Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms

Robert Stad, Natalie A. Little, Dimitris P. Xirodimas¹, Ruth Frenk, Alex J. van der Eb, David P. Lane¹, Mark K. Saville¹ & Aart G. Jochemsen⁺

Leiden University Medical Centre, Department of Molecular and Cell Biology and Centre for Biomedical Genetics, PO Box 9503, 2300 RA Leiden, The Netherlands and ¹University of Dundee, Department of Surgery and Molecular Oncology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

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The p53 protein maintains genomic integrity through its ability to induce cell cycle arrest or apoptosis in response to various forms of stress. Substantial regulation of p53 activity occurs at the level of protein stability, largely determined by the activity of the Mdm2 protein. Mdm2 targets both p53 and itself for ubiquitylation and subsequent proteasomal degradation by acting as an ubiquitin ligase, a function that needs an intact Mdm2 RING finger. For efficient degradation of p53 nuclear export appears to be required. The Mdmx protein, structurally homologous to Mdm2, does not target p53 for degradation, but even stabilizes both p53 and Mdm2, an activity most likely mediated by heterodimerization of the RING fingers of Mdm2 and Mdmx. Here we show that Mdmx expression leads to accumulation of ubiquitylated, nuclear p53 but does not significantly affect the Mdm2-mediated ubiquitylation of p53. In contrast, Mdmx stabilizes Mdm2 by inhibiting its self-ubiquitylation.

INTRODUCTION

The p53 tumour suppressor protein plays a key part in maintaining the integrity of the genome through its ability to impose cell cycle arrest or apoptosis following diverse stresses or inappropriate activation of oncogenes. Mdm2 is a crucial negative regulator of p53. Besides inhibiting the transcriptional regulatory properties of p53 directly, Mdm2 also regulates p53 protein levels (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Mdm2 is a RING-type E3 ubiquitin ligase (Honda *et al.*, 1997; Fang *et al.*, 2000; Honda and Yasuda, 2000), which directs the ubiquitylation of itself and p53. The poly-ubiquitylated Mdm2 and p53 are subsequently degraded by the 26S proteasome. The RING finger of Mdm2 is essential for its ubiquitin ligase activity,

although for the subsequent degradation of p53 other Mdm2 domains are also required (Argentini *et al.*, 2001; Maya *et al.*, 2001). Several reports suggest that nuclear export of p53, mediated through the nuclear export signal (NES) of Mdm2 and/or through the NES of p53 itself, is required for its efficient degradation (Roth *et al.*, 1998; Tao and Levine, 1999; Boyd *et al.*, 2000; Geyer *et al.*, 2000).

The Mdmx protein is structurally homologous to Mdm2 (Shvarts *et al.*, 1996). However, Mdmx does not target p53 for degradation but even prevents Mdm2-mediated degradation of p53 and also stabilizes Mdm2 (Sharp *et al.*, 1999; Jackson and Berberich, 2000; Stad *et al.*, 2000). Heterodimerization of the RING fingers of Mdmx and Mdm2 appears to be required for these effects, but the exact mechanism by which Mdmx operates has not been determined. Here we show that Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. Mdmx expression does not necessarily alter the overall pattern of Mdm2-mediated ubiquitylation of p53 but rather results in the accumulation of ubiquitylated p53 in the nucleus. In contrast, stabilization of Mdm2 by Mdmx is correlating with an inhibition of Mdm2 poly-ubiquitylation.

RESULTS

Different effects of Mdmx on p53 ubiquitylation and self-ubiquitylation by Mdm2

We investigated the effect of Mdmx on Mdm2-dependent ubiquitylation of p53 and on Mdm2 auto-ubiquitylation *in vivo*. To specifically analyse the level of ubiquitylated proteins, an *in vivo* ubiquitylation assay was carried out (Xirodimas *et al.*,

⁺Corresponding author. Tel: +31 71 5276136; Fax: +31 71 5276284; E-mail: A.G.Jochemsen@lumc.nl

Fig. 1. Mdmx distinctly affects ubiquitylation of p53 and Mdm2. (A–C) H1299, *p53/mdm2* double knockout and Saos-2 cells were transfected with 500 ng of wild-type p53, 2 μ g mouse *mdm2*, 5 μ g human *mdmx* (as indicated) and 2 μ g His₆-tagged ubiquitin expression vectors. (D) H1299 cells were transfected with 2 μ g *mdm2* or *hdm2* and 5 μ g human *mdmx* expression vectors. Cells were untreated (–) or treated (+) with 20 μ M MG132 for 4 h, starting 40 h after transfection and subsequently harvested. Western blot analysis was performed on ubiquitylated proteins (His purified) or total cell lysates (total lysate). p53 was detected with DO-1, mouse Mdm2 with 4B2 and human Mdm2 with SMP14.

2001; see Methods). The level of ubiquitylated p53 and Mdm2 was analysed by western blotting. p53-negative H1299 cells were transfected with expression vectors for p53 alone, or in combination with expression vectors for Mdm2 and/or Mdmx. Co-expression of Mdm2 resulted in a decrease in p53 levels (Figure 1A, total lysate) and a clear increase in the levels of ubiquitylated p53 (Figure 1A, His purified). The band running at ~60 kDa is a background band consistently detected in this assay, also in the absence of transfected p53. Mdm2-dependent accumulation of ubiquitylated p53 was enhanced by addition of the proteasome inhibitor MG132 4 h prior to harvesting the cells (Figure 1A, His purified). As observed previously, Mdmx inhibited the Mdm2-dependent degradation of p53 (Figure 1A, total lysate). Surprisingly, this stabilization was accompanied by an accumulation of ubiquitylated p53. While Mdmx caused a general increase in the level of ubiquitylated p53 it did not detectably change the overall pattern of Mdm2-dependent ubiquitylation of p53 (Figure 1A, His purified, lanes 5, 6 compared to lanes 3, 4), indicating that Mdmx does not block the formation of a normally short-lived species of ubiquitylated p53. In cells co-transfected with Mdm2 and Mdmx, MG132 had no further effect on the level of ubiquitylated p53, indicating that Mdmx blocks proteasome-mediated degradation of ubiquitylated p53. Co-expression of Mdmx with p53, in the absence of exogenous Mdm2, resulted in some increase in p53 expression and in the level of ubiquitylated p53, although the amount of

ubiquitylated p53 was considerably less than that observed in the presence of co-transfected Mdm2 (Figure 1A, total lysate and His purified, lanes 7 and 8). In order to investigate whether this latter effect of Mdmx on p53 ubiquitylation was through regulation of endogenous Mdm2 or whether Mdmx itself possesses ubiquitin ligase activity, an *in vivo* ubiquitylation assay was performed in *p53/mdm2* double knockout (DKO) cells. In these cells, lacking endogenous Mdm2, no ubiquitylated p53 could be detected after transfection of an expression vector of p53 alone (Figure 1B, His purified, lanes 1 and 2). Co-expression of Mdm2 resulted in ubiquitylation of p53 that was detectable after inhibition of the proteasome (Figure 1B, His purified, lane 4). In the absence of endogenous Mdm2, Mdmx did not result in the accumulation of ubiquitylated p53 (Figure 1B, His purified, lanes 7, 8), indicating that Mdmx by itself does not have ubiquitin ligase activity towards p53. As in H1299 cells, Mdmx prevents the degradation of p53 by Mdm2, (Figure 1B, total lysate, lanes 5, 6 compared with lanes 3, 4). However, in contrast to H1299 cells, in these cells this effect was accompanied by some reduction in the amount of slow migrating ubiquitylated forms of p53 relative to the faster migrating forms (Figure 1B, His purified, lanes 5, 6). To investigate which effect of Mdmx would be the more common, the same experiment was performed in Saos-2 cells (Figure 1C) and p53-deficient mouse embryo fibroblasts (not shown). In both cases, some relative reduction of the slower migrating ubiquitylated forms of p53

could be observed, although somewhat less pronounced compared to the DKO cells.

Together, the data in Figure 1A–C show that, depending on the cell line tested, Mdmx does not (H1299) or partly (Saos-2, DKO) prevent Mdm2-mediated (poly-)ubiquitylation of p53. In all cases, the amount of ubiquitylated p53 in the presence of both Mdm2 and Mdmx is higher than after transfection of only p53, indicating a stabilization of ubiquitylated p53. Mdmx does not have intrinsic ubiquitin ligase activity and Mdmx can modestly affect p53 ubiquitylation in the absence of co-transfected Mdm2 only when endogenous Mdm2 is expressed, which is probably stabilized by the transfected Mdmx.

The effect of Mdmx on ubiquitylation of Mdm2 was also investigated. H1299 cells were transfected with an Mdm2 expression vector alone or in combination with an Mdmx expression vector and an *in vivo* ubiquitylation assay was performed. Mdm2 is normally degraded very rapidly and poly-ubiquitylated Mdm2 was only clearly detectable in the presence of MG132 (Figure 1D, His purified, compare lanes 1 and 2). Poly-ubiquitylated Mdm2 does not appear as a distinct ladder pattern but rather as a high molecular smear, as has been observed by others (Fang *et al.*, 2000; Honda and Yasuda, 2000). Co-expression of Mdmx significantly reduced the amount of poly-ubiquitylated Mdm2 (Figure 1D, His purified, lane 3 and 4), despite the total levels of Mdm2 being increased (Figure 1D, total lysate, compare lanes 1, 2 with 3, 4). In contrast to the poly-ubiquitylated forms, the amount of the fastest migrating lower ubiquitylated forms of Mdm2 was increased. A similar effect was observed with Hdm2, although the pattern of ubiquitylated Mdm2 and Hdm2 is somewhat different (Figure 1D, lanes 5–8). In addition, the same effect was observed in Saos-2 cells (not shown). It has been shown that poly-ubiquitylated rather than mono- or di-ubiquitylated proteins are efficiently recognized and degraded by the proteasome. Thus, the inhibition of poly-ubiquitylation of Mdm2 would provide a mechanism by which Mdmx prevents the targeting of Mdm2 for proteasomal degradation.

Mdmx inhibits Mdm2-mediated nuclear export of p53

Since Mdmx could stabilize p53 without inhibition of ubiquitylation, we next sought to investigate the main mechanism by which Mdmx stabilized ubiquitylated p53. Two recent reports suggest that co-expression of Mdm2 with p53 in Saos-2 or U2OS cells results in enhanced nuclear export of p53, required for its efficient degradation (Boyd *et al.*, 2000; Geyer *et al.*, 2000). Since Mdmx is unable to shuttle from the nucleus to the cytoplasm (Jackson and Berberich, 2000), we tested in a similar experiment whether Mdmx could affect this relocalization of p53. Saos-2 cells were transfected with plasmids encoding p53, Mdm2 and Mdmx and examined by immunofluorescence. In each case at least 100 cells in three independent experiments were scored for p53 localization. In these experiments we primarily made use of a mutant p53 (V143A) to prevent induction of the endogenous *mdm2* gene. We have previously found that this mutant can be efficiently degraded and ubiquitylated by Mdm2 and stabilized by Mdmx (Stad *et al.*, 2000 and data not shown). Figure 2A shows examples of p53 localization. When mutant p53 was transfected alone into Saos-2 cells, 49% of the p53 positive cells showed exclusively nuclear and 50% strong

Fig. 2. Mdmx expression leads to nuclear accumulation of p53. Saos-2 cells were transfected with 1 μ g p53, 4 μ g human mdm2 and 6 μ g human mdmx as indicated. Cells were fixed 24 h after transfection. In each experiment, more than 100 cells were counted for their p53 localization and categorized as only nuclear (N; (A), panel 1), strong nuclear and weak cytoplasmic [N>C; (A), panel 3], equal [N=C; (A), panel 5] and weak or no nuclear and strong cytoplasmic [N<C; (A), panel 6] staining. (A) Panels 2, 4 and 6 show DAPI stain of the same fields. (B) A graphic representation of the results. Error bars indicate the variation in three independent experiments. p53 was detected with FL393, Mdm2 with 4B2 and Mdmx with 6B1A.

nuclear and weak cytoplasmic staining (Figure 2B, left). Co-expression of Mdm2 resulted in a clear increase in cytoplasmic p53 in the Mdm2-positive cells. Additional co-expression of Mdmx reversed this shift significantly, suggesting that Mdmx inhibited the Mdm2-mediated nuclear export of p53. With the use of a wild-type p53 expression vector, the effect of Mdm2 on p53 localization is only very limited in our hands, but still in this case co-expression of Mdmx leads to an increase in nuclear localization of p53 (Figure 2B, right). Similar results were obtained in U2OS cells (data not shown).

Mdmx does not prevent Mdm2-mediated degradation of a cytoplasmic mutant of p53

To further investigate the importance of subcellular localization on the activity of Mdmx we determined the effect of Mdmx on p53 and Mdm2 proteins containing a mutation in their respective nuclear localization signal (NLS⁻), which makes them mainly cytoplasmic. Cytoplasmic p53 is efficiently ubiquitylated and degraded by cytoplasmic Mdm2. If Mdmx stabilizes p53 primarily by inhibiting its nuclear export, then it could be predicted that Mdmx would not prevent the Mdm2-dependent degradation of cytoplasmic p53. On the other hand, if the partial inhibition of p53 ubiquitylation as observed in Saos-2 cells would be

sufficient for stabilization, Mdmx should also prevent the degradation of cytoplasmic p53, provided that the same inhibition of p53 ubiquitylation occurs in the cytoplasm. Therefore, transfections were performed both in H1299 and Saos-2 cells. In Figure 3A examples of transfected H1299 cells are shown. Expression of the p53(NLS⁻) mutant was mainly confined to the cytoplasm (Figure 3A, panel 1) and co-expression of Mdm2(NLS⁻) resulted in the degradation of p53(NLS⁻) (Figure 3A, panels 3, 4, and Figure 3B). However, Mdmx did not stabilize cytoplasmic p53(NLS⁻), neither in H1299 nor in Saos-2 cells (Figure 3A, panel 7, and Figure 3B), although in a parallel control transfection it prevented the degradation of wild-type nuclear p53 by wild-type Mdm2 (Figure 3A, panel 18 and Figure 3B).

The effects of Mdmx on the ubiquitylation of cytoplasmic p53 by cytoplasmic Mdm2 was determined in parallel with wild-type p53 and Mdm2. In Saos-2 cells (Figure 3C, left panel), Mdmx again marginally reduced the poly-ubiquitylation of wild-type p53. Ubiquitylation of p53(NLS⁻) is hardly detected in the absence of proteasome inhibition, most likely because the rate of degradation of ubiquitylated cytoplasmic p53 is very high. In the absence of MG132, Mdmx expression does not lead to a significant increase in the amount of ubiquitylated cytoplasmic p53, reflecting the fact that Mdmx is not stabilizing p53(NLS⁻). When degradation is prevented by MG132, the expression of Mdmx does lead to some reduction of ubiquitylation of cytoplasmic p53, comparable or even stronger to that observed with wild-type p53. These results strongly indicate that the partial inhibition of Mdm2-mediated p53 ubiquitylation by Mdmx in Saos-2 is not sufficient to prevent the degradation of p53, but that stabilization of p53 by Mdmx is dependent on a nuclear localization. Also in H1299 cells (Figure 3C, right panel), Mdmx expression does not result in a significant accumulation of ubiquitylated cytoplasmic p53 in the absence of proteasome inhibition. Upon inhibition of the proteasome, Mdmx expression does not significantly alter the ubiquitylation pattern of p53(NLS⁻). These results support the observation that Mdmx can not stabilize cytoplasmic p53 (see also Figure 3C, total lysate) and does not affect ubiquitylation of p53 by Mdm2 in H1299 cells.

In marked contrast to p53, cytoplasmic Mdm2 was stabilized by Mdmx (Figure 3A, panel 6 and Figure 3B) to the same extent as wild-type nuclear Mdm2 (Figure 3A, panel 17 and Figure 3B). The effects of Mdmx described here are not the result of Mdmx being expressed in another cellular compartment compared to the p53(NLS⁻) and Mdm2(NLS⁻), or because Mdmx is not able to bind Mdm2(NLS⁻), since Mdmx localization was largely dependent on the localization of Mdm2 (Figure 3A panels 9, 10 and 20, 21) and co-immunoprecipitation studies indicate that Mdmx binds to both wild-type Mdm2 and the Mdm2(NLS⁻) mutant (Figure 3D). These data are consistent with the previous results showing that Mdmx prevents Mdm2-dependent degradation of p53 by stabilization of nuclear, ubiquitylated p53, whereas Mdmx stabilizes Mdm2 independent of its localization, most likely by repressing its auto-ubiquitylation.

DISCUSSION

In this report we show that Mdmx can block Mdm2-dependent degradation of p53 without significantly affecting the pattern of p53 ubiquitylation. Although some inhibition of p53 ubiquitylation

is observed in some cell lines, this effect appears neither necessary nor sufficient for the stabilization by Mdmx. For Mdm2 to ubiquitylate p53 its RING finger is essential (Honda *et al.*, 1997). Since the RING finger of Mdmx is needed for stabilization of p53, and Mdmx and Mdm2 interact via their RING finger domains (Sharp *et al.*, 1999; Stad *et al.*, 2000), this interaction appears not to block Mdm2's ubiquitin ligase activity towards p53. We provide evidence that the main mechanism by which Mdmx stabilizes p53 is through sequestration of ubiquitylated p53 in the nucleus, consistent with the model in which nuclear export of ubiquitylated p53 is essential for its degradation (Roth *et al.*, 1998; Tao and Levine, 1999; Boyd *et al.*, 2000; Geyer *et al.*, 2000). However, the mechanism by which Mdmx prevents the relocalization of p53 into the cytoplasm remains unclear. p53, Mdm2 and Mdmx can form a trimeric complex (Stad *et al.*, 2000). The presence of Mdmx, lacking a nuclear export signal, in this complex may interfere with the nuclear export of p53. Our results indicate that ubiquitylation of p53 is not sufficient for its nuclear export. This conclusion is supported by a recent observation that a Mdm2 mutant, in which a constitutive phosphorylation at the major ATM-phosphorylation site, ser395, was mimicked by replacing the serine for a glutamic acid (S395D), is impaired in degradation of p53 (Maya *et al.*, 2001). This effect was explained by the observation that the Mdm2 S395D mutant was strongly inhibited in its ability to stimulate nuclear export of p53, while preliminary data suggested that the ubiquitin ligase activity was not decreased. Thus, a domain just N-terminal of the RING finger in Mdm2 might be involved in p53 nuclear export. Possibly, Mdmx interferes with the interaction of Mdm2 with other protein(s), which is essential for the stimulation of p53 nuclear export. In contrast to the way Mdmx stabilizes p53, Mdm2 appears to be stabilized due to the inhibition of auto-ubiquitylation. It may be that poly-ubiquitylation of Mdm2 is required for the export of a Mdm2-p53 complex and, therefore, inhibition of Mdm2 poly-ubiquitylation by Mdmx results in stabilization of both p53 and Mdm2. It is intriguing that Mdmx differentially affects the ubiquitylation of Mdm2 and p53. An interesting model that could explain how Mdmx is able to inhibit Mdm2 auto-ubiquitylation without affecting Mdm2-mediated p53 ubiquitylation is that for efficient Mdm2 auto-ubiquitylation, homo-dimerization and ubiquitylation *in trans* is necessary. Mdmx-Mdm2 heterodimers are preferentially formed over their respective homodimers (Sharp *et al.*, 1999), so expression of Mdmx would prevent Mdm2 homo-dimerization and subsequent auto-ubiquitylation. We have previously observed that *in vivo* p14ARF also affects Mdm2-mediated ubiquitylation of p53 and itself differently. While p14ARF also stabilizes Mdm2 and blocks Mdm2-mediated degradation of p53, its effects on p53 and Mdm2 ubiquitylation are the opposite of Mdmx. p14ARF inhibits the poly-ubiquitylation of p53 while the ubiquitylation of Mdm2 is unaffected (Xirodimas *et al.*, 2001). Further evidence that ubiquitylation of p53 and Mdm2 is not necessarily coupled was provided by experiments in which the RING finger domain of Mdm2 was exchanged for the RING finger domain of the Praja 1 protein. This switch did not affect Mdm2 ubiquitylation but prevented the ubiquitylation of p53 (Fang *et al.*, 2000). In conclusion, these observations suggest that the targeting of ubiquitin ligase activity of Mdm2 towards p53 or itself is strongly dependent on the cellular environment, i.e. expression levels of ARF and Mdmx. The molecular mechanism of this and its

Fig. 3. Mdmx does not stabilize cytoplasmic p53. (A and B) H1299 and Saos-2 cells were transfected with 100 ng p53, 2 μ g mouse mdm2, either wild-type or NLS mutants, 5 μ g human mdmx and 2 μ g LacZ expression vectors. Cells were fixed or harvested 44 h after transfection. (A) Immunofluorescence analysis of the transfected H1299 cells. p53 was stained with FL393, Mdm2 with 4B2 and Mdmx with p56. (B) Western blot analysis of simultaneously transfected H1299 and Saos-2 cells. p53 was detected with DO-1, Mdm2 with 4B2, Mdmx with 6B1A and LacZ with D19-2F3-2. (C) Comparison of the effect of Mdmx on Mdm2-mediated ubiquitylation of nuclear and cytoplasmic p53. Transfections of H1299 and Saos-2 cells with the indicated plasmids and Western blot analysis of ubiquitylated p53 (His purified) and total p53 (total lysate) were performed as described for Figure 1. (D) Mdmx interacts both with wild-type and NLS-mutant of Mdm2. H1299 cells were transfected with 4 μ g of Mdm2 and Mdmx expression vectors as indicated. Lysis and immunoprecipitations were performed as described by Stad *et al.* (2000). Eighty percent of the lysate was immunoprecipitated with anti-Mdmx serum p56, and 10% of the lysate was directly loaded on gel. Mdm2 expression was detected with 4B2 antibody.

R. Stad *et al.*

implications for therapeutic intervention are the subject of ongoing investigation.

METHODS

Plasmids. Expression vectors for wild-type and mutant (V143A) p53, mouse Mdm2 and human Mdmx were as described (Stad *et al.*, 2000). The Mdm2(NLS⁻) mutant was constructed by using the QuickChange kit for site-directed mutagenesis (Stratagene) with two complementary oligonucleotides, 5'-GCTACCTGGGGAGCGGCACACGACGCTCCGCAGGTC-CCTGTCCTTTG-3', 5'-CAAAGGACAGGGACCTGCGGAGCGTC-GTGTGCCGCTCCCCAGGTAGC-3' and as template the pCOC-X2 plasmid (which encodes for wild-type Mdm2). This resulted in mutating amino acids R178, K179 and R180 (within the putative nuclear localization signal) into threonine, threonine and leucine, respectively. The same approach was used to make the p53(NLS⁻) mutant with the following primers: 5'-CTCTC-CCCAGCCAAAGACGAAACCACTGGA and 5'-CTCCAT-CCAGTGGTTTCGCTCTTTGGCTGGG. This resulted in mutating amino acid 320 (within the major nuclear localization signal of human p53) from lysine to threonine. The His₆-tagged ubiquitin expressing construct was a kind gift from Dr S. Mitnacht.

Cell lines, cell culture and transfection. H1299 cells were cultured in RPMI, 8% fetal bovine serum (FBS). Approximately 3 h prior to transfection, the medium was changed to DMEM, 10% FBS. Saos-2 and U2OS cells were cultured in DMEM, 10% FBS. Cells were transfected by the calcium phosphate coprecipitation method as described previously (Stad *et al.*, 2000). p53/mdm2 double knock out (DKO) cells were cultured in F15, 10% FBS and transfected with Eugene6 transfection reagent (Roche).

In vivo ubiquitylation assay. Purification of His-tagged ubiquitylated conjugates was performed essentially as described previously (Xirodimas *et al.*, 2001). Forty-four hours post transfection cells were washed 2× with PBS and scraped in PBS. 20% of cell suspension was lysed in IPB 0.7 (20mM TEA, pH 7.4, 0.7 M NaCl, 0.5% NP-40, 0.2% sodium deoxycholic acid) and analysed by western blot as described (Stad *et al.*, 2000). Lysis of the remaining 80% of the cells and the subsequent isolation of ubiquitylated proteins was performed exactly as described by Xirodimas *et al.* (2001). Eluates were analysed by western blot.

Immunofluorescence and antibodies. To perform immunofluorescence analyses, the cells were seeded onto coverslips before transfection. Cells were fixed for 15 min in 4% paraformaldehyde and subsequently permeabilized in 100 % methanol, 5 min, -20 °C. Detection of the expressed proteins was performed as described (Stad *et al.*, 2000). p53 was detected with rabbit serum FL393 or mouse monoclonal DO-1 (Santa Cruz Biotechnology), Mdm2 with mouse monoclonals 4B2 or SMP14, Mdmx with rabbit serum p56 or mouse monoclonal

6B1A, and LacZ with mouse monoclonal D19-2F3-2 (Roche Molecular Diagnostics). Nuclei were visualized by DAPI staining.

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