Proteasome activator PA28 γ regulates p53 by enhancing its MDM2-mediated degradation

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Downregulation of p53 by MDM2-mediated proteasomal degradation makes cells resistant to apoptosis. The MDM2–p53 interaction is well characterized, but the mechanisms that regulate the interaction are not well understood. Here, we show that PA28 γ , a proteasome activator that inhibits apoptosis and promotes cell cycle progression through unknown mechanisms, exerts an effect as a cofactor in the MDM2–p53 interaction. The polymer form of PA28 γ interacts with both MDM2 and p53 proteins and facilitates their physical interaction. This promotes ubiquitination- and MDM2-dependent proteasomal degradation of p53, limiting its accumulation and resulting in inhibited apoptosis after DNA damage. Elimination of endogenous PA28 γ in human cancer cells abrogates MDM2-mediated p53 degradation, increases the activity of p53, and enhances apoptosis. These findings reveal the mechanism by which PA28 γ affects apoptosis and proliferation. Manipulation of the level of PA28 γ , an approach that would regulate the cellular content of p53, may improve the efficacy of current cancer therapies.

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Introduction

Tumor suppressor p53 is involved in controlling cell growth and differentiation and in the maintenance of genomic integrity, with high levels of p53 activity inducing apoptosis, cell cycle arrest, or senescence (Lane, 1992). The regulation of p53 protein expression and activity in cells is of particular interest in the field of cancer research, both for the development of more effective therapeutic strategies and for ongoing efforts to gain a better understanding of tumorigenesis. The level of p53 protein is elevated upon exposure of cells to stress, such as DNA damage, hypoxia, nutrient deprivation, or oncogene activation (Levine, 1997). In unstressed cells, the level of p53 (hence its activity) is largely controlled

by MDM2, an oncoprotein containing an ubiquitin E3 ligase (Haupt *et al*, 1997; Brooks and Gu, 2006). MDM2 promotes the polyubiquitination of p53, which is an essential step in its degradation (Haupt *et al*, 1997; Brooks and Gu, 2006).

Although it is known that the MDM2–p53 interaction is controlled by an autoregulatory loop (Haupt *et al*, 1997; Brooks and Gu, 2003, 2006; Zhang *et al*, 2005) and that the MDM2-mediated degradation of p53 is dependent upon the cellular level of MDM2 (Li *et al*, 2003), the mechanisms regulating this interaction appear to be complex and are poorly understood. It was suggested that a cofactor may be required for MDM2-mediated p53 degradation because low levels of MDM2 fail to catalyse polyubiquitination of p53 (Li *et al*, 2003).

PA28 γ (REG γ , PSME3, Ki antigen) belongs to a family of activators of the 20S proteasome (Rechsteiner and Hill, 2005). Unlike its family members, PA28 α and PA28 β , PA28 γ is localized within the nucleus and forms a homoheptamer (rather than a heterocomplex). PA28 γ has been implicated in the regulation of cell cycle progression and apoptosis, and embryonic fibroblasts of PA28 γ nullizygous mice demonstrate spontaneous apoptosis and G1 arrest (Rechsteiner and Hill, 2005). These mice also have a reduced adult body mass and show growth retardation (Rechsteiner and Hill, 2005). The mechanism by which PA28 γ exerts these effects has not been elucidated. These findings prompted us to explore the possibility that PA28 γ may regulate p53. Here, we report that PA28 γ has an integral function in the degradation of p53, and that it does so by acting as an essential cofactor that promotes the binding of MDM2 and p53.

Results

PA28 γ negatively regulates p53

Overexpression of PA28 γ , but not PA28 β , reduced the level of p53 protein and its transactivational activity (measured by p21^{Waf1} protein) in LNCaP, MCF-7, and U2OS cells (p53 wild type), but not in PC3 cells (p53 null) (Figure 1A). Knockdown of endogenous PA28 γ using a small interfering RNA (siRNA) pool (Dharmacon) increased the levels of endogenous p53 and p21^{Waf1} in LNCaP, HCT116, A549, MCF-7, and MCF10A (a normal breast epithelial line) cells, but not PC3 cells (Figures 1B–D and Supplementary Figure S1). Although PA28 γ levels were decreased in all eight cell lines treated with PA28 γ siRNA (Figures 1B and C and Supplementary Figures S1 and S2); levels of the homologue PA28 β showed little or no change, indicating that the off-target effects of the siRNA pool were minimal (Figure 1B and Supplementary Figure S1).

Confirming that induction of p21^{Waf1} after PA28 γ knock-down is p53 dependent, when MCF10A cells were transfected

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with an siRNA pool targeting PA28 γ , p53, or both, the induction of p21^{Waf1} was largely inhibited when p53 was knocked down (Figure 1C). Furthermore, an increase in p21^{Waf1} mRNA after PA28 γ knockdown by siRNA was observed in HCT116 cells, but not in p53^{-/-} HCT116 cells (Figure 1D). At the post-translational level, the extent of the induction of p21^{Waf1} in p53^{-/-} HCT116 cells was much less than in HCT116 cells (Figure 1D). These results suggest that the induction of p21^{Waf1} after PA28 γ knockdown occurs largely through p53. However, modulation of p21^{Waf1} directly by PA28 γ at the post-translational level is also possible.

Two individual PA28 γ siRNA duplexes and one control siRNA duplex were randomly selected from the previous siRNA pools to confirm that PA28 γ negatively regulates p53. Each of the PA28 γ siRNA duplexes was capable of down-regulating endogenous PA28 γ , resulting in dose-dependent accumulation of p53 in HCT116 cells (Figure 1E, left panel). In contrast, randomly selected PA28 β siRNA duplexes from the PA28 β siRNA pool (Dharmacon) did not affect the level of p53, although endogenous PA28 β was knocked down (Figure 1E, right panel). Additionally, p53 was elevated in PA28 γ ^{-/-} mouse embryonic fibroblasts (MEFs) (with low passage numbers) compared with wild-type MEF (Figure 1F). This elevation in p53 was decreased by overexpressed PA28 γ (Figure 1G).

Several p53 targets (including p21^{Waf1}, Puma, and 14-3-3 σ), and p53 itself, were downregulated by ectopic PA28 γ in wild-type MEF cells (Figure 1H). In HCT116 cells, knockdown of PA28 γ led to induction of p53 at the protein level and increases in p21^{Waf1}, Puma, and 14-3-3 σ at both the mRNA and protein levels (Figure 1I). Similar results were obtained in MCF-7 cells (Supplementary Figure S3). Overexpression of PA28 γ , but not PA28 β , in MCF-7 cells co-transfected with a luciferase reporter under the control of the p21^{Waf1} promoter, inhibited p53-dependent transcriptional activity (Figure 1J, left panel). Co-transfection with p53 reversed the PA28 γ -mediated inhibition of p53-dependent transcriptional activity (Figure 1J, right panel).

PA28 γ promotes ubiquitination-dependent proteasomal degradation of p53

We hypothesized that PA28 γ downregulates p53 by promoting its degradation. Confirming this hypothesis, overexpression of PA28 γ increased degradation of endogenous p53 protein in LNCaP cells (Figure 2A), and increased the amount of proteasome-bound p53 in PA28 γ -overexpressing U2OS cells (Figure 2B). In MCF-7 cells, overexpression of PA28 γ enhanced the ubiquitination of p53 (Figure 2C).

The stability of p53 in ts20b cells, which express a temperature-sensitive E1 ubiquitin-activating enzyme, was examined to determine whether the promotion of p53 degradation by PA28 γ is ubiquitination dependent. PA28 γ lost its capacity to promote p53 degradation when the ts20b cells were cultured at 39°C. However, its promotion of ubiquitin-independent proteasomal degradation of p21^{Waf1} was retained (Figure 2D).

To exclude the possibility that PA28 γ may have a post-ubiquitination effect in promoting degradation of ubiquitinated p53 through the proteasome, ubiquitinated p53 was incubated with either 20S or 26S proteasome in the presence

of PA28 γ . The 20S proteasome alone did not degrade ubiquitinated p53, and PA28 γ did not activate the 20S proteasome to degrade ubiquitinated p53 (Figure 2E, upper panel). Although the 26S proteasome degraded ubiquitinated p53, PA28 γ did not enhance this activity (Figure 2E, lower panel). In contrast, PA28 γ enhanced the degradation of p21^{Waf1} by the 20S proteasome (Figure 2F).

PA28 γ is a cofactor for MDM2-mediated p53 degradation

The PA28 γ protein does not have a motif characteristic of an ubiquitin E3 ligase, suggesting that PA28 γ may promote the ubiquitinating activity of another molecule. To test this hypothesis, we examined whether PA28 γ promotes MDM2-mediated p53 ubiquitination and degradation. Co-transfection of low amounts of MDM2 and PA28 γ into MCF-7 cells enhanced the downregulation of p53 (Figure 3A). Although low amounts of PA28 γ or MDM2 transfected alone into LNCaP cells (Figure 3B) or A549 cells (Figure 3C) minimally induced p53 ubiquitination, co-transfection with both PA28 γ and MDM2 enhanced its ubiquitination (Figures 3B and C). The possibility that the enhanced ubiquitination was a result of changes in the pool of cellular-free ubiquitin, E1 or E2 was excluded using purified recombinant proteins in *in vitro* ubiquitination assays. Incubation of a lower amount of MDM2 protein with purified p53 protein, along with purified E1, E2 and ubiquitin *in vitro*, resulted in minimal ubiquitination of the p53 protein (Figure 3D). When recombinant PA28 γ was included in the reaction, the ubiquitination of p53 was increased (Figure 3D).

Exposure to a high concentration of Nutlin-3 (10 μ M), a known inhibitor of the MDM2–p53 interaction, 12 hours after transfection of MCF-7 cells with PA28 γ reversed the effects of PA28 γ on p53 (Figure 3E). Exposure of LNCaP cells to an anti-MDM2 antisense oligonucleotide (AS) (Zhang *et al.*, 2005), but not its mismatch control (ASM), prevented the induction of p53 degradation by PA28 γ (Figure 3F). Co-transfection of U2OS cells with MDM2 ASM or AS and various amounts of PA28 γ further indicated that the PA28 γ -mediated reduction in the p53 level is dependent on the presence of MDM2 (Figure 3G). When PA28 γ was knocked down in A549 cells by siRNA prior to transfection with MDM2, the down-regulatory effect of MDM2 on p53 was not observed (Figure 3H).

The kinetics of the changes in expression and transactivation activity of p53 after γ -irradiation were compared in A549 cells overexpressing either MDM2 or PA28 γ (Figure 3I). Both MDM2 and PA28 γ limited the accumulation of p53 and the elevation of p53 activity (manifested by the level of p21^{Waf1}) with similar kinetics after DNA damage (Figure 3I), indicating that they work via the same pathway. When PA28 γ was pre-knocked down by siRNA, neither a low concentration of Nutlin-3 (3 μ M) (Figure 3J) nor overexpressed p14^{Arf} (Figure 3K) led to modulation of the level of p53. This is likely due to the fact that the MDM2–p53 interaction (the target of both Nutlin-3 and p14^{Arf}) is disrupted by the absence of PA28 γ . Another cofactor for the MDM2–p53 interaction, Daxx, also lost its capacity to downregulate p53 in the absence of PA28 γ (Figure 3L). In contrast, YY1 retained its capacity for downregulating p53 (Figure 3M), indicating that the mechanism by which YY1 enhances the MDM2–p53 interaction is different from that of PA28 γ .

PA28 γ binds to MDM2 and p53, and promotes the MDM2-p53 interaction

The stability of MDM2 did not change in PC3 cells transfected with either PA28 γ or the control vector (Supplementary Figure S4), suggesting that it is unlikely that PA28 γ modulates the level of p53 by inhibiting the capacity of MDM2 to catalyse self-ubiquitination. As PC3 cells are p53 null,

changes in MDM2 are not a result of alterations in p53 induced by PA28 γ .

Direct physical interactions between endogenous PA28 γ and p53 proteins, as well as between endogenous PA28 γ and MDM2 proteins, were observed in MCF-7 cells (Figure 4A). Confirmation of these direct interactions was obtained by use of pull-down assays (Figure 4B), and immunoprecipitation

with overexpressed PA28 γ -GFP and p53-HA (haemagglutinin) (Figure 4C, upper panel) and PA28 γ -GFP and MDM2-T7 (Figure 4C, lower panel) in COS7 cells.

To determine if PA28 γ promotes the MDM2–p53 interaction, lysates from U2OS cells overexpressing different amounts of PA28 γ were immunoprecipitated with p53 antibody, and it was observed that the MDM2 level was higher in the p53 immunoprecipitates from cells that were transfected with PA28 γ (Figure 4D, left panel). Moreover, the amount of MDM2 in p53 immunoprecipitates from A549 cells treated with the PA28 γ siRNA pool was lower than that from cells treated with the control siRNA pool (Figure 4D, right panel). The amount of p53 bound to GST-MDM2 was also enhanced by the addition of PA28 γ in an *in vitro* pull-down assay (Figure 4E). Finally, the amount of MDM2 bound by p53 in PA28 $\gamma^{-/-}$ MEF cells was higher than in wild-type MEF cells (Figure 4F).

To determine which form of PA28 γ (monomer/polymer) mediates the MDM2–p53 interaction, gel filtration chromatography was performed. The elution profile of the lysates from COS7 cells transfected with GFP-PA28 γ , p53-HA, and MDM2-T7 showed a peak that was absent in the profile from the cells transfected with GFP, p53-HA, and MDM2-T7 (Figure 4G, left panel). The molecular weight of this peak was estimated to be 560 kDa, corresponding to a complex of heptameric GFP-PA28 γ –MDM2–p53 (the molecular weight of GFP-PA28 γ is 60 kDa) (Figure 4G, left panel). The eluted fractions were resolved on 10% SDS–PAGE and immunoblotted using T7, HA, and GFP antibodies (Figure 4G, right panel).

In GFP-PA28 γ -transfected cells, GFP-PA28 γ , MDM2-T7, and p53-HA appeared simultaneously in the fractions with molecular weights from about 440 kDa to more than 669 kDa (Figure 4G, right panel). Moreover, GFP-PA28 γ barely appeared in the fractions with molecular weights less than 232 kDa, suggesting that GFP-PA28 γ primarily exists in the

polymer form in cells. In GFP-transfected cells, p53-HA and MDM2-T7 appeared simultaneously only in the fractions with lower molecular weight, about 400–440 kDa (Figure 4G, right panel). To assess the capacity of GFP-PA28 γ to form polymers, an *in vitro* approach employing glutaraldehyde (to covalently crosslink any polymers that form) was performed (Supplementary Figure S5). GFP-PA28 γ and three mutants, P245Y, G150S, and N151Y (incapable of activating the proteasome), were all capable of forming polymers (Supplementary Figure S5).

PA28 γ specifically downregulates p53

To confirm that PA28 γ does not regulate p53 directly through its proteasome-binding or -activating functions, we tested the activity of three mutants of PA28 γ ; P245Y (incapable of binding the 20S proteasome; Zhang *et al.*, 1998b), and G150S and N151Y (unable to activate, but still able to bind the proteasome; Zhang *et al.*, 1998b). Similar half-lives for endogenous p53 protein were found in cells that were transfected with PA28 γ and cells transfected with any of the three mutants (Figure 5A). Notably, the homologue-specific ‘insert region’ of PA28 γ (amino acids 71–103) that differentiates it from other PA28 family members (Zhang *et al.*, 1998b) was demonstrated to be essential for the promotion of poly-ubiquitination and degradation of p53 (Figures 5A–C).

We have further defined the regions that are essential for the binding of PA28 γ to MDM2 and p53, both of which are within the insert region. A PA28 γ mutant without amino acids 76–103 did not bind to MDM2, but PA28 γ 66–161, PA28 γ 86–255, and PA28 γ 96–255 all bound MDM2 (Figures 5D, F and Supplementary Figure S6A), indicating that amino acids 96–103 are essential for PA28 γ binding to MDM2 (Figures 5D, F and Supplementary Figure S6A). We also determined that amino acids 86–96 are required for binding p53 (Figures 5E, F and Supplementary Figure S6B).

Figure 1 PA28 γ regulates the expression and transactivational activity of p53. (A) p53 is downregulated by overexpressed PA28 γ , but not by PA28 β . LNCaP, MCF-7, U2OS, and PC3 cells were transfected to overexpress PA28 γ (0, 1.5, 3 and 5 μ g), or PA28 β (5 μ g), or their corresponding control vectors. After 24 h, whole cell lysates were collected for detection of target proteins by immunoblotting. The p53 protein level is elevated after PA28 γ knockdown in human cancer cells (B) and normal cells (C). (B) LNCaP, HCT116, and PC3 cells were transfected with a control or a PA28 γ siRNA pool (1.25 nM for each duplex) and incubated for 24 h, target proteins were detected by immunoblotting. (C) Human normal breast epithelial MCF10A cells were incubated with a siRNA pool targeting PA28 γ or p53, or a control siRNA pool (1.25 nM for each duplex) for 24 h. Target proteins were detected by immunoblotting. The numbers under the p21^{Waf1} band indicate expression as a percentage of the control siRNA, normalized to the corresponding β -actin level. (D) HCT116 or HCT116 p53^{-/-} cells were treated with siRNA pools (1.25 nM for each duplex). Cells were harvested 24 h later for extraction of either protein or total RNA. The levels of target proteins including p53 and p21^{Waf1} were examined by immunoblotting. The numbers under the p21^{Waf1} band indicate expression as a percentage of the control siRNA, normalized to the corresponding β -actin level. The mRNA level of p21^{Waf1} was analysed by RT–PCR in which p21^{Waf1} mRNA was co-amplified with β -actin mRNA in the same reaction. The number under each band is expressed as a percentage of control siRNA, normalized by the corresponding co-amplified β -actin mRNA level. (E) siRNA duplexes targeting PA28 γ , but not those targeting PA28 β , elevate the cellular level of p53. HCT116 cells were treated with two siRNA duplexes targeting PA28 γ , or PA28 β , or with a control siRNA duplex, at various concentrations for 48 h before whole cell lysates were collected for detection of target proteins. (F, G) Knockdown of PA28 γ in transgenic mice results in an increase in p53. (F) Target proteins in low passage mouse embryonic fibroblasts (MEFs) isolated from wild-type or PA28 γ knockdown mice were examined by immunoblotting. (G) PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ MEFs were transfected with a plasmid expressing PA28 γ (3 μ g), 24 h later, whole cell lysates were collected for detection of target proteins. (H–J) PA28 γ negatively regulates p53 transactivational activity. (H) PA28 $\gamma^{+/+}$ MEFs were transfected with a plasmid expressing PA28 γ (3 μ g), 24 h later, cell lysates were collected for detection of target proteins. (I) HCT116 cells were treated with control siRNA duplex, PA28 γ siRNA duplex, or transfection reagent (Dharmafectin), or left untreated (negative). Cells were harvested 24 h later for extraction of either protein or total RNA. The levels of target proteins including p53 and p21^{Waf1} were examined by immunoblotting. The mRNA expression of p21^{Waf1}, *Puma*, and 14-3-3 σ was analysed by RT–PCR in which p21^{Waf1} mRNA, *Puma* mRNA, or 14-3-3 σ mRNA was co-amplified with β -actin mRNA in the same reaction. The number under each band is expressed as a percentage of the negative control, normalized to the corresponding co-amplified β -actin mRNA level. (J) MCF-7 cells were co-transfected with various amounts of PA28 γ or PA28 β with p21^{Waf1} luciferase reporter (1.5 μ g) and *Renilla* luciferase reporter (0.3 μ g) (internal control; Promega). After 28 h, the luciferase activity of the p21^{Waf1} promoter reporter was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The p21^{Waf1} reporter activity was normalized to the corresponding *Renilla* luciferase reporter activity (left panel). In a separate experiment, MCF-7 cells were co-transfected with PA28 γ or PA28 β with p53, p21^{Waf1} luciferase reporter, and *Renilla* luciferase reporter. After 28 h, the luciferase assay was performed as above (right panel). The relative luciferase units (RLUs) represent the means $\pm$ s.d. of two independent samples.

Figure 2 PA28 γ promotes ubiquitination-dependent p53 proteasomal degradation. (A) PA28 γ destabilizes p53 protein in cells. LNCaP cells were transfected with PA28 γ (4 μ g) or corresponding empty vector for 24 h, followed by exposure to cycloheximide (CHX) (10 μ g/ml) for different times. Target proteins in whole cell lysates were detected by immunoblotting. The intensity of the p53 protein band was analysed by densitometry (Bio-Rad Model GS-670 Imaging Densitometer; Bio-Rad). The relative densitometry data at each time point are expressed as a percentage of the density at time 0, after normalization to the corresponding β -actin level. The average relative densitometry data of three independent experiments are shown in the right panel. * P < 0.05. (B) PA28 γ induces proteasome binding of p53. U2OS cells with PA28 γ (0 or 4 μ g) were evaluated for proteasome-bound p53 by immunoprecipitation with an antibody to the proteasome α/β subunits followed by detection of p53 by immunoblotting with p53 antibody DO-1. Proteasomal ATPase subunit Rpt1/S7 was included to verify equal protein loading. (C) p53 ubiquitination is enhanced in cells with PA28 γ overexpression. HA-Ub (3 μ g) and PA28 γ (3 μ g) were overexpressed in MCF-7 cells followed by exposure to MG132. Ubiquitinated p53 was detected by direct immunoblotting with the DO-1 antibody. (D) The promotion of p53 degradation by PA28 γ depends on the integrity of the ubiquitin-proteasome pathway. ts20b cells were incubated at 32 or 39°C for 12 h before being transfected with plasmids to overexpress PA28 γ (3.5 μ g) or control vector. The stability of p53 and p21^{Waf1} proteins was determined 24 h later as described in (A). (E) PA28 γ does not promote proteasomal degradation of ubiquitinated p53. Ubiquitinated p53 was incubated with 20S or 26S proteasome in the presence or absence of PA28 γ at 37°C for 5 h. The reaction was terminated by boiling with SDS sample buffer for 5 min. Ubiquitinated p53 was detected by immunoblotting. (F) PA28 γ promotes p21^{Waf1} degradation by the 20S proteasome. p21^{Waf1} was incubated with 20S proteasome in the presence or absence of PA28 γ at 37°C for 45 min. The reaction was terminated by boiling with SDS sample buffer for 5 min. p21^{Waf1} was detected by immunoblotting.

Figure 3 PA28 γ functions as a critical cofactor for MDM2-mediated p53 degradation. (A) MDM2 and PA28 γ synergistically decrease p53 protein. MCF-7 cells were transfected with low amounts of MDM2 (0, 1, or 1.5 μ g) in the presence or absence of PA28 γ (0 or 1.5 μ g) for 24 h. Endogenous p53 protein expression in whole cell lysates was examined by immunoblotting. (B, C) MDM2 and PA28 γ synergistically induce p53 ubiquitination in cells. (B) Different combinations of MDM2 (0 or 2 μ g), Ub-HA (0 or 2 μ g), and/or PA28 γ (0 or 2 μ g) were expressed in LNCaP cells. Ubiquitinated p53 was immunoprecipitated using p53 antibody DO-1, and the precipitates were immunoblotted using antibodies against HA and ubiquitin. *Ubiquitinated p53. (C) A549 cells were transfected with MDM2, Ub-HA, and PA28 γ as described in (B). 24 h later, cells were incubated with MG132 (15 μ M) for 4 h. Cell lysates were immunoprecipitated by p53 antibody FL393, and the precipitates were immunoblotted with an antibody against the polyubiquitin chain, FK1. (D) *In vitro* ubiquitination of p53 by MDM2 is enhanced by PA28 γ . His-p53 (6 ng) was incubated with His-MDM2 (3 ng) and GST-PA28 γ (30 ng) for 3 h in the presence of E1 (10 ng), E2 (40 ng), and ubiquitin (5 μ g). Ubiquitinated p53 was detected by immunoblotting with the p53 antibody, DO-1. (E) The downregulation of p53 by PA28 γ is reversed by Nutlin-3. MCF-7 cells were transfected with the PA28 γ plasmid (3.5 μ g) or control vector (3.5 μ g), and incubated for 24 h. At 12 h before the termination of the incubation, cells were exposed to 10 μ M Nutlin-3 or the solvent, DMSO. Target proteins in whole cell lysates were detected by immunoblotting. (F, G) PA28 γ loses the capacity to induce p53 degradation when MDM2 is knocked down by MDM2 antisense oligonucleotides. (F) LNCaP cells were co-transfected with PA28 γ (4 μ g) or control vector (4 μ g) with an MDM2 antisense oligonucleotide (AS) (150 nM) or a mismatch control oligonucleotide (ASM) (150 nM). After 24 h, p53 stability was determined as described above. (G) U2OS cells were co-transfected with MDM2 AS (150 nM) or ASM (150 nM) with various amounts of PA28 γ (0, 1.5, 3, or 5 μ g). The p53 protein level in cell lysates was determined by immunoblotting. (H) PA28 γ is required for MDM2-mediated p53 degradation. A549 cells were transiently transfected with a control siRNA pool (1.25 nM for each duplex) or a PA28 γ siRNA pool (1.25 nM for each duplex) to knockdown endogenous PA28 γ . Cells were transfected with a plasmid to overexpress MDM2 (3 μ g) or a control vector (3 μ g) 24 h later. After an additional 24 h, target proteins were detected by immunoblotting. (I) PA28 γ and MDM2 exhibit similar kinetics in limiting the accumulation of p53 induced by γ -irradiation. A549 cells were transfected with plasmids overexpressing MDM2 or PA28 γ (3.5 μ g for each). 24 h later, cells were exposed to 8 Gy γ -irradiation, and whole cell lysates were collected at different time points after irradiation for detection of target proteins. (J) The effect of Nutlin-3 on p53 requires PA28 γ . A549 cells pretreated with the PA28 γ siRNA pool (1.25 nM for each duplex for 12 h) were incubated with 3 μ M Nutlin-3 for another 12 h. Target proteins were examined by immunoblotting. (K) p14^{Arf} cannot induce accumulation of p53 when PA28 γ is knocked down. HCT116 cells pre-incubated with the PA28 γ siRNA pool or control siRNA pool (1.25 nM for each duplex) for 24 h were transfected with plasmids overexpressing p14^{Arf} (3 μ g) or a control vector (3 μ g). After another 24 h, target proteins in the whole cell lysates were examined by immunoblotting. (L) Daxx loses its capacity to downregulate p53 when PA28 γ is absent. MEF cells (PA28 γ ^{+/+} or PA28 γ ^{-/-}) were transfected with a plasmid to overexpress Daxx (3 μ g) or a control plasmid (3 μ g). Whole cell lysates were collected for the examination of target proteins 24 h later. (M) PA28 γ is not involved in the downregulation of p53 by YY1. HCT116 cells pretreated with the PA28 γ siRNA pool or control siRNA pool (1.25 nM for each duplex) for 24 h were transfected with a plasmid to overexpress YY1 (3 μ g) or a control plasmid (3 μ g). After another 24 h, whole cell lysates were collected for examination of target proteins.

Supporting the specificity of the effect of PA28 γ on p53, PA28 γ knockdown did not result in apparent changes in the levels of Numb or MDM4 (two other substrates of MDM2) in HCT116 cells, although upregulation of p53 was observed (Figure 5G).

PA28 γ regulates apoptosis through p53

To demonstrate that PA28 γ regulates apoptosis through modulation of p53, we compared survival and apoptosis of cells treated with a PA28 γ siRNA pool or a control siRNA pool. The tested cancer cell lines included: A549 (wt p53); a pair of MCF-7 cell lines (one with wt p53 (p53^{+/+}) and one nullizygous for p53 (p53^{-/-}) by stable knockdown of p53 by siRNA; Li *et al.*, 2005); a pair of HCT116 cell lines (one with wt p53 (p53^{+/+}) and one nullizygous for p53 (p53^{-/-}) by

deletion of p53 through homologous recombination); and PC3 cells (p53 null). Knockdown of PA28 γ resulted in a reduction in the survival of A549, MCF-7 (p53^{+/+}), and HCT116 (p53^{+/+}) cells (Figure 6A and Supplementary Figure S7) and in higher numbers of apoptotic cells (Figure 6B and Supplementary Figure S7). In contrast, there were no effects on the survival or apoptosis of MCF-7 p53^{-/-}, HCT116 p53^{-/-}, or PC3 (p53^{null}) cells (Figures 6A and B and Supplementary Figure S7).

Similarly, γ -irradiation-induced accumulation of p53 was reduced by overexpression of PA28 γ in MCF-7 cells (Figure 6C), and overexpressed PA28 γ stabilized the MDM2-p53 interaction in the presence of γ -irradiation (Figure 6D). The extent of apoptosis in MCF-7 cells with overexpressed PA28 γ was also lower than in control cells

following γ -irradiation (Figure 6E), and knockdown of p53 in HCT116 cells inhibited the radiosensitizing effect of PA28 γ siRNA on cells (Figure 6F).

MDM2 is considered a target for human cancer therapy (Bond *et al*, 2004; Zhang *et al*, 2005; Poyurovsky and Prives, 2006). Several experimental therapeutic molecules, including

a specific anti-MDM2 antisense oligonucleotide (AS), have been developed (Zhang *et al*, 2003, 2005). However, the response of p53 to inhibition of MDM2 by AS varies in different cell lines. These differences might reflect variations in the basal levels of endogenous PA28 γ . Analysis of the basal expression of PA28 γ in six cancer cell lines with wild-type p53

demonstrated that U2OS cells had the lowest level of PA28 γ (Figure 6G). Interestingly, MDM2 inhibition in U2OS cells did not result in any appreciable enhancement of the level of p53 (Figure 6H). In contrast, p53 accumulated following inhibition of MDM2 in the other cells (Figure 6H).

To examine whether downregulation of PA28 γ enhances the effects of MDM2 inhibition in cancer cells with a high level of PA28 γ , HCT116 and A549 cells were exposed to a combination of Nutlin-3 (3 μ M) and PA28 γ siRNA. The combination led to a synergistic upregulation of p53 (Figure 6I), inhibited survival, and enhanced apoptosis (Figure 6J). This suggests a new strategy for sensitizing human cancers to MDM2 inhibitors.

Discussion

The role of MDM2 in regulating p53 is supported by transgenic animal studies (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Several regulators of the MDM2–p53 interaction have been identified, including p14^{Arf} (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998a), YY1 (Sui *et al.*, 2004), MDMX (Francoz *et al.*, 2006), gankyrin (Higashitsuji *et al.*, 2005), L11 (Lohrum *et al.*, 2003), and Daxx (Tang *et al.*, 2006). This large number of regulatory molecules indicates the complexity of the MDM2–p53 interaction. The pattern of p53 degradation depends on the cellular level of MDM2 (Li *et al.*, 2003). Further, in different human cancer cell lines, the extent of p53 accumulation varies even when a similar level of MDM2 inhibition has been achieved (Zhang *et al.*, 2005). The identification of PA28 γ as a cofactor for the degradation of p53 suggests a new model for p53 degradation, in which PA28 γ regulates the MDM2–p53 interaction and MDM2-dependent degradation. Without PA28 γ , MDM2 induces p53 monoubiquitination and nuclear export; subsequent polyubiquitination is catalysed by cytoplasmic factors. When PA28 γ is present, p53 is efficiently polyubiquitinated by low levels of MDM2 within the nucleus, and the p53 level remains under stringent control by MDM2. This could provide advantages to cells in that it would circumvent the need for elevated levels of MDM2 and the need for nucleus-to-cytoplasm transport.

Recent studies have shown that MDM2 can form polymers to increase the local concentrations of components of the ubiquitination reaction and provide a binding surface for multiple E2s (Poyurovsky *et al.*, 2007). We have observed by FPLC analysis that in lysates of GFP-PA28 γ -transfected cells, a portion of MDM2 appeared with GFP-PA28 γ and p53 in the fractions with molecular weights higher than 669 kDa (Figure 4G, right panel). It is possible that polymerized PA28 γ mediates the interactions not only between p53 and monomeric MDM2 (appearing in fractions $\sim$ 560 kDa) but also between p53 and polymeric MDM2. This high-order structure would allow for efficient MDM2-dependent polyubiquitination of p53. The protein complex may also provide a scaffold to orient the substrates, in addition to increasing the local concentrations of the components, especially when the amounts of cellular E2 are low.

MDM2 is being considered as a target for human cancer therapy. We have provided evidence that PA28 γ has an integral function in regulating the MDM2–p53 interaction by facilitating the interaction of the two proteins. This observation may have clinical implications. In designing future trials of agents targeting MDM2, screening for the expression of PA28 γ in cancer patients should be taken into account.

Another interesting observation is that the change in MDM2 expression in different cells is not the same after PA28 γ overexpression. MDM2 is a target of p53, and PA28 γ is a negative regulator of p53. Therefore, overexpression of PA28 γ in cells may result in a decrease in the level of MDM2. This may occur through the inhibition of the transactivation activity of p53 by PA28 γ . Although this seems to be the case in A549 cells, at the current stage we cannot completely rule out the possibility that PA28 γ may directly regulate MDM2 or may regulate the oncoprotein through other pathways. These mechanisms may be cell-type dependent. Future studies investigating the possible regulatory function of PA28 γ on MDM2 expression either in the presence or absence of p53 are warranted.

This study reveals a previously unrecognized mechanism underlying the effects of PA28 γ on apoptosis and cell cycle regulation. Although one PA28 γ substrate, SRC-3/AIB1, had been identified previously (Li *et al.*, 2006), its pro-growth

Figure 4 PA28 γ protein interacts with both MDM2 and p53 proteins and promotes the MDM2–p53 interaction. (A) Endogenous PA28 γ , MDM2, and p53 interact in intact cells. Lysates of MCF-7 cells were immunoprecipitated by PA28 γ (Invitrogen) (left panel), MDM2 (N-20) (middle panel), or p53 (FL393) (right panel), and target proteins in the immunocomplexes were separated by SDS–PAGE and detected by antibodies against MDM2 (SMP14), p53 (DO-1), or PA28 γ (BD Biosciences). (B) PA28 γ interacts with MDM2 and p53 *in vitro*. GST-MDM2 (upper panel, left) or GST-PA28 γ (lower panel, left) was incubated with *in vitro*-translated PA28 γ or MDM2. The complex was pulled down by GST beads, and the target proteins were detected by PA28 γ or MDM2 antibody (SMP14). GST-p53 (upper panel, right) or GST-PA28 γ (lower panel, right) was incubated with *in vitro*-translated PA28 γ or p53. The complex was pulled down by GST beads, and the target proteins were detected by PA28 γ or p53 antibody (DO-1). (C) Exogenous MDM2, p53, and PA28 γ interact in COS7 cells. COS7 cells were transfected with p53-HA or PA28 γ -GFP, or both (upper panel), and cell lysates were immunoprecipitated by GFP or HA antibody. The binding between p53 and PA28 γ was examined by immunoblotting with HA or GFP antibody. In a separate experiment, COS7 cells were transfected with MDM2-T7 or PA28 γ -GFP, or both (lower panel), then cell lysates were immunoprecipitated by GFP or T7 antibody. The binding between MDM2 and PA28 γ was examined by immunoblotting with T7 or GFP antibody. (D–F) PA28 γ promotes the binding between endogenous MDM2 and p53 in cells and *in vitro*. (D) The interaction between MDM2 and p53 in U2OS cells with overexpression of PA28 γ (0, 3, and 5 μ g) (left panel), or in A549 cells with PA28 γ knockdown by PA28 γ siRNA pool (1.25 nM for each duplex) (right panel), was examined by immunoprecipitation with p53 antibody (FL393) followed by immunoblotting with MDM2 antibody (SMP14). (E) GST-MDM2 was incubated with *in vitro*-translated p53 in the presence or absence of PA28 γ at 4°C for 4 h. p53 bound by GST-MDM2 was pulled down by GST beads, eluted by SDS sample buffer at 100°C for 10 min, and detected by immunoblotting with p53 antibody, DO-1. (F) Cell lysates of low passage MEF were immunoprecipitated with p53 antibody FL393, and the immunoprecipitates were separated on SDS–PAGE. Target proteins were detected by immunoblotting with specific antibodies. (G) The polymer of PA28 γ mediates the interaction with MDM2 and p53. COS7 cells were transfected with plasmids overexpressing GFP or GFP-PA28 γ with MDM2-T7 and p53-HA (5 μ g for each plasmid per 100 mm dish). Cell lysates were collected and subjected to gel filtration using the Superose 6/Superdex 200 system 24 h later. The absorbance profiles are shown in the left panel. Eluted fractions were subjected to 10% SDS–PAGE, and target proteins were detected by immunoblotting (right panel).

Figure 5 PA28 γ specifically downregulates p53. (A–C) PA28 γ mutants lacking the capacity to activate the proteasome still promote p53 degradation in cells, whereas a mutant lacking the insert region is incapable of facilitating p53 degradation. U2OS cells were transfected with GFP-PA28 γ , mutants P245Y, G150S, N151Y, Δ 76–103, or GFP (4 μ g for each plasmid). The stability of endogenous p53 was determined by immunoblotting (left panel). The relative densitometry data at each time point are expressed as a percentage of the density of p53 at time 0, normalized to the corresponding β -actin level. The average of the relative densitometry data in the three independent experiments are shown in the right panel. * P < 0.05. (B) U2OS cells were transfected with ubiquitin (2 μ g per 60 mm dish) with either GFP, GFP-PA28 γ , or GFP-PA28 γ - Δ 76–103 (4 μ g per 60 mm dish) for 28 h, followed by the exposure to proteasome inhibitor MG132 (15 μ M) for 4 h. Cell lysates were immunoprecipitated with p53 antibody FL393. The immunoprecipitates were separated on 10% SDS-PAGE and immunoblotted with polyubiquitin chain antibody, FK1. (C) His-p53 (6 ng) was incubated with His-MDM2 (6 ng) and GST-PA28 γ (30 ng), or GST-PA28 γ - Δ 76–103 (30 ng) for 3 h in the presence of E1 (10 ng), E2 (40 ng), and ubiquitin (5 μ g). Ubiquitinated p53 was detected by immunoblotting with the p53 antibody, DO-1. A series of plasmids expressing mutants of PA28 γ were transfected with MDM2-T7 (D) or p53-HA (E) into COS7 cells. Sequential assays of immunoprecipitation with GFP antibody and immunoblotting with T7 (D) or HA (E) antibody were employed to determine the region essential for the binding of PA28 γ to MDM2 or p53. (F) A summary of the capacities of the PA28 γ mutants to bind to MDM2 and p53. (G) HCT116 cells were treated with a control siRNA duplex or a PA28 γ siRNA duplex (10 nM, 48 h). Target proteins were detected by immunoblotting.

nature is difficult to reconcile with the pro-apoptotic and antiproliferative phenotypes following PA28 γ knockdown. Recently, p21^{Waf1} was also demonstrated to be a substrate of PA28 γ (Chen *et al.*, 2007; Li *et al.*, 2007). Although the cell cycle arrest phenotype of PA28 γ knockdown could be explained by its effects on p21^{Waf1}, the mechanisms responsible for its induction of apoptosis were not clear. We have identified an interaction among PA28 γ , MDM2, and p53, which have established activities in apoptosis and cell proliferation. This interaction could provide the missing link between PA28 γ and apoptosis.

In support of this theory, PA28 $\gamma^{-/-}$ mice, although viable, have a phenotype similar to transgenic mice with hypomorphic MDM2. We also observed that PA28 γ has a negative regulatory function in the level of p53 in both MEF cells and normal breast epithelial MCF10A cells. It is possible that the viability of PA28 $\gamma^{-/-}$ mice may depend on the presence of other p53-regulatory molecules when PA28 γ is knocked down. For example, we have observed that YY1 is still capable of downregulating p53 in the presence of PA28 γ knockdown (Figure 3M). Recent efforts have identified several cellular factors that selectively regulate the transactiva-

Figure 6 See page 862 for caption.

tional activities of p53 (Das *et al*, 2007; Tanaka *et al*, 2007). These molecules may also regulate the activities of p53 in the absence of PA28 γ .

MDM2 knockout results in lethality early in development in mice (Jones *et al*, 1995; Montes de Oca Luna *et al*, 1995). This may result from a p53-mediated hypoxic response due to the lack of regulation by MDM2. It is possible that the regulation of MDM2–p53 by PA28 γ may not be involved in this stage of early development, or that other cofactors have a more important function at this stage, which could explain the viability of PA28 $\gamma^{-/-}$ mice. Nonetheless, in support of the effects of PA28 γ on p53 regulation, PA28 $\gamma^{-/-}$ mice, although viable, have reduced adult body mass, growth retardation, and demonstrate spontaneous apoptosis and G1 arrest in embryonic fibroblasts.

In conclusion, the present study (a) identifies a novel factor involved in the regulation of the MDM2–p53 interaction; (b) indicates that a previously undefined partner of MDM2 is required for the degradation of p53 protein; (c) suggests a novel mechanism by which PA28 γ regulates apoptosis and cell proliferation; (d) demonstrates that PA28 γ mediates the degradation of intact proteins in addition to peptides; and (e) provides a possible new target for human cancer therapy.

Materials and methods

Cells

LNCAp, A549, MCF-7, and PC3 cells were purchased from the ATCC. U2OS, and HCT116 p53 $^{+/+}$ and HCT116 p53 $^{-/-}$ cells were provided by Dr X Chen (UC Davis) and Dr B Vogelstein (Johns Hopkins), respectively. MCF-7 cells with stable p53 knockdown have been described previously (Li *et al*, 2005). All cells were cultured under standard conditions described by the ATCC. PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ MEFs and immortalized MEF cells (3 passages after immortalization, ~20 passages from initial culture) were kindly provided by Dr L Barton (Austin College). The temperature-sensitive Balb/c 3T3 cell line, ts20b, was a gift from Dr O Harvey (UMDNJ-New Jersey Medical School).

Antibodies

Antibodies were obtained from Affinity (proteasome α/β subunit, S7/Rpt1, and PA28 β), BD Biosciences (mouse PA28 γ), Calbiochem

(p53 DO-1, T7, and human p21^{Waf1} Ab-1), Cell Signaling (Puma and Numb), Covance (HA.11), Invitrogen (rabbit polyclonal PA28 γ), Santa Cruz (p53 FL393, 14-3-3 σ , MDM4, MDM2 SMP14, and MDM2 N-20), and Sigma (Flag M2 and GFP-N-terminal).

Plasmids, oligonucleotides, and reagents

Various plasmids were kind gifts from other investigators, including Flag-tagged pcDNA3-PA28 β and pEF-PA28 γ (Dr Y Matsuura, Osaka University, Japan), pcDNA3-p53-HA, pcGT-T7-MDM2, p21^{Waf1} luciferase reporter, and pBabe-U6-p53 (Dr X Chen, UC Davis), pcMV-MDM2 and pcMV-p53 (Dr J Chen, Moffitt Cancer Center), HA-Ub and YY1-HA (Dr Y Shi, Harvard), and Daxx-HA (Dr X Yang, U Penn). The GFP-C1-PA28 γ , PGEX-2T-PA28 γ and pcDNA3-PA28 γ vectors were generated by proof-reading PCR, and mutants GFP-C1-PA28 γ -P245Y, -G150S and -N151Y were produced using the QuikChange[®] site-directed mutagenesis kit (Stratagene) and verified by sequencing. MDM2 antisense and mismatch oligonucleotides have been described previously (Zhang *et al*, 2003, 2005). Nutlin-3, cycloheximide (CHX), and MG132 were purchased from Sigma-Aldrich. E1, E2 (UbcH5b), and Myc-Ubiquitin for *in vitro* ubiquitination assays were purchased from Boston Biochem, and 20S and 26S proteasomes were purchased from Biomol.

Immunoprecipitation and immunoblotting

Cells were lysed in NP-40 buffer, then lysates were immunoprecipitated with specific antibodies and examined by immunoblotting. The Supplementary data can be consulted for additional details.

Generation of GST fusion proteins and pull-down assays

Direct physical binding *in vitro* between the MDM2 protein and PA28 γ , or between p53 and PA28 γ , was assessed using GST fusion proteins. The bound proteins were captured by glutathione-agarose beads and resolved on SDS-PAGE. Details are available in the Supplementary data section.

Purification of His-tagged recombinant proteins and *in vitro* ubiquitination assay

MDM2 and p53 fusion proteins were generated in *Escherichia coli*, then purified on Ni-NTA Superflow Columns. We utilized a previously described protocol to evaluate ubiquitination (Li *et al*, 2003). Ubiquitinated p53 was detected by immunoblotting with the DO-1 p53 antibody. For more details about these experiments, please see the Supplementary data.

In vitro proteasome degradation assay

A modification of a published protocol was used to evaluate proteasomal degradation of p53 and p21 (Dai *et al*, 2006; see Supplementary data). An aliquot of the reaction was analysed by immunoblotting.

Figure 6 PA28 γ regulates apoptosis through p53. (A, B) PA28 γ knockdown results in p53-mediated cell killing. (A) A549, MCF-7 p53 $^{+/+}$, and MCF-7 p53 $^{-/-}$ cells were treated with a control siRNA pool (5 nM of each duplex) or a PA28 γ siRNA pool (1.25, 2.5, or 5 nM of each duplex), and cell viability was determined by MTT assay. (B) A549, MCF-7 p53 $^{+/+}$, and MCF-7 p53 $^{-/-}$ cells were treated with a PA28 γ siRNA pool or control siRNA pool as above, and apoptotic cells were detected by flow cytometry. The relative percentages of surviving cells and apoptotic indices are presented as the means $\pm$ s.d. of three independent samples. (C–F) PA28 γ inhibits p53 accumulation and apoptosis following γ -irradiation (IR). (C) MCF-7 cells were transfected with PA28 γ (3 μ g) or control vector (3 μ g), 24 h later cells were exposed to γ -irradiation (8 Gy). Target proteins in whole cell lysates collected at different time points after irradiation were detected by immunoblotting. (D) A549 cells were transfected with a plasmid overexpressing PA28 γ (3 μ g) or the control vector (3 μ g). Cells were exposed to γ -irradiation (8 Gy) 24 h later. After different time periods, cell lysates were immunoprecipitated by p53 antibody FL393, and the immunoprecipitates were separated on 10% SDS-PAGE. Target proteins were detected by immunoblotting. (E) MCF-7 cells with transiently overexpressed GFP-PA28 γ (3 μ g) or GFP (3 μ g) were exposed to γ -irradiation. After 8 h, cells were collected and fixed in 70% ethanol at 4°C overnight. After staining with PI, GFP-positive cell populations were counted by flow cytometry to determine DNA content, and the sub-G1 populations were selected to represent the apoptotic cells. Relative apoptotic indices are presented as the means $\pm$ s.d. of two independent samples. (F) HCT116 p53 $^{+/+}$ and HCT116 p53 $^{-/-}$ cells were pretreated with the PA28 γ siRNA or control siRNA pool (0.625 nM for each siRNA) (24 h) or were exposed to γ -irradiation (8 Gy). After another 24 h, cell viability was determined by MTT assay. The relative percentages of surviving cells are presented as means $\pm$ s.d. of three independent samples. (G, H) The enhanced levels of p53 in different cancer cell lines upon MDM2 inhibition is related to the level of endogenous PA28 γ . Cancer cells were examined for basal PA28 γ protein (G), then treated with MDM2 AS (50 and 150 nM) or ASM (150 nM). After 24 h, target proteins were detected by immunoblotting (H). (I, J) PA28 γ knockdown in human cancer cells with high levels of endogenous PA28 γ sensitizes them to Nutlin-3. (I) HCT116 and A549 cells were transfected with a control or PA28 γ siRNA pool (1.25 nM for each duplex). 3 h after transfection, 3 μ M Nutlin-3 or the solvent, DMSO, was added to the culture media, and 24 h after transfection, target proteins were detected by immunoblotting. (J) A549 cells were treated as described in (G), then cell viability was determined by MTT assay, and apoptotic cells were detected by flow cytometry. The relative percentages of surviving cells and apoptotic indices are presented as the means $\pm$ s.d. of three independent samples. MTT and apoptosis assays were performed at least twice, and similar results were observed. * $P < 0.05$; † $P < 0.0001$.

siRNA transfection

The PA28 γ , p53, and control siRNA pools, individual siRNA duplexes targeting PA28 γ or PA28 β , the control siRNA duplex randomly selected from the siRNA pool, and the transfection reagent DharmaFECT™ 1 were purchased from Dharmacon. siRNA transfection was performed according to the manufacturer's protocol. For immunoblotting assays using the siRNA pool, cells were incubated with 1.25 nM of each siRNA for 24 h; for MTT and apoptosis assays, 0.625, 1.25, 2.5, or 5 nM of each siRNA was incubated with cells for 48 h.

Analytical gel filtration chromatography

Analysis of protein profiles of lysates from COS7 cells transfected with MDM2-T7, p53-HA, GFP, or GFP-PA28 γ by the FPLC method was accomplished following a previously described protocol (Garber *et al.*, 2000). Detailed information is provided in the Supplementary data.

RT-PCR

The extraction of total RNA, reverse transcription, and PCR were performed according to the procedures described previously (Zhang *et al.*, 2003). Details are available in the Supplementary data.

Luciferase assay

Cells were co-transfected with full-length p21^{Waf1} promoter vectors with the *Renilla* luciferase reporter (as an internal control; Promega) for 24 h. The luciferase activity of the p21^{Waf1} promoter reporter was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the provided protocol, and normalized to the corresponding *Renilla* luciferase reporter activity.

Cell survival, apoptosis, and cell cycle distribution assays

MTTs assay to measure the cell viability, and apoptosis and cell cycle distribution assays were accomplished according to methods described previously (Li *et al.*, 2005).

γ -Irradiation

Cells were placed in a ⁶⁰Co Picker unit irradiator (1.56 Gy/min) and exposed to 8 Gy γ -irradiation.

Statistical analysis

The means of relative densitometry of p53 bands, survival indices, and apoptotic indices between two treatment groups were analysed using the two-tailed paired *t*-test. The significance of the variances of means of indices in multiple treatment groups was initially analysed by ANOVA. If positive (*P* < 0.05), the means between the control and each treatment group were analysed using the two-tailed paired *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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