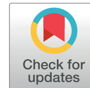

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PIG3 Regulates p53 Stability by Suppressing Its MDM2-Mediated Ubiquitination

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Abstract

Under normal, non-stressed conditions, intracellular p53 is continually ubiquitinated by MDM2 and targeted for degradation. However, in response to severe genotoxic stress, p53 protein levels are markedly increased and apoptotic cell death is triggered. Inhibiting the ubiquitination of p53 under conditions where DNA damage has occurred is therefore crucial for preventing the development of cancer, because if cells with severely damaged genomes are not removed from the population, uncontrolled growth can result. However, questions remain about the cellular mechanisms underlying the regulation of p53 stability. In this study, we show that p53-inducible gene 3 (PIG3), which is a transcriptional target of p53, regulates p53 stability. Overexpression of PIG3 stabilized both endogenous and transfected wild-type p53, whereas a knockdown of PIG3 lead to a reduction in both endogenous and UV-induced p53 levels in p53-proficient human cancer cells. Using both *in vivo* and *in vitro* ubiquitination assays, we found that PIG3 suppressed both ubiquitination- and MDM2-dependent proteasomal degradation of p53. Notably, we demonstrate that PIG3 interacts directly with MDM2 and promoted MDM2 ubiquitination. Moreover, elimination of endogenous PIG3 in p53-proficient HCT116 cells decreased p53 phosphorylation in response to UV irradiation. These results suggest an important role for PIG3 in regulating intracellular p53 levels through the inhibition of p53 ubiquitination.

Key Words: PIG3, p53, MDM2, Ubiquitination, Apoptosis

INTRODUCTION

PIG3 (p53-inducible gene 3) is encoded by a gene located downstream of the gene encoding the tumor suppressor protein p53, and was originally discovered during a study aimed at identifying genes induced by p53 prior to the onset of apoptosis in human colorectal cancer cells (Polyak *et al*., 1997). p53 binds to a penta-nucleotide microsatellite sequence within the PIG3 promoter and thereby transactivates the expression of PIG3 prior to the onset of p53-initated apoptosis (Contente *et al*., 2002). The major cellular role of PIG3 is in the generation of reactive oxygen species (ROS), which then act as downstream mediators of p53-dependent apoptosis (Polyak *et al*., 1997; Porte *et al*., 2009). The amino acid sequence of PIG3 shows significant homology to that of NADH quinine oxidoreductase 1 (NQO1), which is a protein known to contribute to the generation of ROS (Polyak *et al*., 1997). It has also been reported that PIG3 directly inhibits catalase, an antioxidant enzyme that mitigates hydrogen peroxide (H_2O_2) toxicity, resulting in increased intracellular ROS levels (Kang *et al*., 2013). Under genotoxic conditions, high levels of both p53 and PIG3 act cooperatively to inhibit catalase activity, and the subsequent shift toward an oxidative intracellular environment leads to the induction of apoptotic cell death (Kang *et al*., 2013). Recently, PIG3 has been shown to play an important role in the cellular response to DNA damage, particularly in checkpoint signaling and DNA repair. PIG3 knockdown cells had increased sensitivity to DNA-damaging agents, impaired DSB repair, and ineffective intra-S and G2/M phase checkpoint activation (Lee *et al*., 2010; Kotsinas *et al*., 2012; Li *et al*., 2013). In addition, the involvement of PIG3 in glutathione peroxidase 3 (GPx3)-mediated cell death was shown through experiments in which either a knockdown of PIG3 or loss of the PIG3 binding motif in GPx3 abrogated the increase in ROS generation and caspase-3 activity that are normally observed (Wang *et al*., 2012).

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The cellular response to genotoxic damage is also influenced by p53 (Lane, 1992), and the levels and activity of p53 are controlled mainly by MDM2 (Kubbutat *et al*., 1997). When MDM2 binds to p53, ubiquitination and subsequent proteasomal degradation are triggered. In an elegant feedback loop, p53 controls the expression levels of this negative regulator by acting as a transcription factor for MDM2 (Barak *et al*., 1993; Oliner *et al*., 1993; Perry *et al*., 1993). In non-stressed cells, p53 is kept at low levels by directly associating with MDM2, which represses its ability to act as a transcription factor and promotes its proteolytic degradation (Momand *et al*., 1992; Oliner *et al*., 1993; Haupt *et al*., 1997). Induction of p53 involves several mechanisms leading to post-translational modifications, such as phosphorylation and acetylation (Ashcroft *et al*., 2000). DNA damage-induced phosphorylation of serine and threonine residues at the amino terminus of p53 prevents MDM2 from binding and renders p53 more stable (Shieh *et al*., 1997; Unger *et al*., 1999).

Although the relationship between PIG3 and p53 has been investigated previously, the role of PIG3 in p53 stability was still unclear. It is of particular interest that PIG3 shares significant homology with NQO1 (Polyak *et al*., 1997; Porte *et al*., 2009), a protein that plays an important role in regulating p53 functions by inhibiting its degradation (Asher *et al*., 2001, 2002a, 2002b). We therefore investigated whether PIG3 could regulate p53 stability in a manner similar to that of NQO1. Our results clearly show that PIG3 suppresses p53 degradation. However, unlike NQO1, which regulates p53 stabilization via MDM2- and ubiquitin-independent proteasomal degradation, our results indicate that PIG3 suppresses MDM2-mediated p53 ubiquitination through direct association with MDM2. Our investigation advances the understanding of mechanisms regulating p53 stability and the role of PIG3 in p53-dependent apoptotic cell death.

MATERIALS AND METHODS

Cell culture and treatment

The human osteosarcoma U2OS cells and human embryonic kidney HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen). The human colorectal carcinoma HCT116 cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS, penicillin and streptomycin. The human lung carcinoma H460 cells were maintained in RPMI-1640 medium containing 10% FBS, penicillin and streptomycin. These cells were from the American Type Culture Collection (ATCC). The $p53^{-/-}MDM2^{-/-}$ mouse embryonic fibroblasts (MEFs) cells were kindly provided by Prof. Chin Ha Chung (Seoul National University, Seoul, Korea), and were maintained in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained in a humidified incubator with an atmosphere of 5% CO_2 at 37°C. Cycloheximide (CHX, protein synthesis inhibitor, Sigma, St. Louis, MO, USA) was used at a concentration of 80 μg/ml. For UV radiation, cells were exposed to light from a 254-nm UVC lamp (UVP; Model UVGL-25, Upland, CA, USA) in a minimal volume of serum-free culture medium at a 10 J/m^2.

Plasmids, PIG3 siRNA and transfection

To generate the full length PIG3 cDNA, cDNA was amplified from human fibroblast GM00637 cells by RT-PCR using PIG3 oligo primers of the following sequences: sense, 5'-accgaattcatgttagccgtgcac-3' and antisense, 5'-aatctcgagtcactggggcagttc-3'. The amplified PIG3 PCR products were cloned into pcDNA3-HA vector, and confirmed sequences and orientation were by automated DNA sequencing. pcDNA-Myc-p53, pcDNA3-MDM2 and pcDNA-His-Ubiquitin constructs were obtained from Prof. Chin Ha Chung (Seoul National University). Cells were transfected with the indicated plasmids or siRNA using TurboFect (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Knockdown of PIG3 expression was achieved by transfecting siRNA using Lipofectamine RNAiMAX (Invitrogen) as mentioned before (Lee *et al*., 2010).

Immunoprecipitation assay and western blot analysis

Cells were lysed in ice-cold RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin] and amount of protein was determined using dye-binding microassay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by 6-12% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h with TBS-t [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20] containing 5% nonfat milk and then incubated for overnight at 4°C with appropriate primary antibodies. The blots were washed four times for 15 min with TBS-t and then incubated for 1 h with peroxidase-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The blots were washed four more times with TBS-t and developed using an enhanced chemiluminescence detection system (ECL; intron). The intensity of protein bands were quantified using Scion Image software (Scion Corp., Frederick, MD, USA) For the immunoprecipitation assay, lysates were precleared with protein A-Sepharose beads (GE Healthcare, Little Chalfont, UK) prior to adding the antibody. After removing the protein A-Sepharose by centrifugation, the supernatant was then incubated at 4°C overnight with appropriate antibodies. After the addition fresh protein A-Sepharose bead, the incubation was continued for an additional one hour, and then beads were washed five times with RIPA buffer. Immunoprecipitated proteins were denatured in SDS sample buffer, boiled for 5 min and analyzed by western blotting using the appropriate antibodies.

Antibodies

All antibodies used in this study are anti-human anti-p53 polyclonal antibody, anti-phospho-p53-(Ser15) polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA); anti-α-tubulin monoclonal antibody (BD Phamingen, San Jose, CA, USA); anti-PIG3 (H300) polyclonal antibody, anti-p53 (DO-1) monoclonal antibody, anti-MDM2 (SMP14) monoclonal antibody, anti-myc polyclonal antibody, anti-HA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We followed manufacturer's protocol for dilution of all primary antibodies.

In vitro transcription and translation

In vitro transcription and translation were carried out using TNT Quick coupled Transcription and Translation system (Promega, Amedison, WI, USA). For *in vitro* translation, pcDNA-HA-PIG3, pcDNA-Myc-p53 and pcDNA3-MDM2 expression vector were used. One microgram of each construct was used per reaction of the TNT-Quick coupled reticulocyte lysate system (Promega), following the manufacturer's instructions. The synthesized proteins were used *in vitro* ubiquitination and *in vitro* protein binding assay.

In vitro protein binding assay

Synthesized PIG3 and MDM2 by TNT system was incubated in PBS buffer at 4°C for overnight. After incubation, reaction solution was added antibody for PIG3 and the precipitated with protein G-Sepharose beads (GE Healthcare). The immunoprecipitates were then subjected to Western blot analysis with anti-PIG3 or anti-MDM2 antibody.

In vitro ubiquitination assay

Synthesized p53, MDM2 and PIG3 proteins were mixed with UBE1 (E1 enzyme), UbcH5c (E2 enzyme) and Ubiquitin (Ub) in reaction solution (R&D systems, Minneapolis, MN, USA) following to the manufacturer's instructions. The mixture was incubated in 37°C for 30-60 minutes and stopped by addition of SDS-PAGE sample buffer. The reaction mixture was analyzed by Western blotting with anti-p53 antibody.

In vivo ubiquitination assay

Cells were transfected with PIG3 siRNA or indicated constructs and treated with 20 μM MG132 (Sigma) for 4 h before harvest for inhibition of proteasome-mediated protein degradation. After 48 h, the cells were harvested and split into two aliquots, one for immunoblot and the other for ubiquitination assays. For ubiquitination assay, cells were lysed in RIPA buffer and then perform immunoprecipitation with anti-p53 or MDM2 antibodies, and the immunocomplexes were detected with anti-ubiquitin antibody. For NI-NTA pull down assay, cells were lysed in buffer I [6 M guanidinium-HCl, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L β-mercaptoethanol] and incubated with Ni-NTA beads at room temperature for 4 h. Beads were washed once each with buffer I, buffer II [8 mol/L urea, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L β-mercaptoethanol], and buffer III [8 mol/L urea, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl (pH 6.3), 10 mmol/L β-mercaptoethanol]. Proteins were eluted from the beads in buffer IV [200 mmol/L imidazole, 0.15 mol/L Tris-HCl (pH 6.7), 30% (v/v) glycerol, 0.72 mol/L β-mercaptoethanol, and 5% (w/v) SDS]. Eluted proteins were analyzed by immunoblot with monoclonal anti-p53 or anti-MDM2 antibodies.

Statistical analysis

Data in all experiments are represented as mean ± SD. Statistical comparisons were carried out using two-tailed paired *t*-test. We considered $p<0.01$ (indicated** in figures) as significant. Analyses were carried out with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) and Excel (Microsoft, Redmond, WA, USA).

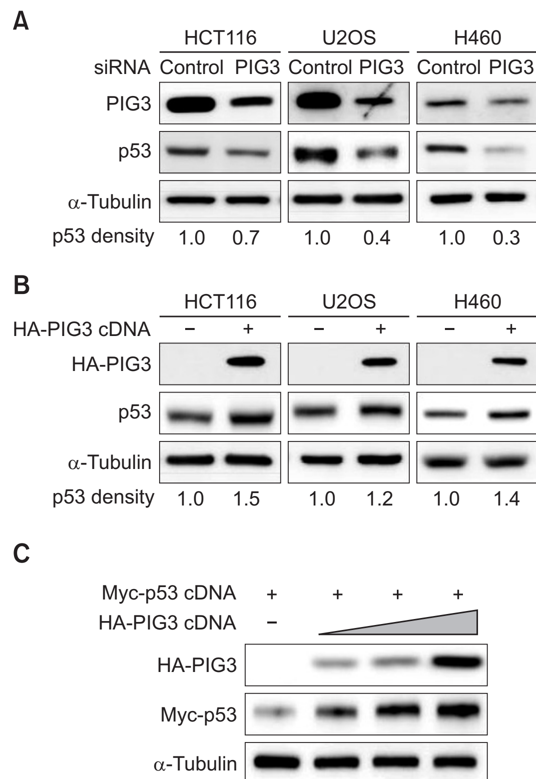

Fig. 1. PIG3 affects the stability of p53. (A) HCT116, U2OS, and H460 cells were transfected with control siRNA or PIG3 siRNA. At 48 h after transfection, the levels of endogenous PIG3 and p53 were analyzed by Western blotting. α-tubulin was used as a positive control. Relative p53 expression levels are reported below each panel. (B) HCT116, U2OS, and H460 cells were transfected with either control vector or the HA-PIG3 expression vector. At 48 h after transfection, the levels of HA-PIG3 and p53 were analyzed by Western blotting with the indicated antibodies. Relative p53 expression levels are reported below each panel. (C) HEK293 cells were co-transfected with Myc-p53 and an increasing expression of the HA-PIG3 plasmid and harvested 48 h after transfection for Western blotting with antibodies as indicated.

RESULTS

PIG3 controls the stability of p53

To explore the role of PIG3 in p53 stability, we started by investigating the effects of a PIG3 knockdown on the expression of p53 protein. To this end, we used a PIG3-specific siRNA to deplete protein levels in three different cell lines that contain endogenous wild-type p53: HCT116 colorectal carcinoma, U2OS osteosarcoma, and H460 lung carcinoma cells. The expression of PIG3 was reduced by approximately 70% in cells transfected with PIG3-specific siRNA as compared to control siRNA-transfected cells (Fig. 1A, first row). We then measured p53 protein levels and observed that lower levels of PIG3 corresponded to lower levels of endogenous p53 (Fig. 1A, second row, and quantification below). The same three cell lines were transiently transfected with a vector expressing HA-tagged PIG3 such that PIG3 would be overexpressed. p53 expression levels were higher in PIG3-expressing cells as compared to the vector controls (Fig. 1B). Moreover, when Myc-tagged p53 and HA-tagged PIG3 were co-transfected

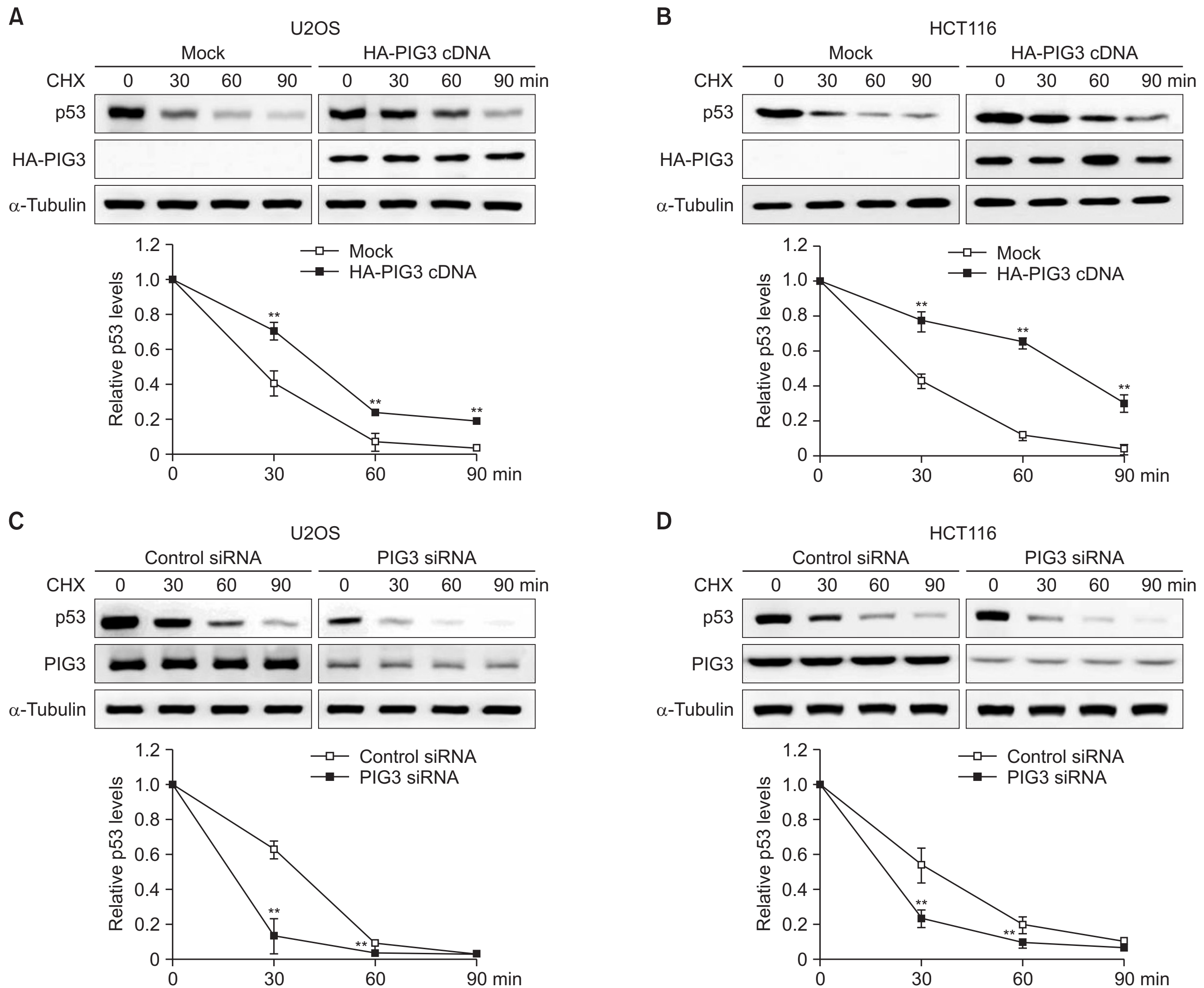

Fig. 2. PIG3 regulates the half-life of endogenous p53. (A, B) U2OS (A) or HCT116 (B) cells were transfected with either control vector or HA-PIG3 expression vector, treated with 80 μg/ml cycloheximide (CHX) at 48 h after transfection and harvested at the indicated time points for Western blotting with antibodies against p53, PIG3 and α-tubulin. Relative p53 levels at different time point were plotted in lower panel. Relative values represent the mean ± SD. ***p*<0.01. (C, D) U2OS (C) or HCT116 (D) cells were transfected with control siRNA or PIG3 siRNA, treated with CHX at 48 h after transfection and harvested at the indicated time points for Western blotting using the indicated antibodies. Relative p53 levels at different time point were plotted in lower panel. Relative values represent the mean ± SD. ***p*<0.01.

into HEK293 cells, there was a significant increase in p53 protein levels when we observed an increase in PIG3 expression levels (Fig. 1C). These results support the idea that PIG3 contributes to p53 stability under normal conditions.

PIG3 inhibits p53 proteasomal degradation

Proteasome-mediated degradation is important for the turnover of many cellular proteins, including p53 (Burger and Seth, 2004). To determine whether the observed increase in p53 levels in the presence of PIG3 was due to protein stabilization or new protein synthesis, p53 levels in both control and PIG3-expressing U2OS and HCT116 cells were measured with and without exposure to a protein synthesis inhibitor. Cells were treated with cycloheximide for 0, 30, 60, and 90 minutes, and p53 levels were measured using Western blot analysis. The half-life of p53 increased 2-3-fold in cells overexpressing PIG3 (Fig. 2A, 2B). We then measured the half-life of p53 in PIG3 knockdown cells following the same protocol. As shown in Fig. 2C, 2D, in PIG3 knockdown cells, p53 levels rapidly decreased after cycloheximide treatment, suggesting that PIG3 promotes p53 stability by inhibiting proteasomal degradation.

PIG3 suppresses MDM2-mediated p53 ubiquitination

One mechanism by which PIG3 could have stabilized p53 protein levels was to inhibit ubiquitination. To test this possibility, we measured levels of ubiquitinated p53 in HCT116 cells with and without overexpression of PIG3. At 48 h after transfection with the HA-PIG3 expression vector, cells were either directly lysed to measure p53 and PIG3 protein levels by Western blotting, or were first treated with the proteasome inhibitor MG132 prior to lysis and immunoprecipitation with a p53 antibody. Overexpression of PIG3 suppressed the ubiqui-

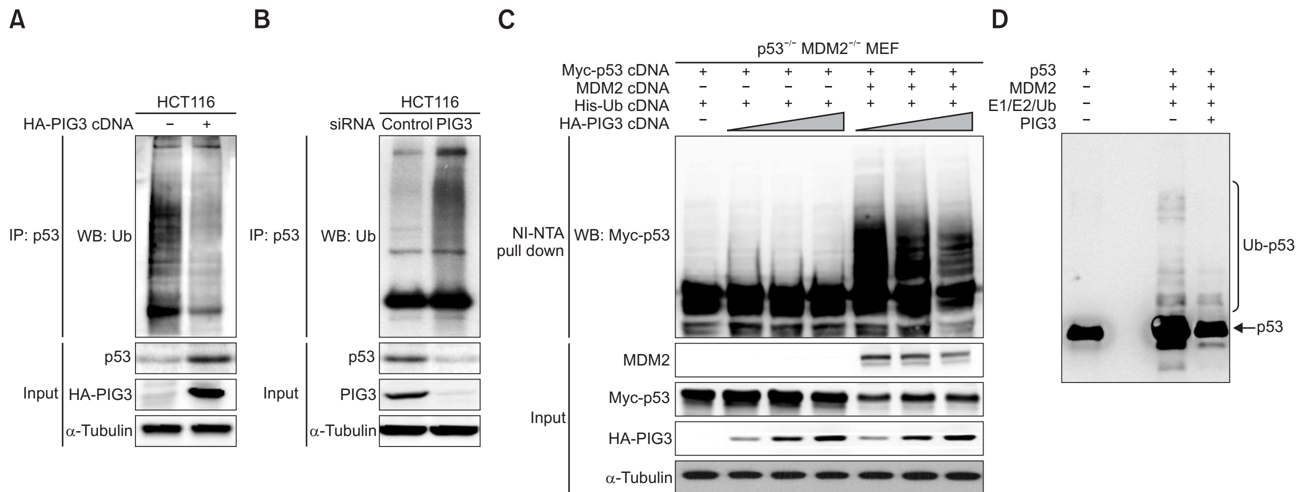

Fig. 3. PIG3 inhibits MDM2-mediated p53 ubiquitination *in vivo* and *in vitro*. (A) HCT116 cells were transfected with either control vector or HA-PIG3 expression vector. At 48 h after transfection, cells were treated with 20 μM MG-132 for 4 h before harvesting. Cell lysates were subjected to immunoprecipitation with anti-p53 antibody, followed by Western blotting with anti-ubiquitin antibody. The expression levels of p53 and HA-PIG3 are shown in the lower panels. (B) PIG3 knockdown decreases p53 stability in HCT116 cells. The same assay as shown in panel (A) was performed, except that control and PIG3 siRNA were used. (C) p53$^{-/-}$ MDM2$^{-/-}$ MEF cells were transfected with a combination of plasmid encoding Myc-p53, MDM2, His-Ub, and an increasing amount of the HA-PIG3. At 48 h after transfection, the cells were subjected to a pulldown using Ni-NTA bead, followed by immunoblotting using the anti-Myc antibody. The expression of MDM2, Myc-p53 and HA-PIG3 was shown in lower panels. (D) PIG3 inhibits MDM2-mediated p53 ubiquitination *in vitro*. The *in vitro* ubiquitination experiment was conducted as described in Materials and Methods, and analyzed by immunoblotting using anti-p53 antibodies.

tination of p53, as observed by a decrease in the population of high molecular weight p53 protein levels (Fig. 3A). Conversely, in PIG3 knockdown cells, there was an increase in p53 ubiquitination (Fig. 3B).

MDM2 is a key regulator of p53 because it can both inhibit p53 transcriptional activity and target it for degradation (Haupt *et al*., 1997; Kubbutat *et al*., 1997). We therefore explored whether the PIG3-mediated increase in p53 protein levels was caused by an inhibition in MDM2-mediated ubiquitination and degradation of p53. To test this hypothesis, an *in vivo* ubiquitination experiment was set up in which p53$^{-/-}$ MDM2$^{-/-}$ MEF cells were co-transfected with constructs expressing His-tagged Ub, MDM2, Myc-tagged p53, and increasing doses of HA-tagged PIG3. The level of ubiquitinated p53 was determined by Ni-NTA purification of proteins conjugated to His-Ub followed by immunoblotting with an anti-Myc antibody. As shown in Fig. 3C, ubiquitination of p53 was higher in the presence of MDM2, as expected. However, ubiquitination was significantly lower when PIG3 was co-expressed. This effect was dose-dependent, meaning that increasing amounts of PIG3 corresponded to decreased ubiquitination of p53. We confirmed this same result *in vitro* by combining recombinant UBE1 (E1), UbcHc5 (E2), MDM2, p53, and Ub in the presence or absence of PIG3. The results of this assay indicated that in the presence of MDM2 and the absence of PIG3, there was greatly stimulated ubiquitination of p53 (Fig. 3D). However, when PIG3 was added, MDM2-mediated ubiquitination was drastically suppressed. These results indicate that PIG3 directly suppresses MDM2-mediated p53 ubiquitination.

PIG3 binds directly to MDM2 and promotes MDM2 ubiquitination

Because other MDM2 binding proteins are also involved in the regulation of p53 degradation, our next question was whether PIG3 associates directly with MDM2 or acts indirectly through MDM2 binding proteins. Extracts of HCT116 cells, which endogenously express PIG3, were immunoprecipitated using an anti-PIG3 antibody and Western blot analyses revealed that MDM2 co-purified with PIG3 (Fig. 4A). We then looked for the same effect *in vitro* by premixing synthesized PIG3 with MDM2 and subjecting the precipitates to Western blot analysis. Immunoprecipitation with an anti-PIG3 antibody immunoprecipitated MDM2 from the PIG3-MDM2 mixture (Fig. 4B), suggesting a direct interaction between these two proteins.

Knowing that PIG3 and MDM2 interact directly, we next asked whether PIG3 regulates protein levels of MDM2. Our results showed that ectopic expression of PIG3 in HCT116, U2OS, or H460 cells led to reduced MDM2 (Fig. 4C), whereas the downregulation of PIG3 increased MDM2 levels (Fig. 4D). Because MDM2 is a negative regulator of p53, we hypothesized that the role of PIG3 is to regulate p53 stability and function by counteracting the E3 ubiquitin ligase activity of MDM2. We tested this by comparing MDM2 ubiquitination levels in HCT116 cells 48 h after transfection with either control siRNA or PIG3 siRNA. Endogenous MDM2 ubiquitination was lower in PIG3 knockdown cells (Fig. 5A) and higher when PIG3 was over-expressed (Fig. 5B). To further confirm these results, we introduced combinations of ectopic His-tagged Ub, MDM2, and HA-tagged PIG3 into p53$^{-/-}$ MDM2$^{-/-}$ MEF cells. As shown in Fig. 5C, ectopic expression of PIG3 indeed promoted MDM2 ubiquitination in a dose-dependent manner. Collectively, these data suggest that PIG3 interacts with MDM2, which in turn enhances the level to which MDM2 is ubiquitinated and is subject to degradation.

PIG3 regulates p53 phosphorylation

To demonstrate the physiological impact of PIG3 on the p53

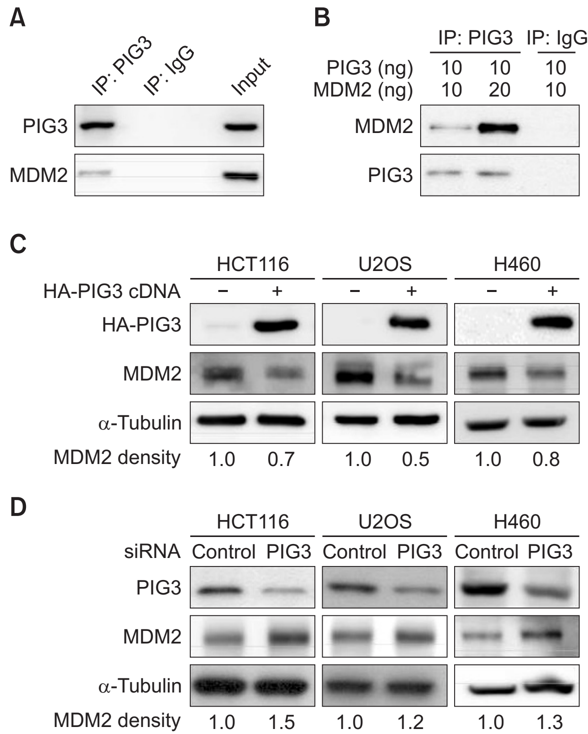

Fig. 4. PIG3 binds to MDM2 and regulates MDM2 stability. (A) HCT116 cell lysates were immunoprecipitated with anti-PIG3 antibody and the immunoprecipitated proteins were probed with anti-MDM2 and anti-PIG3 antibodies. (B) Immunoprecipitation of human PIG3 from a mixture of PIG3 and MDM2. The precipitates were subjected to immunoblotting using anti-MDM2 antibody. The levels of precipitated PIG3 were assessed using a human anti-PIG3 antibody. (C, D) HCT116, U2OS, and H460 cells were transfected with either control vector or HA-PIG3 expression vector (C), or with either control siRNA or PIG3 siRNA (D). At 48 h after transfection, the levels of HA-PIG3 and p53 were analyzed by Western blotting. α-tubulin was used as a positive control. Relative MDM2 expression levels are reported below the panels.

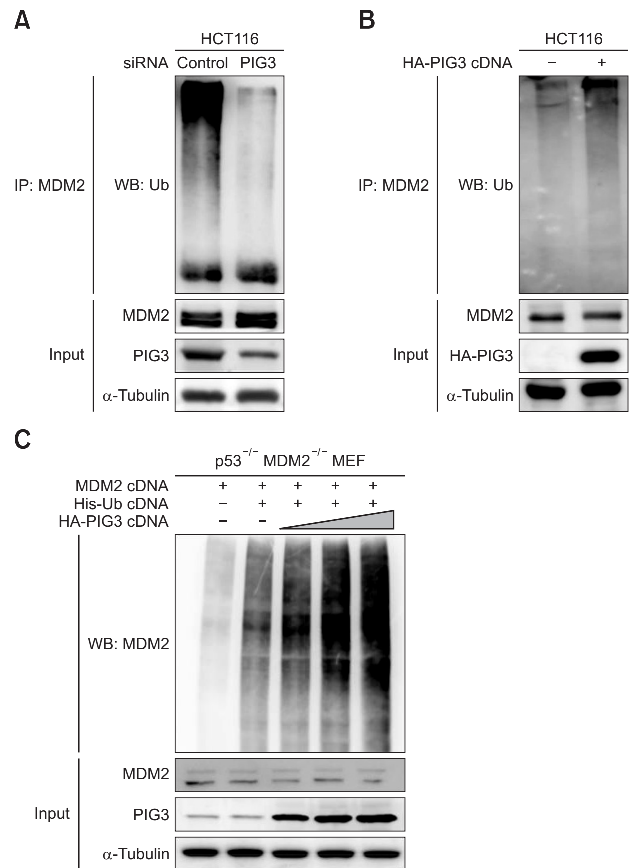

Fig. 5. PIG3 promotes MDM2 ubiquitination. (A, B) HCT116 cells were transfected with either control siRNA or PIG3 siRNA (A) or with either control vector or HA-PIG3 expression vector (B). At 48 h after transfection, cells were treated with 20 μM MG-132 for 4 h before harvesting. Cell lysates were subjected to immunoprecipitation with anti-ubiquitin antibody, followed by Western blotting with anti-MDM2 antibody. The expression levels of MDM2 and PIG3 are shown in the lower panels. (C) $p53^{-/-}$ $MDM2^{-/-}$ MEF cells were transfected with a combination of plasmids encoding MDM2, His-Ub, and an increasing expression of the HA-PIG3. The level of MDM2 ubiquitination was determined by Ni-NTA purification, followed by immunoblotting using the anti-MDM2 antibody.

ubiquitination pathway, we examined the role of PIG3 in the p53 phosphorylation in response to DNA damage. To test this, p53-proficient HCT116 cells were transfected with either control or PIG3 siRNA and then exposed to 10 J/m^2 UV to induce DNA damage. The control cells showed a significant increase in p53 expression levels and p53 phosphorylation at Ser15 in a time-dependent manner, reaching a maximum at 3 h after UV irradiation (Fig. 6A). In contrast, UV-induced p53 expression was markedly lower in PIG3-knockdown cells, indicating that PIG3 indeed contributes to p53 stability and activation in response to DNA damage. Taken all together, these results indicate that after DNA damage has occurred, p53 induces the expression of PIG3, which then enhances MDM2 ubiquitination, further increasing the amount of cellular p53 protein, and thereby promoting p53 activation (Fig. 6B).

DISCUSSION

In the present study, we have identified and validated PIG3 as a novel regulator of the p53 degradation that typically occurs in the MDM2-p53 pathway. Our studies not only identify a novel participant in this pathway, but also suggest a mechanism by which p53 activation occurs in response to DNA damage.

p53 is often referred to as the ‘cellular gatekeeper’ or the ‘guardian of the genome,’ and its importance is substantiated by the discovery of p53 mutations in >50% of all human tumor cells (Levine, 1997). The stabilized and activated p53 that responds to DNA damage transactivates a large battery of genes that can mediate apoptosis, DNA repair, or multiple points of cell cycle arrest (Levine and Oren, 2009). Assuming that p53 successfully carries out these functions, a subsequent mechanism for returning p53 to basal levels is required. Therefore, careful regulation of p53 levels is critical for both proper response of the cell to a stressed state and appropriate return of the cell to a normal state. In the present study, we observed that the PIG3 protein regulated p53 degradation. A knockdown of PIG3 accelerated intracellular p53 degradation, and decreased the half-life of p53 in several different p53-proficient human cell lines. Moreover, p53 steady-state level and

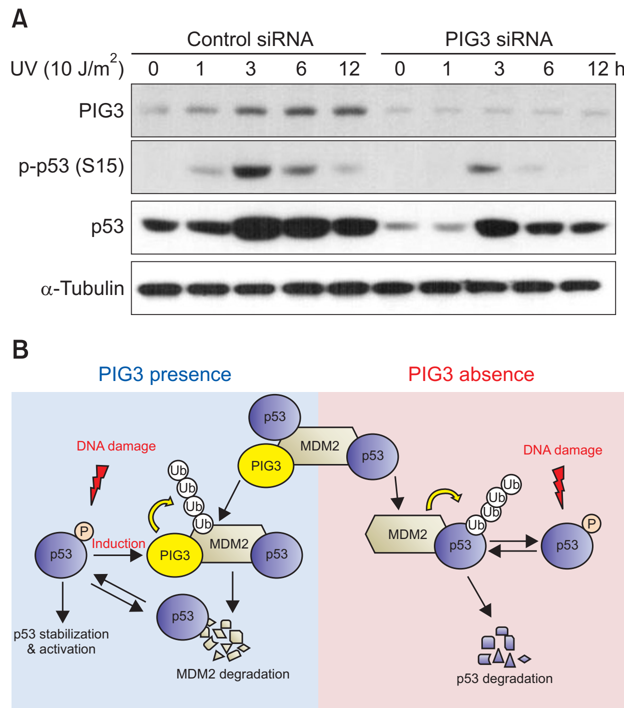

Fig. 6. A PIG3 knockdown inhibits UV-induced p53 phosphorylation in HCT116 cells. (A) Control siRNA- and PIG3 siRNA-transfected HCT116 cells were irradiated with 10 J/m^2 UV, and whole-cell lysates were prepared at the indicated times. Western blot analysis was carried out using anti-PIG3, anti-p53, anti-p53-P (Ser15) and α-tubulin antibodies. (B) Model recapitulation the role of PIG3 in regulating of p53 stability in response to DNA damage.

half-life were significantly enhanced in PIG3-overexpressed cells. We further demonstrated that the effect of PIG3 on the level of p53 protein is due to a change in the ubiquitination state of p53, suggesting that PIG3 acts by suppressing the degradation of p53.

MDM2 is an ubiquitination E3 ligase that promotes ubiquitination of itself, p53, and several other cellular proteins, including androgen receptor, Tip60, glucocorticoid receptor, and the MDM2 homolog MDMX (de Graaf *et al*., 2003; Kawai *et al*., 2003; Pan and Chen, 2003). Ubiquitination by MDM2 is a primary mechanism by which p53 levels are regulated in the cell. Additionally, MDM2 can inactivate p53 by directly concealing the p53 transactivation domain, inhibiting p53 acetylation by coactivators, and promoting p53 deacetylation by recruiting histone deacetylases (Kobet *et al*., 2000; Ito *et al*., 2001, 2002). Although some of these functions can be carried out by MDM2 alone, cooperation with other MDM2-interacting proteins is likely as well. Here we reveal that PIG3 is a new player in the MDM2-p53 pathway. Supporting this statement are several lines of evidence. First, PIG3 interacted directly with MDM2 both *in vivo* and *in vitro*, and formed a PIG3-MDM2 complex. Moreover, PIG3 inhibited MDM2-mediated p53 ubiquitination and degradation. In addition, PIG3 influences the levels of MDM2 protein, as evidenced in two ways: ectopic expression of PIG3 led to a decrease in MDM2 expression and a knockdown of PIG3 increased MDM2 expression. Finally, PIG3 promoted ubiquitination of MDM2 itself.

Several cellular proteins that regulate the MDM2-p53 pathway have been identified; these include p14^{ART} (Pomerantz *et al*., 1998), YY1 (Sui *et al*., 2004), MDMX (Francoz *et al*., 2006), DAXX and HAUSP (Tang *et al*., 2006), PA28γ (Zhang and Zhang, 2008), and a number of ribosomal proteins (Zhang *et al*., 2013; Zhou *et al*., 2013). This large number of regulatory molecules highlights the complexity of the MDM2-p53 pathway. Why would mammalian cells need so many proteins to overcome the negation of p53 by MDM2? One possibility might be that MDM2 functions as a multiple subunit complex, such as a homohexamer (Poyurovsky *et al*., 2007; Uldrijan *et al*., 2007), to inactivate p53 in cells. Thus, individual proteins would need to work together or to independently bind to different subunits in the homohexameric MDM2 complex in order to efficiently inactivate this protein and consequently activate p53. Alternatively, different MDM2-binding proteins may act either sequentially or in response to different signals when inactivating MDM2 in response to stresses. The identification of PIG3 as regulator of MDM2 stability suggests that PIG3 is a new important player in maintenance of cellular p53 levels. When PIG3 is present, MDM2 cannot initiate p53 ubiquitination, but when PIG3 is absent, p53 levels remain under stringent control of MDM2 (Fig. 6B). This mechanism could be an advantage to the cells because it would circumvent the need for elevated levels of MDM2.

Apoptosis is a vital process for tissue homeostasis that is frequently disturbed in several pathological conditions (Fernald and Kurokawa, 2013). PIG3 is believed to be one of the major factors involved in p53-induced apoptosis through ROS generation (Polyak *et al*., 1997). However, because PIG3 expression alone is insufficient to induce apoptosis (Polyak *et al*., 1997), and because PIG3 knockdown cannot efficiently induce apoptosis in response to DNA damage (Lee *et al*., 2010), it is assumed that several other factors cooperate to cause apoptotic cell death. p53 plays a critical role in initiating an early response to genotoxic stress (Lane, 1992; Levine and Oren, 2009). It has an arsenal of target genes at its disposal and may even possess some selectivity towards a particular fate. For example, with the assistance of ASPP proteins, p53 exhibits a striking preference for the promoters of pro-apoptotic genes (Samuels-Lev *et al*., 2001). However, the initial activation of its function as a transcription factor is key to its ability to drive these particular downstream pathways. The gene encoding PIG3 is induced by p53 prior to the onset of apoptosis (Polyak *et al*., 1997). Thus, after initial expression and activation of PIG3 in response to DNA damage, a role for PIG3 might be to amplify p53-mediated apoptotic signals via the suppression of p53 degradation. The data presented here show that phosphorylation of p53 at Ser15 in response to UV was significantly decreased in PIG3 knockdown cells as compared to control cells. Together these results suggest that PIG3 is important in the amplification of p53 signaling in response to DNA damage.

In conclusion, our study brings to light an important role for PIG3 in the positive regulation of intracellular p53 levels. By promoting degradation of MDM2, and thus the stabilization of p53, PIG3 may be important in the early stages of the p53-mediated cellular response to genotoxic stress.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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