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PIG3 regulates the stability of p53 via Mdm2

조선대학교 대학원

의 학 과

김 명

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pig3 에 의한 p53 stability 연구

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ABSTRACT

PIG3 regulates the stability of p53 via MDM2

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The tumour suppressor p53 induces apoptosis or cell-cycle arrest in response to genotoxic and other stresses. One of the most important regulators of p53 is mouse double minute 2 (Mdm2), a RING domain E3 ligase that ubiquitinates p53, leading to both proteasomal degradation and relocation of p53 from the nucleus to the cytoplasm1. The p53-inducible gene 3 (Pig3) is a transcriptional target of p53 and is thought to play a role in apoptosis, but its other functions are not clear now. In this study, we showed that knocking down Pig3 by siRNA accelerated p53 and Mdm2 degradation, and decreased the half-life of wild-type p53 in several of the human cell lines; overexpression of PIG3 inhibited ubiquitination of exogenous p53 in a dose-dependent manner, whereas knocking down Pig3 by siRNA enhanced ubiquitination of endogenous p53. Using a coimmuno-precipitation assay, the interaction between endogenous PIG3 and Mdm2 and p53 was detected, it indicated that PIG3 binded with Mdm2 in normal condition, and released after UV treatment, but PIG3 binded with p53 after UV treatment. And furthermore, PIG3 and Mdm2 competitve binded with p53 in vitro. To confirm the function of PIG3 in the p53

system, the effect of PIG3 on p53-mediated apoptosis was examined. FACS and Western Blotting results showed that downregulation of PIG3 strongly decreased UV-induced apoptosis in HCT116. These results suggest that as the transcriptional target of p53, PIG3 regulated p53 stability via it and Mdm2 competitive binding with p53, which decreaed the ubiquitinatin of p53; and PIG3 promote apoptosis in p53-dependent manner, it is a feedback loop.

Keywords: PIG3; p53; stability; ubiquitination

Ⅰ. INTRODUCTION

The tumour-suppressor protein p53 is important in the cellular response to genotoxic damage. In non-stressed cells, p53 is kept silent and at low levels after association with Mdm2, which represses transcription when associated with p53 and promotes its proteolytic degradation(1–4). Because *mdm2* is a p53 target gene, both proteins are mutually regulated in a negative feedback loop(5). Adverse agents such as irradiation with UV and γ-rays, withdrawal of growth factors, hypoxia and oncogenes such as Ras induce signalling events that result in the transient stabilization of the p53 protein and the onset of p53 function as a transcriptional activator or repressor within the cell nucleous (6,7). The induction of p53 involves several mechanisms including post-translational modifications such as phosphorylation and acetylation(8). DNA damage- induced phosphorylation of serines and threonines within the p53 amino terminus contributes to p53 stability by preventing Mdm2 from binding and by rendering p53 more resistant to Mdm2 (refs 9–11). Five serines in the carboxy-terminal portion and seven serines and one threonine within the N-terminal 46 amino acids are known to be inducibly phosphorylated(12), but the exact order of phosphorylations and their specific contribution to p53 effector function remain to be elucidated. For example, Ser 6, Ser 9, Ser 15, Ser 20 and Ser 37 are phosphorylated in response to DNA damage, probably by protein kinases including ATM, Chk2, DNA-PK and ATR(13–16). Phosphorylation of Ser 15 is observed only in response to DNA damage(17), but not after p53 activation in response to E1A expression(18), showing that the specific roles of individual phosphorylation sites depend on the stimulus and probably the cell type. In addition, phosphorylation of Ser 46 is functionally important, because

mutation of this site within the p53 protein decreases sensitivity towards UV-induced apoptosis(19,20). Acetylation of the p53 protein occurs in response to many different p53-activating stimuli *in vivo(*21,22). Lys 373 and Lys 382 are inducibly acetylated by cAMP-responement-binding (CREB)- binding protein (CBP) and the closely related p300 protein(21,23), whereas Lys 320 is acetylated *in vitro* by the acetylase P/CAF (p300/CBP-associated factor) (24). Acetylation stimulates DNA-binding activity of p53 *in vitro* and thus enhances p53-dependent gene expression(21,23), p53-mediated apoptosis and growth arrest(25), whereas overexpression of a dominant-negative form of p300 counteracts p53 function.

The activation of p53 not only involves post-translational modifications and protein stabilization, but is also linked to localization into distinct subnuclear structures: expression of the promyelocytic leukaemia (PML) protein isoform PML3, Rasderived signals, γ-irradiation or treatment of cells with UV radiation and As2O3 lead to the recruitment of p53 into PML nuclear bodies (PML-NBs)(26–29).

The levels and activity of p53 are controlled largely by MDM2, the product of a p53- inducible gene. MDM2 can bind to p53 and promote its ubiquitination and subsequent degradation by the proteasome. MDM2 was initially identified as an amplified oncogene in murine cell lines (30) and in sarcomas(31) and was subsequently found to act as a ubiquitin ligase promoting proteasome dependent degradation of p53(32-34). MDM2 is also a transcriptional target of p53 (35-37), such that p53 activity controls the expression and protein level of its own negative regulator, providing for an elegant feedback loop. MDM2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein(38,39). The MDM2–p53 complex

also inhibits p53 mediated transactivation (40).

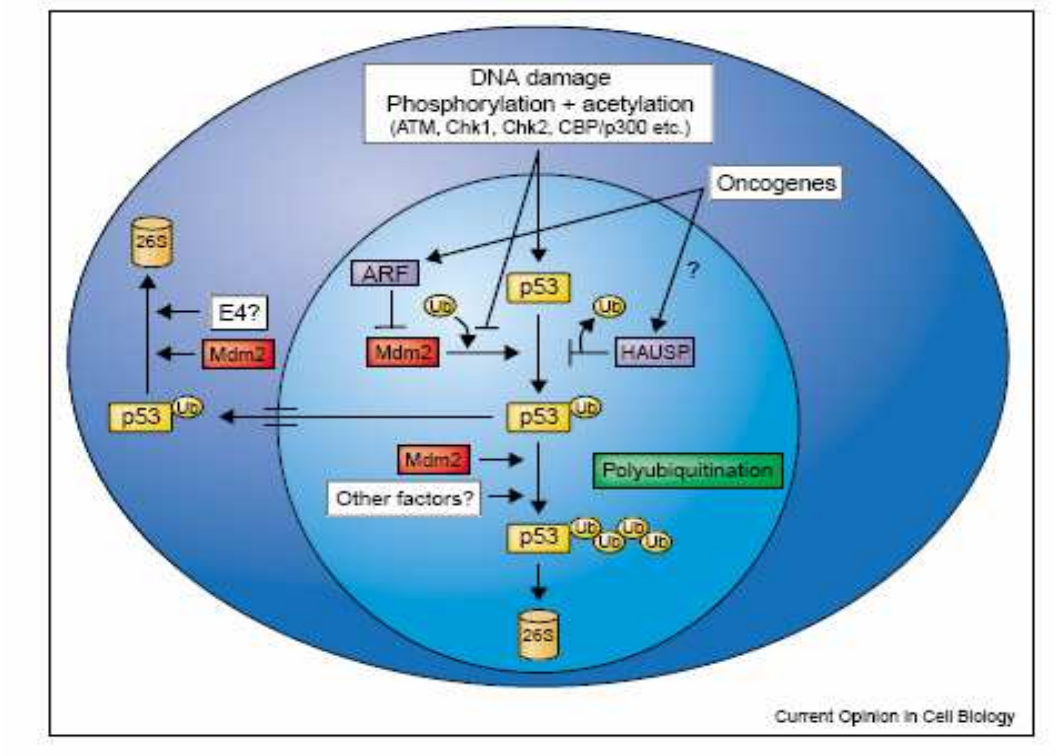

Fig.1 Mdm2-depedent mechanism of p53 stabilization. Several mechanisms for regulating p53 protein levels are depicted here. p53 is ubiquitinated within the nucleus by Mdm2 and possibly other factors, which leads to its degradation by the 26S proteosome either in or out of the nucleus. DNA damage-induced upstream factors can block this cascade of events by targeting Mdm2 and p53. ARF and HAUSP represent antagonists of Mdm2 in this process. The mechanism of oncogenic activation of HAUSP and the role of other factors including E4 ligases in Mdm2-mediated ubiquitination of p53 are yet to be elucidated. 26S, 26S proteosome; ATM, ataxia telangiectasia mutated; Chk, checkpoint kinases; E4, E4 ligases; Ub, ubiquitination.

Apoptosis is a vital process for tissue homeostasis that is frequently disturbed in several pathological conditions, including cancer. It has been shown that p53-dependent apoptosis involves the induction of a series of genes called PIGs (p53-inducible gene(s)) (41). These genes participate in this process through reactive oxygen species (ROS) modulation Interestingly, for one of them, assigned as PIG3 (41) , induction has been shown to be mediated by p53 mainly through a microsatellite present in its 5'-upstream regulatory region and not through a classical El-Deiry consensus sequence (42) . This microsatellite acquired its polymorphic structure and p53-dependent functional ability to induce this gene, recently in the evolutionary history of biological species (43) . Specifically, although it is present in lower primates its full length and function appears only in *Hominoidea* (apes and humans).

In humans this microsatellite represents a pentanucleotide (TGYCC)*n* polymorphism, where Y = C or T, and with four alleles of 10, 15, 16 and 17 repetitions (42) . Furthermore, the extent of transcriptional activation of *PIG3* by p53 correlates directly with the number of repeats. Given that *PIG3* is associated with reactive oxygen species-dependent apoptosis (41), the recent evolutionary control of this gene by p53 raises two important points, as Contente et al. comment (43). First, they suggest that the evolutionary appearance of this polymorphism represents a continuous adaptation of tumor suppressor mechanism(s), and second, they draw caution on the comparative interpretation of gene expression results from animal models and human cancers. However, what is not clear yet, is first if there is any potential mechanism(s) by which this gene may be implicated in human carcinogenesis, and second what is its exact role during tumorigenesis.

As previously hypothesized, the nature of this polymorphism indicates two possible

ways that may account for *PIG3* involvement in tumorigenesis (42). In neoplasias, as it is well known, microsatellites are frequently prone to alterations emerging from instability at these loci. Microsatellite instability (MI) usually involves a change in the number of repetitions at a polymorphic locus in the neoplastic areas. Therefore, any aberrations affecting the structural integrity of this polymorphism should affect the ability to induce *PIG3* by p53 and maybe consequently the ROS-dependent apoptotic process. A second mechanism that is cancerprone represents the genetic constitution of an individual. Taking into consideration that alleles with low number of repetitions (specifically the 10 repetitions one) are less able to induce PIG3 expression by p53 (42), it is expected that combination(s) of such alleles provide an increased cancer risk for an individual. Therefore, cancer patient cohorts may have a different frequency distribution as compared to control cases, with low repetition alleles expected to have a higher penetrance.

In this study, We have now investigated the relationship between PIG3 and p53. Our results suggest that as the transcriptional target of p53, PIG3 regulated p53 stability via it and Mdm2 competitive binding with p53, which decreaed the ubiquitinatin of p53; and PIG3 promote apoptosis in p53-dependent manner, it is a feedback loop.

Ⅱ. MATERIALS AND METHODS

1. Maintenance of Cell Lines

HEK 293 (Coriell Institute for Medical Research, Camden, NJ) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA). Human breast cancer epithelial cell lines MCF-7 and (Coriell Institute for Medical Research, Camden, NJ) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA). U20s was cultured in McCoys 5A medium/10% FCS. HCT116(p53+/+) were maintained in IMDM medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified incubator in an atmosphere containing 5 % CO_2 at 37 ^{o}C.

2. Plasmid Constructs of PIG3

uman PIG3 cDNA was amplified by RT-PCR using the PIG3 oligo primer: sense 5'-ATGTTAGCCGTGCACTTTGACAA-3' and antisense 5'-TCA CTG GGG CAG TTC CAG GAC-3' from human fibroblast GM00637 cells. The amplified PIG3 cDNA construct was inserted into a pcDNA3.1/ mammalian expression vector driven by the CMV promoter (Invitrogen Life Technologies), and confirmed the DNA sequence and orientation.

3. PIG3-siRNA design, synthesis and transfection

Three target sites within human OGG1 genes were chosen from the human PIG3 mRNA sequence (Gene Bank accession number NC_000002), which was extracted from the NCBI Entrez nucleotide database. After selection, each target site was searched with NCBI BLAST to confirm the specificity only to the human PIG3. The sequences of the 21-nucleotide sense- and antisense-RNA are as follows: PIG3-siRNA, 5'-AAA UGU UCA GGC UGG AGA CUA-3' (sense) and 5'-UAG UCU CCA GCC UGA ACA UUU-3'(antisense) for the PIG3; LacZ siRNA, 5'-CGUACG-CGGAAUACUUCGAUU-3' (sense), 5'-AAUC GAAGUAUUCCGCGUACGUU-3' (antisense) for the LacZ gene. These siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. LacZ siRNA was used as the negative control. Cells were transiently transfected with siRNA duplexes using Oligofectamine (Invitrogen). The siRNA expression vector(pSilencer hygro) for hOGG1 and a control vector were employed. The construction of siRNA-expression plasmid was base on the pSilencer hygro vector(Ambion,Texas,USA). The vector included a human U6 promoter, a hygromycin resistance gene. We purchased synthetic oligo-nucleotides(Xenotech, Korea). After anneling, DNA fragments were ligated into the pSilencer hygro. Cells were transfected with the siRNA vector by using Lipofectamine(Invitrogen. Carlsbad.CA). After transfection with the hygromycin-resistance vector, resistant colonies were grown in the presence of Hygromycin(100ug/ml)(Invitrogen. Carlsbad.CA).

4. Other Plasmids

myc3-p53, HDM2 and His6-ubiquitin from weregifts from Chin Ha Chung, NRL of Protein Biochemistry, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea.

5. Western blot analysis

The cell were washed with phosphate-buffered saline(PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added protease Inhibitor Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 20ug of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBST and then incubated with appropriate primary antibodies in TBST at 4℃ overnight. All antibodies used in this study are anti-human anti-p53 pAb, anti-p53-P(Ser15) pAb, anti-p53-P(Ser20) pAb , anti-cleaved caspas-3 pAb, anti- cleaved caspas-7 pAb, anti-cleaved caspas-9 pAb, (Cell Signaling Technology, Danvers, MA); anti-p21 monoclonal antibody (mAb) (BD Phamingen, San Jose, CA); anti-Noxa mAb (Calbiochem, Darmstadt,

Germany); anti-a-tubulin mAb (BD Phamingen, San Jose, CA); anti-PIG3(N-20), anti-Bax, anti-Bcl2 (Santa Cruz Biotechnology, Santa Cruz, CA). We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 2 hours at room temperature , and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

6. Nuclear-cytoplasmic fractionation

Cells were harvested and resuspended in 800 μl buffer A (25 mM Tris−HCl at pH 8.0, 10 mM KCl, 1 mM DTT and 0.5 mM PMSF) supplemented with complete protease inhibitor cocktail (Boehringer Mannheim). After incubation on ice for 15 min, 50 μl of 10% Nonidet P40 was added to the cells and vortexed for 10 sec. After centrifugation at 1,400*g* for 30 sec, the supernatants were collected and subsequently referred to as cytoplasmic extracts. The pellet was resuspended in 100 μl of ice-cold buffer C (50 mM Tris−HCl at pH 8.0, 400 mM NaCl, 1 mM DTT and 1 mM PMSF) supplemented with complete protease inhibitor cocktail (Roche). After centrifugation at 15,000*g* for 30 min, the supernatants were collectd and subsequently referred to as nuclear extracts.

7. Immunoprecipitation (IP)

Whole cell lysates were made in lysis buffer (50 mM HEPES at pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 100 mM NaF, 1 mM PMSF, 1 mM DTT, 1× complete protease cocktail and 10% glycerol) and pre-cleared with protein A−G-coupled Sepharose

beads for 2 h. The lysates were then immunoprecipitated with the indicated antibodies and isotype-matched control antibodies plus protein A−G Sepharose for at least 4 h or overnight. Beads were washed four times with lysis buffer, once with ice-cold PBS and boiled in 2× loading buffer. Protein samples were resolved by SDS−PAGE and transferred onto nitrocellulose membrane, which was blocked in 5% skim milk in PBST and probed with the indicated antibodies. The following antibodies were used for immunoprecipitation and western blotting: Human Mdm2 immunoprecipitation, Ab-1 and Ab-4 (Oncogene, San Diego, CA); human Mdm2 western blotting, SMP-14 (Santa Cruz Biotechnology, Santa Cruz, CA); p53 western blotting and immunoprecipitation, DO-1 or FL-393 (Santa Cruz); PIG3 immunoprecipitation and western blotting, H-300 and N-20(Santa Cruz Biotechnology, Santa Cruz, CA).

8. In Vivo Ubiquitination Assay

In vivo ubiquitination assays were conducted as previously described (33). Briefly, HEK 293 was transfected with combinations of the following plasmids as indicated in the figure legends: His6-ubiquitin (0.8 ug), myc3-p53 (0.4ug),HA-MDM2(0.4ug), PIG3(0.4,0.8, 1.2ug) using Metafectene. For inhibition of proteasome-mediated protein degradation, the cells were treated with 20 mM MG132 for 4 h before harvest. Forty-eight hours after transfection cells from each plate were harvested and split into three aliquots, one for immunoblot and the other two for ubiquitination assays. For pulldown assay, Cell pellets were lysed in buffer I (6 M guanidinium-HCl, 0.1 mol/ liter Na2HPO4/NaH2PO4, 10 mmol/liter Tris-HCl (pH 8.0), 10 mmol/liter 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/liter urea, 0.1 mol/liter Na2HPO4/NaH2PO4, 10 mmol/liter Tris-HCl (pH 8.0), 10 mmol/liter β-mercaptoethanol), and buffer III (8 mol/liter urea, 0.1 mol/liter Na2HPO4 /NaH2PO4, 10 mmol/liter Tris- HCl (pH 6.3), 10 mmol/ liter β-mercaptoethanol). Proteins were eluted from the beads in buffer IV (200 mmol/liter imidazole, 0.15 mol/liter Tris-HCl (pH 6.7), 30% (v/v) glycerol, 0.72 mol/liter β-mercaptoethanol, and 5% (w/v) SDS). Eluted proteins were analyzed by immunoblot with monoclonal anti-p53 (DO-1), anti-HA, or anti-MDM2 (SMP14) antibodies. For Immunoprecipitation, cell lysates were IP with anti-p53(DO-1), and detected with anti-ubiquitin.

9. Immunofluorescence microscopy

Cells cultured on coverslips were washed twice with cold PBS. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% BSA and incubated with anti-PIG3 (H-300) ,anti-p53(DO-1) and anti-Mdm2 (SMP-14) antibodies as indicated, followed by a Texas-red conjugated anti-mouse IgG and a FITC-conjugated anti-rabbit IgG antibody. The cells were mounted and the images were acquired with a confocal microscope.

10.Flow cytometry by PI staining

The floating and trypsin-detached HCT116 cells were collected and washed once with ice-cold PBS, followed by fixing in 70% cold ethanol for 30minutes at 4℃. The cells were then stained in PBS and PI (50 μg/ml), RNase A (50 μg/ml), and 0.05 % Triton X-100.

The DNA content of the GM00673 cells was analyzed by fluorescent-activated cell sorting (FACSort, Becton Dickinson, Franklin Lakes, NJ). At least 10000 events were analyzed, and the percentage of cells in sub-G_1 population was calculated. Aggregates of cell debris at the origin of histogram were excluded from the sub-G_1 cells.

11. Statistical analysis

Results are expressed as mean ± standard deviation (SD). For statistical analysis, ANOVA with P values were performed for both the overall (P) and the pair-wise comparison as indicated by asterisks. Values of $P<0.05$ were considered to be significant.

Ⅲ. RESULTS

1. PIG3 controls the stability of p53.

To determine whether the p53 level is regulated by PIG3 in wild-type p53 cell lines, including a pair of human colorectal carcinoma HCT116 cells that are wild type (*p53*+/+) and WI38, cells were treated with the PIG3 small interfering RNA (siRNA). Treatment for 24 hours resulted in a significant decrease in p53 level (Fig. 2).

The effect of PIG3 on p53 steady-state levels is not due to changes in their transcriptio because PIG3 does not alter the abundance of p53 mRNA in human HCT116 cells (data not shown), but regulates the stability of p53 protein. Knocking down PIG3 by siRNA decreased the half-life of wild-type p53 in HCT116(p53+/+), MCF7 or U2OS cells (Fig. 3).

2. PIG3 regulates p53 stability via ubiquitination pathway

To determine if the effect of PIG3 on p53 steady-state levels is due to changes p53 ubiquitination, HEK 293 cells were transfected with expression DNAs encoding myc3-tagged wild-type p53, MDM2, His6-Ubiquitin or co-transfected with PIG3 DNA. Lysates from the transfected cells were immunoprecipitated with an anti-p53(DO-1) antibody, and the immunoprecipitates were examined with an antibody against ubiquitin, or immuno-precipitated with NTA-Ni bead, and the immunoprecipitates were examined with an antibody against p53(DO-1). As shown in Fig. 4, a high molecular weight ladder of p53 species that are ubiquitin-p53 conjugates was increased in PIG3 transfected cell types when myc3-tagged wild-type p53 was expressed with MDM2, indicating that overexpression of PIG3 increses ubiquitination of p53 in a dose-dependent manner. To

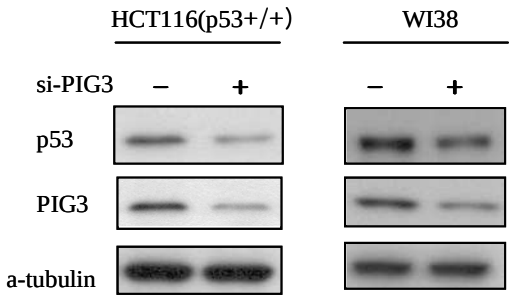

Fig.2 PIG3 controls the stability of p53(1). . Effect of siRNA-mediated downregulation of *PIG3* on the steady-state levels of Mdm2 and p53 in human cells. Lysates from indicated cells were analysed by western blot.

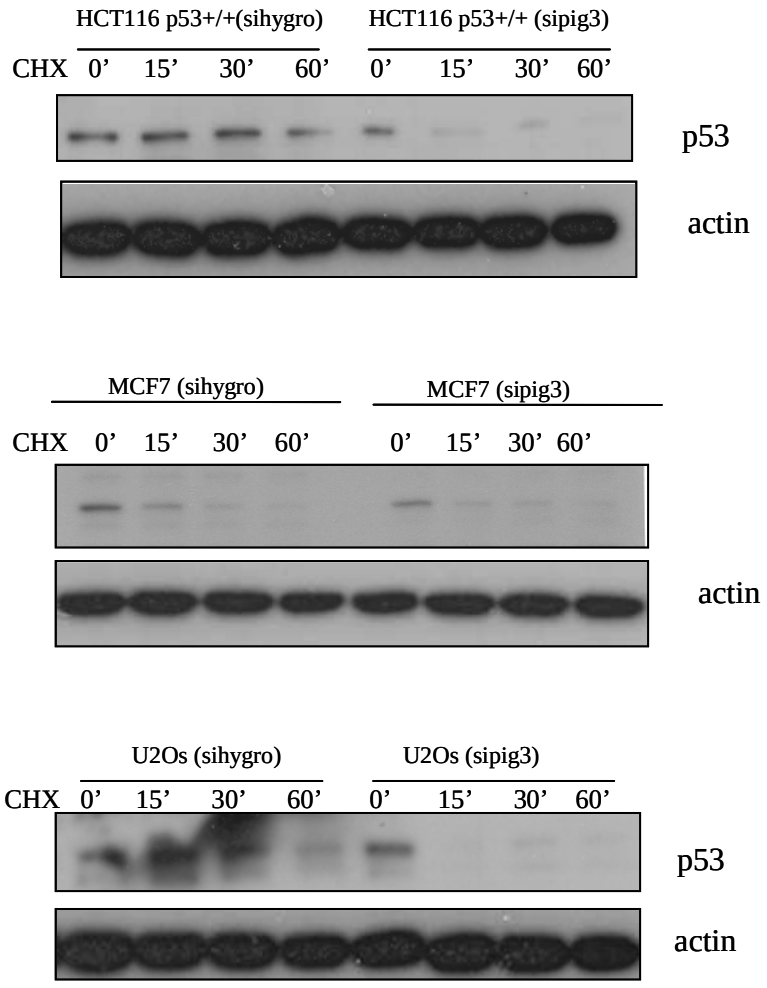

Fig.3 PIG3 controls the stability of p53(2). PIG3 modulates the half-life of p53. HCT116(p53+/+), MCF7 or U2OS cells were treated with PIG3 siRNA and control siRNA. Cells were cultured in the presence of 80 μg/ ml CHX, and were subsequently analyzed by Western blot.

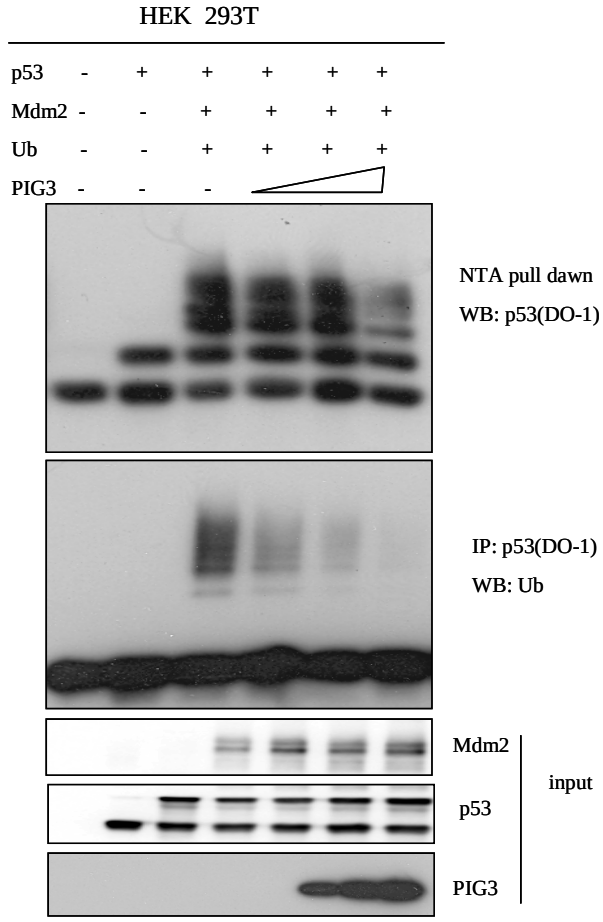

Fig.4 PIG3 regulates p53 stability via ubiquitination pathway(1). Overexpression of PIG3 affects ubiquitination of p53. HEK 293 cells were transfected with increasing amount of PIG3. Twenty-four hours later, cells were treated with 20 uM MG-132 for 4 h and were analysed for p53 ubiquitination.

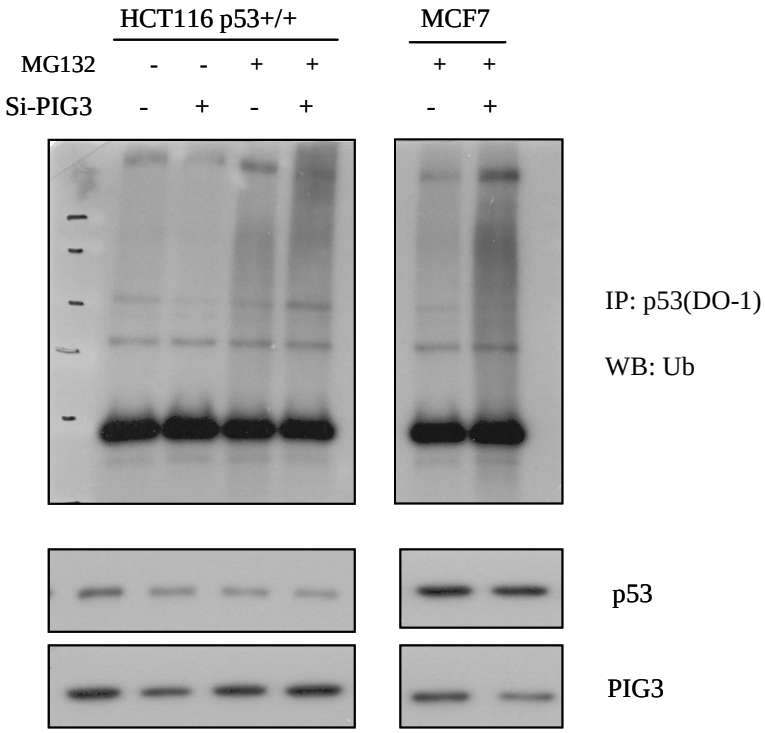

Fig.5. PIG3 regulates p53 stability via ubiquitination pathway(2). Downregulation of PIG3 affects ubiquitination of endogenous p53. MCF7 and HCT116(p53+/+) silencing stable cell line were treated with 20 uM MG-132 for 4h and were analysed for p53 ubiquitination.

conform the effect of PIG3 on endogenous p53 ubiquitination, MCF7 and HCT116 (p53+/+) silencing stable cell line were treated with 20 uM MG-132 for 4h and were analysed for p53 ubiquitination. Similarly, knocking down PIG3 inhibited ubiquitination of endogenous p53 (Fig. 5).

3. PIG3 physically interacts with p53 and mdm2.

MDM2 can bind p53 and promote its ubiquitination and subsequent degradation by the proteasome (45,46). The stabilizing effect of PIG3 on p53 led us to test whether PIG3 binds to Mdm2 and p53. Using a coimmunoprecipitation assay, the interaction between endogenous PIG3, p53 and Mdm2 was detected in U2Os cell lines, indicating that PIG3 interacted with p53 and Mdm2 in normal condition (Fig. 6A). And the PIG3−Mdm2 interaction is likely to be direct, as shown by an *in vitro* binding assay with PIG3 and Mdm2 protein synthesized using TNT kit (Fig. 6B). To test whether PIG3 and Mdm2 competitve binded with p53, in vitro competitive binding assay was used (Fig. 7). Using TNT kit, synthesized p53 and Mdm2 were mixed and IP with anti-Mdm2 antibody, detected with anti-p53, anti-Mdm2 antibody. Or synthesized p53 and PIG3 were mixed and IP with anti-PIG3 antibody, detected with anti-p53, anti-PIG3 antibody. The results showed that PIG3 and Mdm2 competitve binded with p53 in vitro.

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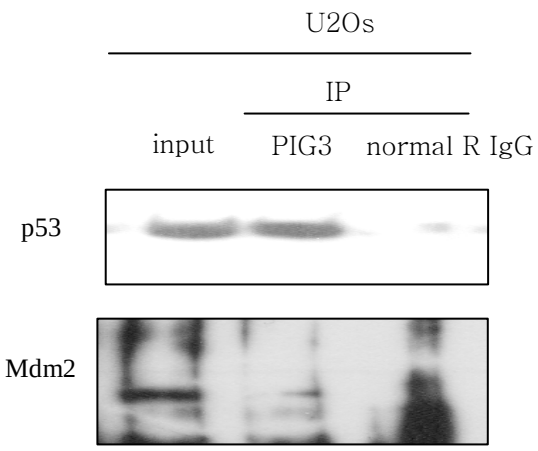
A.



B.

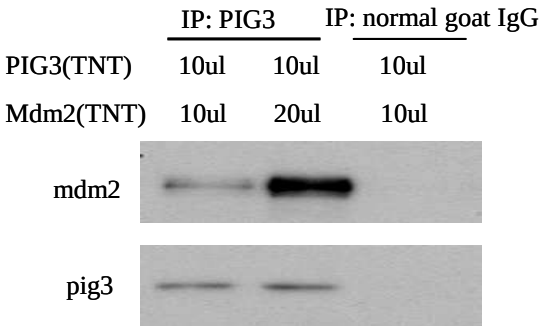


Fig.6. PIG3 physically interacts with p53 and mdm2(1). (A). Association of PIG3 and Mdm2, p53 *in vivo*. Cell lysates from U2Os cells were coimmunoprecipitated with anti-PIG3 and detected with anti-Mdm2 and anti-p53. (B) Direct binding of PIG3 and Mdm2 *in vitro*. PIG3 and Mdm2 protein were synthesized using TNT kit and mixed. The mixtures were coimmunoprecipitated with anti-PIG3 and detected with anti-Mdm2.

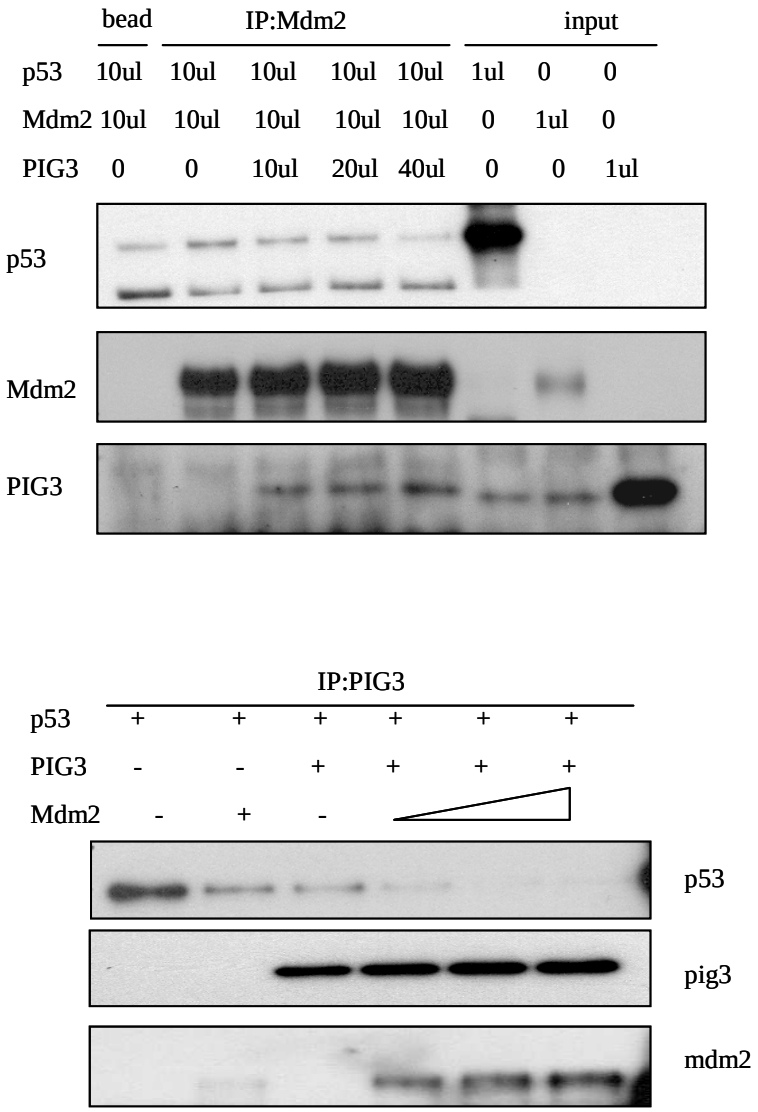


Fig.7. PIG3 physically interacts with p53 and mdm2(2). In vitro Competition assay between PIG3, p53 and Mdm2.

4. DNA damage regulates p53 stability through PIG3.

The effect of PIG3 on p53 stability prompted us to examine whether the DNA-damage signal regulates the p53 stability through PIG3. The results showed that treatment of with UV, compared with control cells, HCT116(p53+/+) siPIG3 cells p53 protein expression level, phosphor-p53 and Mdm2 protein expression level decreased(Fig. 8). To conform it, nucleous and cytoplasm fraction were made. The similar results we got in these two fractions (Fig. 9). To test interaction PIG3, P53 and Mdm2 in damage condition, Immunofluorescence microscopy analysis and immunoprecipition were used. Immunofluorescence microscopy analysis revealed that PIG3, p53 and Mdm2 colocalized in normal condition, but PIG3 and p53 merge increased and PIG3 and Mdm2 merge decreased after UV treatment (Fig. 10). Immunoprecipition showed that Mdm2−PIG3 and PIG3−p53 interactions and subsequently the PIG3−p53-Mdm2 complex in normal condition, but Mdm2 did not binding with PIG3 after UV treatment (Fig. 11).

The disruption of the binary Mdm2−PIG3-p53 interaction by DNA-damage signals seemed to be persistent and was not restored 16 h after treatment with UV , whereas the binary PIG3−p53 interaction was partially recovered by this time. These data show that DNA damage signals differentially and specifically affect the Mdm2−PIG3 and PIG3−p53 interactions and disrupt the ternary complex.

5. PIG3 regulates the apoptotic function of p53.

p53 has an important role in apoptosis. And it is clear that toxic reactive oxygen species are generated with kinetics which follow the p53-dependent induction of PIGs in cells undergoing p53-dependent apoptotic death.(47). It prompted us to examine whether PIG3 regulates the p53 apoptosis fuction. Apoptosis can be detected by flow cytometry

demonstrating a sub-G1 population with decreased staining of DNA by propidium iodide. In addition, we also carried out apoptosis detections with western blot analysis. First, we evaluated the HCT116(p53+/+) siPIG3 stable cell lines to undergo UV-induced apoptosis It showed that compare with control cells, siPIG3 cells showed a low level of apoptosis after exposure to UV in time-dependent and does-dependent manner(Fig. 12). Western blot analysis results showed similar trend of apoptosis between mock and PIG3 knockdown cells. Compare with control cells, the protein expression level of Bcl-2, Bax, cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, NOSA were decreased in siPIG3 cells, but PUMA was not altered(Fig. 13). These results suggested that PIG3 knockdown led to decreased UV-induced apoptosis regardless of p53 status.

In summary, in unstressed cells, PIG3 is stabilized in a complex with Mdm2 and p53 leading to rapid ubiquitination and degradation of p53, thereby preventing p53 activation . . In response to DNA damage, the PIG3–Mdm2–p53 complex is disrupted. Subsequently, Mdm2 dissociates from complex, which allows accumulation of p53, and increase the apoptosis fuction of p53.

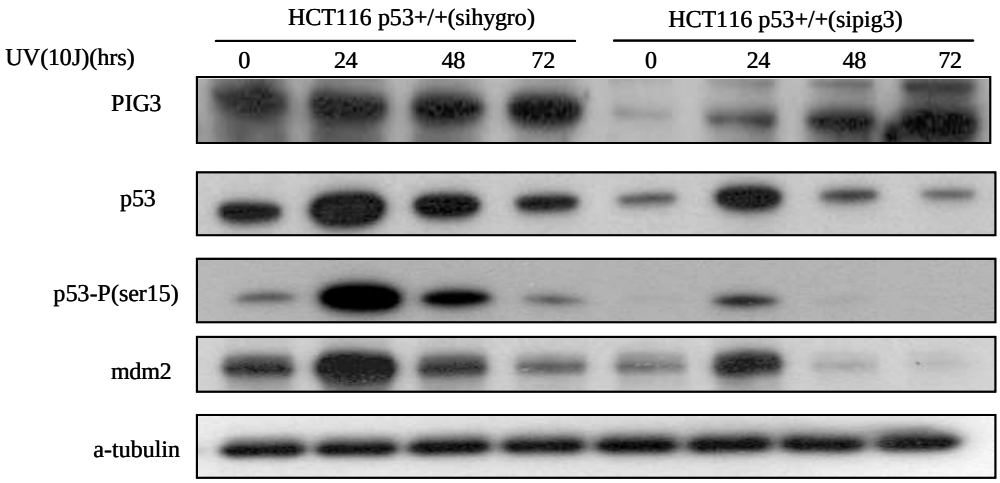


Fig.8. DNA damage regulates p53 stability through PIG3 (1). The protein expression level of p53, p-p53 and Mdm2 in HCT116(p53+/+) control and siPIG3 stable cells. Lysates were analysed by western blot.

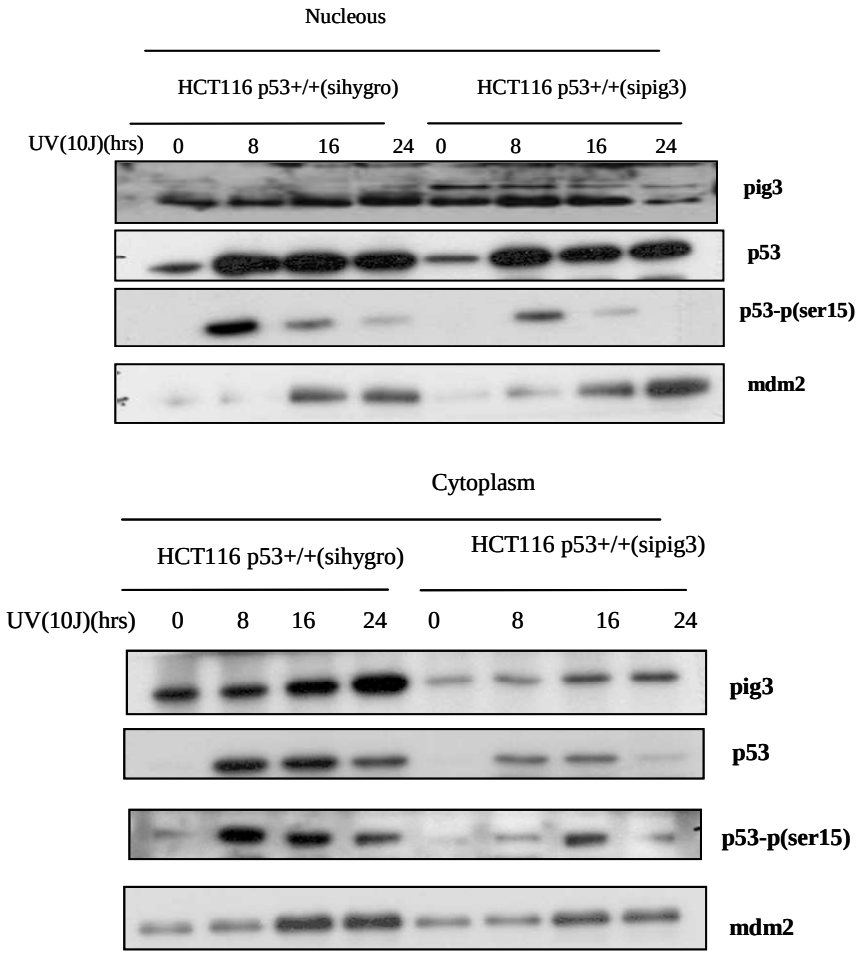


Fig.9. DNA damage regulates p53 stability through PIG3(2). The protein expression level of p53, p-p53 and Mdm2 in HCT116(p53+/+) control and siPIG3 stable cells cytoplasm and nucleous fraction. Lysates were analysed by western blot

HCT116p53+/+ cells : UV 10J

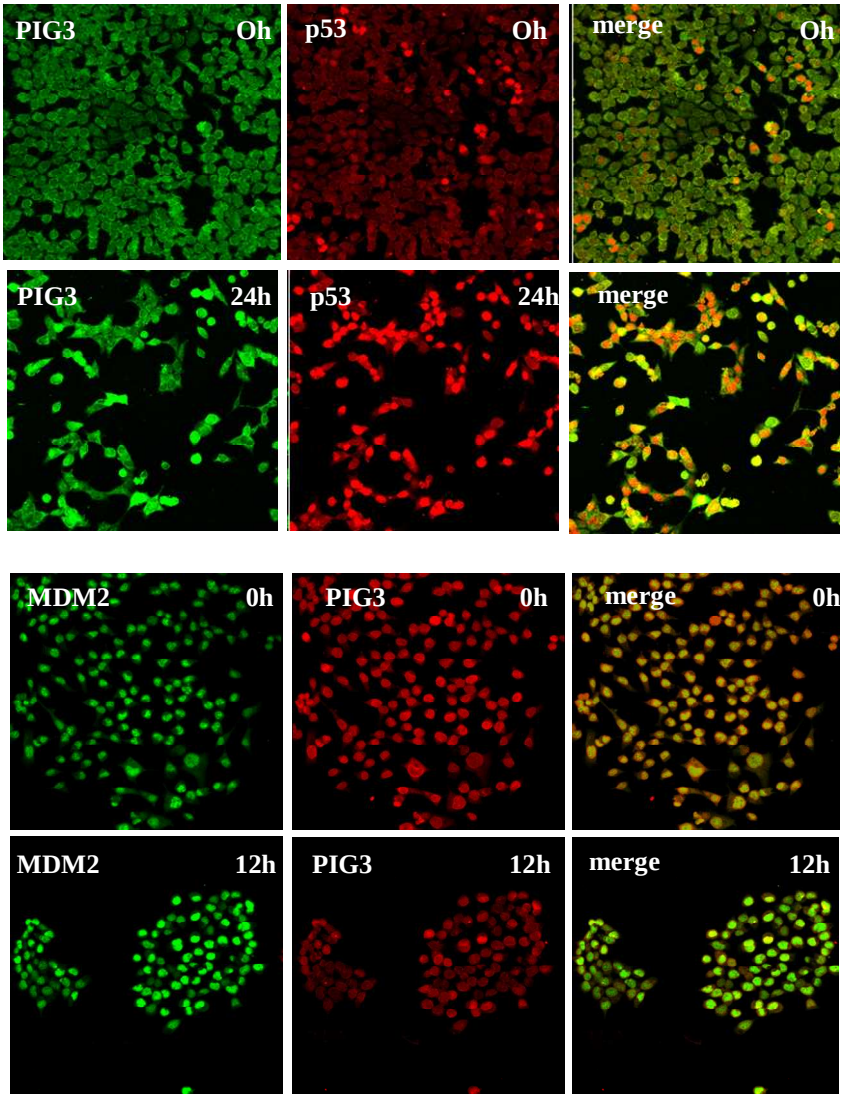


Fig.10. DNA damage regulates p53 stability through PIG3(3). Immunofluorescence microscopy analysis showed PIG3-p53 and PIG3-Mdm2 colocalization.

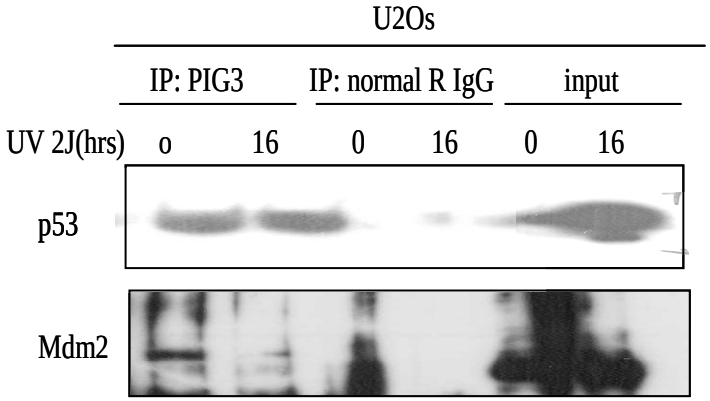
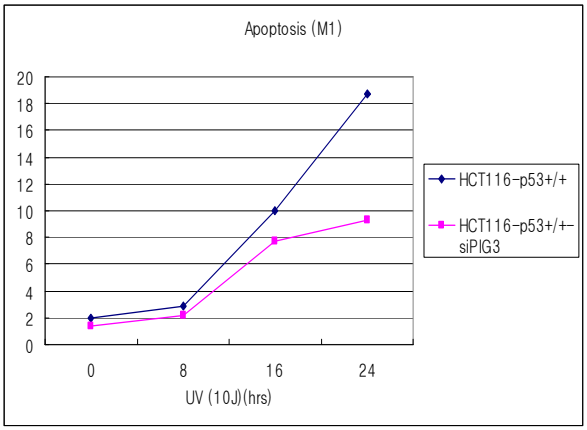


Fig.11. DNA damage regulates p53 stability through PIG3(4). Association of PIG3 and Mdm2, p53 *in vivo* under UV damage. Cell lysates from U2Os cells were coimmunoprecipitated with anti-PIG3 and detected with anti-Mdm2 and anti-p53.



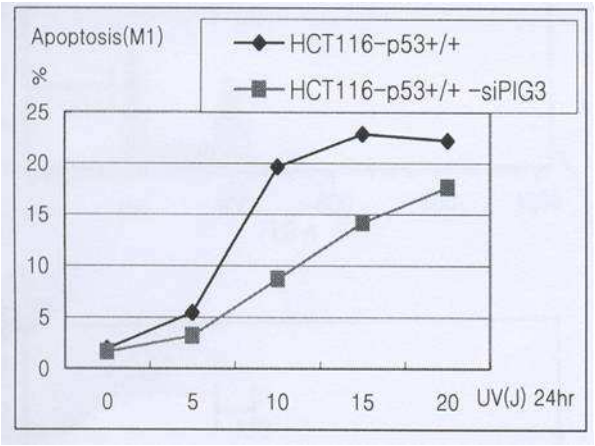


Fig.12. PIG3 regulates the apoptotic function of p53(1). Apoptosis levels were determined by sub-G1 fractions in HCT116(p53+/+) control and siPIG3 stable cells.

(B)

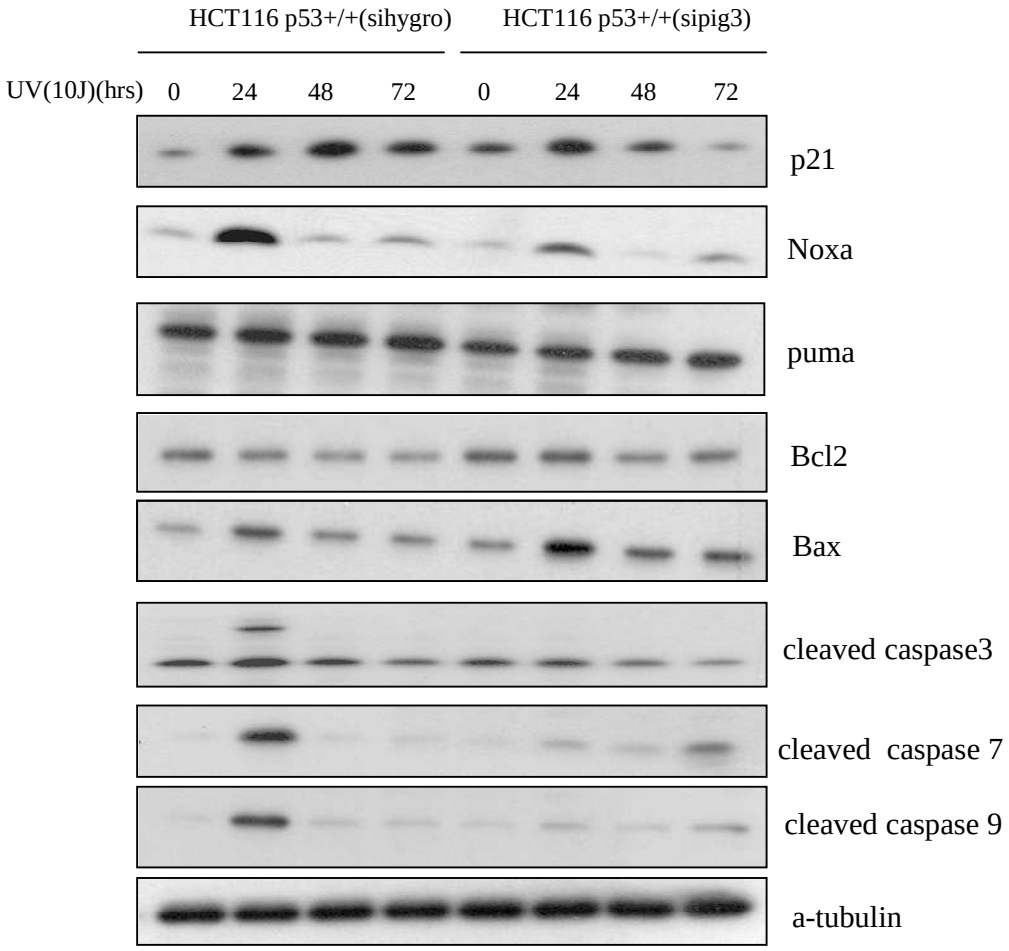


Fig.13. PIG3 regulates the apoptotic function of p53(2). The expression level of apoptosis protein in HCT116(p53+/+) control and siPIG3 stable cells. Lysates were analysed by western blot.

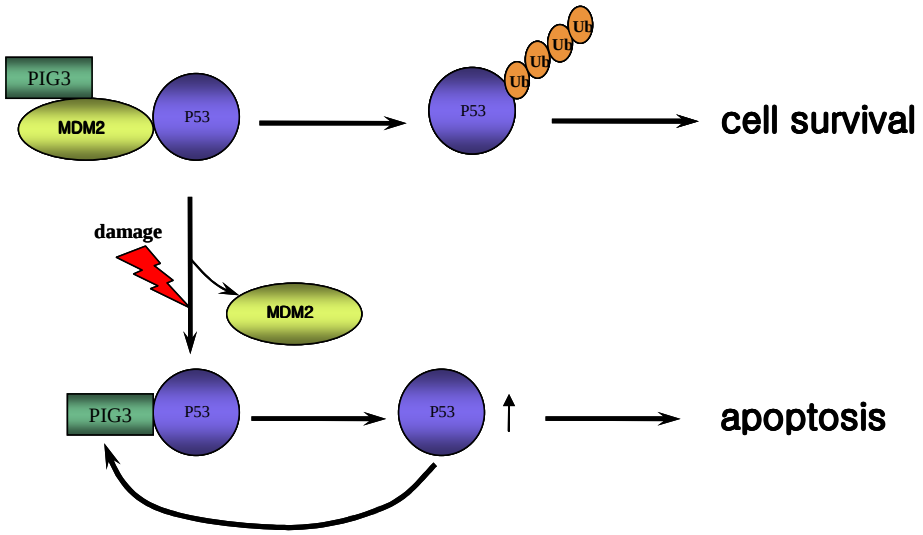


Fig.14. PIG3 regulates the apoptotic function of p53(3). A schematic representation of the role of PIG3 in regulating p53 stability. In unstressed cells, PIG3 is stabilized in a complex with Mdm2 and p53 leading to rapid ubiquitination and degradation of p53. In response to DNA damage, the PIG3–Mdm2–p53 complex is disrupted. Subsequently, Mdm2 dissociates from complex, which allows accumulation of p53.

Ⅳ. DISCUSSION

p53 is often referred to as the ‘cellular gatekeeper’ or the ‘guardian of the genome’ and its importance is emphasized by the discovery of mutations of p53 in over 50% of all human tumors (48). Ubiquitination represents an essential activity of Mdm2 for regulating the amount of p53 protein available at any given time within a cell. Mdm2 acts specifically as an E3 ligase for p53 by linking E2-conjugated ubiquitin molecules to it via an isopeptide bond(49). Details of the events that occur after this reaction, including the recognition of a growing polyubiquitin chain and subsequent degradation by the 26S proteosome, have remained somewhat elusive(51). Apoptosis is a vital process for tissue homeostasis that is frequently disturbed in several pathological conditions. The p53-inducible gene 3 (Pig3) is a transcriptional target of p53 and is thought to play a role in apoptosis(50), but its other functions are not clear now.

In this study, We have now investigated the relationship between PIG3 and p53. knocking down Pig3 accelerated p53 and Mdm2 degradation, and decreased the half-life of wild-type p53 in several of the human cell lines(Fig.2, 3). It showed that PIG3 controls the stability of p53. To determine if the effect of PIG3 on p53 steady-state levels is due to changes p53 ubiquitination, in vivo ubiquitination was used(Fig.4).. The result showed that PIG3 regulates p53 stability via ubiquitination pathway.

Several proteins thwart the p53–Mdm2 interaction by binding directly to p53 (e.g. ING1b) or Mdm2 (e.g. pRb, p19ARF and MdmX) (52-55). The predominant regulator of Mdm2 is p19ARF, a protein derived from the INK4a/ARF locus (56). The stabilizing effect of PIG3 on p53 led us to test whether PIG3 binds to Mdm2 and p53. The

immunoprecipitation assay result showed that PIG3 interacted with p53 and Mdm2 (Fig. 6A,B); PIG3 and Mdm2 competitve binded with p53 in vitro (Fig. 7). And we examine whether the DNA-damage signal regulates the p53 stability through PIG3. The western blot analysis results showed that treatment of with UV, compared with control cells, siPIG3 cells p53 protein expression level, phosphor-p53 and Mdm2 protein expression level decreasedin whole cell lysates, nucleous and cytoplasm fraction (Fig. 8,9). On this condition Mdm2−PIG3−p53 complex was disrupted.

In addition to stabilization, fuction of p53 is critical for initiating an early response to genotoxic stress. p53 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 proapoptotic genes(57). However, the initial activation of its function as a transcription factor is key for its ability to drive particular downstream pathways. So we examined whether PIG3 regulates the p53 apoptosis fuction. Flow cytometry and western blot results demonstrated that compare with control cells, siPIG3 cells showed a low level of apoptosis after exposure to UV in time-dependent and does-dependent manner(Fig. 12,13). All these results suggested that PIG3 knockdown led to decreased UV-induced apoptosis regardless of p53 status.

This study reveals that PIG3 is an integral part of this differential regulation through its ability to prevent p53 degradation. In unstressed cells, PIG3 interacts simultaneously with Mdm2 and p53 and mediates the stabilizing effect of Mdm2 on p53, thereby increase p53 activation . In response to DNA damage, Mdm2 dissociates from PIG3 and p53, which allows accumulation of p53.

Our results suggest that as the transcriptional target of p53, PIG3 regulated p53 stability

via it and Mdm2 competitive binding with p53, which decreaed the ubiquitinatin of p53; and PIG3 promote apoptosis in p53-dependent manner, it is a feedback loop.

Recent advances in understanding the initial accumulation and activation of p53 have added a layer of complexity to this pathway. The number of mechanisms used to quell p53 ubiquitination implicates this process as a key target in the initial response to genotoxic stress. Of particular interest are the specific fates of mono-ubiquitinated and poly-ubiquitinated forms of p53. Is Mdm2 sufficient for p53 degradation or are other factors required? There may be differential consequences for p53 depending on how many ubiquitin molecules are added and what type of ubiquitin linkage has occurred. A related perplexity is exactly how p53 is shuttled in and out of the nucleus, and whether associated factors or the ubiquitination status of p53 play a role.

Insight into the transcriptional activation and regulation of p53 continues to raise interesting questions. Do posttranslational modifications in response to stress pathways create p53 molecules that can preferentially interact with specific promoters and cause different cellular responses? How does a cell choose either a growth arrest or apoptotic pathway? How does the cell sense that DNA repair is complete, and by what mechanism is this linked to shutting-off p53? Considering the breadth of complexity in the regulation of p53 function, advances continue to show the multitude of mechanisms the cell uses to stabilize and activate this essential protein.

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Ming Jin (金明)

저작물 이용 허락서

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논문제목	한글: pig3에 의한 p53 stability 연구 영문: PIG3 regulates the stability of p53 via Mdm2				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2007 년 6 월 29 일

작자: 김명 (서명 또는 인)

조선대학교 총장 귀하