REGγ modulates p53 activity by regulating its cellular localization

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Summary

The proteasome activator REGγ mediates a shortcut for the destruction of intact mammalian proteins. The biological roles of REGγ and the underlying mechanisms are not fully understood. Here we provide evidence that REGγ regulates cellular distribution of p53 by facilitating its multiple monoubiquitylation and subsequent nuclear export and degradation. We also show that inhibition of p53 tetramerization by REGγ might further enhance cytoplasmic relocation of p53 and reduce active p53 in the nucleus. Furthermore, multiple monoubiquitylation of p53 enhances its physical interaction with HDM2 and probably facilitates subsequent polyubiquitylation of p53, suggesting that monoubiquitylation can act as a signal for p53 degradation. Depletion of REGγ sensitizes cells to stress-induced apoptosis, validating its crucial role in the control of apoptosis, probably through regulation of p53 function. Using a mouse xenograft model, we show that REGγ knockdown results in a significant reduction of tumor growth, suggesting an important role for REGγ in tumor development. Our study therefore demonstrates that REGγ-mediated inactivation of p53 is one of the mechanisms involved in cancer progression.

Key words: REGγ, Cancer, p53, Monoubiquitylation

Introduction

REGγ (also known as PA28γ and PSME3) is a member of the 11S family of proteasome activators and has been recently shown to promote the degradation of several important regulatory proteins, including the cyclin-dependent kinase inhibitor p21 (Chen et al., 2007; Li et al., 2007). In line with previous reports that REGγ-knockout mice and cells display reduced body growth, decreased cell proliferation and increased apoptosis (Barton et al., 2004; Murata et al., 1999), REGγ has been reported to be associated with some types of cancers (Mao et al., 2008; Roessler et al., 2006). However, the precise roles and mechanisms by which REGγ is involved in cancer development remain to be explored.

The tumor suppressor p53 responds to diverse forms of cellular stress to regulate many biological processes including cell-cycle arrest, apoptosis, senescence, DNA repair and metabolism (Green and Chipuk, 2006; Harms et al., 2004; Toledo and Wahl, 2006). The profound roles of p53 in promoting growth arrest or cell death are significantly affected by its nuclear protein levels, which are tightly controlled by several mechanisms. It is generally accepted that post-translational modifications, including ubiquitylation (Brooks and Gu, 2006), have a significant role in the regulation of p53. It is now evident that the regulation of p53 ubiquitylation is very dynamic and complex.

A key mediator in the regulation of p53 is MDM2 (mouse double-minute 2 protein) or HDM2 (human ortholog of MDM2), which functions as an important ubiquitin ligase to promote p53 ubiquitylation for proteasomal degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Modulation of the interaction between p53 and MDM2 has been proven to be essential for p53 activation (Vassilev et al., 2004). The complexity of p53 regulation is further demonstrated by the identification of numerous regulators of MDM2 and p53 interaction, including the recently discovered REGγ (Pomerantz et al., 1998; Zhang et al., 1998; Zhang and Zhang, 2008).

REGγ mediates p53 degradation by promoting MDM2-mediated polyubiquitylation and subsequent proteasomal degradation of p53 (Zhang and Zhang, 2008), However, the biological consequences and underlying mechanisms of REGγ-mediated regulation of p53 other than protein degradation remain to be elucidated. This has prompted us to search for additional mechanisms that might provide further insight into REGγ-mediated regulation of p53. Here, we report that REGγ can enhance nuclear export of p53 by facilitating its monoubiquitylation at several sites, alleviating its concentration in the nuclear compartment where active p53 exerts its transcriptional activity. In addition, interference of p53 tetramerization by REGγ might contribute further to nuclear export and attenuation of p53 activity. We also provide evidence that shows the biological significance of REGγ-mediated modulation of p53 activity in tumor development.

Results

REGγ regulates p53 cellular distribution

The human non-small cell lung cancer cell line A549, which expresses wild-type p53 (Nishizaki et al., 2004), was used to

generate stable cell lines constitutively expressing a control shRNA (shN) or a REGγ-specific shRNA (shR). Compared with A549-shN cells, the A549-shR cells displayed significant knockdown of REGγ and a moderate increase in p53 expression (Fig. 1A). Using these stable cells, we assessed the influence of REGγ-mediated regulation of p53 on its target gene expression. Results in Fig. 1A demonstrated upregulation of p21, Bax, and Puma in REGγ-knockdown cells. We also examined transcriptional activity of p53 by transfecting a p21 luciferase reporter into shN or shR cells. There was markedly increased p21 gene expression when REGγ was depleted (supplementary material Fig. S1). To understand the molecular details of this phenomenon, a pair of pooled A549-shN and A549-shR cell lines was examined for the cellular distribution of p53 by immunostaining (Fig. 1B) with antibodies against p53 and REGγ. We found that p53 accumulated in the nucleus in A549-shR cells where REGγ was knocked down. By contrast, ubiquitous distribution of p53 was observed in A549-shN cells bearing the stably integrated control shRNA (Fig. 1B).

To verify our observation, we performed gain-of-function experiments using the previously generated 293-REGγ cells (Li et al., 2007) in which REGγ expression can be induced by doxycycline (Dox). As shown in supplementary material Fig. S2A (upper panel), cellular p53 was mostly colocalized with REGγ in the nucleus before treatment with Dox. Although REGγ remained exclusively in the nucleus, Dox induction resulted in a robust increase of REGγ and led to p53 relocation to the cytoplasm (supplementary material Fig. S2A, lower panel). This observation was further confirmed by cell fractionation, which showed that the concentration of p53 in the cytosol was increased markedly after induction with Dox (supplementary material Fig. S2B).

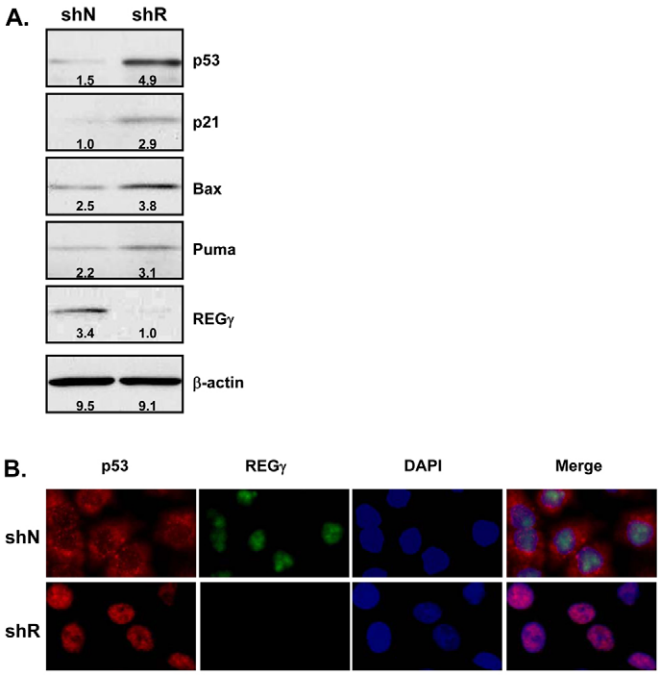

Fig. 1. REGγ regulates p53 activity and its cellular distribution. (**A**) REGγ regulates p53 activity. A549 cells stably integrated with a control shRNA (A549-shN) or shREGγ (A549-shR) were used to examine REGγ-mediated regulation of p53 and its target genes. Whole-cell lysates were prepared for western blot analysis of indicated proteins. The intensity of the protein bands was quantified by densitometry and the relative fold changes are shown (other data in this study were quantified similarly). (**B**) REGγ regulates p53 cellular localization in A549 cells. A549-shN and A549-shR cells were double immunostained with anti-p53 (DO-1, red) and anti-REGγ (green). Cell nuclei were counterstained with DAPI (blue). Quantification of over 200 cells indicates that A549-shN cells have >70% cytoplasmic localization of p53 whereas A549-shR cells have <30% of p53 in the cytosol.

REGγ regulates p53 cellular distribution in an MDM2- and ubiquitin-dependent manner

To understand the role of REGγ in the regulation of p53 localization under physiological conditions, we carried out experiments in non-cancerous REGγ$^{-/-}$ mouse embryonic fibroblasts (MEFs). Since endogenous p53 is extremely low in MEFs, we transfected exogenous p53 into REGγ$^{-/-}$ or REG$^{+/+}$ MEF cells alone or in combination with REGγ. We found that in REGγ$^{-/-}$ MEFs the transfected REGγ was expressed at higher levels than endogenous REGγ in REGγ$^{+/+}$ MEFs. Therefore, we were able to create a series of REGγ expression gradients in MEFs: no expression (–) in REGγ$^{-/-}$ MEFs; mid-level expression (+) in REGγ$^{+/+}$ MEFs; mid- to high-level expression (++) in REGγ$^{-/-}$ MEFs transfected with exogenous REGγ; high-level expression (+++) in REGγ$^{+/+}$ MEFs transfected with exogenous REGγ (Fig. 2A, left). The ratio of the numbers of cells with cytosolic p53 distribution relative to cells with exclusively nuclear localization of p53 increased in a REGγ level-dependent manner (Fig. 2A, right). A rescue experiment is represented in Fig. 2A, in which introduction of exogenous REGγ back into REGγ$^{-/-}$ MEFs resulted in a threefold increase of p53 nuclear export (Fig. 2A, right).

MDM2 has been shown to control p53 nuclear export (Li et al., 2003; Rodriguez et al., 2000). We then investigated whether the nuclear export of p53 regulated by REGγ is MDM2 dependent using p53 and MDM2 double-knockout (p53$^{-/-}$, MDM2$^{-/-}$) MEFs. Although a significant amount of REGγ was present in these cells (Fig. 2B, inset), exogenously expressed p53 was barely seen in the cytoplasm (<5%, Fig. 2B, top). Nuclear export of p53 could only be rescued by introducing MDM2 back to these cells (>35%, Fig. 2B, bottom), suggesting that REGγ-mediated regulation of p53 is MDM2 dependent. To further validate MDM2-dependent REGγ action on p53 nuclear export, we knocked down MDM2 in REGγ$^{-/-}$ MEFs and tested whether restoration of REGγ can rescue p53 nuclear export. We found that in contrast to the control (Fig. 2C, top left), following efficient knockdown of MDM2 (supplementary material Fig. S2C), restoration of REGγ failed to rescue nuclear export of p53 (Fig. 2C, bottom left), which is consistent with our observation that REGγ-mediated nuclear export of p53 requires MDM2 (Fig. 2B).

To determine whether ubiquitylation is required for REGγ-mediated p53 nuclear export, we used a mutant p53 whose six lysine residues in the C-terminal domain were replaced with arginine residues (p53-6KR). This mutant has been demonstrated to be refractory to HDM2-mediated ubiquitylation (Rodriguez et al., 2000). Fig. 2D shows that, although the p53-6KR mutant was mainly restricted to the nucleus (cytoplasmic localization of p53 was in less than 5%) even in the presence of MDM2 (bottom panels), wild-type p53 had significantly increased cytoplasmic expression (>35%) (top panels), indicating that REGγ mediated regulation of p53 is likely to involve ubiquitylation events.

REGγ promotes nuclear export of p53 by facilitating its monoubiquitylation

MDM2-induced monoubiquitylation of p53 and a p53–ubiquitin fusion protein mimicking the monoubiquitylated form of p53 were

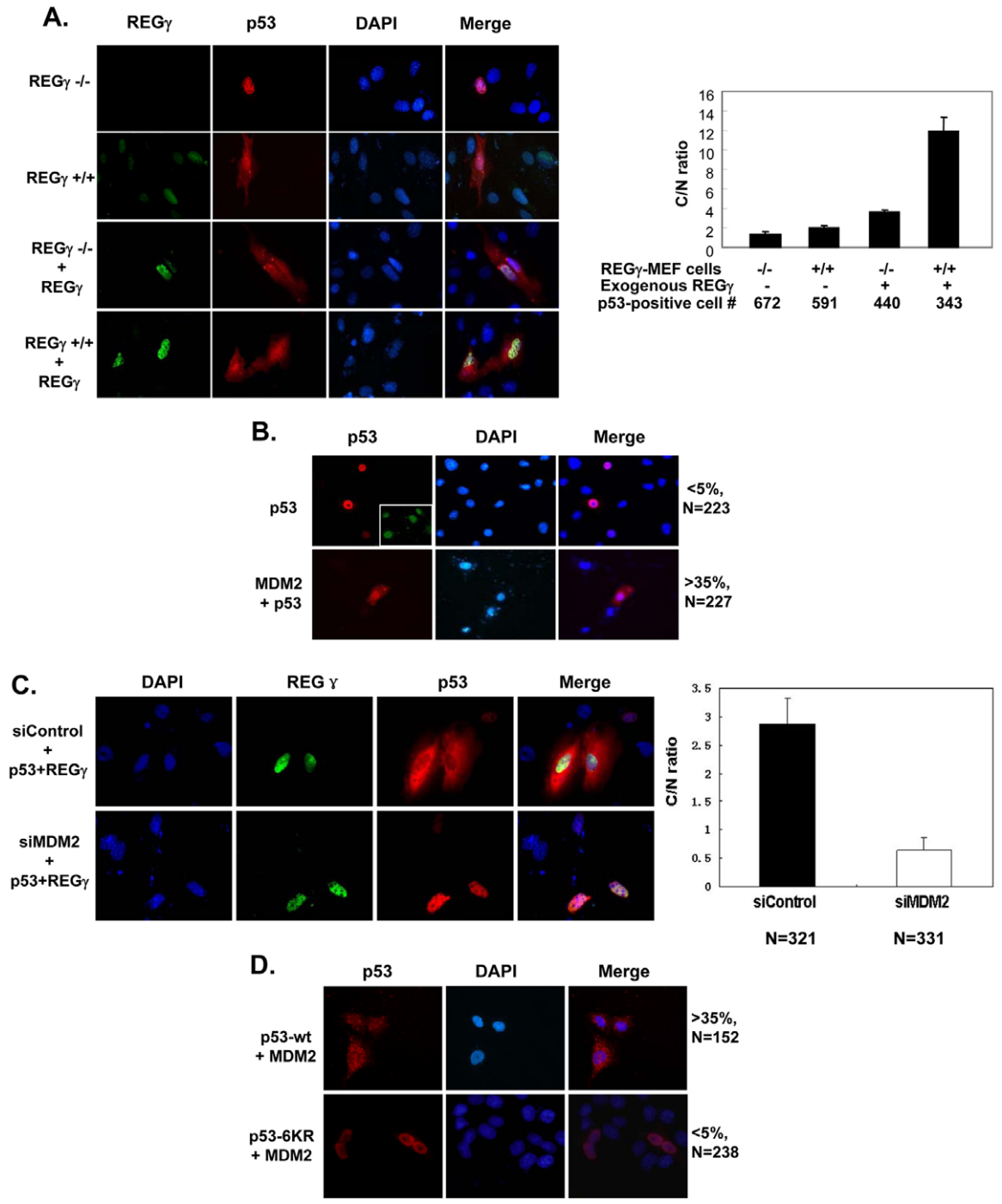

Fig. 2. REGγ regulates p53 distribution in an MDM2- and ubiquitin-dependent manner. (**A**) REGγ regulates p53 cellular localization in MEFs. REGγ$^{-/-}$ or REGγ$^{+/+}$ MEFs were seeded onto coverslips in 12-well plates and transfected with human p53 (0.25 μg) alone or together with REGγ (2 μg). Left panels show representative images of p53 (red) and REGγ (green) staining in different REGγ-expressing groups. The ratio between the numbers of cells with cytoplasmic and exclusively nuclear p53 expression in different groups is displayed on the right. The number of p53-positive cells used in quantification is indicated. Results are means + s.d. (**B**) MDM2 overexpression restores cytoplasmic localization of p53 in MDM2-knockout cells. p53 and MDM2 double-knockout MEFs were transfected with p53 (0.25 μg) alone or together with MDM2 (0.1 μg). Immunocytochemical staining was performed for detection of p53 (red) and REGγ (green, inset). Nuclei are stained with DAPI (blue). The percentage of cells with cytoplasmic localization of p53 over the total number of p53 positive cells (N) is displayed. (**C**) REGγ-mediated nuclear export of p53 requires MDM2. REGγ$^{-/-}$ MEFs were transiently co-transfected with p53 and REGγ plasmids, together with a control siRNA (upper images) or siRNA to knock down MDM2 (lower images). Immunostaining was conducted for p53 (red) and REGγ (green). The efficiency of MDM2 knockdown (more than 90%) is shown in supplementary material Fig. S2C. The ratio of the numbers of cells with cytoplasmic and exclusively nuclear p53 expression in the two groups is shown in the right panel. The number of both p53- and REGγ-positive cells (N) used in quantification is indicated. Results are means + s.d. (**D**) REGγ-mediated nuclear export of p53 requires ubiquitylation. Constructs expressing p53 wild type (wt) or p53-6KR mutant (0.25 μg), were co-transfected with MDM2 (0.1 μg) into p53$^{-/-}$, MDM2$^{-/-}$ MEFs. The cellular distribution of p53 was determined by immunostaining. The percentage of cells with cytoplasmic expression of p53 over the total number of p53 positive cells (N) is listed.

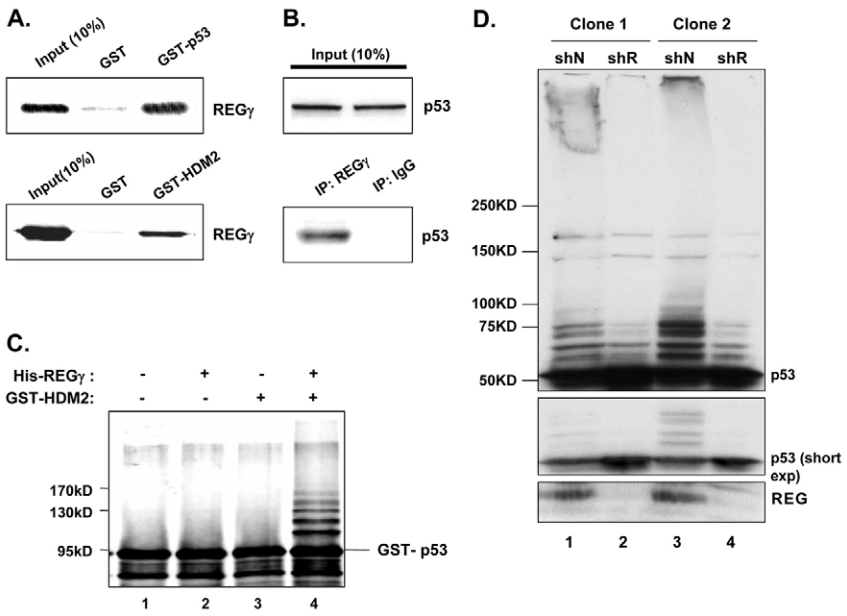

Fig. 3. REGγ promotes multiple monoubiquitylation of p53. (**A**) REGγ interacts with both p53 and HDM2 in vitro. GST–p53 (top) or GST–HDM2 (bottom) protein was incubated with in vitro translated REGγ. The relative amount of GST or GST fusion proteins is shown in supplementary material Fig. S3A. Following incubation for 4 hours at 4°C, the complex was pulled down by GST beads, and REGγ was detected by immunoblotting. GST protein was used as a negative control. (**B**) REGγ interacts with p53 in cells. H1299 cells were co-transfected with p53 (0.5 μg) and REGγ (3 μg) for 36 hours. Immunoprecipitation was performed with whole-cell lysates using anti-REGγ antibody or IgG (control). The immunocomplexes were separated by SDS-PAGE and detected with an antibody against p53. (**C**) REGγ enhances HDM2-mediated monoubiquitylation of p53 in vitro. Ubiquitylation assay was performed using purified GST–p53 (5 ng), E1 (110 ng), E2 (170 ng) and ubiquitin (5 ng) in the presence or absence of GST–HDM2 (10 ng) and His–REGγ (100 ng) as indicated. (**D**) REGγ knockdown results in reduced monoubiquitylation of p53. The pooled (clone 1) or individual (clone 2) clone of A549-shN and A549-shR cells was treated with MG132 at 20 μM for 8 hours. Endogenous monoubiquitylation status of p53 was analyzed by western blot. Note that REGγ expression is inversely correlated with p53 level, but positively correlated with p53 ubiquitylation.

reported to drive its relocation to the cytoplasm (Li et al., 2003). We therefore questioned whether REGγ regulates p53 distribution by enhancing its monoubiquitylation. The interactions between REGγ and p53, as well as REGγ and HDM2, were observed (Fig. 3A,B). Approximately equivalent molar concentrations of purified GST proteins were used in the pull-down experiments (supplementary material Fig. S3A). We further showed that REGγ, HDM2 and p53 can be co-immunoprecipitated simultaneously (supplementary material Fig. S3B), in accordance with the previous report that REGγ might facilitate the interaction of p53 with HDM2 (Zhang and Zhang, 2008). In vitro ubiquitylation assays demonstrated that REGγ significantly enhanced ubiquitylation of p53 (Fig. 3C, lane 4). Furthermore, similar multi-monoubiquitylation patterns were observed in reactions containing wild-type ubiquitin (Ub-wt) (Fig. 3C) or polyubiquitylation-defective ubiquitin (supplementary material Fig. S4A), indicating that REGγ facilitates monoubiquitylation of p53.

To validate our cell-free studies, we examined the ubiquitylation status of endogenous p53 in A549-shN and A549-shR cells after proteasome inhibition. The results from pooled cells and an individual clone demonstrated significant p53 monoubiquitylation in A549-shN cells (Fig. 3D, lanes 1 and 3). By contrast, A549-shR cells with REGγ depletion had dramatically diminished p53 monoubiquitylation, although total p53 levels were higher (Fig. 3D, lanes 2 and 4). We also examined p53 ubiquitylation in H1299 cells transfected with Flag–p53, HDM2 and REGγ or control plasmids. By immunoprecipitation of p53 followed by western blotting with a different anti-p53 or an anti-ubiquitin antibody, we observed an increase in the levels of monoubiquitylated p53 in cells overexpressing REGγ (see supplementary material Fig. S4B). Collectively, our results indicate a significant role for REGγ in the regulation of p53 monoubiquitylation.

Biological consequences of p53 monoubiquitylation

We then further analyzed how REGγ-mediated monoubiquitylation modulates the fate of p53. Monoubiquitylated p53 is shown to be exported to the cytoplasm in a CRM1-dependent manner (Lohrum et al., 2001), so we examined the cellular distribution of p53 following treatment with leptomycin B (LMB), a specific nuclear export inhibitor that inhibits p53 export by targeting CRM1/exportin-1 (Kudo et al., 1999). We performed immunostaining to visualize distribution of p53 in shN and shR cells treated with LMB or MG132 (Fig. 4A). We concomitantly performed cell fractionation experiments and summarized quantitative analysis of cytoplasmic and nuclear p53 expression in shN and shR cells from three independent experiments (Fig. 4B; supplementary material Fig. S5A). In the vehicle-treated group, A549-shN cells had almost equal amount of p53 in nuclear and cytoplasmic fractions whereas A549-shR cells had significantly higher p53 levels in the nucleus and lower p53 in the cytoplasm compared with shN cells (Fig. 4A, top; Fig. 4B). Following LMB treatment, the relative amount of nuclear and cytoplasmic p53 in A549-shN cells was comparable with that in vehicle-treated A549-shR cells (Fig. 4A,B), suggesting that depletion of REGγ had a similar effect as LMB on prevention of p53 nuclear export (Fig. 4A,B). After treatment with MG132, the

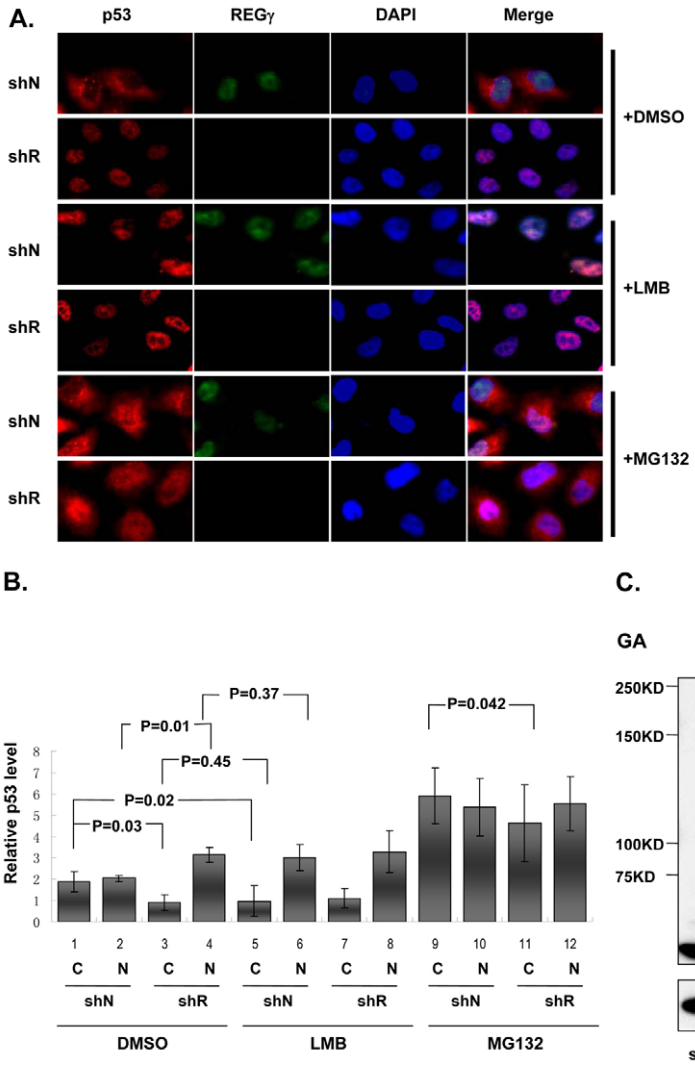

Fig. 4. Biological consequences of p53 monoubiquitylation. (**A**) Depletion of REGγ leads to nuclear accumulation of p53, which mimics the effect of leptomycin B (LMB). A549-shN and A549-shR cells were treated with vehicle (DMSO) or LMB (2 nM) for 2 hours, or MG132 (20 μM) for 8 hours. Immunostaining was performed to examine the expression and distribution of p53 (red) and REGγ (green). Note that localization of p53 following LMB treatment clearly resembles the pattern in shR cells treated with vehicle. (**B**) Fractionation and quantitative analysis of cellular p53 distribution. A549-shN and A549-shR cells were treated with vehicle (DMSO), LMB or MG132. Equal amount of cell extracts were subjected to cytoplasmic (C) and nuclear (N) fractionation, followed by western blot analysis. The p53 levels were normalized against loading controls (supplementary material Fig. S5A), averaged and plotted for quantitative analysis of the differences in cells with or without REGγ knockdown. Data are shown as mean ± s.d. of three independent experiments. Statistical analysis indicates significant differences between lane 1 and lanes 3 and 5, lanes 2 and 4, lanes 9 and 11, but no difference between lanes 3 or 4 and 5 or 6. (**C**) Knockdown of REGγ enhances p53 oligomerization. Cell lysates prepared from A549-shN (with a control shRNA) and A549-shR (with REGγ knockdown) were incubated on ice with glutaraldehyde at a final concentration of 0.09% for 15 minutes to induce oligomerization. The dimerization and tetramerization of p53 as well as other indicated proteins in different cell lysates were analyzed by western blot. Relative fold changes of p53 dimers (~100 kDa) and tetramers (~ 200 kDa) were quantified and displayed.

cytoplamic p53 levels were statistically higher in shN compared with ShR cells (Fig. 4B), indicating that REGγ-mediated p53 cytoplasmic distribution is probably not due to the influence of protein degradation. The patterns of p53 cellular distribution by immunostaining correspond well with the cell fractionation data (Fig. 4A,B).

Of the mechanisms that regulate p53 nuclear export, tetramerization of p53 has been reported to block p53 nuclear export by masking its nuclear export signal (NES) (Stommel et al., 1999). We therefore investigated the effects of REGγ on p53 oligomerization. Because the total levels of p53 are higher in shR than in shN cells, we pre-treated these cells with MG132 to prevent REGγ-mediated degradation of p53. We found distinct patterns of p53 dimerization and tetramerization in A549-shN and A549-shR cells (Fig. 4C). Following different quantitative analysis (supplementary material Fig. S5B,C), we concluded that there were significantly more p53 dimers and tetramers in shR cells than in shN cells. Our results suggest an interference of p53 oligomerization by REGγ, indicating an additional mechanism by which REGγ regulates the cellular distribution and function of p53. In addition to facilitating p53 monoubiquitylation, REGγ probably also triggers cytosolic relocation of p53 by inhibiting its tetramerization, and subsequently exposing its NES.

From the experiments in Fig. 3D, we observed that significant monoubiquitylation of p53 is associated with reduced amount of total p53 level in shN cells treated with MG132 for a short time. We reasoned that monoubiquitylated p53 could also be quickly targeted for degradation, possibly because of an increased affinity for HDM2. To test this, we overexpressed Ub-KO in H1299 cells to increase the population of monoubiquitylated p53 (supplementary material Fig. S6). Reciprocal immunoprecipitation of p53 or HDM2 showed that cells with Ub-KO overexpression displayed significantly enhanced interaction between p53 and HDM2 (Fig. 5A; supplementary material Fig. S6). To further validate the physical interactions between HDM2 and monoubiquitylated p53, we used the ubiquitylation-defective p53-6KR mutant in H1299 cells. Fig. 5B showed that the p53-6KR mutant had a weaker binding affinity with HDM2 compared with wt-p53 when co-expressed with Ub-KO. These results suggest a potential mechanism by which monoubiquitylation in p53 may serve as a signal for further polyubiquitylation and subsequent proteolysis.

REGγ regulates p53 and contributes to oncogenic features in A549 cells

Given the evidence for REGγ regulation of the distribution and expression of p53, we investigated its capacity to modulate biological function of p53. Fig. 6A shows that REGγ depletion sensitized the

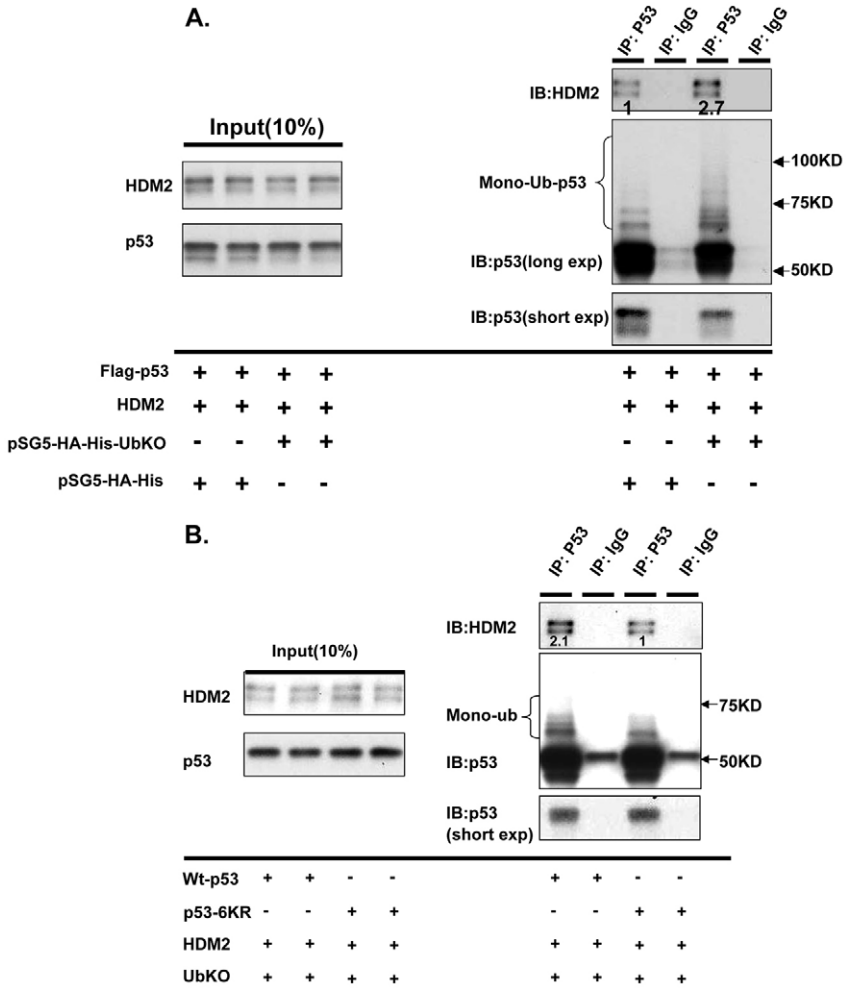

Fig. 5. Monoubiquitylation of p53 enhances the HDM2–p53 interaction. (**A**) Monoubiquitylation of p53 enhances its interaction with HDM2. H1299 cells were transfected with Flag–p53 (0.5 μg), HDM2 (0.25 μg) and Ub-KO (5 μg) or control vectors. 24 hours after transfection, cells were treated with proteasome inhibitors (MG132, 20 μM) for 6 hours and then harvested. Immunoprecipitation was performed with a rabbit polyclonal antibody against p53 (sc-6243) in the presence of protease inhibitor cocktail (Roche) and 5 mM NEM (Sigma) followed by western blot analysis of HDM2 and p53 (DO-1). (**B**) Mutant p53 lacking the C-terminal ubiquitylation sites has attenuated interaction with HDM2 when co-expressed with Ub-KO. The pcDNA3.1 (+)-wt-p53 (0.5 μg) or pcDNA3.1 (+)-p53-6KR (0.5 μg) was transfected with Ub-KO (5 μg) and HDM2 (0.25 μg) into H1299 cells. Immunoprecipitation and western blot analysis were performed as in A using anti-p53 antibody.

A549 cells to cisplatin (an anti-cancer drug)-induced apoptosis as demonstrated by the accumulation of a cleavage fragment of poly (ADP-ribose) polymerase (PARP, lanes 5 and 6). Stress-induced apoptosis was associated with a dramatic increase of p53 and p21 expression in cells with REGγ depletion (Fig. 6A). To address whether REGγ-regulated stress response is p53 dependent, we used a synthetic siRNA to knockdown p53 in shN and shR cells with or without cisplatin treatment. Sensitivity of shR cells to the stress response was attenuated following p53 knockdown (Fig. 6A, compare lanes 6 and 8), suggesting that the impact of REGγ depletion on cell chemosensitivity is at least partially p53 dependent. Furthermore, we characterized the cell viability in response to cisplatin treatment. MTT results showed significantly fewer viable cells in the A549-shR group compared with the A549-shN group (Fig. 6B, lanes 5 and 6), whereas p53 knockdown alleviated such effects induced by cisplatin in A549-shR cells (compare lanes 7 and 8 in Fig. 6B). The MTT results support our data in Fig. 6A that a REGγ-regulated stress response is likely to occur through regulation of p53-mediated apoptosis or cell growth arrest. Consistent with these observations, TUNEL staining revealed increased apoptosis in A549-shR compared with A549-shN cells in response to cisplatin treatment (supplementary material Fig. S7). In line with these results, REGγ depletion resulted in growth reduction in colony foci formation assays (Fig. 6C).

To gain more insight into the role of REGγ in vivo, we examined the consequence of REGγ knockdown on tumorigenicity using a mouse xenograft model. As shown in Fig. 7, tumor volumes were significantly reduced in the mice injected with A549-shR cells compared with those injected with A549-shN cells (Fig. 7A,B), with an increased expression of p53 and p21 in A549-shR tumors (Fig. 7C). These in vivo results suggest an important role for REGγ in cancer cell growth.

Discussion

In this study, we have elaborated the role of REGγ in modulating p53 activity through the following mechanisms: (1) REGγ promotes p53 nuclear export and its cytoplasmic degradation by facilitating p53 monoubiquitylation; (2) p53 monoubiquitylation enhances its interaction with HDM2, which is a new role for monoubiquitylation as a signal for degradation of cellular p53; and (3) REGγ attenuates p53 oligomerization.

The results of current study reveal the finding that multiple monoubiquitylation of p53 is facilitated by REGγ. This process is important for REGγ-mediated inactivation of p53. REGγ regulates the canonical process of p53 monoubiquitylation, nuclear export and cytoplasmic degradation, one of the mechanisms involved in p53 degradation (Freedman and Levine, 1998). Furthermore, REGγ blocks p53 dimerization and tetramerization, which might expose the NES to further enhance p53 nuclear export and reduce active p53 as a functional protein complex in the nucleus. Previous results suggest that REGγ serves as a cofactor for p53 degradation by the ubiquitin–proteasome pathway (Zhang and Zhang, 2008). The present study adds to this finding by demonstrating that

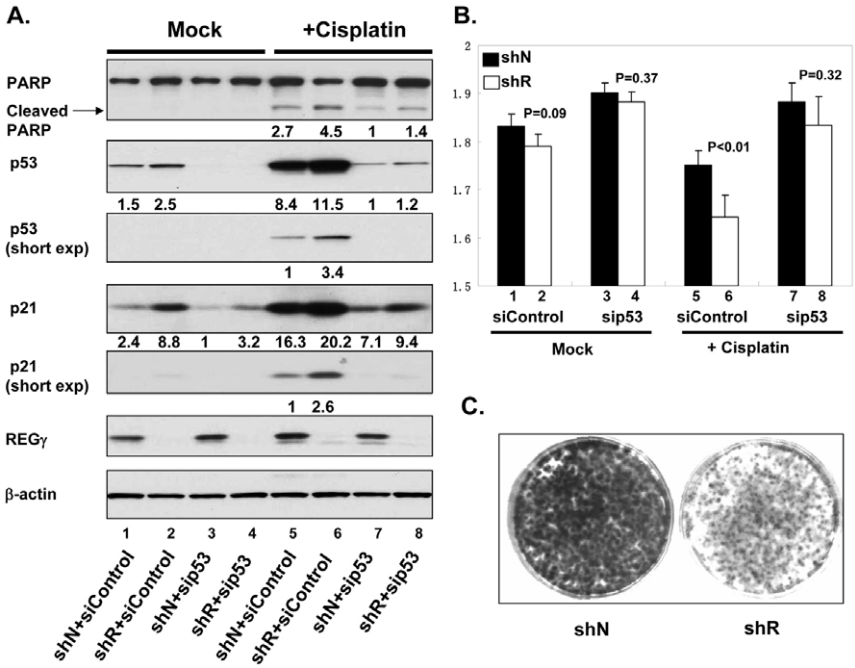

Fig. 6. REGγ regulates p53 and contributes to oncogenic features in human lung carcinoma cells. (**A**) Depletion of REGγ sensitizes tumor cells to stress-induced apoptosis. A549-shN and A549-shR cells were transfected with a control siRNA (siControl, 40 nM) or a synthetic siRNA to knock down p53 (sip53, 40 nM). Approximately 24 hours later, the transfected A549-shN and A549-shR cells were trypsinized and divided equally into two parts for 36 hours of continuous culture, followed by cisplatin treatment (3 μg/ml) for an additional 24 hours. One portion was collected for western bolt analysis and the other was subjected to MTT assay. Western blot results of indicated proteins are displayed. The fragments of PARP cleavage are indicated with an arrow. (**B**) REGγ depletion reduces cell viability in a p53-dependent manner under stress. MTT assays were performed with A549-shN and A549-shR cells treated with a control or siRNA against p53 followed by cisplatin, as described in A. The differences of cell viabilities within each group of shN and shR cells were statistically analyzed by paired *t*-test. Data are shown as mean + s.d. of three independent experiments with *P* values. (**C**) Knockdown of REGγ inhibits cell growth. Colony foci formation assay was performed using A549-shN and A549-shR cells seeded at a density of 10^3 cells per 35-mm dish and cultured for 3 weeks until colony foci were visible. Result of one of three experiments is shown.

monoubiquitylation is an important post-translational modification involved in REGγ-mediated regulation of p53 activity at an additional layer. In addition to protein stability, perhaps the more important impact of REGγ-mediated regulation of p53 is to force p53 away from the most sensitive region of the cell, the nucleus, where promiscuous p53 activity would be disastrous. It is worth mentioning that charge modification of p53 at C-terminal lysine residues regulates p53 nuclear–cytoplasmic trafficking (Kawaguchi et al., 2006), which is independent of MDM2. At this stage, we cannot exclude the contribution of p53 acetylation at the C-terminal lysine residues or p53 ubiquitylation at sites other than the C-terminus.

Monoubiquitylation has been implicated in a number of degradation-independent processes, including endocytosis, virus budding and the regulation of transcription (Brooks and Gu, 2006). Although polyubiquitylation has been known to target proteins for

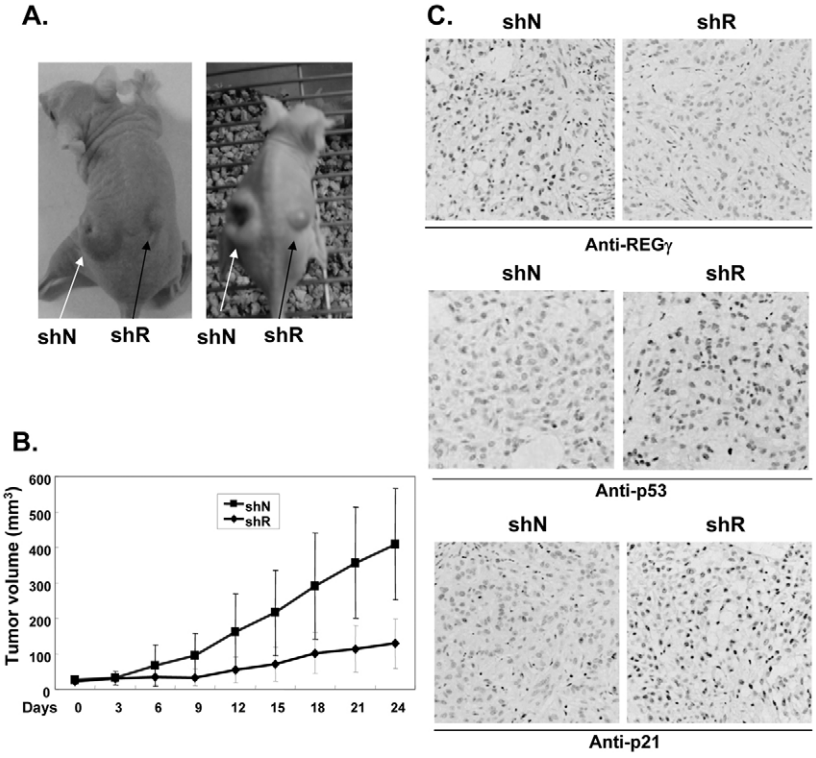

Fig. 7. REGγ knockdown inhibits the growth of A549-derived mouse tumors. (**A**) Representative xenograft tumors originating from A549-shN and A549-shR cells that were injected into dorsal flanking sites. (**B**) Time-course comparative analysis of A549-shN and A549-shR tumor volumes from three independent trials of eight mice per group. Data are presented as means ± s.d. The average volumes between shN and shR tumors are significantly different starting at day 12 ($P<0.05$). (**C**) Immunohistochemical analysis of REGγ, p53 and p21 levels in xenograft tumors.

degradation, the role of monoubiquitylation in regulating protein stability has not been well investigated. In this study, we discovered that monoubiquitylated p53 has enhanced affinity for HDM2, which suggests that monoubiquitylation primes polyubiquitylation events. Although there was a report of monoubiquitylation acting as a signal for proteasomal degradation (Boutet et al., 2007), HDM2-mediated monoubiquitylation in p53 seems to not be a direct signal for p53 destruction because overexpression of Ub-KO, a non-branchable ubiquitin that has mutations in all lysine residues, cannot enhance p53 degradation (Fig. 5A). Our data also support the notion that nuclear export of p53 following monoubiquitylation is one mechanism that regulates the p53 protein level in human cells (Freedman and Levine, 1998). Therefore, we believe that monoubiquitylation of p53 serves as a signal to enhance further polyubiquitylation and its degradation. Taken together, REGγ-mediated multiple monoubiquitylation could initiate an important step toward tight regulation of p53 by the ubiquitin–proteasome pathway.

The biological significance of REGγ-mediated regulation of p53 is highlighted by its effect on stress-induced apoptosis, cell growth arrest and its impact on xenograft tumor growth. Our study is consistent with previous results that REGγ-deficient MEFs have markedly increased levels of apoptosis compared with the wild-type counterparts (Mao et al., 2008) and provides a mechanistic link between REGγ action and its physiological consequences. Our recent study in REGγ-knockout mice (data not shown) reveals a significant increase of endogenous p53 in multiple tissues or cells when compared with wild-type littermates, indicating a crucial role for REGγ in p53 regulation in vivo.

The available evidence strongly supports crucial physiological roles for REGγ in the regulation of several fundamental cellular processes. As a proteasome activator, REGγ has been reported to degrade several proteins such as SRC-3, p21 and hepatitis virus C core protein, in an ATP- and ubiquitin-independent manner (Li et al., 2007; Li et al., 2006). REGγ is therefore suggested to have either a tumor-promoting or tumor-suppressive role, and the overall biological outcome of REGγ might be complicated depending upon the major targets in different tissues or cell types. Because of this, REGγ-mediated regulation of p53 might not be the only process that is involved in its effect on tumor progression. Adding to this complexity is the fact that REGγ also has a proteasome-activator-independent function (Mao et al., 2008), as in the case of p53 degradation. REGγ-mediated regulation of p53 seems to be cell-type and cell-context specific because REGγ-mediated regulation of p53 stability was only observed in cells containing wild-type p53. We also noticed that REGγ-mediated cytoplasmic localization of p53 was more effective when cell culture medium contained calf serum, but not fetal bovine serum, indicating that a specific signaling event might be involved in REGγ-mediated function.

Taken together, the present study describes mechanisms for REGγ-mediated regulation of p53. The step-wise multiple monoubiquitylation and polyubiquitylation processes might be applicable to maintaining the lifespan of other proteins that are regulated in a similar fashion. It is noteworthy that other types of post-translational modification at the C-terminus of p53, such as SUMOylation, which facilitates MDM2 dissociation with p53 (Carter et al., 2007), are also important for p53 regulation. It is possible that ubiquitylation and sumoylation compete for C-terminal modification sites and regulate association–dissociation between MDM2 and p53. Our results substantiate the role of REGγ in the regulation of p53 in vitro and in vivo. Our animal tumor model data have provided further insight into the role of REGγ in the regulation of the important cell cycle regulator p53, and the oncogenic potential of REGγ in human cancer progression.

Materials and Methods

Cell lines and cell culture

A549, HEK293 and H1299 cells were purchased from ATCC and maintained at Cell Culture Core at the Department of Cell Biology, BCM. The A549 stable cell lines were generated by retroviral shRNA vectors specific for REGγ or a control vector from OriGene (Rockville, MD). The 293-REGγ inducible cell line and the REGγ$^{-/-}$ or REGγ$^{+/+}$ MEFs were previously generated (Li et al., 2007). All cells were cultured under standard conditions described by the ATCC.

Plasmids and reagents

The following plasmids were kindly provided by Jiandong Chen (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL): pcDNA3.1-HDM2, p21-luciferase, ubiquitin wild type (Ub-wt), and ubiquitin knockout (Ub-KO). REGγ derivative plasmids were described previously (Li et al., 2007). GST–p53 and GST–HDM2 were generated in pGEX 4T-1 and His-REGγ was generated in pET16b vector. Wt-p53 and p53-6KR were generated in pCDNA3.1(+) vector.

Antibodies were purchased from Invitrogen (PA28γ), Calbiochem (p21Cip/WAF1), Sigma (β-actin, M2 anti-Flag), Santa Cruz (p53, lamin, HSP90, Bax, Puma, Ub and HDM2), and Cell Signaling Technology (PARP). Other purchased reagents include Leptomycin B, cisplatin, glutaraldehyde and MG132 (Sigma); Cycloheximide and MTT assay reagents (Amresco). All the experiments shown in the study were repeated at least three times.

Generation of GST fusion proteins and pull-down assays

GST–REGγ, GST–HDM2 and GST–p53 were purified using glutathione Sepharose affinity chromatography (Bio-Rad) as previously described (Li et al., 2007). Direct physical interactions between p53 and REGγ or HDM2 and REGγ, were assessed using purified GST fusion proteins and in vitro translated proteins as described (Li et al., 2007).

Purification of His-tagged proteins and in vitro ubiquitylation assays

His-REGγ was purified with Mini-Profinity IMAC-Ni column by FPLC (Bio-Rad). Purified GST–p53 was incubated with GST–HDM2 and His–REGγ in the presence of E1, E2 and Ub-wt or Ub-KO in 20 µl reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP and 1 mM DTT) at 30°C for 2 hours. The reactions were stopped in SDS sample buffer and were applied for western blot analysis.

siRNA transfection

siRNAs targeting p53, MDM2, REGγ or control siRNA were transfected into cells using Lipofectamine 2000 (Invitrogen).

Immunostaining

Cells or tissue samples were immunostained to visualize p53, p21 and REGγ as previously described (Li et al., 2007). Antibodies against p53, p21 and REGγ were used at 1:300 dilutions. TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100; Chemicon International; Millipore).

Immunoprecipitation, western blot analysis and cell fractionation

Immunoprecipitation and western blot analysis were performed as described (Li et al., 2007). Nuclear and cytoplasmic fractions were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Product No. 78833). The purity of fractions was determined using Hsp90 as a cytosolic marker and lamin as a nuclear marker.

Colony foci formation and MTT assay

A549-shN or A549-shR cells were seeded at a density of 10^3 cells per 35mm dish. After two to three weeks until colony foci were visible, cells were stained with 0.2 g/l crystal violet (Sigma) for 10 minutes at room temperature and destained with water. MTT assay was performed by seeding A549-shN or A549-shR cells in a 96-well plate at 2.5×10^3 cells per well and were cultured for 36 hours. Following cisplatin treatment (3 µg/ml) for another 24 hours, cells were incubated with MTT solution at 37°C for 2 hours and absorbance (490 nm) was measured and analyzed.

Oligomerization assay

Equal amounts of lysates from A549-shN or A549-shR cells were treated without or with glutaraldehyde at a final concentration of 0.09% for 15 minutes on ice, followed by western blot analysis for p53 dimerization and tetramerization.

Mouse tumorigenicity study

Female BALB/c nude mice at the age of ~5 weeks were randomly divided into three groups, with eight mice in each group. A549 cells (shN and shR) were implanted subcutaneously into both flanks of nude mice at 2×10^6 cells in 100 µl per spot. Ten days after injection, which was arbitrarily set as day 0, tumor size was measured

twice a week using calipers. All animal experiments were approved by the Animal Care and Use Committee.

Data collection and statistical analysis

The intensity of the western blot results was analyzed by densitometry using Bio-Rad Quantity One 4.4.0 software and normalized to the band with the least intensity which was arbitrarily set as 1. The results were expressed as the mean ± s.d. Statistical analysis was performed using the two-tailed, paired Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

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