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Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation

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The p53 tumor suppressor is inhibited and destabilized by Mdm2. However, under stress conditions, this downregulation is relieved, allowing the accumulation of biologically active p53. Recently we showed that c-Abl is important for p53 activation under stress conditions. In response to DNA damage, c-Abl protects p53 by neutralizing the inhibitory effects of Mdm2. In this study we ask whether this neutralization involves a direct interplay between c-Abl and Mdm2, and what is the contribution of the c-Abl kinase activity? We demonstrate that the kinase activity of c-Abl is required for maintaining the basal levels of p53 expression and for achieving maximal accumulation of p53 in response to DNA damage. Importantly, c-Abl binds and phosphorylates Mdm2 *in vivo* and *in vitro*. We characterize Hdm2 (human Mdm2) phosphorylation at Tyr394. Substitution of Tyr394 by Phe394 enhances the ability of Mdm2 to promote p53 degradation and to inhibit its transcriptional and apoptotic activities. Our results suggest that phosphorylation of Mdm2 by c-Abl impairs the inhibition of p53 by Mdm2, hence defining a novel mechanism by which c-Abl activates p53.

Keywords: c-Abl/Mdm2/p53/tyrosine phosphorylation

Introduction

Exposure of cells to stress activates the p53 tumor suppressor protein. This activation is cardinal for the efficient response of cells to stress stimuli, such as DNA damage and hypoxia (Giaccia and Kastan, 1998; Oren, 1999; Vogt Sionov *et al.*, 2001b). The response is either in the form of growth arrest, senescence or apoptosis (Vogt Sionov and Haupt, 1999). A loss of p53 function, either by mutations or by deregulation of regulatory proteins, is often detrimental, leading eventually to the development of cancer (reviewed in Vogt Sionov and Haupt, 1999). The p53 protein is subject to a tight regulation, keeping it labile

and inactive under normal conditions. This regulation occurs at the levels of protein stability, post-translational modifications and subcellular localization (Ashcroft *et al.*, 1999; Jimenez *et al.*, 1999; Gottifredi and Prives, 2001).

The key inhibitor of p53 is the *mdm2* proto-oncogene. Mdm2 (Hdm2 in humans) inhibits the transcriptional activity and growth suppression ability of p53 through an autoregulatory feedback loop (Momand *et al.*, 2000; Michael and Oren, 2002). Importantly, Mdm2 promotes p53 for degradation through the ubiquitin system (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) by acting as an E3-ubiquitin ligase (Honda and Yasuda, 1999; Fang *et al.*, 2000). The nuclear export of p53 is believed to be important for Mdm2-dependent degradation (Roth *et al.*, 1998; Lain *et al.*, 1999; Tao and Levine, 1999), although degradation within the nucleus has also been observed (Yu *et al.*, 2000; Xirodimas *et al.*, 2001). Nucleo-cytoplasmic shuttling depends on one or more nuclear export signals of p53 (Stommel *et al.*, 1999; Zhang and Xiong, 2001) and requires the ubiquitylation of p53 by Mdm2 (Boyd *et al.*, 2000; Geyer *et al.*, 2000).

In order for p53 to be activated in response to stress, the inhibitory activities of Mdm2 on p53 have to be modulated. This modulation is achieved by multiple mechanisms, including specific modifications of both p53 (Ashcroft *et al.*, 1999; Jimenez *et al.*, 1999; Meek, 1999) and Mdm2. Mdm2 is phosphorylated on multiple sites by various kinases (Hay and Meek, 2000; see below). The inhibitory activities of Mdm2 are also neutralized by the action of partner proteins. In response to oncogenic activation, the major activator of p53 is the ARF tumor suppressor protein, which neutralizes Mdm2-mediated ubiquitylation and degradation of p53 (reviewed in Sherr and Weber, 2000). However, in response to ionizing radiation, the ataxia telangiectasia mutant (ATM) kinase plays a key role. ATM induces the phosphorylation of p53 directly and indirectly through the activation of the Chk2 kinase (reviewed by Kastan and Lim, 2000; Shiloh, 2001). Phosphorylation of Mdm2 by ATM also impairs its inhibitory effects on p53 (Khosravi *et al.*, 1999; Maya *et al.*, 2001).

Another pathway that is activated by ATM involves the c-Abl non-receptor tyrosine kinase. ATM phosphorylates and activates c-Abl in response to ionizing radiation (Baskaran *et al.*, 1997; Shafman *et al.*, 1997). In response to stress such as DNA damage, c-Abl promotes cell growth arrest and apoptosis, unlike its oncogenic forms such as Bcr-Abl, which counteracts these cellular responses (reviewed in Kharbanda *et al.*, 1998; Van Etten, 1999; Shaul, 2000). The apoptotic activity of c-Abl is mediated partly via p73 (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999), and to a lesser extent through a p53-dependent pathway (Wen *et al.*, 1996; Yuan *et al.*, 1997). We have recently shown that c-Abl plays an important role

in achieving maximal and rapid accumulation of p53 in response to DNA damage (Vogt Sionov *et al.*, 2001a). c-Abl protects p53 by neutralizing the ability of Mdm2 to promote p53 for degradation (Vogt Sionov *et al.*, 1999), most likely by impairing the ubiquitylation of p53 and its subsequent export to the cytoplasm (Vogt Sionov *et al.*, 2001a). These actions of c-Abl explain how c-Abl enhances the transcriptional and biological activities of p53 (e.g. Goga *et al.*, 1995; Wen *et al.*, 1996; Vogt Sionov *et al.*, 1999).

The regulatory role of c-Abl in the activation of p53 in response to stress, and its ability to neutralize Mdm2, prompted us to propose that these actions of c-Abl involve a direct interplay between c-Abl and Mdm2. We report here that c-Abl and Mdm2 physically interact *in vitro* and *in vivo*. We demonstrate that the kinase activity of c-Abl is important for maintaining the basal level of p53 expression and for its accumulation in response to DNA damage. Furthermore, c-Abl phosphorylates Mdm2 *in vivo* and *in vitro*, and the biochemical and functional characterization of the phosphorylation on Tyr394 is presented. Mutational analysis of Tyr394 suggests that phosphorylation of Tyr394 impairs the ability of Mdm2 to inhibit the transcriptional and apoptotic activities of p53, as well as its efficiency to promote p53 degradation. Thus, our results define a new mechanism by which c-Abl regulates p53.

Results

c-Abl and Mdm2 interact *in vivo* and *in vitro*

In order to understand the mechanism by which c-Abl neutralizes the inhibitory effects of Mdm2 on p53, we investigated whether c-Abl and human Mdm2 (Hdm2) can engage in a protein–protein interaction. To address this question, 293 cells were transfected with expression plasmids for *hdm2* alone or together with *c-abl*. Twenty-four hours post-transfection, cells were harvested and the extracts were subjected to immunoprecipitation using anti-c-Abl antibody (ABL-148; Sigma), followed by western blot analysis using anti-Hdm2 (SMP14). Hdm2 was brought down efficiently with anti-c-Abl antibody when both Hdm2 and c-Abl were expressed, but not in the absence of c-Abl (Figure 1AI, lanes 2 and 3), confirming the existence of an interaction between the two proteins. This interaction was also observed when the co-immunoprecipitation was carried out via Hdm2 (data not shown). To examine whether the interaction between the two proteins is direct, the binding was tested *in vitro*. c-Abl and Mdm2 were synthesized and radioactively labeled with [³⁵S]methionine *in vitro*. Following incubation of the two proteins together, Mdm2 was immunoprecipitated using anti-Mdm2 antibody (2A10), and the amount of bound c-Abl was detected by exposure to film. A co-immunoprecipitation of the two proteins was clearly observed with radiolabeled c-Abl (Figure 1B, lane 4), but not with non-radioactive c-Abl (c-Abl*; Figure 1B, lane 5). Thus, c-Abl and Mdm2 interact both *in vivo* and *in vitro*.

To define the interaction region of c-Abl within Hdm2, a series of Hdm2 mutants lacking different regions of the protein were tested in an *in vivo* binding assay. These deletions cover the entire Hdm2 protein (Figure 2A). 293 cells were transfected with expression plasmids for

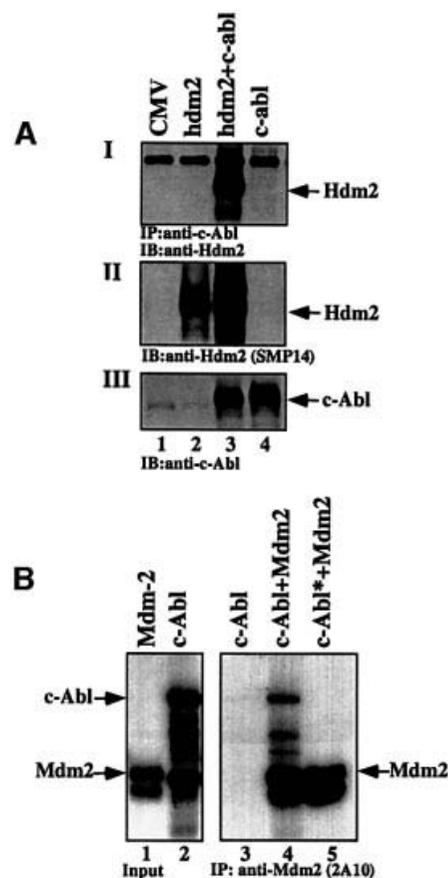


Fig. 1. Interaction between c-Abl and Hdm2 *in vitro* and *in vivo*. (A) 293 cells were transfected with the indicated expression plasmids (5 μ g each). Twenty-four hours post-transfection, cell extracts were subjected to immunoprecipitation using anti-c-Abl antibody (ABL-148) followed by western blotting using anti-Hdm2 (SMP14) (I). The expression level of Hdm2 in the transfected cells was monitored by blotting with anti-Hdm2 antibody (SMP14) (II), and that of c-Abl using anti-c-Abl antibody (ABL-148) (III). (B) Radioactively labeled (³⁵S)methionine) Mdm2 and c-Abl were incubated *in vitro* before being subjected to immunoprecipitation using anti-Hdm2 antibody (2A10). The amount of bound c-Abl was detected by exposure to film. As a control, unlabeled c-Abl was used (lane 5). The amount of input for each protein is shown (lanes 1 and 2).

His-tagged *c-abl* alone, or together with wild-type *hdm2* or deletion mutants of *hdm2*, as indicated in Figure 2A. His-c-Abl was isolated from the cell extracts using a nickel resin, and the amount of Hdm2 bound was determined by immunoblotting with anti-Hdm2 antibodies, SMP14 and Hdm2-323 (Sigma; M7815), which together recognize all the Hdm2 mutants (Figure 2B). All the Hdm2 deletion mutants tested were co-immunoprecipitated with c-Abl, but to varying degrees. A marked reduction in the level of interaction was observed with the Hdm2 6–339 mutant lacking the C-terminus (lane 10), while the Hdm2 Δ 222–437 mutant bound with higher affinity (lane 9). This supports a role for the ring-finger domain of Hdm2 in this interaction. However, other Hdm2 mutants lacking the ring-finger domain (Hdm2 Δ 441–491 and Hdm2 1–200) still bind c-Abl well (lanes 8 and 11), supporting the presence of an additional binding site within the N-terminal half of Hdm2. The reduced binding of Hdm2 Δ 1–50 mutant to c-Abl (lane 4), relative to the other deletions at the N-terminus (lanes 5 and 6), may

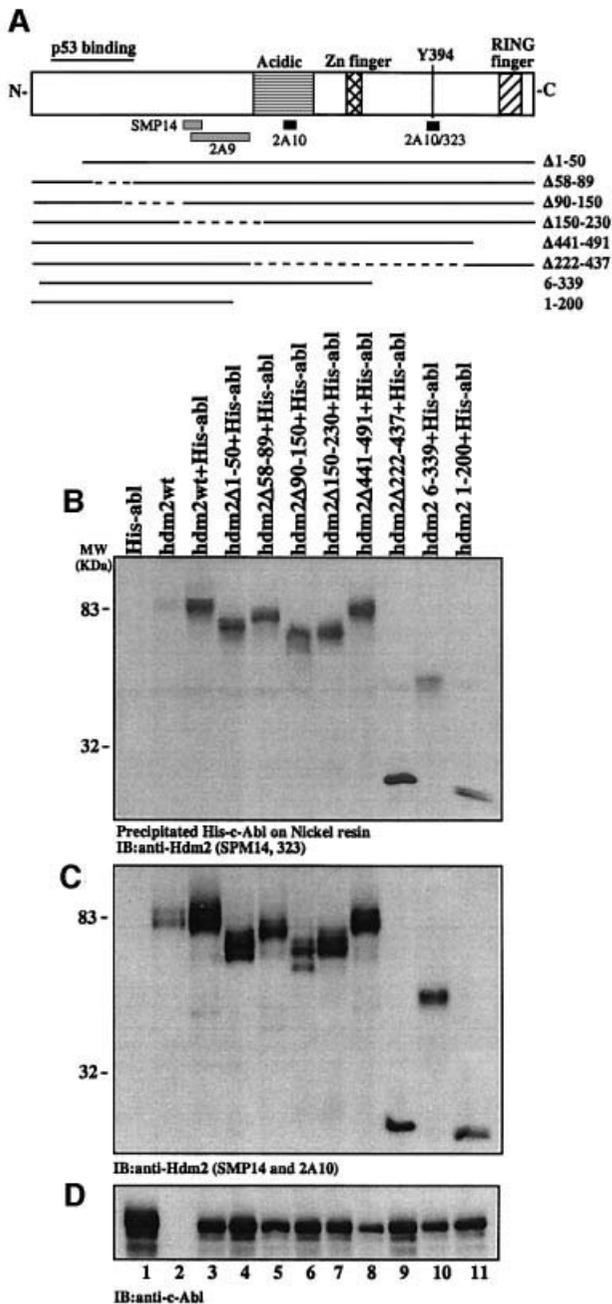


Fig. 2. c-Abl binds Hdm2 at multiple sites. (A) A schematic representation of the Hdm2 protein and the different deletion mutants of Hdm2 used in this study. Solid lines represent the encoded cDNA of the mutants, while the broken lines denote internal deletions. (B) 293 cells were transfected with expression plasmids for His-tagged c-Abl alone (5 μ g, lane 1), Hdm2 alone (5 μ g, lane 2), or His-c-Abl together with Hdm2 wild-type or deletion mutants as indicated (5 μ g each, lanes 3–11). His-c-Abl was isolated from the cell extracts using nickel resin, and the protein complex was subjected to western blotting using SMP14 and 323 anti-Hdm2 antibodies (B). (C) The levels of Hdm2 in the transfected cells were determined prior to immunoprecipitation using SMP14 and 323. (D) The level of c-Abl was monitored with anti-c-Abl antibody (ABL-148).

support a role for the first 50 residues of Hdm2 in this interaction. Furthermore, the stronger interaction of c-Abl with the Hdm2 1–200 mutant than with Hdm2 6–339 (lane 11 versus 10) may support the involvement of the first five residues of Hdm2 in this interaction. Thus, it

appears that the extreme N- and C-termini of Hdm2 are both important for binding c-Abl.

Both c-Abl and Mdm2 have nuclear localization and nuclear export signals (NLS and NES, respectively). Since both Mdm2 and c-Abl affect the subcellular distribution of p53 (Roth *et al.*, 1998; Boyd *et al.*, 2000; Geyer *et al.*, 2000; Vogt Sionov *et al.*, 2001a), it was of interest to define whether the interaction between Mdm2 and c-Abl is confined to a particular compartment within the cell. For this purpose, the effect of the subcellular localization of c-Abl on its interaction with Hdm2 was tested by comparing the binding of Hdm2 to NLS-defective c-Abl (mutant 'K') versus c-Abl that is distributed both in the nucleus and the cytoplasm (mutant 'A') (Wen *et al.*, 1996). The distribution of the latter mutant 'A' is similar to that of wild-type c-Abl. 293 cells were transfected with an expression plasmid for *hdm2*, either alone or together with *c-abl* 'A' or 'K'. Hdm2 bound efficiently to c-Abl mutant 'A', but only weakly to c-Abl mutant 'K' (Figure 3AI, lanes 2 and 3), suggesting that the interaction occurs primarily, but not exclusively, in the nucleus.

This binding assay also revealed that c-Abl increases the steady-state level of Hdm2 (Figure 1AII, lane 2 versus 3). Since c-Abl stabilizes and activates p53 by neutralizing the inhibitory effects of Mdm2 (Vogt Sionov *et al.*, 1999, 2001a), it is plausible that this action of c-Abl on Mdm2 expression is mediated through the induction of p53 transcriptional activation of the *mdm2* gene. Therefore, the requirement for p53 for the elevation of Mdm2 expression by c-Abl was evaluated in H1299 cells lacking p53 expression. H1299 cells were transfected with expression plasmids for *hdm2*, either alone or together with *c-abl* or *p53*, or all three plasmids together. The steady-state level of Hdm2 was determined by western blot analysis using the anti-Hdm2 antibody SMP14 (Figure 3BI). The data show that accumulation of Hdm2 in response to c-Abl occurs efficiently also in the absence of p53 (Figure 3B, compare lanes 2 and 3). This effect is further enhanced in the presence of p53 (lane 4). Therefore, p53 contributes to, but is not essential for, the elevation of Hdm2 by c-Abl.

c-Abl kinase activity is important for the stabilization of p53 under normal conditions and in response to DNA damage

We have recently shown that the presence of endogenous c-Abl is important for maintaining the basal levels of p53, and for the maximal and efficient stabilization of p53 in response to DNA damage (Vogt Sionov *et al.*, 2001a). To evaluate the role of c-Abl kinase activity in this process, the expression of p53 was compared between wild-type fibroblasts and c-Abl-deficient fibroblasts, either non-manipulated or reconstituted with a kinase-defective c-Abl mutant (K290R; cell lines K8 and K10). To evaluate the role of the c-Abl kinase activity in the basal expression levels of p53, equal numbers of untreated cells of each fibroblastic cell type were harvested and p53 protein levels were determined by western blot analysis. Consistent with our recent findings (Vogt Sionov *et al.*, 2001a), the basal levels of p53 expression in c-Abl-deficient fibroblasts were significantly lower than those in normal cells expressing physiological levels of c-Abl (Figure 4A). Interestingly, the expression levels of p53 in cells expressing kinase-defective c-Abl were significantly lower than in wild-type

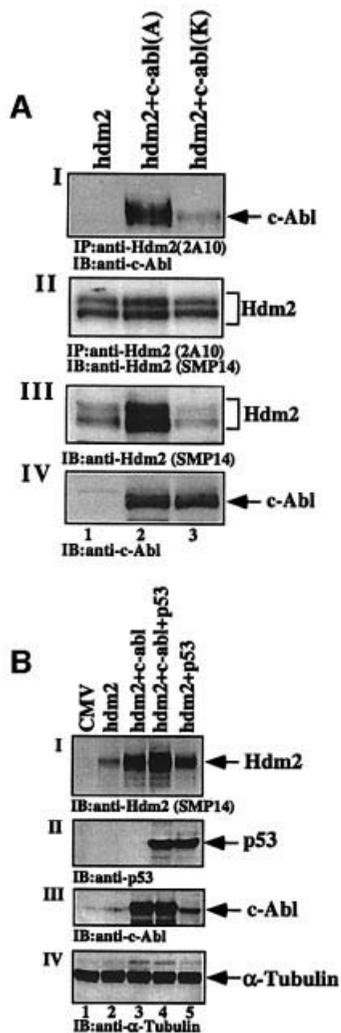


Fig. 3. c-Abl binds Hdm2 in the nucleus and enhances its accumulation in a p53-independent manner. (A) 293 cells were transfected with the expression plasmid for *Hdm2* alone (5 μ g, lane 1), or together with *c-abl* mutants (5 μ g) that are expressed in the cytoplasm (*c-abl* K; lane 3) or in both the nucleus and cytoplasm (mutant 'A'; lane 2). Cell extracts were subjected to immunoprecipitation using anti-Hdm2 antibody (2A10), followed by blotting with anti-c-Abl antibody (ABL-148) (I) or with anti-Hdm2 antibody (SMP14) (II). The levels of Hdm2 and c-Abl in the transfected cells were determined by western blotting with anti-Hdm2 (SMP14) (III) or anti-c-Abl (IV), respectively. (B) H1299 cells were transfected with the indicated expression plasmids Hdm2 (3 μ g), c-Abl (4 μ g) and p53 (1 μ g). Twenty hours post-transfection, cell extracts were subjected to western blotting using anti-Hdm2 (I), anti-p53 (II) and anti-c-Abl (ABL-148) (III). The amount of extracts loaded was monitored with anti- α -tubulin (IV).

cells, but slightly higher than in the c-Abl-negative cells (Figure 4A). Identical results were obtained when c-Abl-deficient fibroblasts reconstituted with wild-type c-Abl at physiological levels (Vogt Sionov *et al.*, 1999) were used instead of wild-type fibroblasts (data not shown). Thus, the kinase activity of c-Abl is important for maintaining the basal levels of p53 expression.

Next, we asked whether the kinase activity of c-Abl is also important for the accumulation of p53 in response to DNA damage. For this purpose, fibroblasts lacking c-Abl and fibroblastic cell lines reconstituted with wild-type or kinase-defective c-Abl were exposed to different types of DNA-damaging agents. Cells were exposed to γ -radiation

(10 Gy) and harvested at the indicated times. Further, cells were incubated with mitomycin C (1 μ g/ml) or doxorubicin (10 μ g/ml) for 6 h. At the indicated times after treatment, cells were harvested and p53 expression levels were determined. As shown in Figure 4, the stabilization of p53 in response to γ -radiation (B), mitomycin C or doxorubicin (C) was greater in cells expressing wild-type c-Abl than in cells lacking c-Abl or expressing a kinase-defective mutant. Taken together, these results demonstrate a clear role for the kinase activity of c-Abl in the stabilization of p53 in response to exposure to various DNA damaging agents, and in the maintenance of p53 expression under non-stressed conditions.

Since c-Abl has been implicated in transcription through the phosphorylation of RNA polymerase II (Baskaran *et al.*, 1999), we examined whether the basal or stress-induced elevation of p53 expression levels by wild-type c-Abl results from enhanced transcription of endogenous p53 mRNA. c-Abl-negative and c-Abl-reconstituted fibroblasts were either not treated or exposed to the proteasome inhibitor ALLN (150 μ M for 2 h), mitomycin C (3 μ g/ml for 6 h) or doxorubicin (3 μ g/ml for 6 h). Total mRNA was prepared from treated and untreated cells, and the expression of endogenous p53 was measured by northern blot analysis using a p53-specific cDNA probe. The amounts of mRNAs loaded were monitored by the expression levels of GAPDH. As shown in Figure 4D, the levels of p53 mRNAs were similar in cells lacking or expressing wild-type c-Abl irrespective of the treatment. Therefore, the presence of c-Abl does not increase the amount of p53 mRNA in untreated cells or in cells exposed to DNA damage. This strongly suggests that the effect of c-Abl on p53 occurs at the protein level.

c-Abl phosphorylates Mdm2 in vivo

The physical and functional link between c-Abl and Mdm2 encouraged us to examine whether Mdm2 is a direct target for phosphorylation by c-Abl. This possibility is attractive, particularly since c-Abl tyrosine kinase activity is induced in response to DNA damage (Kharbanda *et al.*, 1995), and the kinase activity of c-Abl is required for the maximal accumulation of p53 in response to stress (Figure 4). The ability of c-Abl to phosphorylate Hdm2 *in vivo* was examined in 293 cells transfected with expression plasmids for *hdm2*, either alone or together with wild-type *c-abl* or kinase-defective *c-abl*. Twenty-four hours post-transfection, cells were harvested and Hdm2 was immunoprecipitated using anti-Hdm2 antibody (SMP14), followed by immunoblot analysis using anti-phosphotyrosine antibody (4G10; Upstate Biotechnology). Tyrosine phosphorylation of Hdm2 was observed in the presence of wild-type c-Abl but not with the c-Abl kinase-defective mutant (Figure 5AI, lanes 3 and 4). Similar results were obtained in 293 cells transfected with expression plasmids for glutathione S-transferase (GST)-c-Abl fusion proteins. The kinase-defective mutant (GST-c-AblK⁻) did not phosphorylate Hdm2, while an SH3-deficient mutant (GST-c-Abl Δ SH3) enhanced the phosphorylation of Hdm2 at least to the same extent as wild-type c-Abl (GST-c-Abl) (Figure 5B). These results demonstrate that c-Abl can phosphorylate Hdm2 on tyrosine *in vivo*.

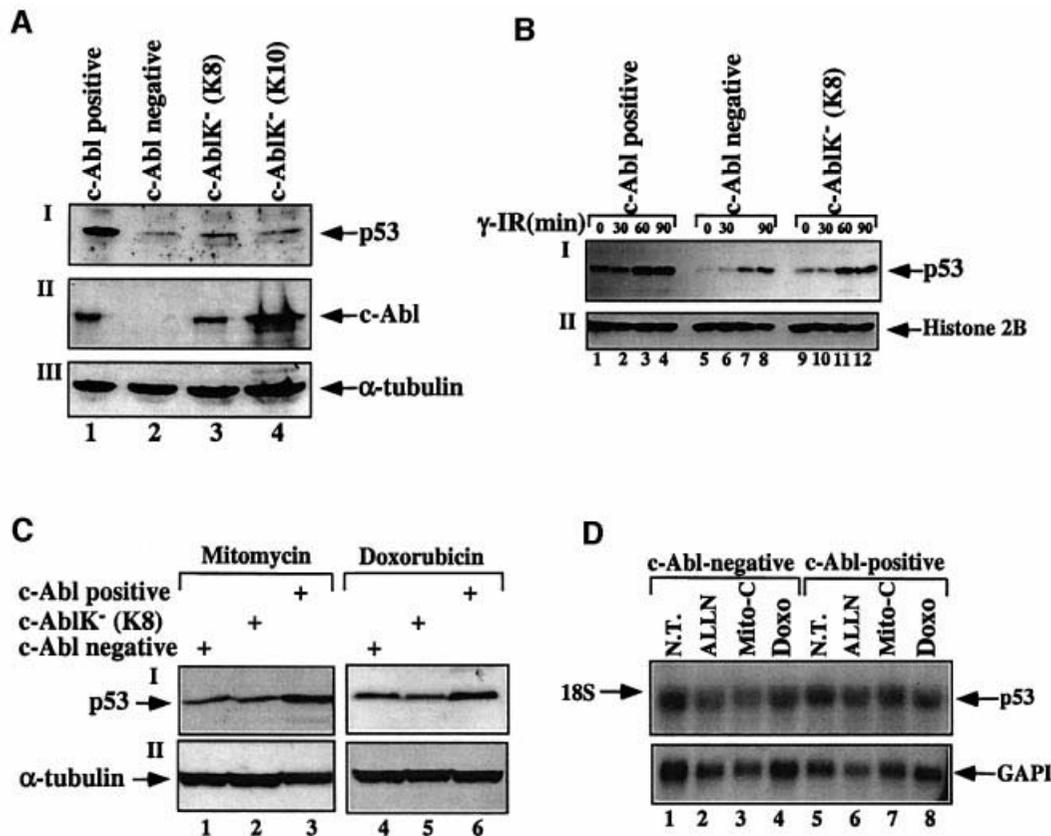


Fig. 4. The kinase activity of c-Abl is important for the stabilization of p53 under basal and stress conditions. (A) Cell extracts were prepared from normal primary fibroblasts (c-Abl positive, lane 1), from c-Abl null fibroblasts (c-Abl negative, lane 2), or from two fibroblastic cell lines that were reconstituted with a c-Abl kinase-defective mutant (K8, lane 3; K10, lane 4). Extracts were subjected to western blotting using anti-p53 antibody (CM5; I) or anti-c-Abl (ABL-148; II). Long exposure of the film was required in order to detect p53 in the c-Abl-negative cells (lane 2), which resulted in an apparent high expression level of p53 in normal cells (lane 1) (I). The amounts of protein loaded were monitored by blotting with anti- α -tubulin (III). (B) Fibroblastic cell lines, either negative for c-Abl (lanes 5–8), or reconstituted with either wild-type c-Abl (c-Abl positive; lanes 1–4) or kinase-defective c-Abl (c-AblK⁻ K8; lanes 9–12), were either untreated (lanes 1, 5, 9) or exposed to γ -radiation (10 Gy) and harvested at the times indicated. The expression levels of p53 were monitored by blotting with anti-p53 antibody (CM5). The amount of protein loaded was determined with anti-histone 2B (II). (C) The three fibroblastic cell lines were treated with mitomycin C (1 μ g/ml; lanes 1–3) or doxorubicin (10 μ g/ml; lanes 4–6) for 6 h. The expression levels of p53 were monitored as in (A)(I), and the amount of protein loaded was determined with anti- α -tubulin (II). (D) Northern blot analysis of c-Abl null primary fibroblasts (c-Abl negative; lanes 1–4) or normal primary fibroblasts (c-Abl positive; lanes 5–8). Cells were either untreated (NT) or exposed to the proteasome inhibitor ALLN (150 μ M for 2 h), mitomycin C (Mito-C; 3 μ g/ml for 6 h) or doxorubicin (3 μ g/ml for 6 h). Total mRNA was prepared from each sample and subjected to northern blot analysis. The levels of p53 mRNA were determined by probing with ³²P-labeled p53 cDNA, and the amount of mRNA loaded was monitored by reprobing with ³²P-labeled cDNA for GAPDH.

c-Abl phosphorylates Hdm2 on Y394

Hdm2 has 14 tyrosine residues that are dispersed along the protein. A search for the relevant tyrosine phosphorylation sites suggested that Tyr394 (Y394) was an attractive candidate. The sequence encompassing Y394 shares the highest homology with the phosphorylation consensus site of c-Abl (Till *et al.*, 1999), and is highly conserved among mice, humans and hamsters (Figure 6A; Momand *et al.*, 2000). Intriguingly, the Y394 site is very similar to the phosphorylation site of c-Abl in p73 (Figure 6A; Yuan *et al.*, 1999). To test whether Y394 is a phosphorylation site, this tyrosine was substituted by phenylalanine (F), to prevent phosphorylation. The effect of this substitution on the phosphorylation of Hdm2 by c-Abl was measured *in vivo* and *in vitro*. For the *in vivo* assay, 293 cells were transfected with expression plasmids for wild-type *hdm2* or *hdm2* F394 alone, or together with an expression plasmid for *c-abl*. Hdm2 phosphorylation was examined by immunoprecipitation of Hdm2 followed by immunoblotting with anti-phosphotyrosine antibody (4G10). The

Y394F substitution reduced significantly the extent of Hdm2 phosphorylation, as compared with wild-type Hdm2 (Figure 6BI, lanes 3 and 5). This reduction supports the notion that Y394 is a major phosphorylation site for c-Abl. However, the data leave open the possibility that c-Abl may phosphorylate Hdm2 on additional site(s). One potential site is Y489 (LTYFP). However, unlike F394, the Hdm2 F489 substitution mutant was effectively phosphorylated by c-Abl *in vivo* (Figure 6BI, lanes 3 and 7), arguing that Y489 is not a relevant target site of c-Abl.

To substantiate the phosphorylation of Hdm2 on Y394, we applied an anti-phospho-Y394 polyclonal antibody raised against a phosphorylated Y394-bearing peptide. For the *in vivo* assay, two experimental approaches were used. In the first, 293 cells were transfected with expression plasmids for c-Abl together with either wild-type Hdm2 or Hdm2 F394. Twenty-four hours after transfection, Hdm2 was immunoprecipitated from the cell extracts using a mixture of anti-Hdm2 antibodies (SMP14, 2A9 and 2A10), followed by western blot analysis using anti-

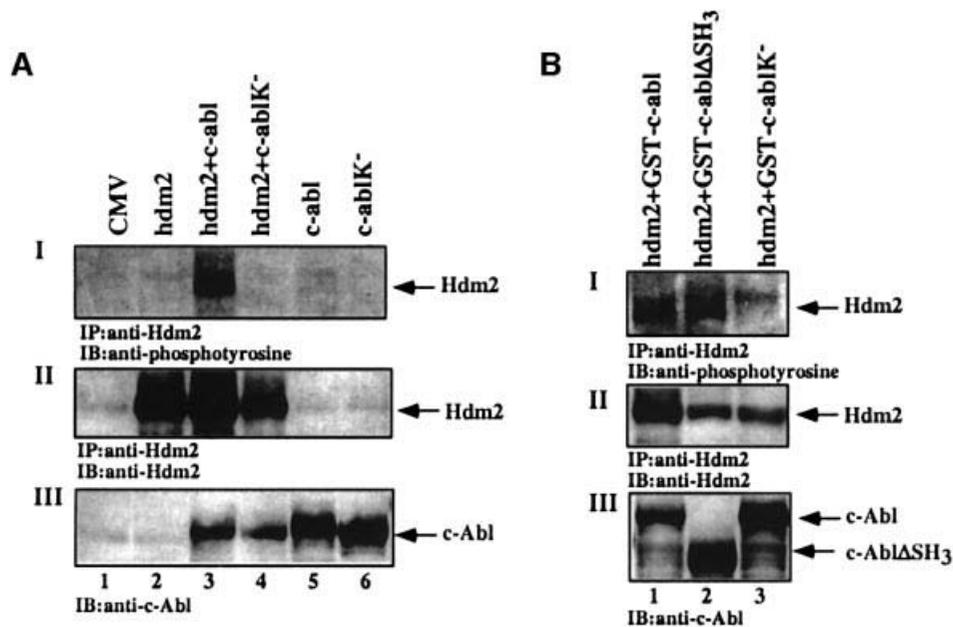


Fig. 5. c-Abl phosphorylates Hdm2 *in vivo*. (A) 293 cells were transfected with the indicated expression plasmids (5 μ g each). Twenty-four hours later, cell extracts were subjected to immunoprecipitation using SMP14, followed by western blotting with 4G10 (I) or SMP14 (II). The levels of c-Abl in the transfected cells were monitored with anti-c-Abl antibody (ABL-148) prior to immunoprecipitation (III). (B) 293 cells were transfected with expression plasmids for Hdm2 together with GST fused to wild-type c-Abl (lane 1) or to SH3-deletion mutant (lane 2) or to a kinase-defective mutant (lane 3). The phosphorylation of Hdm2 (I) and the expression levels of Hdm2 (II) and c-Abl (III) were monitored as described in (A).

phospho-Y394 polyclonal antibodies. As shown in Figure 6C, specific phosphorylation was detected with anti-phospho-Y394 only in cells expressing wild-type Hdm2 (lane 3) but not Hdm2 F394 (lane 2). Furthermore, to examine whether endogenous Hdm2 is also phosphorylated on Y394, SJSA-1 osteosarcoma cells were either non-treated, or treated with the tyrosine phosphatase inhibitor sodium orthovanadate alone or together with H₂O₂, which prevents dephosphorylation of Hdm2 and activates c-Abl (Sun *et al.*, 2000). Endogenous Hdm2 was immunoprecipitated as in 293 cells, and the immune complex was subjected to western blot analysis using anti-phospho-Y394 antibodies. Specific phosphorylation of endogenous Hdm2 was detected only in cells treated with orthovanadate and H₂O₂ (Figure 6D, lane 2). These results support the phosphorylation of Hdm2 on Y394 *in vivo*, and further demonstrate that phosphorylation of endogenous Hdm2 occurs at physiological levels of c-Abl under conditions that trigger its kinase activity (Sun *et al.*, 2000).

For the *in vitro* assay, wild-type or a kinase-defective c-Abl was isolated by immunoprecipitation from 293 cells transfected with the respective expression plasmids. The kinase activity of c-Abl was verified using bacterially derived GST-c-Crk (120–225), or GST-c-Crk (120–212), the latter lacking Tyr221 (Shafman *et al.*, 1997). Wild-type c-Abl, but not the kinase-defective mutant, phosphorylated c-Crk (120–225) but not c-Crk (120–212) (data not shown). To test the *in vitro* phosphorylation of Hdm2 on Y394, GST was fused to full-length Hdm2 or to the C-terminus of Hdm2 (Hdm2 Δ N; amino acids 358–493), with either Y or F at position 394. The proteins were expressed and purified from bacteria, and subjected to a phosphorylation assay using wild-type or a kinase-defective c-Abl immunoprecipitated from transfected 293 cells. Reaction products were subjected to immunoblotting

using anti-phospho-Y394 antibodies. A significant and specific phosphorylation of wild-type Hdm2, both full length and Δ N, but not their equivalent F394 mutants, was observed (Figure 6E, lanes 1 and 5 versus lanes 3 and 7). Taken together, these results demonstrate the phosphorylation of Hdm2 on Y394 by c-Abl both *in vivo* and *in vitro*.

Modifications of the ATM phosphorylation site at Ser395 do not affect Y394 phosphorylation by c-Abl

Our finding of Hdm2 phosphorylation by c-Abl at Y394 is particularly striking as this site is adjacent to the ATM-mediated phosphorylation site, Ser395 (S395) (Khosravi *et al.*, 1999; Maya *et al.*, 2001). This raised the question whether phosphorylation of S395 by ATM affects the phosphorylation of Y394 by c-Abl. To address this question, the effect of modifications of S395 on the phosphorylation of Y394 was measured *in vitro* and *in vivo*.

The *in vitro* assay was carried out with purified His-tagged c-Abl, comparing wild-type with the kinase-defective c-Abl mutant. The phosphorylation of four different GST-fused Hdm2 proteins by c-Abl was compared. These include wild-type Hdm2 and Hdm2 F394, and two Hdm2 mutants bearing substitutions at S395: serine to alanine substitution (Hdm2 S395A) to prevent phosphorylation at S395, and serine to aspartate (Hdm2 S395D) to mimic the phosphorylated state of S395 (Maya *et al.*, 2001). Following the *in vitro* phosphorylation assay, the reaction products were subjected to immunoblot analysis using anti-phospho-Y394 antibodies. A strong and specific phosphorylation of wild-type Hdm2, Hdm2 S395A and Hdm2 S395D was observed in the presence of wild-type c-Abl, but not with the kinase-defective mutant (Figure 7A). Therefore, the phosphoryl-

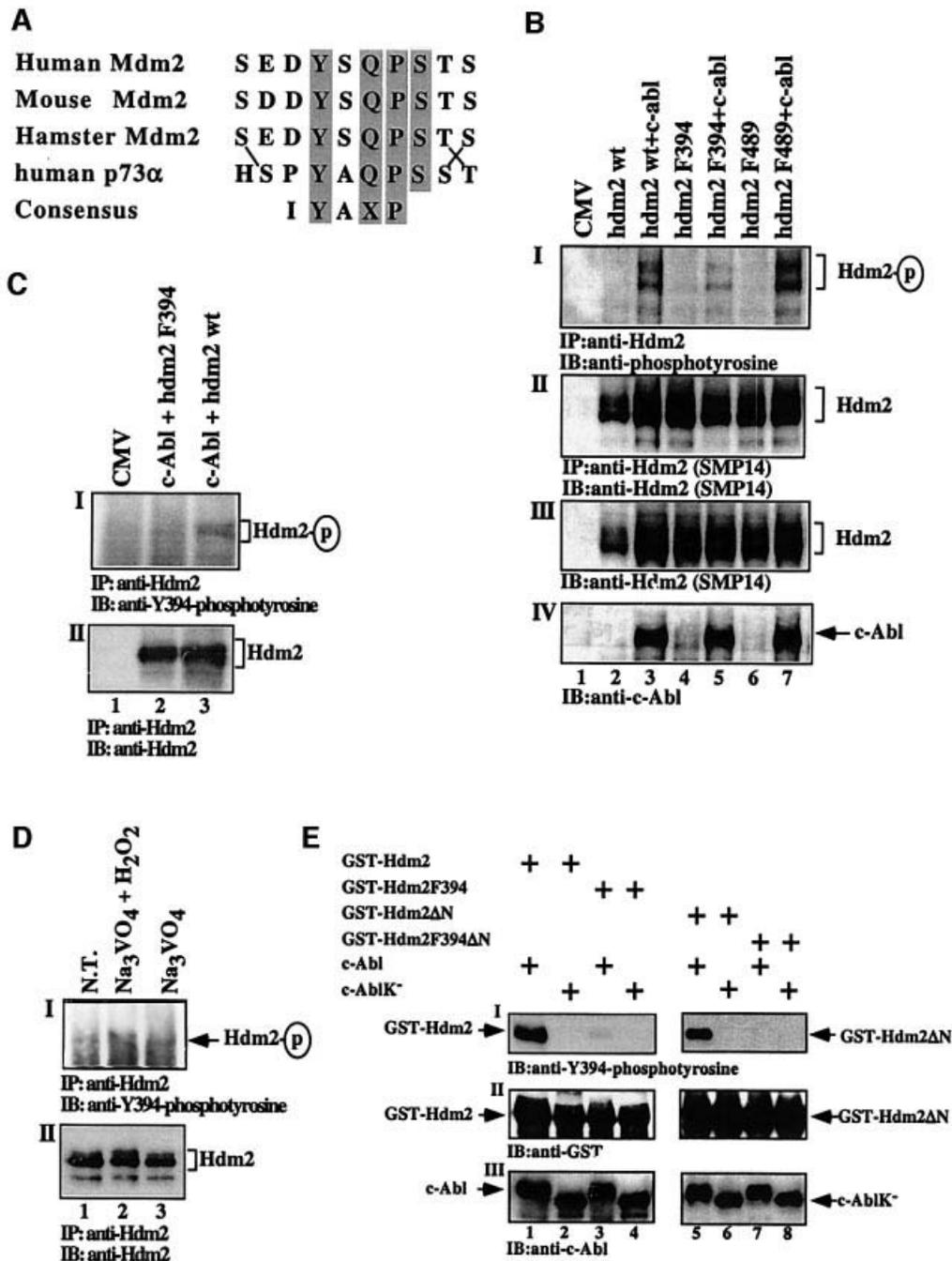


Fig. 6. c-Abl phosphorylates Hdm2 on Tyr394 *in vivo* and *in vitro*. (A) Alignment of the amino acid sequence around Y394 showing the sequence homology in the Mdm2 sequence among three species and to the c-Abl phosphorylation site in p73 (Y99). The c-Abl consensus phosphorylation site (Till *et al.*, 1999) is shown at the bottom. Gray boxes represent conserved amino acids and lines point to position substitution of conserved residues. (B) 293 cells were transfected with the indicated expression plasmids (5 μ g each). Phosphorylation of Hdm2 (I), the levels of Hdm2 after (II) and before immunoprecipitation (III), and the levels of c-Abl (IV) were determined as described in Figure 5A. (C) 293 cells were transfected with expression plasmid for *c-abl* (5 μ g) together with *Hdm2 F394* (lane 2) or wild-type *Hdm2* (lane 3). Hdm2 was immunoprecipitated using a mixture of antibodies (SMP14, 2A9 and 2A10), followed by immunoblotting with anti-phospho-Y394 antibodies (I) or anti-Hdm2 antibody (SMP14) (II). The position of phosphorylated Hdm2 and total Hdm2 is indicated. (D) SJSA cells were either non-treated (NT) or treated with the tyrosine phosphatase inhibitor sodium orthovanadate (Na₃VO₄) alone (lane 3) or together with H₂O₂ (lane 2) for 20 min. The detection of Hdm2 phosphorylated on Y394 was as described in (C). (E) An *in vitro* phosphorylation assay of Hdm2 by c-Abl. GST fused to full-length wild-type Hdm2 (lanes 1–4) or to N-terminally truncated mutants (Hdm2 Δ N, lanes 5–8) bearing a Y394 (lanes 1, 2, 5 and 6) or an F394 substitution (lanes 3, 4, 7 and 8) were incubated with either wild-type c-Abl (lanes 1, 3, 5 and 7) or a kinase-defective mutant (lanes 2, 4, 6 and 8). The phosphorylation of Hdm2 on Y394 was determined by western blot analysis using anti-phospho-Y394 polyclonal antibodies (I). The amount of GST–Hdm2 proteins was determined with anti-GST antibody (II) and the levels of c-Abl were monitored using anti-c-Abl antibody (ABL-148) (III). Note, the phosphorylated form of c-Abl migrates slower on SDS–PAGE.

ation at Y394 by c-Abl is independent of the modifications at S395, and neither the lack nor the mimicking of S395 phosphorylation prevented phosphorylation at Y394 (lanes 1, 3 and 4). If anything, the S395A substitution slightly enhanced Y394 phosphorylation (lanes 1 and 4). Nevertheless, neither substitution at S395 affected the reactivity of the anti-Y394 antibody with Hdm2 in the absence of phosphorylation by c-Abl (lanes 5–8).

The effect of S395 modifications on the phosphorylation of Y394 was also examined *in vivo*. 293 cells were transfected with expression plasmids for wild-type *hdm2*, *hdm2 F394*, *hdm2 S395A* and *hdm2 S395D* together with an expression plasmid for *c-abl*. Twenty-four hours after transfection, cells were treated with sodium orthovanadate for 1 h. The phosphorylation of Hdm2 on Y394 was examined by immunoprecipitation of Hdm2 followed by immunoblotting with anti-phospho-Y394 antibodies. As in the *in vitro* phosphorylation assay, Y394 phosphorylation was specific for Y394 and was unaffected to any significant level by the modifications at S395 (Figure 7B). Taken together, these results demonstrate that phosphorylation of Hdm2 at Y394 by c-Abl occurs independently of ATM-mediated phosphorylation at S395. Furthermore, modifications at S395 do not affect the reactivity or the specificity of the anti-phospho-Y394 for the phosphorylated Y394 site.

Substitution of Tyr by Phe at position 394 enhances the ability of Hdm2 to promote p53 degradation

The demonstration that the kinase activity of c-Abl contributes to the accumulation of p53, and that Hdm2 is phosphorylated by c-Abl on Y394, begged the question as to whether phosphorylation of this site modulates the regulation of p53 by Hdm2. To address this question, the effect of substitution of Tyr by Phe at position 394 on the ability of Hdm2 to promote p53 degradation was assessed. For this purpose, p53/mdm2 double knockout (2KO) fibroblasts were transfected with the expression plasmid for wild-type p53, either alone or together with expression plasmids for either wild-type *Hdm2* or *Hdm2 F394*. As seen in Figure 8A, Hdm2 F394 was significantly more efficient than wild-type Hdm2 in promoting the degradation of p53. This difference was observed despite equal expression levels of Hdm2 F394 as compared with wild-type Hdm2 (Figure 8B). These results strongly support the notion that the Y394 substitution, which prevents the phosphorylation of Hdm2 on Y394, enhances the ability of Hdm2 to promote p53 degradation.

Substitution of Tyr by Phe at position 394 enhances the ability of Hdm2 to inhibit the transcriptional activity of p53

The effect of the substitution of Tyr by Phe at position 394 on the enhanced degradation of p53 encouraged us to examine the effect of the substitution on the inhibition of p53 transcriptional activity by Hdm2 (Chen *et al.*, 1996). For this purpose, H1299 cells were transfected with wild-type p53 expression plasmid, either alone or together with expression plasmids for either wild-type Hdm2 or Hdm2 F394. The transcriptional activity of p53 was monitored by measuring the activity of the luciferase gene under the control of the cyclin G promoter. The inhibition

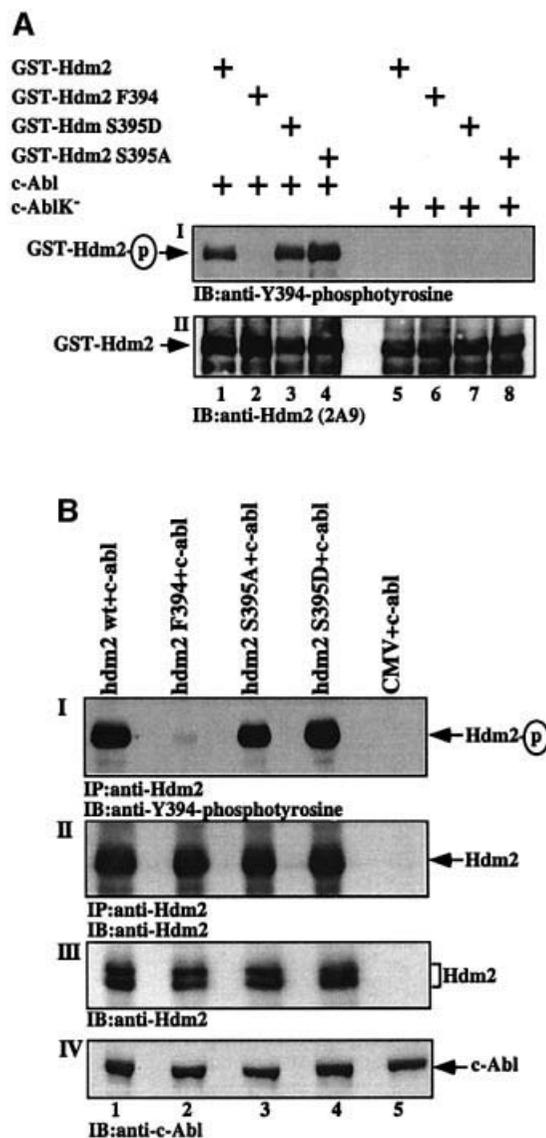


Fig. 7. Modifications at S395 do not affect phosphorylation of Y394 by c-Abl. (A) An *in vitro* phosphorylation of Hdm2 mutants by c-Abl. GST fusions to wild-type Hdm2, Hdm2 F394, Hdm2 395D and Hdm2 395A were incubated with either His-tagged wild-type c-Abl (lanes 1–4) or a kinase-defective mutant (lanes 5–8). The phosphorylation of Y394 was determined by western blot analysis using anti-phospho-Y394 antibodies (I). The amount of GST–Hdm2 proteins loaded was monitored with anti-Hdm2 antibody (2A9). (B) An *in vivo* phosphorylation assay in 293 cells transfected with expression plasmids for *c-abl* alone (5 μ g; lane 5) or together with either wild-type *Hdm2* (lane 1) or *Hdm2 F394* (lane 2), or *Hdm2 S395A* (lane 3) or *Hdm2 S395D* (lane 4) (6 μ g of each mutant). Twenty hours post-transfection, cells were treated with sodium orthovanadate for 1 h. From triplicates of each plasmid combination, Hdm2 was immunoprecipitated with a mixture of anti-Hdm2 antibodies (SMP14, 2A9, 2A10 and 4B11), followed by immunoblotting with anti-phospho-Y394 (I). The amount of Hdm2 loaded was determined with SMP14 (II). The amount of Hdm2 and c-Abl proteins expressed in the transfected cells was also determined before the immunoprecipitation by western blotting using anti-Hdm2 (2A9; III) or anti-c-Abl (ABL-148; IV) antibodies, respectively.

of p53 transcriptional activity by Hdm2 F394 was more efficient than that by wild-type Hdm2. While the difference was moderate, it was highly reproducible in three independent experiments using three to seven replicate

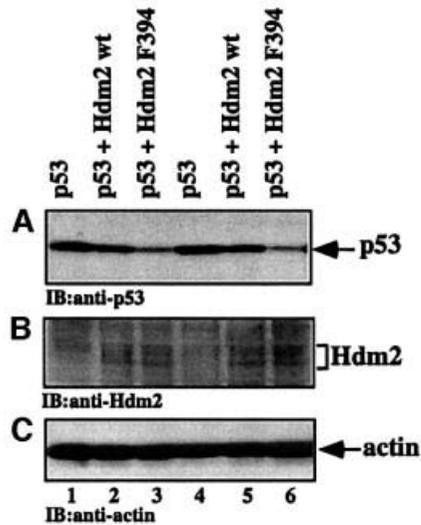


Fig. 8. Enhanced degradation of p53 by the Hdm2 F394 mutant. *p53/mdm2* 2KO fibroblasts were transfected with expression plasmids for wild-type *p53* (25 ng), either alone (lanes 1 and 4), or together with expression plasmids for either wild-type *Hdm2* (lanes 2 and 5) or *Hdm2 F394A* (lanes 3 and 6; 500 ng each). The experiment was carried out in replicate, from which a representative result is shown. The expression levels of p53 (A) and Hdm2 (B) were monitored by western blot analysis using anti-p53 (PAb1801 and DO1) and anti-Hdm2 (SMP14, SMP15, 2A9) antibodies, respectively. (C) The amount of protein loaded was monitored with anti-actin.

samples for each plasmid combination. Figure 9 represents one such experiment, carried out in seven replicate samples. These results demonstrate that the Y394 substitution enhances the ability of Hdm2 to inhibit p53 transcriptional activity.

Substitution of Tyr by Phe at position 394 enhances the ability of Hdm2 to inhibit p53-mediated apoptosis

Hdm2 inhibits the apoptotic activity of p53 (Chen *et al.*, 1996; Haupt *et al.*, 1996), even under conditions where cells are matched for equal expression levels of p53 (Haupt *et al.*, 1996; Unger *et al.*, 1999). To examine whether the Tyr to Phe substitution at position 394 affects this inhibitory activity of Hdm2, H1299 cells were transfected with a wild-type *p53* expression plasmid, either alone or together with increasing amounts of expression plasmid for wild-type *Hdm2* or *Hdm2 F394*. Apoptosis was measured 55 h later (Haupt *et al.*, 1996). As with the transcriptional activity, here too one could observe a mild but consistent increase in the inhibitory effect of Hdm2 F394 relative to wild-type Hdm2, at both the low and high levels of Hdm2 expression. The results of a representative experiment, carried out in triplicate, are summarized in Figure 10A. This difference in the inhibitory activity between wild-type Hdm2 and Hdm2 F394 was maintained at different ratios of Hdm2 and p53 (data not shown). A more robust effect of the Y394 to Phe substitution on the inhibitory effect of Hdm2 was revealed in Saos-2 cells (Figure 10B) transfected with the expression plasmid for wild-type *p53*, either alone or together with expression plasmids for wild-type *Hdm2* or *Hdm2 F394*. While wild-type Hdm2 reduced the apoptotic activity of p53 by 42%, the same amount of Hdm2 F394

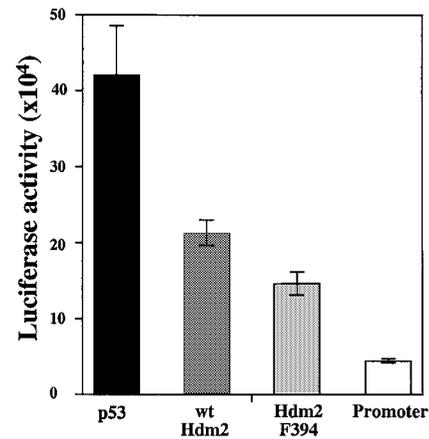


Fig. 9. Enhanced inhibition of p53 transcriptional activity by Hdm2 F394. H1299 cells were transfected with expression plasmids for wild-type *p53* (30 ng; black bar), either alone or together with expression plasmids for wild-type *Hdm2* (400 ng; dark gray bar) or *Hdm2 F394* (400 ng; light gray bar). The transcriptional activity of p53 was monitored by measuring the activity of the luciferase gene under the control of the *cyclin G* promoter (white bar). The results from three independent experiments are shown, each of which represents an average of three to seven independent transfections for each combination of expression plasmids. The standard error is given.

reduced this activity by 65% (Figure 10B). Taken together, these results demonstrate that the substitution of Y394 by F394 increases the inhibitory activity of Hdm2 on p53 apoptotic function.

Discussion

The p53 protein is subject to tight positive and negative regulation. Under normal conditions and during recovery from stress, p53 is very labile and presumably inactive. This regulation is mediated primarily by the Mdm2 protein. By binding to p53, Mdm2 overcomes its transcriptional activity and catalyzes its ubiquitylation and subsequent degradation. Through these combined effects, Mdm2 achieves the efficient and rapid inhibition of p53-mediated signaling. However, under stress conditions, p53 has to be freed from these restrictive effects of Mdm2 in order to exert its growth-inhibitory functions. This relief of p53 from Mdm2-dependent inhibition involves multiple mechanisms depending on the type of stress signal imposed. For example, in response to oncogenic stimuli, the ubiquitylation and subsequent degradation of p53 by Mdm2 are inhibited by ARF (reviewed in Sherr and Weber, 2000).

In response to DNA damage, the inhibitory effect of Mdm2 is relieved in part by phosphorylation of p53. For instance, the phosphorylation of p53 on Ser20 renders it more resistant to the destabilizing activity of Mdm2 (Chehab *et al.*, 1999; Shieh *et al.*, 1999; Unger *et al.*, 1999) and also inhibits its nuclear export (Zhang and Xiong, 2001). However, under certain conditions, the phosphorylation of p53 does not appear to exert a pronounced effect on its stability (Ashcroft *et al.*, 1999; Blattner *et al.*, 1999; Dumaz and Meek, 1999), lending support to the notion that additional mechanisms are involved in this regulation. An attractive candidate for DNA damage-induced modification is Mdm2 itself. Indeed, Mdm2 is phosphorylated on

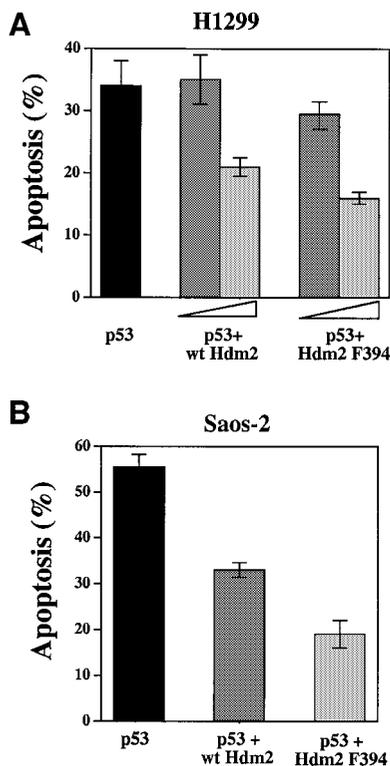


Fig. 10. Hdm2 F394 inhibits p53-mediated apoptosis more efficiently than wild-type Hdm2. **(A)** H1299 cells were transfected with expression plasmids for wild-type p53 (3 μ g, black bar), either alone or together with increasing amounts (4 μ g, dark gray bar; 8 μ g light gray bar) of expression plasmids for wild-type Hdm2 or Hdm2 F394. At 55 h post-transfection, floating and adherent cells were collected, fixed and stained for p53 expression. Cells were simultaneously stained for DNA with propidium iodide. Stained cells were subjected to flow cytometric analysis as described previously (Haupt *et al.*, 1996). Cells transfected with p53 were collected and their cell cycle distribution was determined. The sub-G₁ region represents apoptotic subpopulation (Haupt *et al.*, 1996). The results of one representative experiment, in triplicate, are summarized. **(B)** The same assay as in (A), but in Saos-2 cells. In this experiment, 3 μ g of expression plasmid for p53 and 5 μ g of expression plasmid for Hdm2 were used. The result of one representative experiment, in triplicate, is shown. The standard error is also given.

multiple sites (Fakharzadeh *et al.*, 1991; Hay and Meek, 2000). Mdm2 is phosphorylated by casein kinase-2 (CK2) (Guerra *et al.*, 1997; Gotz *et al.*, 1999; Hjerrild *et al.*, 2001) and by DNA-dependent protein kinase (DNA-PK); the phosphorylation by the latter prevents p53-Mdm2 interaction (Mayo *et al.*, 1997). The *in vivo* relevance of these phosphorylation sites awaits future studies. Mdm2 is also phosphorylated by the PI3-kinase/Akt pathway, and this phosphorylation promotes the nuclear entry of Mdm2 and subsequently the inhibition and degradation of p53 (Mayo and Donner, 2001; Zhou *et al.*, 2001; Gottlieb *et al.*, 2002). Of note, the exposure of cells to DNA damage triggers the *in vivo* phosphorylation of Hdm2 by ATM (Khosravi *et al.*, 1999). Phosphorylation of Hdm2 on S395 by ATM impairs the ability of Hdm2 to promote the degradation of p53 and to promote its nuclear export (Maya *et al.*, 2001). This is consistent with the role of ATM in the stabilization of p53 in response to ionizing radiation (Kastan *et al.*, 1992). Thus, phosphorylation of

Hdm2 can affect the autoregulatory feedback loop between Hdm2 and p53.

A pivotal phosphorylation target of ATM is the c-Abl tyrosine kinase (Baskaran *et al.*, 1997; Shafman *et al.*, 1997). We have recently demonstrated an important role for c-Abl in the accumulation of p53 in response to DNA damage (Vogt Sionov *et al.*, 2001a). Taken together with the ability of c-Abl to abrogate the inhibitory effects of Mdm2 (Vogt Sionov *et al.*, 1999, 2001a), this raised the question to what degree this action of c-Abl is mediated by direct physical and functional interaction with Hdm2. We found that c-Abl and Hdm2 physically interact *in vivo* and *in vitro* (Figure 1). Importantly, the kinase activity of c-Abl contributes to the maximal accumulation of p53 in response to DNA damage. Moreover, we show that this activity of c-Abl is also required for the regulation of p53 expression at the basal level.

The kinase activity of c-Abl is essential for the induction of growth arrest and apoptosis by c-Abl in response to different stress signals (Sawyers *et al.*, 1994; Goga *et al.*, 1995; Wen *et al.*, 1996; Yuan *et al.*, 1996b, 1997; Huang *et al.*, 1997). These biological activities of c-Abl, at least to some extent, are p53 dependent (Goga *et al.*, 1995; Wen *et al.*, 1996; Yuan *et al.*, 1997). Our findings implicating a role for the kinase activity of c-Abl in the cooperation with p53 were surprising in view of previous studies suggesting that this activity of c-Abl is not essential for this cooperation (Goga *et al.*, 1995; Yuan *et al.*, 1996a,b; Vogt Sionov *et al.*, 1999).

How can this apparent contradiction be resolved? One possible explanation is based on differences between the experimental systems used. In previous studies, the c-Abl kinase-defective mutant was either transiently overexpressed (Goga *et al.*, 1995; Vogt Sionov *et al.*, 1999), or constitutively expressed in stable cell lines (Yuan *et al.*, 1996b, 1997). In both cases, this mutant was introduced into cells that express endogenous wild-type c-Abl. In these systems, it was assumed that the kinase-defective mutant of c-Abl neutralizes the kinase activity of the endogenous protein in a dominant-negative fashion (Sawyers *et al.*, 1994). However, it remains possible that in these experimental systems the p53 protein is kept active by residual kinase activity of endogenous c-Abl. On the other hand, in our experimental system, the c-Abl kinase-defective mutant was reconstituted in c-Abl null cells. Another possibility is that c-Abl activates p53 by multiple mechanisms. It is possible that when c-Abl is expressed at high levels, a single pathway may suffice to achieve the maximum effect of c-Abl on p53 activation. For example, c-Abl interacts with the C-terminus of p53 and stabilizes its interaction with DNA (Nie *et al.*, 2000), and this interaction is independent of the kinase activity (Yuan *et al.*, 1996a). This interaction is also likely to stabilize the tetrameric form of p53 and consequently hinder nuclear export and/or interfere with the ubiquitylation of p53 on lysines residing within the C-terminus (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). These two explanations are not mutually exclusive.

Importantly, c-Abl phosphorylates Hdm2 on multiple tyrosine residues both *in vivo* and *in vitro*. We propose that this phosphorylation underlies an important mechanism by which c-Abl neutralizes the inhibitory effect of Hdm2 on p53. We characterized the phosphorylation of Hdm2 on

Y394, which fits the c-Abl consensus site (Till *et al.*, 1999) and shares high homology with the c-Abl phosphorylation site (Y99) in p73 α (Yuan *et al.*, 1999). To evaluate the functional role of Y394 phosphorylation, we substituted tyrosine by phenylalanine at position 394 in order to prevent phosphorylation while imposing minimal structural impact on the Hdm2 protein. This substitution enhanced the ability of Hdm2 to promote p53 degradation, as well as its ability to inhibit the transcriptional activity of p53 and its apoptotic activity. Therefore, the prevention of Hdm2 phosphorylation on Y394 increases the inhibitory activities of Hdm2 on p53 in multiple experimental systems. Our results support the model whereby phosphorylation of Hdm2 on Y394 is important for the regulation of Hdm2 function by c-Abl. The characterization of other c-Abl phosphorylation site(s) in Hdm2 and their contribution to the regulation of Hdm2 activities require further study.

The enhanced ability of the Hdm2 F394 mutant to promote the degradation of p53 is consistent with our finding that c-Abl impairs the ability of Hdm2 to promote the ubiquitylation of p53 and inhibit its nuclear export (Vogt Sionov *et al.*, 2001a). These two effects of Hdm2 on p53 may be linked, where ubiquitylation precedes nuclear export (Boyd *et al.*, 2000; Geyer *et al.*, 2000). However, ubiquitylation assays of p53 *in vivo* and *in vitro* did not reveal major differences between wild-type Hdm2 and Hdm2 F394 (data not shown). Interestingly, similar effects were seen when the adjacent phosphorylation site, S395, was substituted by alanine (Maya *et al.*, 2001). Thus, Hdm2 F394 may enhance the degradation of p53 without affecting the extent of its ubiquitylation. It is of note that Argentini *et al.* (2001) described Hdm2 mutants that are impaired in the degradation of p53, but not in its ubiquitylation. Such mutants may be defective in their ability to promote the nuclear export of p53 and/or directing p53 to the 26S proteasome.

The finding of two adjacent phosphorylation sites (Y394 and S395) being targeted by two types of protein kinases (Maya *et al.*, 2001; this study) is intriguing. Since ATM acts upstream of c-Abl in the cellular response to DNA damage (Baskaran *et al.*, 1997; Shafman *et al.*, 1997), we asked whether the phosphorylation of Y394 by c-Abl requires the prior phosphorylation of Hdm2 on S395 by ATM. Phosphorylation assays, both *in vivo* and *in vitro*, revealed that the phosphorylation of Hdm2 on Y394 by c-Abl occurs independently of the phosphorylation of S395 by ATM (Figure 7). Nevertheless, it is tempting to speculate that ATM and c-Abl cooperate in the regulation of Hdm2 in response to DNA damage. This speculation is particularly attractive because, in response to double-strand DNA breaks, ATM binds, phosphorylates and activates c-Abl (Baskaran *et al.*, 1997; Shafman *et al.*, 1997). Furthermore, in response to ionizing radiation, both ATM and c-Abl play important roles in the accumulation of p53 (Kastan *et al.*, 1992; Vogt Sionov *et al.*, 2001a). The phosphorylation of Hdm2 by these two kinases may occur simultaneously in a single complex, or sequentially. Either way, the phosphorylation of these two adjacent residues has an inhibitory effect on Hdm2 activity, thereby contributing to the rapid accumulation of p53 in response to DNA damage.

Materials and methods

Cell culture and transfection assays

H1299 lung adenocarcinoma cells, Saos-2 and SJSA-1 osteosarcoma cells were cultivated in RPMI-1640 medium supplemented with 10% FCS. 293 kidney epithelial cells, the three fibroblast cell lines [c-Abl negative (Abl^{-/-} fibroblasts reconstituted with LacZ); c-Abl positive (Abl^{+/+} fibroblasts reconstituted with c-Abl); and c-AblK⁻ (c-Abl fibroblasts reconstituted with the c-Abl kinase-defective mutant K290R)] (Vogt Sionov *et al.*, 1999), primary fibroblasts from normal and c-abl null mice, and fibroblasts (174-2) derived from p53/mdm2 2KO mice (McMasters *et al.*, 1996) were grown in DMEM supplemented with 10% heat-inactivated FCS. The c-Abl-positive fibroblasts expressed physiological levels of c-Abl (Vogt Sionov *et al.*, 1999). Transfections by the calcium phosphate precipitation method, and western blot analyses, were carried out as described previously (Haupt *et al.*, 1996). A constant amount of plasmid DNA in each sample was maintained by supplementing with empty plasmid.

The antibodies used were: anti-human p53 monoclonal antibodies PAb1801 and DO1; anti-mouse p53 polyclonal antibody CM5 (Novocastra); anti-Hdm2 SMP14, SMP15, 2A9 and 2A10, 4B11, Hdm2-323 (Sigma; M7815), anti-c-Abl ABL-148 (Sigma), anti- α -tubulin DM1A (Sigma), anti-histone H2B (LG2-2; kindly provided by Dr D.Eilat), anti-phosphotyrosine 4G10 (Upstate Biotechnology, NY), anti-GST polyclonal rabbit immunosera (purified on GST column), HRP-conjugated affinity-purified goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), HRP-conjugated protein A (Jackson ImmunoResearch Laboratories), EnVision peroxidase anti-mouse or anti-rabbit IgG (DAKO Corporation), and FITC-conjugated affinity-pure F(ab')₂ fragment goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The anti-phosphoY394 polyclonal antibodies were raised against a phosphorylated peptide, ESEDY(PO3H2)SQPSTC, as described previously for anti-phospho-Ser15 antibody against human p53 (Shieh *et al.*, 1997).

Plasmids

Expression plasmids were: human wild-type p53 (pCMV-Neo-Bam-p53), human wild-type p53 (pRC-CMV-p53), human wild-type mdm2 (pCMV-Neo-Bam-hdm2), human wild-type mdm2 (pCHDM1A-hdm2; kindly provided by Dr A.Levine), hdm2 1–200, hdm2 Δ Ring, mouse wild-type c-abl (pCMV-c-abl, type IV), mouse kinase-defective c-abl (pCMV-c-ablK290H, type IV), mouse c-abl^{-/-} mutant 'A' [pcDNA-K290RC4 INLS^Q (Q5) (Wen *et al.*, 1996)], mouse cytoplasmic c-Abl^{-/-} mutant 'K' [(pcDNA-K290RC4 INLS^{Q2}NLS^{Q3}NLS^Q, type IV) (Wen *et al.*, 1996)], mouse GST-c-Abl (EBG-Abl, type IV), mouse c-Abl kinase-defective GST-c-AblK290M, mouse GST-c-Abl Δ SH3 (the GST-c-Abl fusion constructs were kindly provided by Dr B.Mayer), p53-responsive Cyclin G-luciferase (Haupt *et al.*, 1996), and empty vectors (pCMV-Neo-Bam, pCDNA3 and pCOC). His-tagged c-Abl was generated by fusing six His residues to mouse c-Abl using PCR. The following truncated forms of hdm2 were kindly provided by Dr A.Levine: pCHDM Δ 1–50, pCHDM Δ 58–89, pCHDM Δ 90–150, pCHDM Δ 1–200, pCHDM Δ 150–230, pCHDM Δ 222–437, pCHDM Δ 441–491 and pCHDM 6–339 (Freedman *et al.*, 1997). The Tyr to Phe substitutions at 394 and 489 of Hdm2 were generated by using site-directed mutagenesis of wild-type pCMV-Neo-Bam-hdm2 (Stratagene). The generation of Hdm2S395A and Hdm2S395D was described previously (Maya *et al.*, 2001). The following plasmids were used for *in vitro* translation–type c-abl (SP6) and mouse mdm2 (T7 pGEM-X2); for *in vitro* GST-conjugated protein production: pGEX-c-CRK 120–225 and pGEX-c-CRK 120–212 (lacking Tyr221) (kindly provided by Dr K.K.Khanna, Australia), C-terminal mouse mdm2-GST (amino acids 356–482) and pGEX (encoding GST alone). The C-termini (amino acids 356–493) of wild-type hdm2, hdm2Y394F and hdm2Y389F were generated by PCR and fused to pGEX. Fragments of full-length wild-type hdm2, hdm2Y394F, hdm2S395A and hdm2S395D were generated by PCR and fused to pGEX. pGEX fusion plasmids were expressed in bacteria and the GST fusion proteins were isolated from the bacterial extract on glutathione beads (Sigma).

Immunoprecipitation and nickel pull down

Immunoprecipitation was carried out essentially as described previously (Haupt *et al.*, 1996), where the lysis buffer contained 50 mM Tris pH 8.0, 150–300 mM NaCl, 5 mM EDTA, 0.5% NP-40, 2 mM PMSF, 20 μ g/ml aprotinin, 25 mM NaF and 0.2 mM sodium orthovanadate. Samples were taken from the cell extracts prior to immunoprecipitation for analysis of

the steady-state levels of the relevant proteins by western blotting. Proteins were synthesized *in vitro* by the TNT transcription-translation-coupled reticulocyte lysate system (Promega), either non-radiolabeled or labeled with [³⁵S]methionine. Immunoprecipitation of the *in vitro* synthesized proteins was carried out in the same way.

For nickel pull down, 293 cells were transfected with wild-type or mutant Hdm2 together with His-tagged c-Abl. Cell extracts were prepared in the above lysis buffer but in the absence of EDTA. Binding to nickel resin was carried out according to the manufacturer's instructions (CytoSignal) in the presence of 15 mM imidazole for 90 min, followed by three washes in washing buffer containing increasing concentrations of imidazole (20–60 mM). His-c-Abl was eluted from the resin in elution buffer (20 mM Tris pH 8, 500 mM NaCl and 300 mM imidazole).

In vivo kinase assay

SJSA-1 cells were treated with 3 mM H₂O₂ (Merck, Germany) and/or 1 mM sodium orthovanadate (tyrosine phosphatase inhibitor; Sigma) for the indicated period. Endogenous Hdm2 was immunoprecipitated by using a combination of SMP14, 2A9 and 2A10 anti-Hdm2 antibodies, followed by immunoblotting using the anti-phosphotyrosine monoclonal antibody (4G10) or anti-phospho-394Y polyclonal antibodies.

293 cells were transfected with 5 µg of *hdm2* and/or 5 µg of *c-abl* or *c-ablK*. Twenty-four hours post-transfection, cells were either harvested directly or when indicated cells were treated with sodium orthovanadate before harvest. Cell extracts were immunoprecipitated with SMP14 or a combination of SMP14, 2A9 and 2A10, followed by immunoblotting using 4G10 or anti-phospho-Y394 antibodies as indicated.

In vitro kinase assay

The *in vitro* kinase assay was carried out with c-Abl isolated from 293 cells transfected with 5 µg of expression plasmids, for either His-tagged or non-tagged wild-type c-Abl or a kinase-defective c-Abl (*c-ablK*). Non-tagged c-Abl was isolated by immunoprecipitation, while the His-tagged c-Abl was isolated by a nickel pull down. Purified His-c-Abl was incubated with 30 µl of substrate in kinase buffer (25 mM Tris pH 7.4, 10 mM MgCl₂, 1 mM MnCl₂, 0.5 mM DTT, 10 µM ATP) for 15 min at room temperature. The incubation mix was subjected to western blot analysis using 4G10 or anti-phospho-Y394 antibodies as indicated.

Luciferase assay

H1299 cells were transfected with 30 ng of expression plasmid for wild-type *p53*, either alone or together with 400 ng of expression plasmids for wild-type *hdm2* or *hdm2Y394F*. All the samples contained 1 µg of the reporter plasmid encoding the luciferase gene under the control of the *Cyclin G* promoter. Post-transfection (17–24 h), the luciferase activity was measured in the cell extract with the Promega luciferase kit using the Lumat luminometer (LB9507; Berthold).

Apoptosis assay

H1299 cells and Saos-2 cells were transfected with the indicated amount of expression plasmid for wild-type *hp53*, either alone or together with the indicated amount of expression plasmids for *hdm2* or *hdm2Y394F*. At the indicated time post-transfection, cells were collected, fixed, stained for p53, and analysed by flow cytometry as described previously (Haupt *et al.*, 1996) using the FACSCalibur (Becton Dickinson).

Northern blot analysis

Northern blot analysis of fibroblasts, either non-treated or treated as indicated in the text, was carried out essentially as described previously (Haupt *et al.*, 1997).

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