p53-dependent upregulation of *PIG3* transcription by γ -ray irradiation and its interaction with KAP1 in responding to DNA damage

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PIG3 (p53-inducible gene 3), originally identified as one of a set of genes induced by p53 before the onset of apoptosis, was assumed to contribute to early cellular response to DNA damage. Here, we studied the relation between p53 status and the increased expression of *PIG3* by ionizing radiation (IR), and the related clues regarding the involvement of *PIG3* in the cellular response to IR-induced DNA damage signaling. We demonstrated that the pentanucleotide microsatellite sequence was responsible for the p53-dependent induction of *PIG3* transcription after irradiation, while sequence upstream of *PIG3* promoter could maintain the basal level of expression which was not inducible by irradiation. The interaction of *PIG3* and the KRAB-ZFP-associated protein 1 (KAP1), a DNA damage response protein, was revealed. *PIG3* nucleus foci were formed 15 min after γ -ray irradiation, and which were found to partially colocalize with the phospho-KAP-1 foci as well as γ -H2AX foci. Although the lac operator tagged EGFP based reporter system revealed that *PIG3* does not remodel chromatin in large scale in the cells under normal growing condition, it indeed prompted the chromatin relaxation in the cellular response to DNA damage signaling. All these data suggest that *PIG3* is involved in IR-induced DNA damage response, and which maybe partially attribute to its interaction with KAP1.

PIG3, KAP1, p53, DNA damage response, inducible expression, large-scale chromatin remodeling

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The p53-inducible gene 3 (*PIG3* or *TP53I3*) was originally identified as one of a set of genes induced by p53 before the onset of apoptosis [1]. The closest relative of *PIG3* in mammals is zeta-Crystallin, an NADPH-quinone oxidoreductase and a potent generator of reactive oxygen species (ROS) [2]. As consistent with its classification in the quinone oxidoreductase (QOR) family, *PIG3* was confirmed an activity of NADPH dependent reductase with *ortho*-quinones [3]. In addition, *in vitro* activation and *in vivo* overexpression of *PIG3* was demonstrated to accumulate reactive oxygen species (ROS), while an inactive *PIG3* mutant (Ser151Val) did not produce ROS in cells, indicating

that enzymatic active protein is necessary for its function [3]. *PIG3* was also recently demonstrated to contribute to early cellular response to DNA damage induced by UV radiation and the DNA damaging-chemical neocarzinostatin through recruiting 53BP1, Mre11, Rad50 and Nbs1 to the sites of DNA break lesions and modulating intra-S and G2/M phase checkpoint [4].

A p53 dependent upregulation of *PIG3* way demonstrated during p53-mediated growth arrest or under genotoxic stress [5]. Szak et al. [6] reported that increased p53 binding to *PIG3*, p21 and MDM2 promoters occurred in the human colorectal carcinoma cell line RKO within 2 h after p53 activation, but a lower p53 affinity was demonstrated for the consensus binding site in the *PIG3* promoters compared to

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its consensus sites in its another two downstream target genes p21 and MDM2. Moreover, significant increases in *PIG3* transcription did not occur until 15 h after p53 binding [6]. Therefore, it was suggested that additional factor or element may be required to stabilize the interaction of p53 with the *PIG3* promoter [6]. Interestingly, the human cellular apoptosis susceptibility protein (hCAS/CSE1L) has been shown to associate with the *PIG3* promoter and affects p53-dependent apoptosis by regulating *PIG3* expression [7]. Previously, we have also found an increased expression of *PIG3* gene in human lymphoblastoid AHH-1 cells 4 h after exposed to ionizing radiation through the transcriptional profiling of cDNA chips analysis, and this inducible expression is radiation dose-dependent [8]. It is important to understand how irradiation regulates *PIG3* expression. A single 20-bp sequence of P53 binding motif was identified to locate at 308 nucleotides upstream of *PIG3* transcription start site [1]. However, another report demonstrated that the -308 region was completely dispensable for p53-mediated transcriptional activation of the *PIG3* promoter [9]. Interestingly, p53 was revealed to interact with a pentanucleotide microsatellite sequence, (TGYCC)_n where Y=C or T, downstream the start site of *PIG3* promoter, and which is necessary and sufficient for transcriptional activation of the *PIG3* promoter by p53 [9]. Moreover, low frequency loss of heterozygosity of the polymorphic *PIG3* microsatellite was found in *de novo* acute myeloid leukemias [10] and microsatellite instability may occur in the pentanucleotide repeat of the *PIG3* promoter in bcr/abl acute lymphoblastic leukemia [11]. Therefore, the mechanistic model and biological significance of *PIG3* expression regulated by p53 in the processing of DNA damage response may be more complicated than we assumed.

In this study, we have further detailed the induced expression pattern of *PIG3* gene by ionizing radiation, and the regulation elements and its dependency on p53 function. The pentanucleotide microsatellite sequence was found to be responsible for the induction. Sequences upstream the pentanucleotide microsatellite maintained basal expression of *PIG3* which is not inducible. Interestingly, the interaction of *PIG3* and the KRAB-ZFP-associated protein 1 (KAP1) was elucidated in this study. To investigate the potential effect of *PIG3* on large-scale chromatin dynamics in mammalian cells, we employed a lac repressor-based system [12]. Lac operator-repressor conjugated and EGFP based reporter system revealed that *PIG3* can remodel the chromatin in large scale in the cellular response to ionizing radiation.

1 Materials and methods

1.1 Cell culture

A03_1 CHO cells were kindly provided by Dr. Andrew S. Belmont. Briefly, multiple copies of the lac operator were engineered into the genome of DG44 CHO cells with a

double deletion for the dihydrofolate reductase (DHFR) locus, and together with the surrounding genomic sequences, were amplified to produce a 90-Mb heterochromatic region [13]. A03_1 cells were grown in selective media consisting of Ham's F12 media without thymidine and hypoxanthine (Specialty Media) and dialyzed FCS (Hyclone Laboratories, Inc.). A549, C33A-C, C33A-E6 were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37°C in 5% CO₂. AHH-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin.

1.2 Plasmids construction

LacR-EGFP was made by inserting the full length PCR product of wild type *lac* repressor fused with a nuclear localization signal (NLS) into pEGFP-N1 vector with *Xho* I and *Eco*R I restriction sites [12]. LacR-*PIG3*-EGFP was constructed by inserting the full length PCR product of *Homo sapiens* *PIG3* cDNA into Lac-EGFP with *Eco*R I and *Kpn* I restriction sites.

1.3 Confocal microscopy of expression plasmids

A03_1 CHO cells were grown on slide covers in tissue culture dishes. The cells were transfected with the plasmid by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, the cells were irradiated with 4Gy γ-ray or not. At 24 h after irradiation, the cells were rinsed with PBS and subjected to fixation using 4% (v/v) paraformaldehyde for 30 min. The cells were stained with DAPI or PI. Images were viewed with a confocal fluorescent microscope connected to a Bio-Rad Radiance2100 laser scanner (Bio-Rad, Richmond, CA, USA).

1.4 siRNAs and transfection

For the knockdown of p53 expression, the small interfering RNA (siRNA) molecules used are adopted from previous reports: annealed p53 siRNAs sense strand 5'-CUACUCCUGAAAACAACGdTdT-3', antisense strand 5'-CGUUGUUUCAGGAAGUAGdTdT-3'; and the silencer negative control siRNA sense strand 5'-UUCUCCGAACGUGUCACGUTT-3', antisense strand 5'-ACGUGACACGUUCGGAGAATT-3'. The siRNA molecules were synthesized and purified by Shanghai GenePharma Co (Shanghai, China), siRNA transfection was performed using Lipofectamine 2000 reagent. The final acting concentration for each siRNA is 50 nmol/L.

1.5 Quantitative real-time PCR

Total RNA was prepared by standard TRIzol-based

methods and was reversely transcribed with M-MLV reverse transcriptase (Promega, Madison, USA) and oligo-dT. Quantitative real-time-PCR was performed in an Chromo 4 (Bio-Rad, Hercules, CA) using SYBR green expression assays (Finnzymes, Espoo, Finland) and the M-MLV (Invitrogen, Carlsbad, CA) with primers 5'-TTAAGCGAGGAA-GTCTGATC-3' and 5'-CTTGCCTATGTTCTTGTGG-3', according to the manufacturers' specifications. Relative gene expression levels were calculated using the method, normalizing to the expression of the actin housekeeping gene. All assays were performed at least in triplicate.

1.6 Northern blotting analysis

To analyze *PIG3* mRNA expression, total RNA was prepared from cells with or without different doses irradiation or H₂O₂, cisplatin treatment using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Subsequently, 20 µg of total RNA was separated on denaturing 1% agarose gel with formaldehyde and electro-transferred onto positively charged Nylon membranes (Qiagen, Ambion). After UV-cross-linking, membranes were hybridized at 58°C overnight in 1 mol L⁻¹ sodium phosphate pH 6.2, 7% SDS with ³²P-labeled *PIG3* specific fragments probes prepared by T4 polynucleotide kinase reaction. After hybridization, membranes were washed twice with 1× SSC, 0.1% SDS for 20 min at 58°C and exposed to MS film (Kodak) at -80°C overnight. For probes, the *PIG3* cDNAs were radio-labelled with γ-³²P-ATP (Furui, Beijing, China).

1.7 Luciferase reporter assay

Different deleted mutants of *PIG3* promoter region (DEL A-E) were cloned into the upstream of firefly luciferase in the plasmid pGL-basic. A549 cells were cotransfected with pGL-basic (firefly luciferase) and pRL-TK. The luciferase reporter assay was carried out by using the Dual-Luciferase Reporter Assay System (Promega).

1.8 Detection of *PIG3*/pKAP 1/γ-H2AX foci by confocal immunofluorescence

After 2 Gy irradiation, the cells were cultured for 15 min, then fixed in 2% paraformaldehyde for 15 min and washed three times with PBS, permeabilized for 15 min on ice in 0.2% Triton X-100, and blocked in PBS with 1% bovine serum albumin for 3 × 10 min at room temperature. Samples were incubated with anti-*PIG3* antibody and anti-phospho-KAP 1/S824 (Bethyl Laboratories, Montgomery, TX, USA), or anti-γ-H2AX antibody (Upstate, Charlottesville, VA) at a 1:130 dilution for 1 h, washed in PBS with 1% FCS for 3 × 10 min, and incubated with Rhodamine (Rd)-conjugated or Alexa Fluor 488/TRITC-conjugated secondary antibodies (Invitrogen) or at a dilution of 1:400 for 1 h, all at room temperature. Cells were

then washed in PBS for 4 × 10 min at room temperature and mounted using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI)(Vector Laboratories, Burlingame, CA). DNA were stained with DAPI in mounting solution. Confocal immunofluorescence microscopy was performed using an LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

1.9 Co-immunoprecipitation and immunoblotting assay

The co-immunoprecipitations (CoIP) were performed using the Immunoprecipitation Kit (Protein A/G, Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, A549 cells were washed twice with ice-cold PBS and collected by centrifugation. The cell pellets were resuspended in pre-chilled lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and the requisite amount of the protease inhibitor tablet provided in the Kit) and homogenised. The supernatants were collected by centrifugation at 12000×g for 10 min at 4°C to remove debris, and then subjected to immunoprecipitation. After being precleared with protein A/G-agarose, the supernatants were incubated for 3 h with 2 µg of the anti-*PIG3* antibody, or anti-KAP-1 antibody at 4°C, followed by an overnight incubation with protein A/G-agarose at 4°C. The immunoprecipitates were collected by centrifugation, washed twice with wash buffer 1 (50 mmol/L Tris-HCl, pH 7.5, 500 mmol/L NaCl, 1% Nonidet P40, and 0.05% sodium deoxycholate) and once with wash buffer 2 (10 mmol/L Tris-HCl, pH 7.5, 0.1% Nonidet P40, and 0.05% sodium deoxycholate). The immunoprecipitates were denatured by heating to 100°C for 3 min in gel-loading buffer and centrifuged at 12000×g for 20 s to remove the protein A/G-agarose.

For immunoblotting (Western blotting) analysis, the above Co-IP products were denatured, resolved by SDS-PAGE, and subjected to Western blotting analyses. Otherwise, the cells were harvested and washed twice in ice-cold PBS. Cell pellets were treated with lysis buffer indicated above, one protease inhibitor cocktail tablet in a 50-mL solution, and total protein was isolated. The protein (50 µg) was resolved using SDS-PAGE (8%), and then transferred onto a polyvinylidene fluoride (PVDF) membrane for Western blotting analysis.

1.10 MS analysis and database searching of *PIG3* interacting proteins

After SDS-PAGE of the immunoprecipitation products of *PIG3* antibody, the gel was immersed in 250 mL of fixation solution (50% methanol, 10% acetic acid) with gentle agitation at least twice for 30 min each time, and stained with Coomassie brilliant blue staining mixture. The peptide

bands of interest were cut out of the gel, and minced into pieces. The gel pieces were destained with 50% ACN in 25 mmol/L ammonium bicarbonate until transparent, and then dried in a vacuum centrifuge. The gel pieces were incubated with 60–80 μ L trypsin (0.01 mg/mL, 25 mmol/L ammonium bicarbonate) for 16–18 h at 37°C, and then immersed in 100 μ L of 0.5% TFA (trifluoroacetic acid), 50% ACN (acetonitrile) for 1 h at 37°C, followed by another 100 μ L of 0.5% TFA, 50% ACN for 1 h at 37°C. The supernatant was combined and vacuum-dried. The peptides were resuspended in 2 μ L of 0.1% TFA, and then immediately subjected to the MS/MS analysis using the Synapt High Definition Mass Spectrometry. The resulting data were analyzed by PLGS v2.3 software and Mascot Search was performed. The search parameters were as follows: 10 μ L/L mass tolerance; trypsin digestion with two missed tryptic cleavage site; MH^+ and monoisotopic; and carbamidomethyl as a fixed modification for cysteines, oxidation as a variable modification for methionines. The criteria for protein identification were as follows: the protein score was significant ($P < 0.05$) and higher than 60.

2 Results

2.1 Upregulation of *PIG3* by γ -ray is at transcriptional level and in dose-dependent

To reveal the pattern of *PIG3* transcriptional alterations in response to DNA damage induced ionizing radiation (IR), we analyzed the expression of *PIG3* in human lymph-

oblastoid AHH-1 cell line irradiated with γ -rays at doses from 0.05 to 10 Gy. Northern blotting showed that *PIG3* mRNA in AHH-1 cells was induced by γ -ray irradiation at dose as small as 0.05–0.2 Gy (Figure 1(a)), and this upregulated expression was in a dose dependent manner (Figure 1(b)). Real-time PCR further confirmed the dose-dependent pattern and revealed a close correlation between *PIG3* mRNA level and irradiation doses from 0–10 Gy (Figure S1(a)). The regression of dose response data was done using Origin 5 software, and the regression equation is $Y = 1.466 + 3.403D - 0.171D^2$, here the “ D ” represents the radiation dose, and the “ Y ” represents the mRNA expression level (fold change). $P = 0.016$. The dose-dependent increased protein level was also observed by Western blotting analysis in AHH-1 cells (Figure S1(b)). Furthermore, to elucidate the kinetics of *PIG3* expression changes with the time post irradiation, we analyzed the mRNA level of *PIG3* by real-time PCR at different time points after 4 Gy irradiation. We found that the induction of *PIG3* transcription at least started from 4 h post-irradiation and reached a peak at 10 h. A stably increased level of mRNA expression remained until 48 h post irradiation (Figure S1(c)). We have also confirmed the IR-induced upregulation of *PIG3* mRNA in human lung cancer line A549 cells, and which is in a dose-dependent manner at least up to 6 Gy (Figure 1(c) and (d)). Moreover, the Western blotting analysis also confirmed that the increased protein level in A549 cells occurred as early as 2 h after 4 Gy irradiation, and persisted to at least 48 h (Figure S1(d)).

To prove if *PIG3* can be regulated in response to DNA

Figure 1 mRNA expression of *PIG3* gene in the cells exposed to γ -ray radiation. (a) Northern blotting hybridization pattern of *PIG3* mRNA expression in AHH-1 cells detected at 4 h after irradiation. (b) Dose-dependent upregulation of *PIG3* mRNA in AHH-1 cells. The data are the means and deviations from 3 independent experiments. (c) Northern blotting hybridization pattern of *PIG3* mRNA expression in A549 cells detected at 4 h after irradiation. (d) Dose-dependent upregulation of *PIG3* mRNA in A549 cells. The data are the means and deviations from 3 independent experiments. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as sample loading control. The relative mRNA levels were quantified as the ratio of hybridization intensity of *PIG3* to *GAPDH*.

damage induced by the reactive oxygen species (ROS), we added different concentration H_2O_2 to the A549 cells medium. As shown in Figure 2(a) and (b), *PIG3* expression increased with increasing concentration of H_2O_2 , at least up to 200–500 $\mu\text{mol/L}$. On the other hand, the induction of *PIG3* expression was declined when H_2O_2 concentration is over 1000 $\mu\text{mol/L}$. However, cisplatin, a platinum-based chemotherapy drug, did not up-regulate *PIG3* in A549 cells (Figure 2(c) and (d)). The relative higher intensity of *PIG3* hybridization signals at the concentrations of 2.0 and 5.0 $\mu\text{mol/L}$ could be contributed to much more sample loaded as shown in the *GAPDH* loading control.

2.2 γ -ray induced upregulation of *PIG3* is p53 dependent

To delineate the role of p53 on the ionizing radiation-induced upregulation of *PIG3*, the siRNA strategy was used to deplete p53 in A549 cells (Figure 3(a)). Obviously, siRNA-mediated depletion of p53 largely abrogated the induced expression of p53 by radiation (Figure 3(a) and (b)). H1299 lung cancer cells is a p53 deficient cell line, *PIG3* expression was also investigated. Although a certain level of constitutive expression of *PIG3* was detected in this cell line, no IR-induced expression was observed (data not shown). E6, an oncogene product encoded by human papillomavirus type 16 (HPV16), was reported to interact with p53 and promote its proteolysis by ubiquitin-proteasome dependent pathway [14,15]. We then employed two human cervical cancer cell lines C33A-C and C33A-E6, with or without expression of HPV16-E6, to further verify if

IR-induced upregulation of *PIG3* depends on p53 status. As shown in Figure 3(c), p53 level in C33A-E6 cells was much lower than that in C33A-C cells. Northern blotting analysis confirmed that the irradiation-increased *PIG3* was only observed in C33A-C cells, but not in C33A-E6 cells (Figure 3(d)).

2.3 The pentanucleotide microsatellite element is responsible for the radiation-induced upregulation of *PIG3*

An early research reported that the –328 to –308 region of *PIG3* promoter is responsible for p53 binding and gene activation [1]. Subsequent research proved that p53 induces *PIG3* expression by interacting with a pentanucleotide microsatellite sequence (TGYCC)_n within the *PIG3* promoter downstream the transcription start point (+442 – +516) but not with the DNA element described previously [9]. In order to reveal which promoter element is dominantly responsible for ionizing radiation-induced upregulation of *PIG3* gene, we constructed a series of deletion mutants of *PIG3* promoter (Figure 4(a)) and dual luciferase assay was performed. As shown in Figure 4(b), the transactivation was activated by γ -ray irradiation only on the promoter constructions comprising the pentanucleotide microsatellite sequence (DEL A and DEL C) (Figure 4(b)). Deletion of the pentanucleotide microsatellite sequence almost completely abrogated this IR-induced transactivation (construction DEL B). Taken together, these results suggested that the pentanucleotide microsatellite sequence is responsible for the upregulation of *PIG3* by ionizing radiation.

Figure 2 mRNA expression of *PIG3* gene in A549 cells after the treatment of H_2O_2 or cisplatin. (a) Northern blotting hybridization pattern of mRNA expression in A549 cells treated with H_2O_2 . (b) Dose-dependent upregulation of *PIG3* mRNA in A549 cells treated with H_2O_2 . The data are the means and deviations from 3 independent experiments. (c) Northern blotting hybridization pattern of mRNA expression in A549 cells treated with cisplatin. (d) The quantification of *PIG3* mRNA expression in A549 cells treated with cisplatin. The data are derived from the hybridization intensity signals of (c). A549 cells were treated with different concentrations of H_2O_2 or cisplatin for 12 h, and then harvested for Northern blotting analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as sample loading control. The relative mRNA levels were quantified as the ratio of hybridization intensity of *PIG3* to *GAPDH*.

Figure 3 Upregulation of PIG3 by ionizing radiation is p53-dependent. (a) siRNA-mediated depletion of p53 abrogated the induced expression of PIG3 by γ -ray irradiation. After treated with p53 siRNA molecules for 48 h, A549 cells were irradiated with 6 Gy of γ -ray and harvested 6 h later for Western blotting analysis of p53 and PIG3 proteins. (b) The intensity of Western blotting hybridization signals was quantified for p53 and PIG3 proteins. (c) The p53 protein levels detected in C33A-E6 cells in which p53 degradation is mediated by HPV-E6 protein and the control C33A-C cells. β -actin was used as sample loading control. (d) mRNA levels of *PIG3* in C33A-C and C33A-E6 cells. The cells were irradiated with indicated doses of γ -ray and mRNA of *PIG3* and *GAPDH* were assayed 4 h post-irradiation by Northern blotting analysis.

Figure 4 Reporter-gene system analysis confirmed that irradiation-induced upregulation of PIG3 is p53-dependent. (a) Schematic representation of deletion mutation of PIG3 promoter vectors. (b) A549 cells were co-transfected with pGL-basic comprising different mutants of *PIG3* promoter and pRL-TK. At 24 h post-transfection, cells were treated or untreated with 6 Gy γ -ray. At 48 h post-transfection, dual luciferase activity was measured, and the ratio of 2 luciferase activities was determined. Fold changes = the transactivation activity in a given group/the transactivation activity in DEL A vector transfection without irradiation group. Data are means and deviations from 3 independent experiments. *, $P < 0.01$ as compared with the corresponding non-irradiated group. Statistical analysis was performed using student's *t*-test method.

2.4 Interaction of PIG3 with KAP1 and its involvement in the large scale chromatin remodeling in the processing of DNA damage response

Up to now, the mechanistic information regarding the

involvement of PIG3 in the cellular response to DNA damage induced by ionizing radiation is very few. In order to clarify the role of PIG3 in ionizing radiation-induced DNA damage, we performed a co-immunoprecipitation (CoIP)-MS/MS experiment to identify PIG3 interacting proteins (Figure 5(a)–(c)). Fortunately, we identified KAP1 (KRAB-associated protein, also known as TIF1 β , KRIP-1 or TRIM28) as an interacting protein of PIG3 (Figure 5(c)). To verify the interaction of PIG3 and KAP1, we firstly performed glutathione S-transferase (GST) pull-down assay with the recombinant GST-PIG3. A direct interaction was observed between KAP1 and GST-PIG3 but not GST (Figure 5(d)). To identify the interaction of KAP1 and PIG3 in vivo, we transfected a HA-PIG3 recombinant vector or HA vector into A549 cells and performed the immunoprecipitation using an antibody against HA. KAP1 was shown to be co-immunoprecipitated with HA-PIG3 recombinant protein in the cells but not HA protein (Figure 5(e)). Further more, the immunoprecipitation using an antibody against KAP1 was performed to pull down PIG3 in both AHH-1 cells and A549 cells. As shown in Figure 4(f), PIG3 can be co-immunoprecipitated with KAP1 protein.

KAP1 was reported to be different from the other DNA damage response (DDR) proteins examined as it is not required for the implementation of cell-cycle checkpoints but for chromatin relaxation after induction of DNA double strand break (DSB) [16]. Phosphorylated KAP1 also forms foci overlapping with γ -H2AX in heterochromatin [17]. DDR proteins need to overcome the barrier of condensed chromatin to gain access to DNA lesion site to detect and repair damaged DNA [18]. ATP-dependent chromatin remodeling is one of the fundamental mechanisms used by cells to relax chromatin in DNA repair [19]. Based on these

Figure 5 Identification of the interaction of PIG3 and KAP1. (a) Coomassie brilliant blue staining pattern of the SDS-PAGE of the co-immunoprecipitation products of PIG3 antibody. (b) The probability based morse score of Mascot search of the MS analysis data of the "Band 1" fragment indicated in panel (a) of this figure. (c) KAP1 was recognized based on the peptides report of the MS analysis of the "Band 1". (d) GST pull-down assay shows direct interaction between KAP1 and GST-PIG3 but not GST. (e) Interaction of KAP1 with HA-PIG3 but not HA *in vivo*. A549 cells were transfected with HA-PIG3 recombinant vector or HA vector, immunoprecipitations were performed using antibody against HA to pull down KAP1. (f) Interaction of KAP1 with PIG3 *in vivo*. The immunoprecipitation was performed using an antibody against KAP1 to pull down PIG3 protein in both AHH-1 cells and A549 cells.

facts, we hypothesized that PIG3 may participate in chromatin decondensation in the cellular response to DNA damage. To prove the hypothesis, we have investigated the potential subcellular co-localization of PIG3 and phospho-KAP1. As shown in Figure 6, PIG3 protein is distributed over the nuclei and cytoplasm, while a tiny level of pKAP1 is homogeneously scattered over the nuclei in non-irradiated A549 cells. PIG3 foci and phospho-KAP1/S824 foci were observed in the nuclei 15 min after 2 Gy-irradiation, and a small number of PIG3 foci were colocalized with pKAP1 foci. However, much more PIG3 foci were colocalized with γ -H2AX foci (Figure 6).

We then employed a lac repressor-based system to investigate the role of PIG3 in large scale chromatin remodeling. In this system, multiple copies of the lac operator were engineered into the genome of CHO cells, and together with the surrounding genomic sequences, were amplified to produce a 90-Mb heterochromatic region [12,13]. The lac repressor conjugated target gene and tagged EGFP reporter system (Figure 7(a)) can drive relaxation of the lac operator and surrounding genomic DNA if the target gene has this function. Using this system, we have firstly confirmed the chromatin relaxation (decondensation) effect of the Tip 60 protein, an important molecule functioning in chromatin remodeling (Figure S2) [12]. As shown in Figure 7(b) and (c), PIG3 expression seems not to induce large scale chromatin relaxation in the cells at the normal growing condition. However, PIG3 significantly prompts the large scale

chromatin relaxation in the cells after 10-Gy irradiation. The green dot size of the heterochromatic region in the group PIG3 + 10 Gy IR is significant larger than that in the control group EGFP + 10 Gy IR ($P = 0.02$) (Figure 7(c)).

3 Discussion

It is well documented that the end-point outcomes and severities of IR-induced biological effects in mammalian cells are closely associated with the changed expressions of a set of radiation-inducible genes. In this study, we confirmed that IR-induced upregulation of PIG3 gene is p53 dependent. Besides, we demonstrated that the pentanucleotide microsatellite sequence mediated the p53-dependent induction after ionizing radiation. The region from -328 to -308 seems not to respond to ionizing radiation, and could be partially responsible for the basal level expression of PIG3. It is controversial from different reports that which region in PIG3 promoter is responsible for p53 induction [1,9]. Our results supported the notion that the pentanucleotide microsatellite region mediates the p53-associated induction of PIG3. However, the upstream element (-328 to -308) could contribute to the basal expression which might be independent of p53 since PIG3 expressed at relatively high level in p53 deficient H1299 cells although no obvious further increased expression after irradiation (data not shown).

Early research conjectured that a group of PIGs (p53-

Figure 6 PIG3 nuclear foci were formed after irradiation and partially colocalized with pKAP1 foci or γ -H2AX foci. A549 cells were irradiated or untreated with 2 Gy γ -ray, fixed 15 min post-irradiation and confocal immunofluorescence analysis was carried with antibodies against PIG3 and pKAP1/S824 or γ -H2AX. 4'-6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei.

Figure 7 Effect of PIG3 on large scale chromatin remodeling. (a) Schematic representation of constructs LacR-EGFP and LacR-PIG3-EGFP. (b) A549 cells which contain heterochromatic 256 lac operator repeats were transfected with expression vectors for the lac repressor conjugated PIG3 and tagged EGFP fusion protein or the control lac repressor tagged EGFP fusion protein as indicated above. At 48 h after transfection, cells were irradiated with or without 10 Gy γ -ray irradiation. At 45 min after irradiation, cells were fixed, stained with DAPI for DNA and prepared for observation with a fluorescence microscopy. (c) Data analysis of the chromatin remodeling observations. 15 to 25 EGFP positive cells images were captured with laser confocal fluorescence microscopy and the green dot size was calculated with Image-Pro Plus software. Statistical analysis was performed using student's *t*-test method.

induced genes) including PIG3 participate in the induction of ROS but not any single one of them. Recent research reported that overexpression of PIG3 accumulates ROS *in vivo* and *in vitro*; while an inactive PIG3 mutant (Ser151Val) does not [3]. Our result, H_2O_2 stimulating PIG3 expression, implied that ROS may also contribute to PIG3 induction by

ionizing radiation via the oxidative DNA damage mechanism.

PIG3 has been shown to play a role in the activation of DNA damage checkpoints after UV irradiation or the treatment of genotoxic chemical neocarzinostatin (NCS) [4]. PIG3 depletion results in a reduced Chk1 and Chk2

phosphorylation, and decreased recruitment of 53BP1, Mre11, Rad50 and Nbs1 to the sites of DNA break lesions in response to DNA damage induced by UV or NCS. Although it is clear that *PIG3* is also a sensitive gene in transcriptional response to ionizing radiation exposure, its detail function, especially the mechanistic pathway, is still unclear in the cellular response to IR. In this study, we uncovered the interaction of *PIG3* and KAP-1. Moreover, a part of *PIG3* nuclear foci in responding to IR-induced DNA damage were shown to colocalize with KAP1 foci or γ -H2AX foci. KAP1, which was primarily identified as a corepressor of gene transcription, has been demonstrated as an effector in the pathway of ATM-dependent chromatin relaxation in response to DNA double-strand breaks (DSB) [16]. KAP1 is phosphorylated on Ser 824 in an ATM-dependent manner after DSB induction. Moreover, KAP1 is phosphorylated exclusively at the damage sites, from which the phosphorylated KAP1 spreads rapidly throughout the chromatin. Ablation of the phosphorylation site of KAP1 leads to loss of DSB-induced chromatin decondensation and renders the cells hypersensitive to DSB-inducing agents [16]. ATM promotes DSB repair within heterochromatin by phosphorylating KAP1 [20]. KAP1 phosphorylation is also critical for 53BP1-mediated repair, and 53BP1-dependent robust localized KAP1 phosphorylation is essential for heterochromatic DNA double-strand break repair. Cells that do not form 53BP1 foci fail to form pKAP-1 foci [17]. Identification of the interaction of *PIG3* with KAP 1 has provided a new clue for understanding the crucial effect of *PIG3* playing in the cellular response to DNA double-strand breaks. More and more researches depict the correlation between DNA repair and chromatin remodeling [18,19,21–23]. Many DNA chromatin remodeling factors were proved to be involved in multiple pathways of DNA repair. As KAP1 plays a role in the heterochromatin relaxation, we have detected the possibility of *PIG3* involving in the large scale chromatin remodeling. By using a lac operator and GFP based reporter system, we showed that *PIG3* seems not to play a significant role in large scale chromatin decondensation in cells at the normal growing condition. However, *PIG3* indeed promoted the chromatin decondensation in the cells after exposure of ionizing radiation. In fact, KAP1 is a multi-functional protein. In addition to its actions as a corepressor of gene transcription and effector of heterochromatin relaxation mentioned above, KAP1 can also stimulate formation of p53-HDAC1 complex and inhibits p53 acetylation by interacting with MDM2 [24]. Therefore, it is worth to go further on the significance and mechanistic study of the interaction of *PIG3* with KAP-1, and its crucial role in the cellular response to ionizing radiation or DNA damage.

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Supporting Information

Figure S1 The dose-dependent changes of PIG 3 expression induced by ionizing radiation.

Figure S2 The effect of Tip60 on large scale chromatin relaxation.

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