human p53 (hp53) at amino acids 315, 378 and 392. Ser315 has been shown to be a target of p34<sup>cdc2</sup> kinase and of cyclin-dependent kinase 2 (cdk2) (Addison *et al.*, 1990; Bischoff *et al.*, 1990; Price *et al.*, 1995). Ser392 is phosphorylated *in vitro* by purified casein kinase II (Meek *et al.*, 1990), and Ser378 is a site for phosphorylation by protein kinase C (PKC) (Baudier *et al.*, 1992). Phosphorylation of these C-terminal serines can enhance the *in vitro* specific DNA-binding activity of p53 (Hupp *et al.*, 1992; Takenaka *et al.*, 1995; Wang and Prives, 1995; Lu *et al.*, 1997). Similarly, dephosphorylation of human p53 Ser376 enhances binding to 14-3-3 protein and increases specific DNA binding (Waterman *et al.*, 1998). The substitution of mouse p53 Ser389 (equivalent to Ser392 in human p53) to glutamate activates p53 *in vivo* (Hao *et al.*, 1996), but substitution to Ala did not affect the ability of p53 to bind DNA or to suppress cell growth (Fiscella *et al.*, 1994). This discrepancy can be explained

by the recent observation that phosphorylation of Ser392 occurs in response to some environmental stresses but not others, suggesting independent mechanisms of activating p53 (Kapoor and Lozano, 1998; Lu *et al.*, 1998).

At the N-terminus of p53, several potential phosphorylation sites have been identified including Ser6, 9, 15, 20, 33, 37 and Thr18 of human p53 (hp53) (reviewed in Steegenga *et al.*, 1996; Milczarek *et al.*, 1997; Fuchs *et al.*, 1998a; Giaccia and Kastan, 1998; Meek, 1998; Prives, 1998). Ser15 and 37 of hp53 can be phosphorylated *in vitro* by the DNA-dependent protein kinase (DNA-PK) (Lees Miller *et al.*, 1992). Ser7, 9 and 12 of mouse p53 can be phosphorylated *in vitro* by casein kinase I (CKI) and CKI-like PK270 (Milne *et al.*, 1992). *In vitro* phosphorylation of Ser34 of mouse p53 by the UV-induced Jun kinase 1 (JNK) has been shown (Milne *et al.*, 1995). Further, the Raf-1 serine/threonine kinase phosphorylates p53 *in vitro* on one or more sites within the first 27 residues (Jamal and Ziff, 1995). Although multiple sites of phosphorylation have been identified in p53, relatively little is known about their role in the regulation of p53 activity *in vivo*. The phosphorylation of Ser15, which partially alleviates the inhibitory effect of Mdm2 on p53 (Shieh *et al.*, 1997), was shown to be induced by DNA damage (Siliciano *et al.*, 1997). Recent evidence strongly implicates the ATM (ataxia telangiectasia mutated) kinase in Ser15 phosphorylation (Banin *et al.*, 1998; Canman *et al.*, 1998). Phosphorylation of Ser33 and Ser37 also enhances C-terminal acetylation of p53 with a consequent increase in specific DNA binding (Sakaguchi *et al.*, 1998).

The *mdm2* oncogene was found to be amplified in sarcomas and several other types of cancers (reviewed by Momand and Zambetti, 1997; Lozano and Montes de Oca Luna, 1998). Mdm2 binds p53 within its transactivation domain and blocks its transcriptional activity (Oliner *et al.*, 1993; Chen *et al.*, 1995). Moreover, Mdm2 abrogates the ability of p53 to induce growth arrest and apoptosis (Chen *et al.*, 1996; Haupt *et al.*, 1996). Since the *mdm2* gene itself is a target gene for activation by p53, a negative autoregulatory loop exists between the two proteins (reviewed in Piette *et al.*, 1997). A lag is sometimes found between activation of p53 and the consequent induction of *mdm2*, defining a time window within which p53 is allowed to exert its inhibitory activities. Alternatively, p53 may continue to operate even after the induction of *mdm2* has occurred. In this case, however, p53 must be rendered immune to the inhibitory effects of Mdm2, possibly through covalent modification of either or both proteins, and perhaps also through the action of additional partner proteins. In addition, Mdm2 has more recently been shown to promote p53 for degradation in a ubiquitin-dependent manner (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), by enhancing the ubiquitination of p53 (Honda *et al.*, 1997). The interaction between the two proteins is obligatory for the inhibitory activities of Mdm2 (Chen *et al.*, 1996; Haupt *et al.*, 1996, 1997; Kubbutat *et al.*, 1997), and facilitates nuclear-cytoplasmic shuttling of p53 (Roth *et al.*, 1998). Blocking the interaction between p53 and Mdm2 by specific mutations, or by peptides and antibodies directed towards the binding site, all prevent the degradation and inactivation of p53 by Mdm2 (Bottger *et al.*, 1997; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Midgley and Lane, 1997). Thus, through parallel mechanisms,

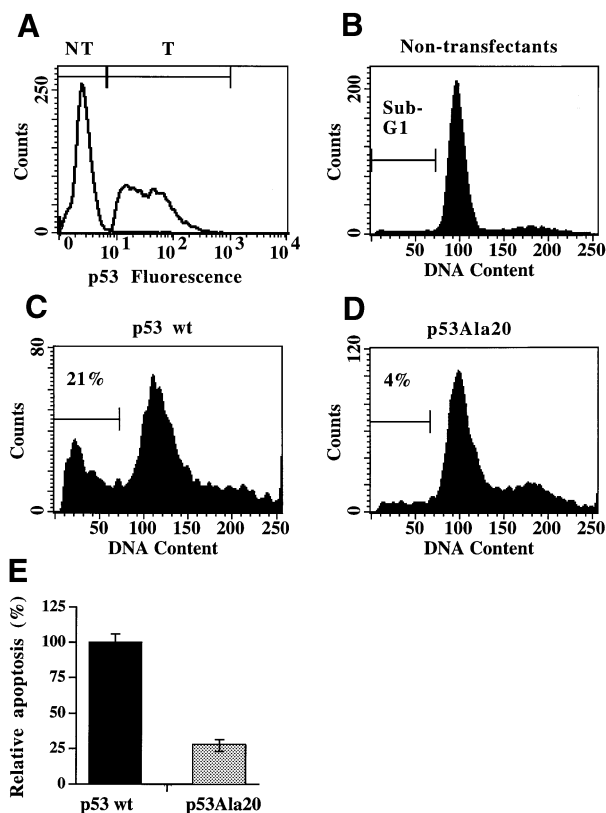
Mdm2 shuts off p53 activity and terminates the propagation of its growth inhibitory signal. This tight regulation of p53 by Mdm2 is essential for normal embryonic development (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

In this study we investigated the role of serine 20 in the regulation of p53 activity and stability. We have recently shown that substitution of serine to alanine at residue 20 (p53-Ala20) partially impaired the apoptotic activity of p53 (Unger *et al.*, 1999). We now report that this reduced apoptosis is due to enhanced susceptibility of p53-Ala20 to negative regulation by Mdm2. Similarly, the transcriptional activity of p53-Ala20 is more sensitive than that of wild-type (wt) p53 to inhibition by Mdm2. Notably, substitution of Ser20 to alanine renders p53 more susceptible to Mdm2-directed proteolytic degradation. All of these observations indicate that mutation of Ser20 to alanine enhances the interaction of p53 with Mdm2. In support of this conclusion, analysis of p53-Mdm2 complexes within transfected cells revealed a much more prominent coprecipitation of Mdm2 with p53-Ala20 than with wt p53. However, this increased interaction is not due to the Ala mutation itself, as p53-Ala20 did not exhibit any enhanced association with Mdm2 when the binding was performed *in vitro*. Hence, the differential binding *in vivo* must be due to a modification which occurs specifically on residue 20 of p53 within living cells. That this modification is the phosphorylation of Ser20 is strongly supported by the finding that p53-derived peptides with a non-phosphorylated Ser20 or with Ala20 were able to compete efficiently with wt p53 for Mdm2 binding, while a similar peptide with phosphorylated Ser20 failed to compete. Our results provide the first *in vivo* demonstration for an important role played by Ser20 in the modulation of p53 activity and stability, and imply that phosphorylation of p53 on this residue interferes with its ability to associate with Mdm2 and be negatively regulated by it. This, in turn, might allow p53 to retain its activity under conditions of extended stress, even after the *mdm2* gene has become maximally induced.

## Results

### **Mutation of Ser20 to Ala renders p53-mediated apoptosis excessively sensitive to inhibition by Mdm2**

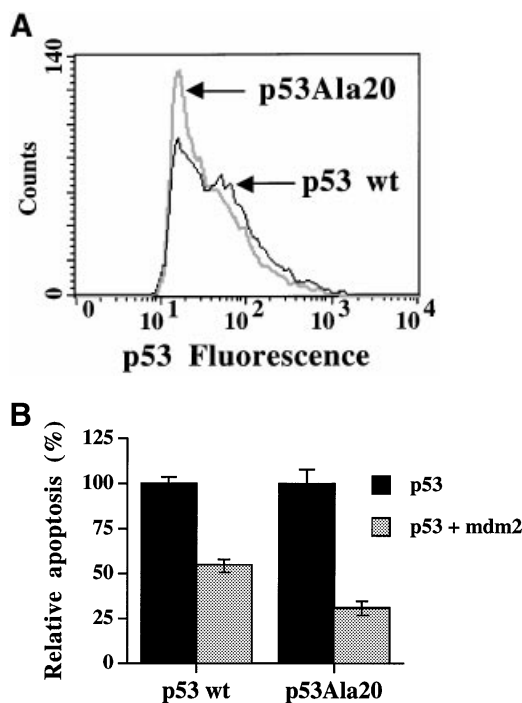
If Ser20 of hp53 is a site of phosphorylation *in vivo*, mutation to Ala should prevent this phosphorylation. Thus, whereas wt p53 may be phosphorylated to a variable extent on Ser20, the p53-Ala20 mutant remains completely non-phosphorylated at this position at all times. To assess the possible consequences of p53 phosphorylation on Ser20 and of its abrogation, we compared the behavior of wt p53 and p53-Ala20 in a variety of biological and biochemical assays. These analyses revealed that p53-Ala20 possesses a partially impaired apoptotic activity, as measured in transient transfection assays (Unger *et al.*, 1999). Further support for this conclusion is provided in Figure 1, where H1299 cells were transfected with expression plasmids encoding either wt p53 or p53-Ala20. Seventy-two hours post-transfection cells were harvested, stained for p53 and subjected to flow-cytometric analysis (Haupt *et al.*, 1996). The non-transfected sub-population



**Fig. 1.** p53-Ala20 has impaired apoptotic activity in H1299 cells. H1299 cells were transfected with 3  $\mu$ g of either wt p53 or p53-Ala20 expression plasmid DNA. Seventy-two hours post-transfection, cells were harvested, fixed and stained for p53 using a mixture of the DO-1 and PAb1801 antibodies, and detected by FITC-conjugated goat-anti-mouse antibodies. Stained cells were then subjected to flow cytometric analysis. Equal numbers (5000) of transfected cells from each population were analyzed separately. (A) Levels of p53 fluorescence in the transfected culture; the non-transfected sub-population (NT) and the transfected one (T) are indicated. Note that fluorescence is plotted on a logarithmic scale. (B) Cell cycle distribution of the non-transfected population as determined by propidium iodide staining. The region of apoptotic cells is marked (Sub-G1). (C) DNA content distribution of cells transfected with wt p53 [sub-population T of (A)]; the percent of apoptosis is indicated. (D) As in (C), except cells were transfected with p53-Ala20 instead of wt p53. (E) Cumulative data from seven independent transfections measuring the relative apoptotic activity of wt p53 and p53-Ala20. The rate of apoptosis induced by wt p53 was taken as 100%. The standard deviation is indicated.

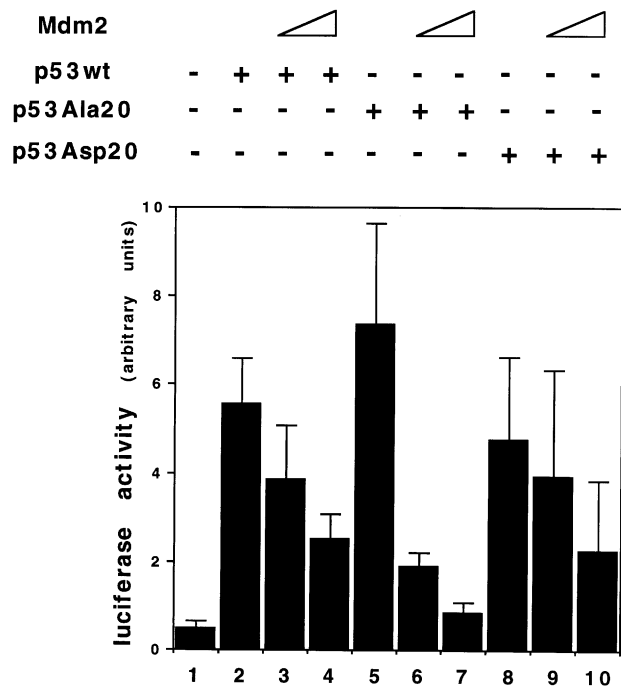
(NT in Figure 1A) exhibits low fluorescent intensity, while the transfected sub-population (T) exhibits high levels of p53 fluorescence (Figure 1A). The cell-cycle distribution of each sub-population was analyzed separately. Cells with DNA content of less than 2N (Sub-G1 in Figure 1B) are considered apoptotic (Darzynkiewicz *et al.*, 1992). As seen in Figure 1C and D, while wt p53 induced 21% apoptosis (after subtraction of the background obtained in the non-transfected cells), p53-Ala20 induced only 4%. A comparison of cumulative data, representing a total of seven independent experiments (Figure 1E), clearly supports the conclusion that p53-Ala20 has impaired apoptotic activity.

The apoptotic activity of p53 is efficiently inhibited by Mdm2 in H1299 cells (Haupt *et al.*, 1996). Ser20 resides within the Mdm2-binding site of p53 (residues 19–26; Picksley *et al.*, 1994; Kussie *et al.*, 1996), which engages



**Fig. 2.** The apoptotic activity of p53-Ala20 is more sensitive than that of wt p53 to inhibition by Mdm2. H1299 cells were transfected with expression plasmids for *mdm2* (1  $\mu$ g), wt p53 (3  $\mu$ g) or p53-Ala20 (3  $\mu$ g), as indicated. Seventy-two hours post-transfection cells were harvested, treated and analyzed as described in Figure 1. (A) Histogram showing the p53 fluorescence intensity distribution among cells transfected with wt p53 together with *mdm2* (thin line), or with p53-Ala20 together with *mdm2* (thick line). (B) Relative apoptotic activity of wt p53 and p53-Ala20 alone or together with *mdm2*. The level of apoptosis obtained with each p53 expression plasmid without *mdm2* was taken as 100% relative apoptosis for this p53 type (black bars), and the residual apoptotic activity in the presence of *mdm2* was calculated relative to this value (gray bars). Standard errors from three independent experiments are indicated.

in tight interactions with a hydrophobic cleft within Mdm2. It is conceivable that phosphorylation of Ser20 may interfere with this binding. In that case, substitution of Ser20 by Ala, by preventing phosphorylation, is expected to augment the inhibitory effect of Mdm2. This might render the mutant protein less competent for induction of apoptosis in cells such as H1299, in which endogenous human Mdm2 (Hdm2) is induced in response to p53 activation (Haupt *et al.*, 1996). This notion was tested by comparing the extent by which limiting amounts of transfected Mdm2 inhibit the apoptotic activity of p53-Ala20 relative to wt p53. H1299 cells were transfected with 3  $\mu$ g of wt p53 or p53-Ala20 DNA either alone or together with 1  $\mu$ g of *mdm2* DNA, and the extent of apoptosis within the transfected sub-population was determined as above. Notably, expression levels of p53 were slightly lower in cells cotransfected with p53-Ala20 than in those with wt p53 (Figure 2A; see below). As expected (Haupt *et al.*, 1996), co-transfection with *mdm2* blocked the apoptotic activity of wt p53 by 45% (Figure 2B). Remarkably, the apoptotic activity of p53-Ala20 was inhibited to a significantly higher extent (70%). A similar difference was observed when different ratios of p53 to *mdm2* were used (data not shown). Presumably, the exogenous Mdm2 acts in concert with the endogenously induced Hdm2 to block p53-mediated apoptosis. Our data



**Fig. 3.** Increased inhibitory effect of Mdm2 on p53-Ala20 transcriptional activity. H1299 cells were transfected with expression plasmids for wt p53 (5 ng), p53-Ala20 (5 ng), p53-Asp20 (5 ng) or with empty vector control, either alone or together with *mdm2* expression plasmid (5 or 10 ng) as indicated above each bar. The *cyclin G* luciferase plasmid (300 ng) served as reporter. Luciferase activity is shown in arbitrary machine units, along with the standard deviation calculated from five independent transfection dishes. Results were essentially identical in different experiments using varying amounts of p53 and *mdm2* expression plasmids.

indicate that this block is significantly more pronounced in the case of p53-Ala20, implying that mutation of Ser20 to Ala indeed causes the apoptotic activity of p53 to become more sensitive to inhibition by Mdm2.

#### **Transcriptional activity of p53-Ala20 is excessively sensitive to inhibition by Mdm2**

The inhibition of p53-mediated apoptosis by Mdm2 in H1299 cells was shown to be largely due to inhibition of the transcriptional activity of p53 (Haupt *et al.*, 1996). Therefore, the extent by which Mdm2 blocks the transcriptional activity of p53 mutated at residue 20 was evaluated using the luciferase assay. The effect of Mdm2 on wt p53 was compared with that seen with p53-Ala20 or with p53 carrying a substitution to Asp at position 20 (p53-Asp20). Asp is negatively charged and in some instances may mimic a phosphorylated state. H1299 cells were transiently transfected with 5 ng of either wt p53, p53-Ala20 or p53-Asp20 expression plasmid DNA, together with increasing amounts of *mdm2* expression plasmid. The transcriptional activity of p53 was deduced from the activity of the luciferase reporter gene driven by the p53-responsive *cyclin G* promoter (Zauberman *et al.*, 1995). Low concentrations of *mdm2* expression plasmid were chosen such that the amounts of encoded Mdm2 protein will be limiting, resulting in only partial inhibition of p53 activity. In the absence of exogenous Mdm2, the transcriptional activity of p53-Ala20 was comparable with that of wt p53 (Figure 3, columns 2, 5 and 8). However, marked differences in the transcriptional activities of the

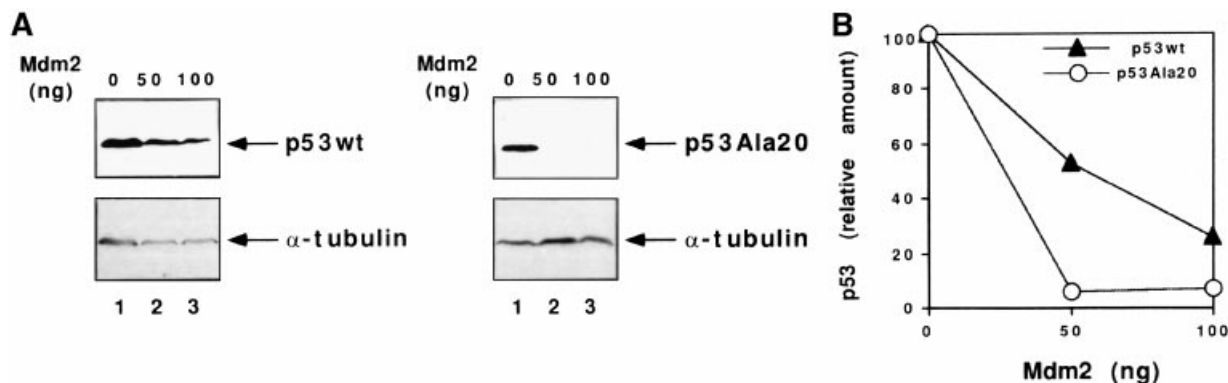
three forms of p53 were revealed in the presence of exogenous Mdm2. Co-transfection of either 5 or 10 ng of *mdm2* expression plasmid inhibited p53-Ala20 activity by as much as 74% (Figure 3, column 6) and 89% (column 7), respectively. On the other hand, the activities of wt p53 and p53-Asp20 were inhibited more mildly by either 5 ng (Figure 3, wt p53 by 31% and p53-Asp20 by 18%, columns 3 and 9) or 10 ng (Figure 3, wt p53 by 55% and p53-Asp20 by 53%, columns 4 and 10) of *mdm2* DNA. Hence, as in the case of apoptosis, the transcriptional activity of p53-Ala20 is also selectively sensitive to the inhibitory effect of Mdm2.

#### **p53-Ala20 is more susceptible than wt p53 to Mdm2-mediated degradation**

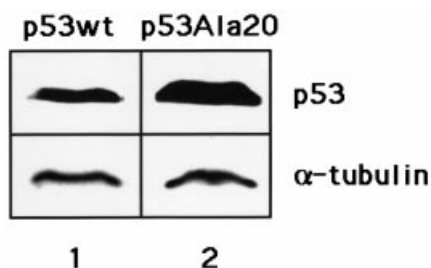
Mdm2 promotes the degradation of p53 through the ubiquitin-proteasome system (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). The observations described above demonstrated that substitution of Ser20 by Ala renders the protein more sensitive to the effects of Mdm2. It was therefore reasonable to expect that p53-Ala20 may also be more susceptible to the destabilizing activity of Mdm2. Evidence supporting this prediction was obtained in the FACS analysis (Figure 2A) which suggested that the average cellular content of p53-Ala20 is lower than that of wt p53 in the presence of co-transfected *mdm2*, and that a distinct sub-population with higher p53 protein content existed in the wt p53-transfected cells under those conditions.

To establish more directly that p53-Ala20 is indeed more susceptible than wt p53 to Mdm2-promoted degradation, the steady-state level of the two proteins was compared by Western blot analysis following transfection with relatively low amounts of expression plasmid DNA. H1299 cells were transfected with wt p53 or p53-Ala20, either alone or together with co-transfected *mdm2*. Using 50 ng of each p53 expression plasmid without any exogenous *mdm2*, the level of p53-Ala20 was only about half that of wt p53 (Figure 4A, lane 1). This difference became much more dramatic when low amounts of exogenous *mdm2* were also included. Inclusion of as little as 50 ng of *mdm2* plasmid was already sufficient to achieve maximum reduction of p53-Ala20 steady-state levels, while the level of wt p53 was reduced only 2-fold (Figure 4A, lane 2, and 4B). Inclusion of 100 ng *mdm2* expression plasmid reduced wt p53 levels by a further 2-fold (Figure 4A, lane 3, and 4B). Similar effects were observed when 10 or 100 ng of the p53 expression plasmids were used (data not shown). These results demonstrate that substitution of Ser20 by Ala destabilizes p53 by rendering it much more sensitive to Mdm2-mediated degradation. Presumably, the relatively lower levels of p53-Ala20 obtained even without exogenous Mdm2 (Figure 4A, lane 1) may also be due to this increased sensitivity, in this case mediated exclusively by the p53-induced endogenous Hdm2 of H1299 cells.

If the above notion is correct, it is expected that in the absence of endogenous Hdm2 the expression levels of wt p53 and p53-Ala20 should be comparable. This prediction was tested in embryonic fibroblasts derived from *p53<sup>-/-</sup>/mdm2<sup>-/-</sup>* double knock-out mice (McMasters *et al.*, 1996). When these cells were transfected with low amounts of the two p53 expression plasmids, p53-Ala20 indeed did not accumulate to lower levels than wt p53 (Figure 5).



**Fig. 4.** Mutation of Ser20 to Ala destabilizes p53 and enhances Mdm2-promoted degradation. (A) H1299 cells were transfected with expression plasmids for wt p53 or p53-Ala20 (50 ng each) either alone or together with the indicated amount of *mdm2* expression plasmid DNA. Twenty-four hours post-transfection, cell extracts were subjected to Western blot analysis using the anti-p53 monoclonal antibody PAb1801. The position of p53 and  $\alpha$ -tubulin are shown by arrows. (B) Identical aliquots of each extract were subjected to Western blot analysis with anti  $\alpha$ -tubulin antibodies, to control for variations in the amount of total protein. The amount of p53 in each lane was quantitated using a densitometer (Molecular Dynamics); wt p53 is indicated by triangles while p53-Ala20 is indicated by open circles. The amount of each type of p53 in the absence of Mdm2 was taken as 100%, and the relative amounts obtained in the presence of Mdm2 were calculated accordingly.



**Fig. 5.** Efficient accumulation of p53-Ala20 in the absence of Mdm2. Double knock-out fibroblasts (*p53*<sup>-/-</sup>/*mdm2*<sup>-/-</sup>) were transfected with 100 ng of expression plasmids encoding either wt p53 (lane 1) or p53-Ala20 (lane 2). p53 protein was visualized as in Figure 4A. Equal loading was confirmed by probing for  $\alpha$ -tubulin.

In fact, the level of p53-Ala20 (Figure 5, lane 2) was even higher than that of wt p53 (lane 1); the reason for this is presently unclear. Thus, the steady-state levels of the Ala20 mutant are compromised only when the cells are capable of expressing endogenous Mdm2, implicating this endogenous protein in the selective down-regulation of p53-Ala20. When the p53 expression plasmids were transfected into the double knock-out fibroblasts together with exogenous Mdm2, the picture was essentially similar to that observed in H1299 cells: p53-Ala20 expression was reduced far more severely than that of wt p53 (data not shown). Collectively, these results demonstrate that Ser20 plays an important role in regulating p53 stability, through dictating the susceptibility of the protein to Mdm2-mediated degradation.

#### **Mdm2 binds preferentially to p53-Ala20 in vivo but not in vitro**

The increased responsiveness of p53-Ala20 to negative regulation by Mdm2 raised the possibility that the Ser20 to Ala substitution altered the binding affinity of p53 to Mdm2 within cells. This conjecture is attractive because Ser20 resides within the Mdm2-binding domain of p53 (Picksley *et al.*, 1994; Kussie, *et al.*, 1996). A co-immunoprecipitation assay was therefore performed in order to compare the two types of p53 with regard to Mdm2 binding. To increase the sensitivity of the assay a mutant Hdm2, Hdm2 $\Delta$  RING, lacking the C-terminal

RING finger domain was used. While this mutant binds p53, it can not promote its degradation (Figure 6AII; R.Vogt Sionov and Y.Haupt, unpublished results; see also Kubbutat *et al.*, 1997). This allows the accumulation of substantial amounts of p53, thereby facilitating detection of stable p53-Mdm2 complexes. Twenty-four hours post-transfection, cell extracts were prepared and Hdm2/p53 complexes were monitored by immunoprecipitation with the anti-p53 monoclonal antibody PAb421, followed by Western blot analysis with the anti-Hdm2 monoclonal antibody SMP14. This revealed that the amount of Hdm2 $\Delta$  RING co-immunoprecipitated with p53-Ala20 was higher (14-fold) than that precipitated with wt p53 (Figure 6AI, compare lanes 1 and 2), despite lower expression level of p53-Ala20 as compared with wt p53 (Figure 6AII, lanes 1 and 2). A similar experiment was also carried out with intact wt Hdm2; this time the effect of Hdm2 on p53 degradation was partially blocked by treating the transfected cells for 2 h with the proteasome inhibitor, ALLN. Again, a higher amount of Hdm2 was co-immunoprecipitated with p53-Ala20 than with wt p53 (Figure 6AI, lanes 3 and 4), although the former was expressed at a substantially lower level (Figure 6AII, lanes 3 and 4). Thus, substitution of Ser20 by Ala increases markedly the association of p53 with Hdm2 within cells.

The lower association of wt p53 with Hdm2 relative to that of p53-Ala20 could be due to the fact that the *in vivo* phosphorylation of the former but not the latter on Ser20 interferes with binding to Hdm2. Alternatively, it could be argued that the Ala substitution itself augments the affinity of p53 for Hdm2, as compared with the wild-type protein with serine at position 20. To distinguish between these two possibilities, the interaction of different p53 forms with Mdm2 was monitored *in vitro*. If reduced Mdm2 binding requires phosphorylation on Ser20, it is likely that such modification will not occur *in vitro* and therefore both p53-Ala20 and wt p53 will bind equally well to Mdm2. p53-Ala20, wt p53 and p53-Asp20 were each translated in a reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. Equal amounts of radiolabeled p53 of each type were reacted with either a glutathione *S*-transferase (GST)-Mdm2 fusion protein or GST alone.

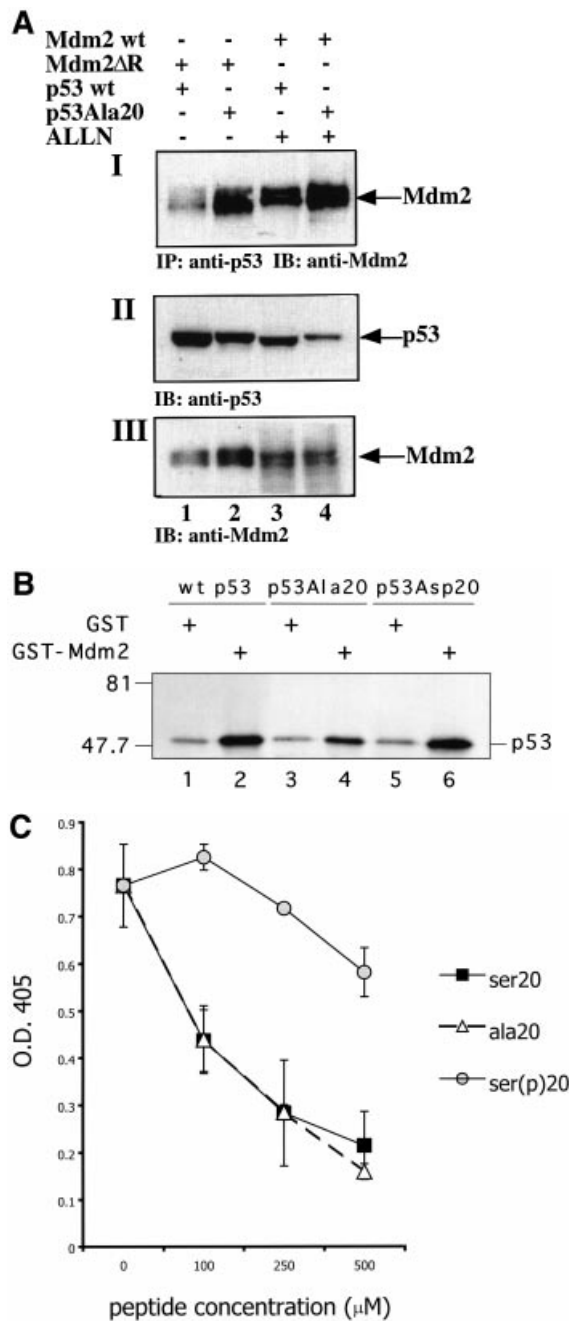
As seen in Figure 6B, all three forms of p53 bound preferentially to GST-Mdm2. However, unlike in the *in vivo* situation, the amount of bound p53-Ala20 was not higher but actually even lower than that of wt p53, while the binding capacity of p53-Asp20 was similar to that of wt p53. Taken together, these results suggest that a specific modification of Ser20 *in vivo*, presumably phosphorylation, compromises the binding capacity of p53 to Mdm2.

To explore this suggestion more directly, we employed a peptide competition assay. Three peptides encompassing the Mdm2-binding domain of human p53 (residues 14–29) were synthesized: one with the wild-type sequence (Ser20), one with Ala at residue 20 (Ala20), and one with a phosphorylated Ser20 [Ser(P)20]. The ability of these three peptides to block binding between human p53 and mouse Mdm2 was tested by ELISA at different peptide

concentrations. Figure 6C shows that both Ser20 and Ala20 peptides, at 100  $\mu$ M as well as 250  $\mu$ M, competed for the binding of wt hp53 to Mdm2 by 43 and 63%, respectively. In contrast, at the same concentrations Ser(P)20 had no significant effect on binding. Similarly, at a peptide concentration of 500  $\mu$ M, p53 binding to Mdm2 was competed markedly by both Ser20 (72%) and Ala20 (79%), while competition by Ser(P)20 was only 24% (Figure 6C). This argues that phosphorylation of p53 on Ser20 significantly reduces the affinity for Mdm2. Overall, our data support the conjecture that the *in vivo* phosphorylation of p53 on Ser20 by a cellular kinase(s) can strongly reduce the tightness of its interaction with Mdm2.

### Discussion

p53 is a key player in the cellular response to environmental stress, including various types of genomic damage. Activation of p53 in response to stress involves post-translational modifications, such as specific phosphorylation at serine residues within the N-terminal transactivation domain (reviewed in Steegenga *et al.*, 1996; Milczarek *et al.*, 1997; Giaccia and Kastan, 1998; Fuchs *et al.*, 1998a; Meek, 1998). A recent study showed that two serines within the first 24 amino acids of p53 are phosphorylated following exposure to ionizing and UV irradiation (Siliciano *et al.*, 1997). This phosphorylation induces accumulation of the p53 protein and enhances its transcriptional activity (Siliciano *et al.*, 1997). One of the two phosphorylation sites has been identified as Ser15 (Siliciano *et al.*, 1997), a potential phosphorylation site for ATM and DNA-PK (Lees-Miller *et al.*, 1992; Shieh *et al.*, 1997; Banin *et al.*, 1998; Canman *et al.*, 1998; Woo *et al.*, 1998). The identity of the second serine is not yet known, leaving Ser6, 9 and 20 as the potential sites (Siliciano *et al.*, 1997). We have recently shown that substitution of serine to alanine at residues 6 and 9 does not alter the biological effects of p53, while mutations in either Ser15 or Ser20 impair the apoptotic activity of p53



**Fig. 6.** Substitution of Ser20 to Ala enhances the binding of Mdm2 to p53 *in vivo* but not *in vitro*. (A) H1299 cells were transfected with expression plasmids encoding wt p53 or p53-Ala20 (2  $\mu$ g each), either alone or together with wt *mdm2* (3  $\mu$ g) or *mdm2* $\Delta$  RING (Hdm2 $\Delta$  R) (3  $\mu$ g) DNA as indicated above each lane. (I) Twenty-four hours post-transfection, cell extracts were subjected to immunoprecipitation with the anti-p53 monoclonal antibody PAb421. Immunoprecipitated (IP) proteins were resolved by 9% SDS-PAGE and immunoblotted (IB) with anti-Hdm2 monoclonal antibody SMP14. Aliquots of cell extracts prior to immunoprecipitation were subjected directly to Western blot analysis using a mixture of the anti-p53 monoclonal antibodies DO-1 and PAb1801 (II), or the anti-Hdm2 monoclonal antibody SMP14 (III). The positions of p53 and Hdm2 $\Delta$  RING are indicated by arrows. (B) *In vitro* interaction of Mdm2 with various forms of p53. Wt p53, p53-Ala20 and p53-Asp20 were translated *in vitro* in the presence of [<sup>35</sup>S]methionine. Equal amounts of radiolabeled p53 of each form were incubated with either GST-Mdm2 or GST alone. Bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE and visualized by exposure to X-ray film after fluorography. Positions of the p53 proteins and molecular markers are indicated. (C) Peptide competition for p53-Mdm2 binding *in vitro*. ELISA plates were coated with GST-Mdm2. Coated plates were incubated with each of the three peptides at the indicated concentrations. His<sub>6</sub>-tagged human p53 was then added, followed by a brief incubation. Bound p53 was identified with monoclonal antibody PAb421, and revealed with HRP-coupled goat-anti-mouse antibodies. HRP activity was determined in the presence of ABTS/H<sub>2</sub>O<sub>2</sub> and color was measured as absorbance at 405 nm. Standard deviations, calculated from triplicates, are shown.

(Unger *et al.*, 1999). These findings suggest that, along with Ser15, Ser20 plays a role in regulating p53 activity; regulation is most probably achieved through phosphorylation of Ser20 in response to stress. Indeed, recent work shows clearly that Ser20 becomes rapidly phosphorylated upon exposure of cells to DNA damage (Shieh *et al.*, 1999).

The ability of Mdm2 to inhibit p53 activities and promote its degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Momand and Zambetti, 1997), together with the fact that Ser20 resides within the Mdm2-binding domain of p53 (Kussie *et al.*, 1996), strongly suggest a role for Mdm2 in mediating the reduced activity of p53-Ala20. This conjecture was confirmed experimentally by the finding that p53-Ala20 is markedly more susceptible than wt p53 to inhibition by Mdm2, as revealed when the apoptotic and transcriptional activities of p53-Ala20 were compared with those of wt p53 in the presence of limiting amounts of Mdm2. Significantly, Mdm2-promoted destabilization of p53 was enhanced by the Ser20 to Ala substitution. This differential susceptibility to Mdm2-promoted proteolytic degradation also explains the observation that p53-Ala20 accumulates less efficiently than wt p53 in cells like H1299 which express endogenous Hdm2, but not in cells lacking Mdm2.

The generality of these findings is supported by several observations. First, the increased sensitivity of p53-Ala20 to inhibition by Mdm2 can be demonstrated in multiple cell types. Secondly, the enhanced inhibition of p53-Ala20 by Mdm2 is seen not only with exogenous overexpressed protein, but also with the endogenously-induced Mdm2. Thirdly, elimination of Mdm2, such as in the *mdm2* null cells, is sufficient to stabilize p53-Ala20. These results support a physiological role for Ser20 in the regulation of p53 stability and activity.

It is rather difficult to assess to what extent the promotion of p53 degradation accounts for the ability of Mdm2 to block p53 activities. In cells expressing relatively low levels of p53, this may well be the major determinant. However, when larger amounts of p53 plasmid were used, resulting in levels of p53-Ala20 more comparable with those of wt p53, the former still induced 4-fold less apoptosis (Figure 1E). Presumably, a lower ratio of Mdm2 to p53 may suffice for inhibiting the apoptotic activity of p53, while a high ratio may be required to promote its degradation as well. Therefore, the promotion of p53 for degradation and the inhibition of its activities through other mechanisms may have additive effects in terminating the p53 signal once the stress has been dealt with properly.

The increased susceptibility of p53-Ala20 to negative regulation by Mdm2 can be explained by the enhanced binding between the two proteins *in vivo* but not *in vitro* (Figure 6A and B). Presumably, phosphorylation of Ser20 reduces the binding of p53 to Mdm2, thereby alleviating the inhibitory effects of Mdm2. Indeed, a peptide with phosphorylated Ser20 was unable to compete for p53/Mdm2 binding while similar peptides with non-phosphorylated Ser20 or with Ala20 efficiently competed with wt p53 for Mdm2 binding (Figure 6C). Since the association between p53 and Mdm2 is mediated primarily through hydrophobic interactions (Kussie *et al.*, 1996), the extra charges contributed by the phosphate group might directly interfere with this association. In addition, phosphorylation on Ser20 may also exert long-range

effects, by augmenting inhibitory interactions between the N-terminal domain of p53 and other parts of the molecule, or by imposing conformational changes on the p53 protein, as suggested for phosphorylation on the adjacent Ser15 (Shieh *et al.*, 1997). Of note, transient transfection of DNA represents an effective DNA damage signal, which induces a strong p53 activation response (Renzing and Lane, 1995; Huang *et al.*, 1996). It is thus conceivable that under the conditions of our transient transfection assays, a significant fraction of the exogenous wt p53 protein might have undergone phosphorylation on N-terminal residues, notably Ser20. Consequently, this phosphorylated sub-population is expected to have become a less favorable Mdm2 target, accounting for the pronounced apoptotic and transcriptional activities of the transfected p53 despite the induction of endogenous Mdm2. On the other hand, mutation of Ser20 to Ala prevents p53 phosphorylation on this residue and retains the protein in a continuously Mdm2-sensitive state. This is reflected in less efficient accumulation of p53-Ala20, coupled with impaired biological activity.

Our findings complement those of Shieh *et al.* (1997), who showed that phosphorylation of Ser15 by DNA-PK attenuates p53-Mdm2 interaction and consequently activates p53. Overall, blocking the Mdm2-p53 interaction can provide an efficient physiological mechanism for activating and stabilizing p53, as also shown by targeting the binding site of p53 and/or Mdm2 using specific mutations, antibodies or short peptides (Bottger *et al.*, 1997; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Midgley and Lane, 1997). Covalent modifications in Mdm2 may also reduce its interaction with p53, and are expected to have a similar effect on p53 activity. In fact, it has been reported that phosphorylation of Mdm2 by DNA-PK within its p53-binding domain interferes significantly with p53 binding and with the ability of Mdm2 to abrogate the transcriptional function of p53 (Mayo *et al.*, 1997). Other mechanisms may also be utilized towards overcoming the negative regulation of p53 by Mdm2. For instance, through direct interaction with Mdm2, the p19<sup>ARF</sup> tumor suppressor protein neutralizes Mdm2 and thereby collaborates with p53 in growth suppression (de Stanchina *et al.*, 1998; Pomerantz *et al.*, 1998; Zhang *et al.*, 1998; Zindy *et al.*, 1998). In addition, it has recently been reported that the adenovirus E1A protein promotes p53 accumulation by blocking the induction of *mdm2* gene expression by p53 (Thomas and White, 1998), as well as by interfering with the association of Mdm2 with p300, which is important for p53 degradation (Grossman *et al.*, 1998). Thus, the interaction between p53 and Mdm2 is a major target for regulation, through which p53 activity and stability are modulated.

Overall, our results strongly implicate Ser20 in the regulation of p53 activity in response to stress. Together with the findings of Shieh *et al.* (1999) who demonstrated that p53 is rapidly phosphorylated on Ser20 *in vivo* in response to ionizing radiation, our results support the following scenario: upon exposure to DNA damage and other types of stress Ser20 is phosphorylated, probably along with Ser15. The newly phosphorylated p53 protein, which is now released from negative regulation by Mdm2, accumulates and becomes activated to elicit its biological effects. Thus, through modification of Ser20, environ-

mental cues are converted into a p53-dependent cellular response. Identifying the kinase(s) that phosphorylates Ser20 will help to delineate the signaling cascade leading to p53 activation.

## Materials and methods

### Cells and transfection

Mouse embryo fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM), while H1299 cells were maintained in Roswell Park Memorial Institute (RPMI). All cells were grown at 37.5°C in the presence of 10% fetal calf serum. The H1299 cell line is derived from a human lung adenocarcinoma and is devoid of any p53 expression. Cells were transfected by the calcium phosphate procedure, which includes a shock with 10% glycerol for 1 min at the end of the transfection (6–16 h from the start). The exact amount of plasmid used in each experiment is indicated in the corresponding figure legends. Whenever needed, an empty vector was used to maintain a constant amount of DNA in each transfection mix.

Luciferase assays, Western blot and immunoprecipitation analyses were carried out as previously described (Haupt *et al.*, 1996). The antibodies used were: anti-human p53 monoclonal antibodies PAb1801, PAb421 and DO-1, anti-Hdm2 SMP14 (Picksley *et al.*, 1994) and anti- $\alpha$ -tubulin antibody (DM 1A, Sigma).

### In vitro binding and peptide competition assay

GST alone and GST-Mdm2 (encoding full-length mouse Mdm2) were purified from bacteria using standard procedures. Human wt p53, p53-Ala20 and p53-Asp20 were transcribed and translated *in vitro* in the presence of [<sup>35</sup>S]methionine using the TNT T7-coupled reticulocyte lysate system (Promega). *In vitro* translated p53 proteins were incubated with GST fusion proteins immobilized on glutathione agarose beads (Sigma) for 2 h at 4°C in 250  $\mu$ l of binding buffer [25 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.2% Nonidet P-40 (NP-40)], with continuous shaking. Following incubation the beads were washed three times with a large excess of washing buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% NP-40) and boiled in protein sample buffer. The separated proteins were resolved by SDS-PAGE and visualized by exposure to X-ray film after fluorography.

For peptide competition assays, GST-Mdm2 (residue 6–121) was purified from bacteria by glutathione agarose beads and human p53 fused to six histidines (p53-His<sub>6</sub>) was purified using nickel column chromatography (Qiagen). Peptides were purified by HPLC (100%; Alpha Diagnostic). The peptides used were:

p53-Ser20: Ac-LSQ-ETF-SDL-WKL-LPE-N-NH<sub>2</sub>  
 p53-Ser(P)20: Ac-LSQ-ETF-S(P)DL-WKL-LPE-N-NH<sub>2</sub>  
 p53-Ala20: Ac-LSQ-ETF-ADL-WKL-LPE-N-NH<sub>2</sub>

ELISA plates were coated with 20 ng of GST-Mdm2 in phosphate-buffered saline (PBS) overnight at 4°C. Plates were blocked in 2% BSA in PBS. One hundred microliters of peptides in PBST (PBS/0.05 Tween 20)/2% bovine serum albumin (BSA), at the indicated concentrations, were added to the blocked plates for 1 h at room temperature. One hundred microliters of purified p53-His<sub>6</sub> in PBST/1% BSA were added to the plates and incubated for an additional 15 min at room temperature. Plates were washed three times with PBST and incubated with anti-p53 PAb421 for 1 h at 4°C. Plates were washed again and incubated with an horseradish peroxidase (HRP)-coupled goat-anti-mouse IgG for 30 min at room temperature and then washed again. A color reaction was established with 100  $\mu$ l of ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-sulfonic acid] (1 mg/ml)/H<sub>2</sub>O<sub>2</sub> (0.01%) in 0.1 M phosphate-citrate buffer pH 4.2 for 15 min at room temperature. The reaction was stopped with 100  $\mu$ l 0.2 M citric acid. Absorbance was measured at 405 nm using Victor2 multilabel counter (Wallac).

### Plasmids

The human p53 cDNA Ser20 to Ala or Asp mutants were generated by PCR-based oligonucleotide-directed mutagenesis. The resultant PCR products were cloned into the *Xba*I-human *Hind*III sites of pRC/CMV vector (Invitrogen). The mutant plasmids were subjected to DNA sequencing to confirm the desired mutation and to ensure that no misincorporations occurred during PCR amplification. Expression plasmids for human wt p53 were pCMV-Neo-Bam-p53 and PRC/CMV wt p53; for mouse wt *mdm2*, pCOC-*mdm2* X2 (Barak *et al.*, 1994; Haupt *et al.*, 1996), and for mutant human *mdm2*, pCMV-Neo-Bam-*Hdm2* RING. All of these cDNAs were placed under the CMV (cytomegalovirus)

immediate early enhancer/promoter. The reporter plasmid used was cyclin G-luciferase (Zauberman *et al.*, 1995). The bacterial expression plasmids for *in vitro* binding were GST alone and GST-Mdm2 (full-length mouse *mdm2*), and for peptide competition assay GST-Mdm2 (6–121) and His<sub>6</sub>-tagged human p53, kindly provided by Z.Ronai (Fuchs *et al.*, 1998b).

### Flow cytometry

At the indicated time post-transfection, adherent and floating cells were collected, fixed in methanol and stained for p53 using a mixture of anti-p53 antibodies DO-1 and PAb1801, followed by an FITC-conjugated secondary antibody. Samples were analyzed in a cell sorter (FACSCalibur) using the CellQuest software (Becton Dickinson). The FITC fluorescent intensity was plotted on a logarithmic scale. Cells were collected separately according to their fluorescent intensity. Cells with high fluorescent intensity represent the successfully transfected sub-population, while cells with low fluorescent intensity represent the non-transfected sub-population. The cell cycle distribution of each sub-population was determined by measuring the DNA content through propidium iodide staining. The apoptotic fraction was determined by quantitating the number of cells possessing a sub-G<sub>1</sub> DNA content (Haupt *et al.*, 1996).

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