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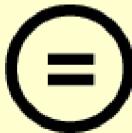

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2015 년 2 월
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PIG3 is involved in the Non-Homologous End Joining through interaction with Ku70

조선대학교 대학원

의과학과

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2015 년 2 월 25 일

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이 논문을 석사학위신청 논문으로 제출함

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CONTENTS

KOREAN ABSTRACT······vii

INTRODUCTION······1

MATERIALS AND METHODS

1. Cell culture······7
2. clonal survival assay······7
3. comet assay ······ 8
4. Immunoprecipitaion assay and western blot······9
5. Immunofluorescence microscopy ······ 10
6. Homologous recombination assay······ 12
7. Non-homologous end joining assay······ 13

RESULTS

1. Deletion of PIG3 leads to decrease DNA double strand break (DSBs) re

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pair ··· 14

2. PIG3 is involved in regulation of the ··· 16

3. PIG3 is not related with expression of NHEJ related protein ···18

4. Identification of a Ku70 as a PIG3 binding protein···19

DISCUSSION

Discussion··· 38

ABSTRACT

Abstract ···42

REFERENCES

References··· 44

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CONTENTS OF FIGURES

Figure 1. Preparation of PIG3 knockdown cells ··22

Figure 2. Knockdown of PIG3 sensitized cells to DNA damage ·····················24

Figure 3. Deletion of Pig3 leads to impaired DSB repair····························· 26

Figure 4. PIG3 is important for DNA damage−induced activation of NHEJ······ 28

Figure 5. PIG3 does not affect the HR ··· 30

Figure 6. Expression of NHEJ−related proteins in PIG3−depleted cells.······ 32

Figure 7. PIG3 interacts with Ku70 ··· 34

Figure 8. Identification of Ku70 binding site in PIG3 ································ 36

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국문초록

PIG3의 비상동결합 활성조절 연구

김 혜 림

지도교수 : 이 정 희

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의과학과

종양 억제 단백질 p53에 의해 유도되는 하위 유전자 PIG3 는 최근 DNA 손상 반응과 연관된 신규 플레이어로 보고되었지만, DNA 손상복구 경로에 관여하는 조절 기전은 명확히 알려져 있지 않다. 따라서 본 연구에서 우리는 DNA 이중가닥 손상 복구에 PIG3 의 분자적 메커니즘에 대해 밝히고자 한다. 세포 내 PIG3의 결핍은 IR 과 UV 등의 DNA 손상에 따른 세포민감도를 증가시키고, comet 분석과 γ-

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H2AX 염색을 통해 PIG3의 감소가 DNA 손상복구의 결함을 나타냄을 확인하였다. 또한, PIG3가 결핍된 세포는 비상동말단 결합에 결함을 보이나 상동재조합 에는 영향이 없음을 관찰하였다. 게다가 PIG3는 Ku70, Ku80, DNA-PK 을 포함하는 비상동결합 관련 단백질과 결합하며 특히, Ku70은 PIG3와 직접적으로 결합함으로써 암과 DNA 손상복구를 조절할 수 있음을 제시하였다. 면역침강 분석을 통해 PIG3 와 Ku70 의 결합에 PIG3의 201-250 아미노산 부위가 중요함을 증명하였다. 이러한 연구결과를 종합해 볼 때, PIG3는 Ku70 과의 결합을 통해 비상동말단 결합 활성을 조절하는 단백질로써 DNA 손상반응에 관여하는 중요한 물질임을 제시한다.

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INTRODUCTION

The DNA damage response is a complex signaling process involving the or-chestration of a variety of cellular events that activate rapidly in response to DNA damage[1]. The DNA double-strand break (DSB) is considered to be the most severe type of DNA damage induced by ionizing radiation, and this form of DNA damage must be repaired immediately to prevent cell death[2]. DSBs are the most catastrophic form of DNA damage and pose great threat to genome stability.

The major sensor of DSBs is ataxia telangiectasia mutated (ATM) kinase, which is critical for the initial steps of the DNA damage response (DDR). In re-sponse to DSBs, ATM phosphorylates and regulates the activity of several sub-strates involved in DNA repair, such as p53-binding protein 1 (53BP1) and his-tone H2AX[3]. To effectively repair a DSB, mammalian cells can choose from two different DDR pathways, namely homologous recombination (HR) and non-

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homologous end joining (NHEJ). HR occurs during S of G2 phase of the cell cycle and provides greater repair fidelity than NHEJ, which is the major pathway for the repair of DSBs in all phases of the cell cycle[4]. HR utilizes the homologous sister chromatid or homologous chromosome as a template, resulting in error-free repair of the missing information in the damaged DNA. In contrast, NHEJ is referred to as an intrinsically error prone repair pathway because this process joins the two broken-DNA ends without using a homologous template[5]. NHEJ can be subdivided into two pathways, the core or classical NHEJ pathway (C-NHEJ), which represents the main end-joining activity in the cell, and alternative NHEJ activities (A-NHEJ) consisting of microhomology mediated repair that functions as backup pathways to join DSB. The C-NHEJ complex in higher eukaryotic cells consists of DNA-dependent protein kinase (DNA-PK), which is

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composed of the Ku heterodimer and DNA-PK catalytic subunit (DNA-PKcs), Artemis, a DNA processing enzyme, a DNA ligase complex, XRCC4/DNA ligase IV. Other accessory factors, including polynucleotide kinase (PNK) and DNA polymerases μ and λ, have been implicated in some aspects of C-NHEJ[6].

Ku proteins are multifunctional proteins that possess deubiquitylation activity and play a key role in DNA repair and transcriptional regulation[7]. The Ku complex has been shown to inhibit apoptosis through an association with the proapoptotic factor Bax[8]. Interactions between Ku70 and p18-cyclin E, and Ku70 and Bax, provide a balance between apoptosis and cell survival in response to genotoxic stress[9]. The human Werner-syndrome protein (WRN), which is a member of the RecQ helicase family, also interacts with Ku, and these proteins are thought to serve a function in one or more pathways of DNA

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metabolism[10,11]. Ku has been shown to interact individually with three of the shelterin members, TRF1, TRF2 and Rap1, all of which have been directly implicated in inhibiting telomeric c-NHEJ[12]. To determine the role of Ku heterotetramerization in NHEJ and shelterins's involvement in inhibiting Ku's NHEJ function at human telomeres[13]. The Ku70 is a main component of the non-homologous end-joining (NHEJ) pathway that repairs DNA double-strand breaks (DSB).

The p53-inducible gene3 (PIG3) was originally identified by Polyak et al in the analysis of p53-induced genes related to the onset of apoptosis. PIG3 has been reported to have oxidoreductase enzymatic activity, this provides direct evidence of PIG3's participation in the generation of ROS in cells[2]. Additionally, human cellular apoptosis susceptibility protein (hCAS/CSEIL) interacts

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with the PIG3 promoter and affects p53-dependent apoptosis by regulating PIG3 expression[14]. PIG3 functions as an upstream component of the DNA damage pathways that are critical for the activation and maintenance of the DNA damage checkpoint-signaling pathways. PIG3 is a critical component of the DNA damage response pathway and has a direct role in the transmission of the DNA damage signal from damaged DNA to the intra-S and G2/M check-point machinery in human cells, but its mechanism involved in DNA repair pathway unknown[1].

In this study, we report that Ku70 associates physically with the DNA damage signal protein PIG3. We show that PIG3-deficient cells have a major DSB repair defect, shown by both the presence of γ-H2AX foci and by com-et-tail analysis. Further biochemical analyses show that PIG3 knockdown leads

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to downregulation of NHEJ, but not HR. Thus, we propose that PIG3 is involved in the NHEJ through a direct physical interaction with Ku70.

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MATERIALS AND METHODS

1. Cell culture

The human osteosarcoma bone morphogenetic cell line U2OS, human cervix adenocarcinoma cell line HeLa were cultured in Dulbecco’ s modified Eagles’ s medium. HCT116 cell were cultured in Iscove’ s Modified Dulbecco’ s Medium. In all cases, the media was supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 unit/ml), and streptomycin sulfate (100mg/ml). All cells were maintained in a humidified incubator containing 5% CO2 at 37℃. Upon reaching 70-80% confluency, cells were digested with 0.5% trypsin-EDTA before being passaged. Cells in exponential growth were harvested for subsequent experiments.

2. Clonal survival assay

After treatment with IR, 5×10^{2} cells were immediately seeded onto a 60mm dish in duplicate and grown for 2-3 weeks at 37℃ to allow colony for-

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mation. Colonies wre stained with 2% methylene blue in 50% ehtanol and counted. the fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated dells to untreated cells,

3. comet assay

DSB repair was assayed by alkaline single-cell agarose-gel electrophoresis as described previously. HCT116 cells , control and shPig3 cells were treated with 10Gy OF IR followed by incubation in culture medium at 37℃. Cells were then harvested, mixed with low-melting temperature agarose, and layered onto agarose-coated glass slides. Slide were submerged in lysis solution 10mM Tris-HCl(pH 10), 2.5M NACL, 01M EDTA, 1% Triton X-100, 10% dimethyl sulfoxide incubated for 30min in alkaline electrophoresis solution (300mM NaOH, 200mM EDTA at pH>13). The electrophoresis ran for 30min (1V/cm tank length), after which the slides were and fixed in 70% ethanol for 5min, air-dried and neutralized slides were stained with 30-50 μl ethidium bromide (20 mg/ml).

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Cells were screened per sample in a fluorescent microscope. Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system (Komet 5.5; Andor Thechnology, South Windsor, CT, USA)

4. Immunoprecipitation assay and Western blot

Cells were lysed in RIPA buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors (Roche Diagnostic Corp.). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hr with TBS-t (10mM Tris-HCl (pH 7.4), 150mM NaCl and 0.1% Tween-20) containing 5% skim milk and then incubated at 4° C with primary antibodies(1:1000). The blots were washed four times for 15 min with 0.1% Tween 20 containing TBS-t and then incubated for 2 hr with peroxidase-conjugated secondary antibodies (1:4000). The membranes were washed four

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more times and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

For the immunoprecipitation assays, aliquots of soluble cell lysates were precleared with protein A/G plus-agarose beads (Santa Cruz Biotechnology), G sepharose and A sepharose (GE Healthcare) as indicated and then incubated at 4℃ for 4h. Next, the appropriate antibody was added, and incubated at 4℃ for 12h. After the addition of fresh protein A/G plus-agarose bead, G sephasros and A sepharose, the reaction was incubated overnight at 4℃ with rotation. The beads were washed three times in RIPA buffer without protease inhibitors, resuspend-ed in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies

5. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were irradiated with 5Gy of ionizing radiation. cells were then washed twice with

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PBS, fixed with 4% paraformaldehyde for 10 min and ice−cold 98% methanol for 5 min, followed by permeabilization with 0.3% Triton X−100 for 15 min at room temperature. Next, the cover slips were washed three times with PBS and then blocked with 5% BSA in PBS for 1hr. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4° C. Next, the cells were washed with PBS and then stained with Alexa Fluor 594 (red, Molecular Probes) conjugated secondary antibodies, as appropriate. After washing, the cells were mounted using Vectashield mounting medium with 4,6−diamidino−2−phenylindole (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken using a Zeiss Axioplan 2 imagingepifluorescent microscope equipped with a charge−coupled device camera and ISIS software (MetaSystems, Altlussheim, Germany).

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6. DR−GFP assay (HR assay)

To measure the HR repair, stable cells lines expressing DR−GFP reports were generated by transfection using lipofectamine 2000. Clones were selected in the selection medium containing 500 μg/ml neomycin for 2 weeks and screened for significant induction of GFP positive cells following infection with I−SceI expressing adenovirus. U2OS−DR−GFP cells were transfected with con−trol or PIG3 siRNA using lipofectamine RNAimax, and then infected with I−SceI−carrying adenovirus at an estimated MOI of 10. After 72 hours, cells were fixed in 307% formaldehyde and stained with 5μg/ml Hoechst 33258 (Sigma). The images were shown at X 10 magnification using an inverted fluorescence microscope (Nikon). 488nm and 405nm wavelength were used to detect the ex−pression of Green Fluorescence Protein (GFP) and Hoechst−33258−stained DNA, respectively. The acquired data are presented as the mean ± s.d. value in three independent experiments.

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7. Non Homologous end joining assay

The NHEJ assay was measured in HeLa EJ5−GFP cells, using methods pre−vious described. EJ5−GFP contains a promoter that is separated from a GFP coding region by puromycin resistance gene, which is flanked by two I−SceI sites that are in the same orientation. When the I−SceI induced DSBs is repaired by NHEJ in HeLa EJ5−GFP cells, the puro gene is removed, and the promoter is rejoined to the rest of the GFP expression cassette, leading GFP expression. Similar to above HR assay, HeLa EJ5−GFP cells were transfected with control or PIG3 siRNA using lipofectamine RNAimax, and then infected with I−SceI−carrying adenovirus at an estimated MOI of 10. After 3days, the percentage of GFP−positive cells which had repaired the DSBs generated by I−SceI was de−termined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

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RESULT

1. Deletion of PIG3 leads to decrease DNA double strand break (DSBs) repair.

To determine whether PIG3 is involved in the DNA damage response, we created the HeLa and HCT116 cell lines containing a stably integrated PIG3-targeting small hairpin RNA (shRNA) expression vector. Immunoblotting confirmed that the expression of PIG3 was reduced by more than 90% in both cell lines stably transfecting with PIG3 shRNA, compared with that of control shRNA transfected cells.

We analyzed control shRNA and PIG3 shRNA expressing cells for their responses to DNA-damaging reagents. Control and PIG3 knockdown HCT116 and HeLa cells were treated with 2Gy and 5Gy IR. PIG3-depleted cells showed significantly compromised clonogenic cell survival after exposure to IR. PIG3 knockdown stable cells formed less colony numbers compare to negative control cells (Fig 2A).

The γ-H2AX is often used as a marker for exploring the spatial distribution and the DNA repair kinetics of cells following ionizing radiation exposure[15]. At DSB sites,

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γ−H2AX foci persist if DSBs are not repaired[16]. We measured the number of DSBs remaining after IR exposure as an indicator of now much of the damaged DNA re−mained unrepaired. As shown in Figure 3A, the depletion of PIG3 with shRNA led to increased level of unrepaired DSBs 24hrs after treatment with IR, as evidenced by the number of γ−H2AX foci remaining. These results suggest that PIG3 is required for DSB repair.

Further evidence of an overt repair defect was shown using the comet assay, which monitors the presence of DSBs in single cells. This very sensitive method can be used to detect low levels of DNA breaks IR treatment induces DSBs, visible as increased DNA mobility or ‘comet tails’[5]. For this, control and both PIG3 knockdown shRNA HCT116 cells were treated 10Gy IR to make DSB and harvested in different time points. In the initial time point's tail movement will be similar between control and PIG3 knockdown cells. But time persist, the DSB repair was measured based on tail movement. The unrepaired DNA strand breaks percentage of control cells de−

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creased in different time (0−6h) whereas PIG3 knockdown cells showed long comet tails. The observation of long comet tails in PIG3 deficient cells indicates impaired DNA DSB repair (Fig 3B). Together, these result suggest that PIG3 is critical for its function in DSB repair.

2. PIG3 is involved in regulation of the NHEJ

DSBs in which both strands in the double helix are severed, are the most dan− gerous type of DNA lesion. If left unrepaired, or repaired incorrectly, DSBs may re− sult in massive loss of genetic information, genomic rearrangements, or cell death[17]. DSBs are repaired by two major mechanisms: non−homologous end joining (NHEJ) and homologous recombination (HR). Plasmid rejoining assays in transfected cells and protein extracts were used initially, and they have yielded enormous insights into DSB repair mechanisms. The majority of these assays are fluorescence based and use the rare cutting endonuclease, I−SceI, to induce a single site specific DSB in

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cells. GFP-based chromosomal reporter assays in two stable cell lines, DR-GFP-U2OS and EJ5-GFP-HeLa, we used to measure HR of NHEJ, respectively.

To assess the involvement of PIG3 in NHEJ-mediated repair, we used EJ5-GFP-HeLa cells, which contain a promoter region separated from a GFP coding cassette by a puromycin (puro) gene flanked by two similarly oriented I-SceI site. Transfection of cells with I-SceI encoding constructs leads to the excision of the puro gene, and restoration of GFP expression relies on efficient NHEJ repair (Fig4A). We examined the levels of NHEJ when PIG3 was depleted and found that the percentage of GFP-positive cells was ~2 fold lower than in control cells (Figure4B and C) suggesting that PIG3 levels impact NHEJ.

To demonstrate the role of PIG3 in the HR, we determined whether depletion of PIG3 is related with HR. In the HR reporter strain, DR-GFP, DSBs are generated through the expression of I-SceI endonuclease, which cleaves a specific recognition site located in the GFP gene. In this system, repair efficiency via HR is monitored by

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measuring the percentage of cells expressing GFP using flow cytometry (Fig5A). The analysis of HR in DR-GFP-U2OS cells showed no difference in the PIG3 knockdown cells compare with control cells (Fig5B and C). We show that NHEJ activity decreased significantly in both PIG3-depleted cell lines in comparison to control cells, but HR activity not decreased. Taken together, these results indicate that PIG3 contributes to DSB repair through regulation of NHEJ but not contributes to HR (Fig 5A).

3. PIG3 is not related with expression of NHEJ-related protein

We next tested the protein levels of NHEJ-related protein, such as Ku70, Ku80, DNA-PKcs, and XRCC4, in PIG3-depleted and control HCT116 cells. We observed no significant difference in Ku70/80, DNA-PKcs protein level between transiently control and PIG3 knockdown HCT116 cells (Fig6A, left panel). The same result can also be seen in PIG3-depltеted HeLa cells (Fig6A, right panel). PIG3 has been found

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to participate in the DNA damage response induced by UV and the radiomimetic drug neocarzinostatin recently. We tested the protein levels of Ku70/80 and DNA-PKcs, in PIG3-depleted and control cell lines. PIG3-depleted HeLa (Fig6B) and HCT116 (Fog6C) cells showed no difference of NHEJ-related protein level at all time points. These finding implicate that PIG3 has role in the NHEJ regardless of NHEJ-related protein level.

4. Identification of a Ku70 as a PIG3 binding protein

PIG3 is required for NHEJ but its mechanism in NHEJ remain to be determined. To determine whether Ku70 interacts with PIG3 in human cells endogenously expressing both proteins, we used immunoprecipitation. For production of DSBs, HCT116 cells were treated with 5Gy of IR. The cells were then lysed, and endogenous PIG3 was immunoprecipitated with a PIG3-specific antibody. Immunoprecipitates were subjected to western blotting with an anti-Ku70 antibody.

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In this reciprocal experiments, Ku70 antibody was able to co-immunoprecipitate PIG3, suggesting that these proteins may interact with each other directly in cells. As control, normal rabbit IgG did not co-immunoprecipitate Ku70 or PIG3, indicating that the co-immunoprecipitation of Ku70 with PIG3 was not due to non-specific antibody binding (Fig 7A). To further confirm this interaction, HCT116 cells were transiently transfected with an expression construct encoding full length Ku70 tagged with HA and a second construct that expressed full length PIG3 tagged with GFP. These results showed us interaction between HA-Ku70 and GFP-PIG3 might be direct exogenously. To identify Ku70 binding domain of PIG3, we used seven PIG3 fragments consisting of residues PIG WT~PIG6. HCT116 cells were transiently co transfected with HA-tagged full length Ku70 and GFP tagged PIG3 constructs. The cells were lysed and, co-immunoprecipitated by using an anti-HA antibody and then immunoblotting was done with anti-GFP antibody. Co-immunoprecipitation results showed us PIG3 constructs PIGWT, PIG1, PIG2, PIG3 and PIG4 binding to HA-

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Ku70 but PIG5, PIG6 did not binding so immunoblotting was not shown any band. These results indicate us binding site of Ku70 is region 201−250 amino acid of PIG3 (Fig 8A, B).

In this study, PIG3 is involved in the repair of DSBs through a direct physical interaction with Ku70. To investigated the biological significance of the PIG3/Ku70 interaction, we assessed the role of PIG3 in NHEJ. Taken together these results indicate that PIG3 contributes to DSB repair through regulation of NHEJ.

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Figure 1

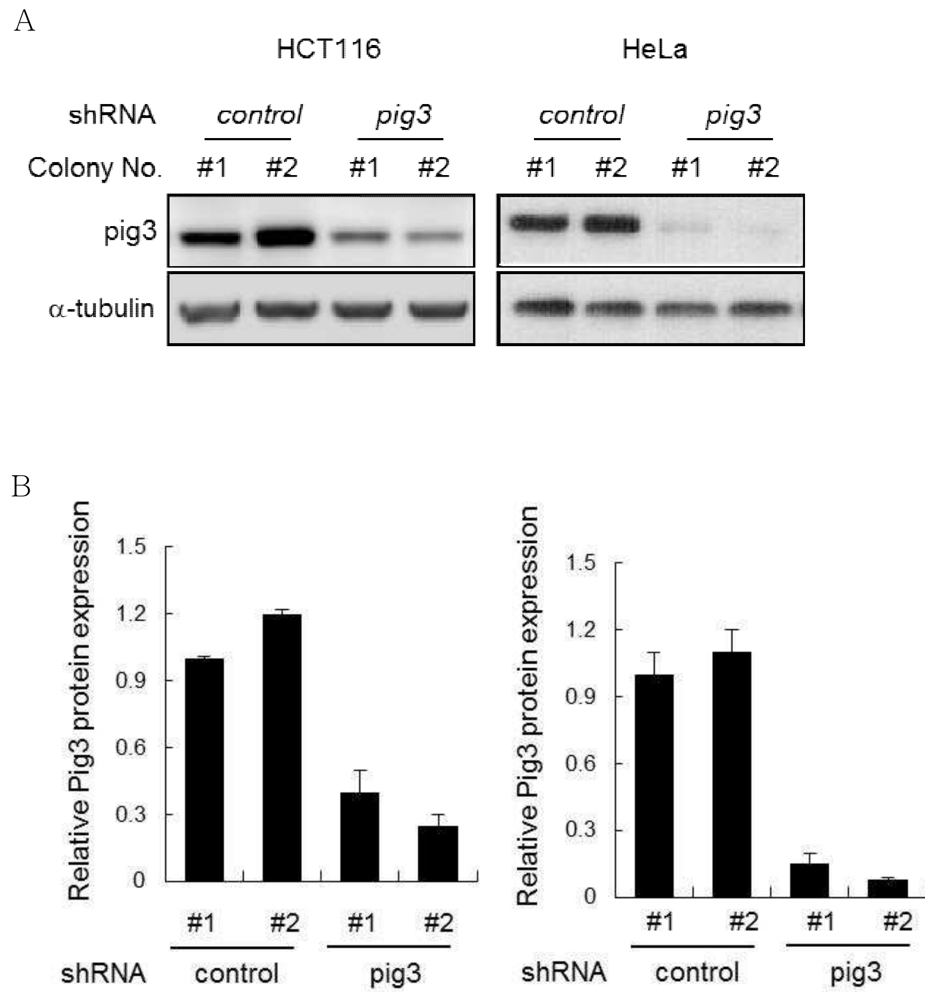

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Figure 1. Preparation of PIG3 knockdown cells A. HCT116 and HeLa cells were transfected with control or two different PIG3 small hairpin RNA (shRNA) expression vectors (PIG3 siRNAs #1 and #2), and selected with 300 μg/ml hygromycin. After three weeks, the expression level of PIG3 protein in each colonies was determined by western blot analysis using PIG3 antibody. B. The graph shows the quantitation of western blot analysis of PIG3. Results are shown as means ±SD (n=3)

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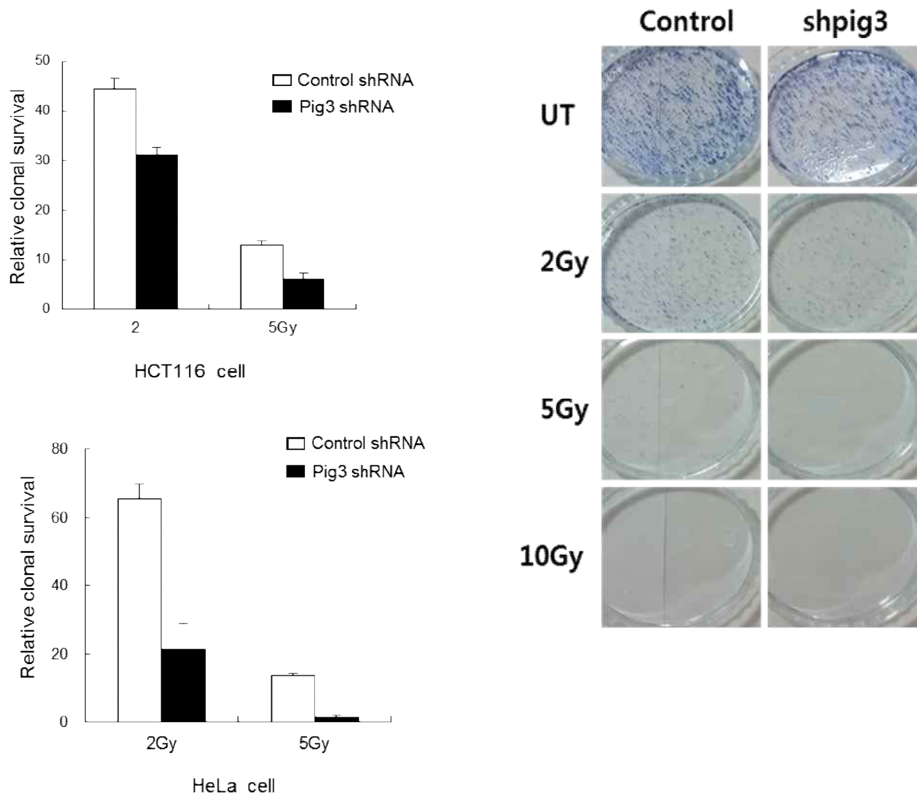
Figure 2

A



B

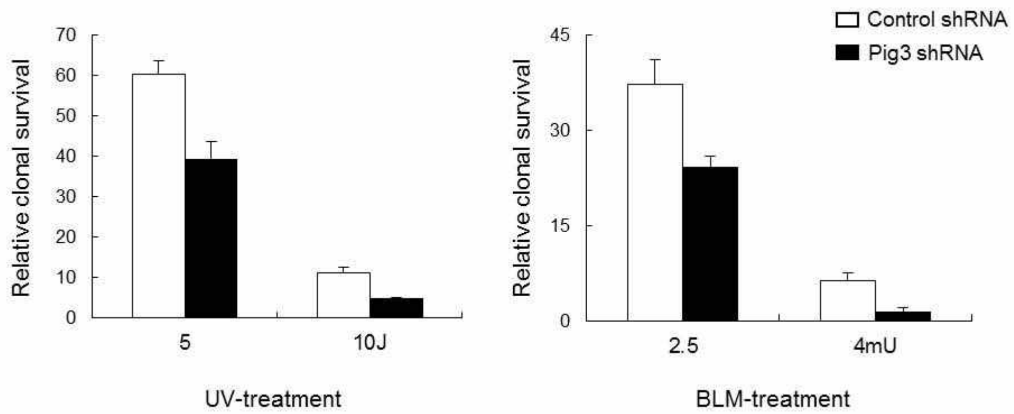


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Figure 2. Knockdown of PIG3 sensitized cells to DNA damage A. Pig3 depletion affects cell viability following exposure to γ-irradiation in HCT116 and HeLa cells. Control and Pig3 depleted cells were untreated of treated with 2 and 5GY ionizing radiations, and the number of surviving colonies was counted using staining of methylene blue. Data are presented as mean ±SD (left panel). Right panel Figure is represented surviving colonies in HCT116 cells. B. Control and PIG3-knokdown HCT116 cells were untreated of treated with the indicated dose of UV (left panel) or Bleomycin (right panel), and survival was assessed by the clonogenic survival assay. Results are shown as means ±SD (n=3)

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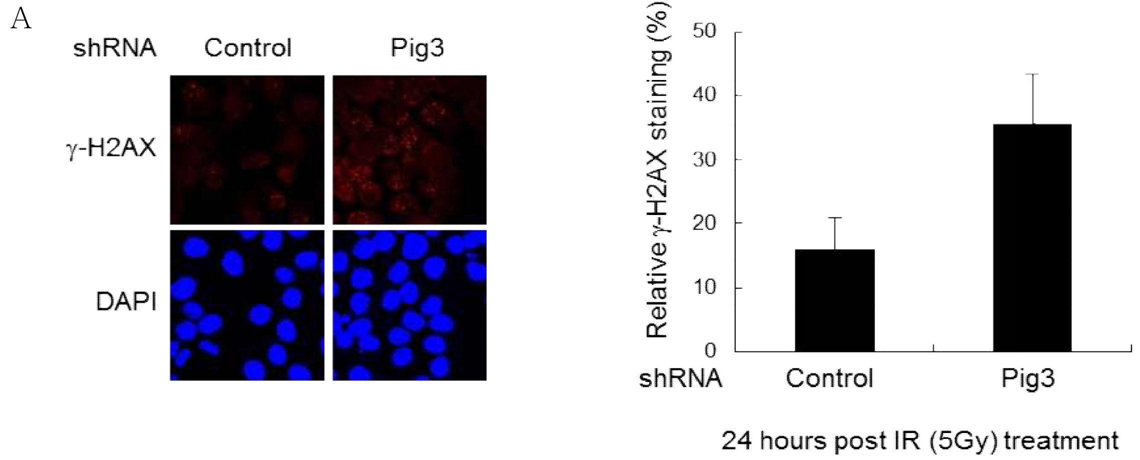
Figure 3



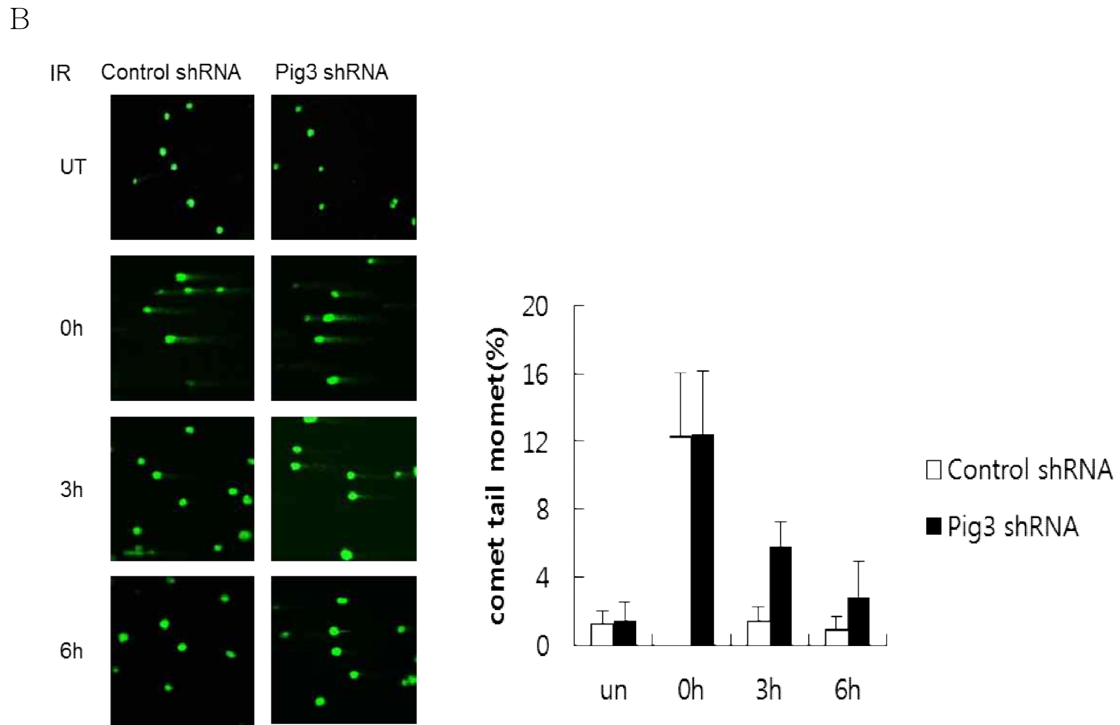


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Figure 3. Deletion of PIG3 leads to impaired DSB repair A. The control and PIG3 depleted HCT116 cells were untreated(UT) or treated with 5Gy ionizing radiation(IR) for 24h, and then fixed and immunofluorescence analysis with anti-body against γ-H2AX was carried out. 4'-6-Diamidino-2-phenylindole (DAPI) staining was performed to indicate the positions of nuclei. The percentage of cells and the respective foci are indicated. Representative images (left panel) and quantification (right panel) are shown. Results are shown as means ±SD (n=3) B. Control and Pig3-depleted HCT116 cells were untreated or treated with 10Gy ionizing radiation. At the indicated time points, cells were harvested for comet tail formation assay under alkaline conditions. Comet images were captured using fluorescence microscopy (left panel). The length and intensity of DNA tails relative to heads is shown as % of the relative tail moment (n=100) in the right panel. Results are shown as means ±SD (n=3)

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Figure 4

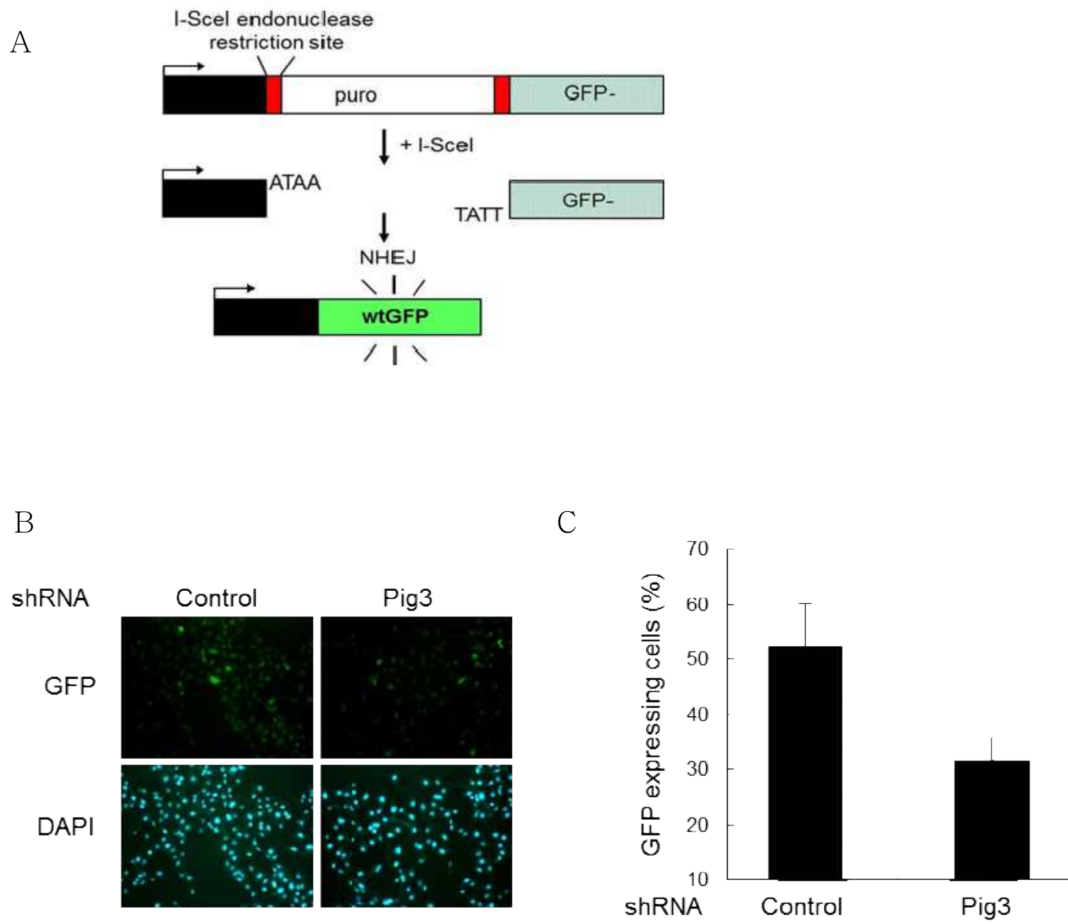

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Figure 4. PIG3 is important for DNA damage−induced activation of NHEJ A. A diagram for the NHEJ assay based on the EJ5−GFP reporter, which contains two tandem recognition site for the I−SceI endonuclease. Once DSBs are generated by I−SceI and the puro gene is excised by NHEJ repair, the promoter is joined to the rest of the expression region, leading to restoration of functional GFP. B. EJ5−GFP−HeLa cells were transfected with the control or PIG3 siRNA for 24hours and then transfected with the I−SceI expressing adenovirus to induce double strand breaks. After 72hours, the GFP−positive cells were visualized us−ing fluorescence staining was performed to indicate the visible cells. C. The population of the cells for GFP expression was measured by flow cytometry. Results are shown as means ±SD.

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Figure 5

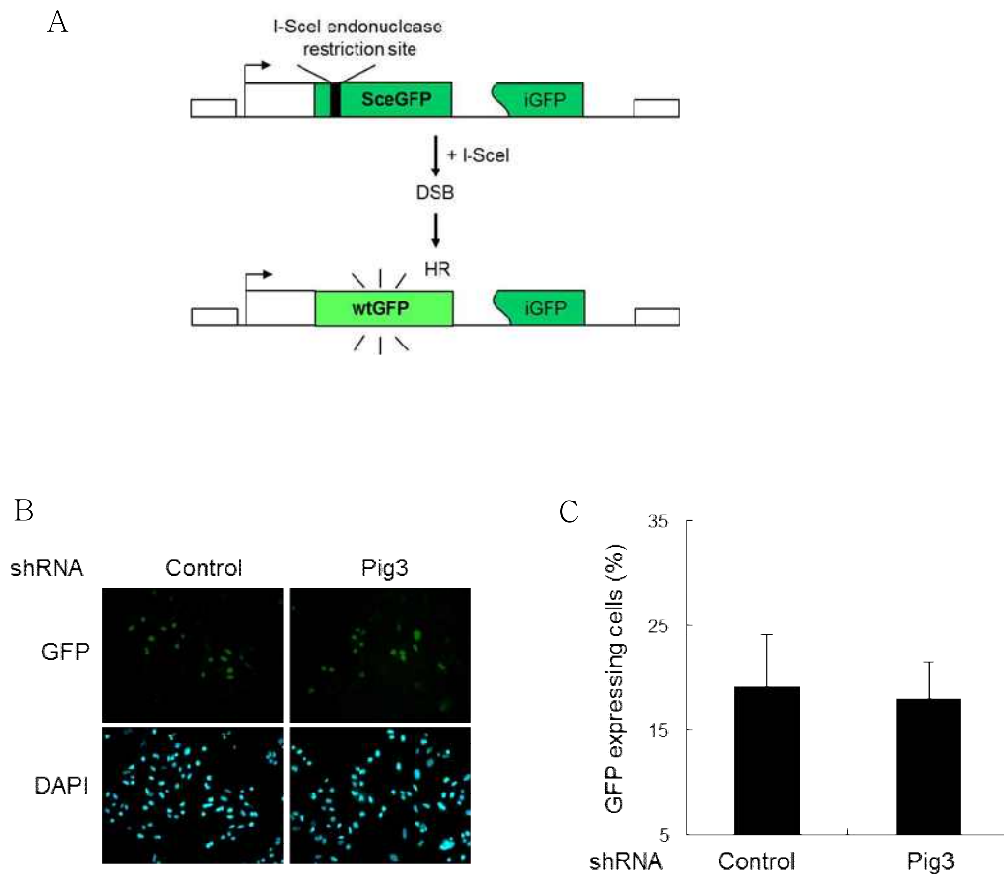

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Figure 5. PIG3 does not affect the HR A. Schematic representation of HR assay. B. The efficiency of HR in control and PIG3-depleted cells was investigated by measuring the fraction of GFP+ cells in the U2OS DR-GFP reporter assay. The presence of an I-SceI site in the GFP reporter gene allows for the introduction of DSB in the presence of the endonuclease I-SceI. When the DSB is repaired, the reporter construct will then express GFP that can bel measured and quantitated. C. The same experiments and quantitation described in Fig4B and C, were performed using DR-GFP-U2OS cells instead of EJ5-GFP-HeLa cells. Results are shown as means ±SD. No significant difference was observed in HR between shPIG3 and control cells.

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Figure 6

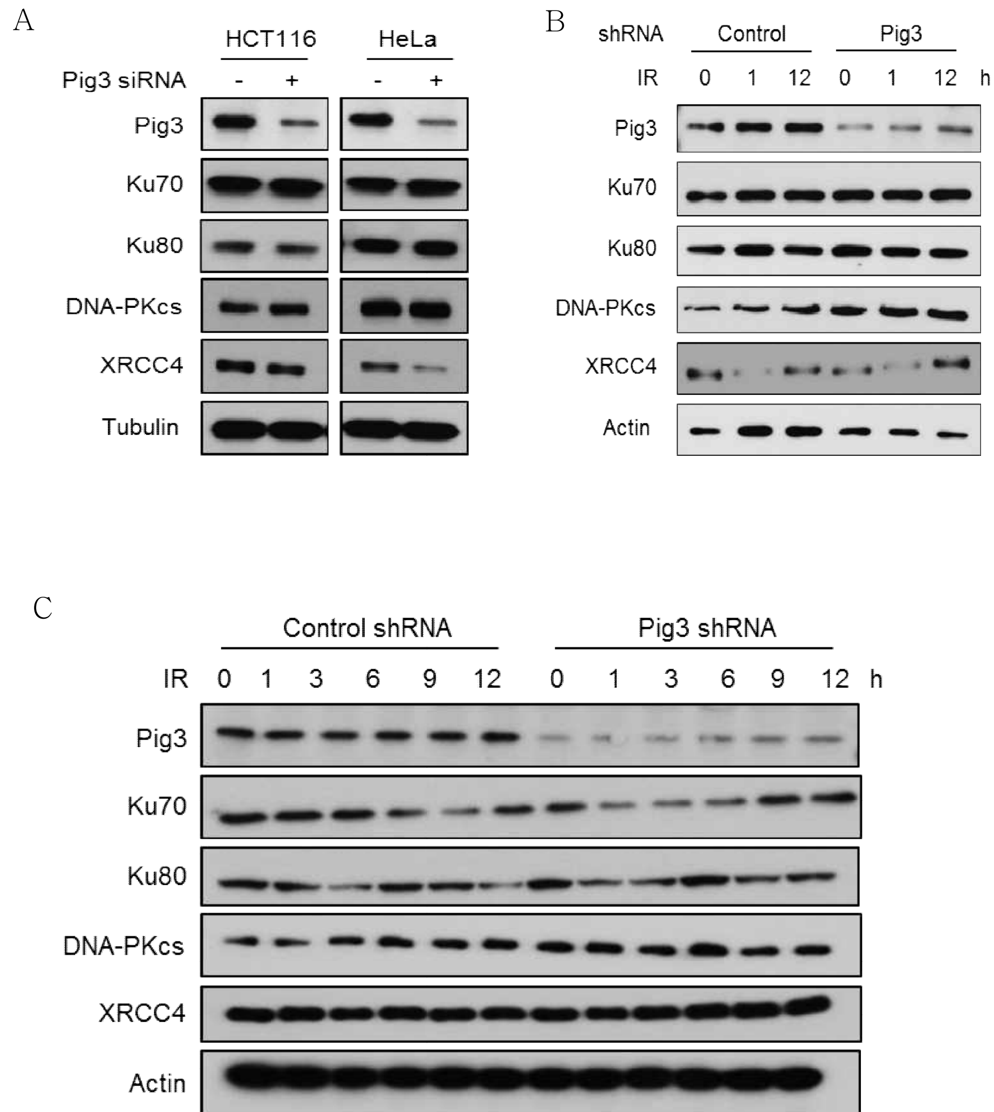

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Figure 6. Expression of NHEJ-related proteins in PIG3-depleted cells A.PIG3 protein levels in control or PIG3 depleted HCT116 and HeLa cells were tested by immunoblotting analysis. B. The NHEJ protein level were detected by immunoblotting analysis at three time points post 10Gy of γ-irradiation in PIG3 depleted (HCT116a-shPIG3) and control (HCT116-shCTL) cell. C. Control and PIG3-depleted HeLa cells were treated with 10Gy of γ-irradiation . Whole cell lysates were prepared at the indicated time points and immunoblot analysis was performed using specific antibodies against PIG3, Ku70/80, DNAPK-cs, XRCC4, and β-Actin.

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Figure 7

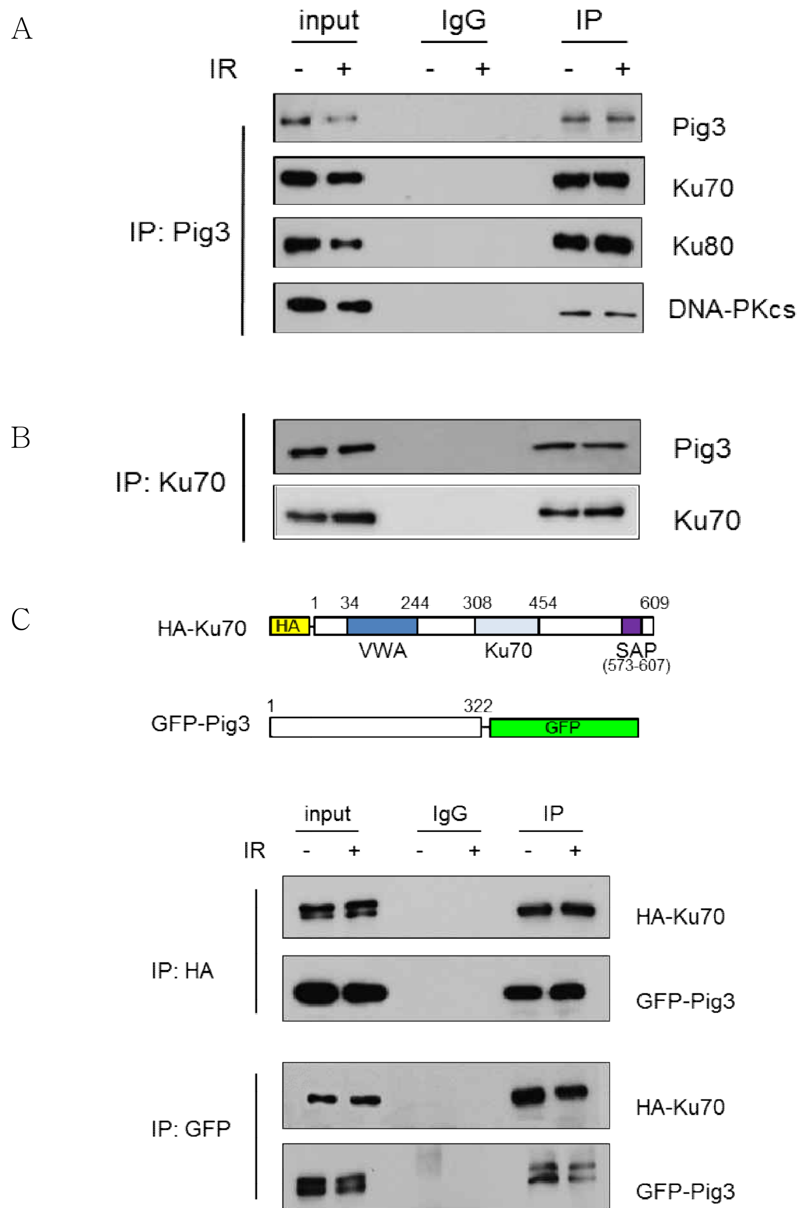

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Figure 7. PIG3 interacts with Ku70 A. HCT116 cells were untreated or treated with 10Gy γ−irradiation for 6h. Proteins were immunoprecipitated from the ly− sates using an anti−PIG3 antibody. Immunoprecipitates were then subjected to western−blot analysis using antibodies specific for Ku70, Ku86, DNA−PKCS and PIG3. Normal rabbit IgG was used for negative control immunoprecipitation. B. HCT116 cells were prepared as in (A), and lysates were subjected to immunoprecipitation for anti−Ku70 antibody followed by western blot analysis with indicated antibodies. C. A schematic representation of HA−tagged wild type Ku70 and GFP tagged Pig3 (upper panel). Lower panel, HA−Ku70 interacts with GFP−Pig3. HCT116 cells were transfected with full−length GFP−Pig3 and HA− Ku70 expression vectors. Proteins were immunoprecipitated from the lysates using an anti−HA Ku70 antibody. Immunoprecipitates were then subjected to western blot analysis using antibodies specific for GFP−Pig3 or HA−Ku70. Nor− mal rabbit IgG was used for negative control immunoprecipitation.

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Figure 8

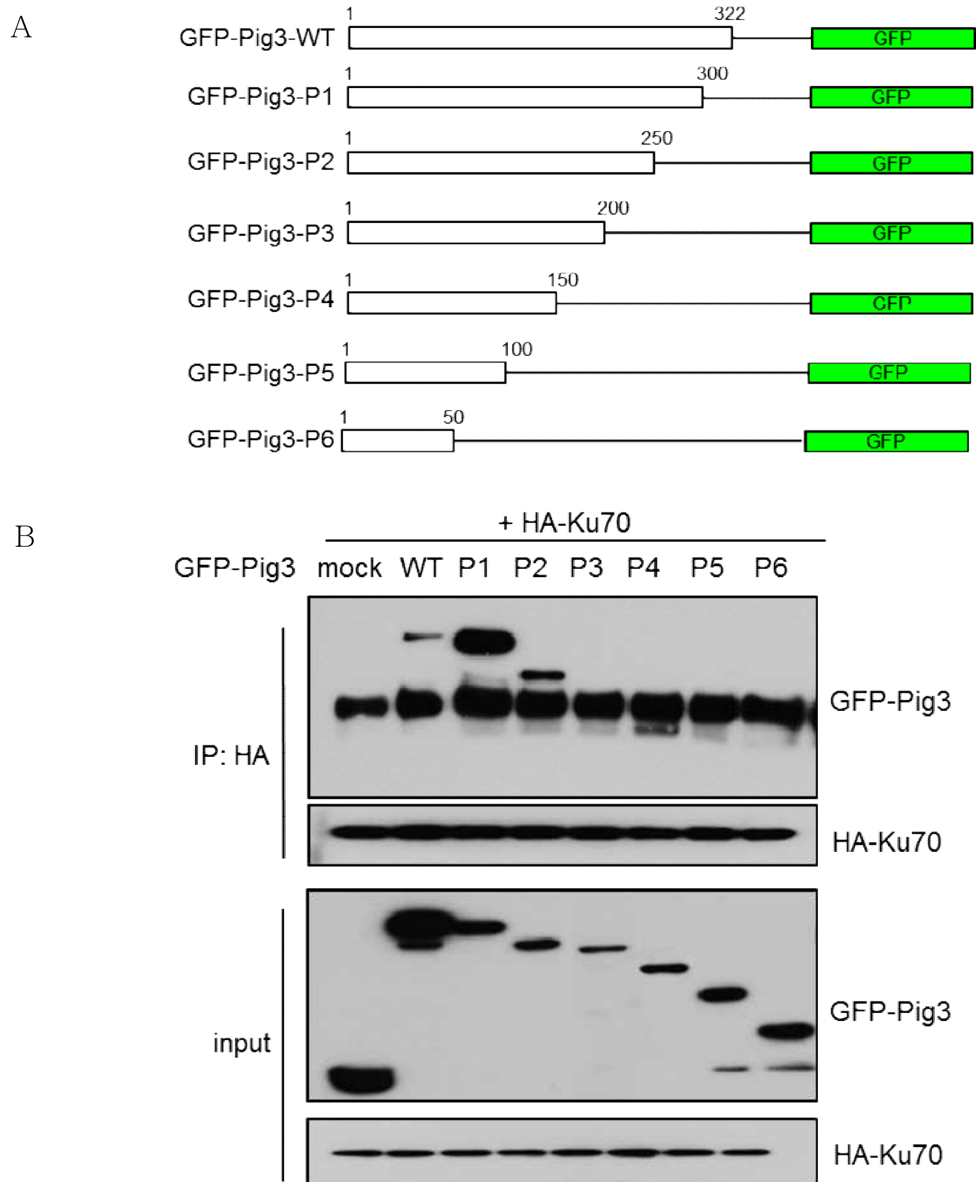

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Figure 8. Identification of Ku70 binding site in PIG3 A. Schematic representation of GFP-tagged PIG3 wild type and various deletion mutants. B. Co-Immunoprecipitation of HA-Ku70 and GFP-PIG3 constructs. HCT116 cells were co-transfected with HA-Ku70 and various GFP-Pig3 constructs named Pig WT~ Pig 6 and mock as control GFP expression vectors. After 48hrs, cells were treated with 10Gy γ irradiation for 3hrs. Proteins were immunoprecipitated from the lysates using and anti-HA antibody. The precipitated proteins were detected by western blotting with anti-HA and anti-GFP antibodies.

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DISCUSSION

The DNA damage response is a comprehensive signaling process including damage sensing, mediators activation, cell cycle arrest and DNA repair. PIG3 was originally identified in a screen for genes induced by p53 before the onset of apoptosis. PIG3 has been recently reported to participate in DNA damage response induced by UV radiation and the radiomimetic drug neocarzinostatin, and by ionizing radiation[1,19].

Many studies have implicated or shown a direct role of ROS in p53-dependent apoptosis[20]. Activated p53 increases cellular ROS levels by enhancing the transcription of proapoptotic genes, such as PIGs and Bax[21]. PIG3 shows sequence similarity with NQO, and influences the production of intracellular[22]. Thus, although PIG3 expression alone is insufficient to induce apoptosis, PIG3 may be one of the factors involved in p53-induced apoptosis through ROS generation. Clonal survival assays indicated that PIG3-deficient cells were more sensitive

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than control cells to DNA damage-induced cell death. This increased sensitivity in PIG3 knockdown cells could be attributed to a defect in the DNA damage repair. In this reports, we also showed that cells exhibiting PIG3 silencing showed a significant reduction in DNA repair.

In this study, we present several observations that relation of PIG3 in DSB repair. First, PIG3 forms IR induced foci that co-localize with γ-H2AX at the sites of DNA breaks. Second, we found that Ku70 co-immunoprecipitates with PIG3 from HCT116 cell extracts. Finally, we showed that the interaction between PIG3 and Ku70 regulates NHEJ, which is critical for repair of DSBs. Recently, several lines of evidence suggest that PIG3 plays a role in repair of DSBs. However, the underlying mechanisms by which PIG3 regulates DSB repair remain unknown. In the present study, we explored whether PIG3 is involved in the repair of DSBs. We utilized PIG3 deficient HCT116 cells to investigate whether PIG3 knockdown affected DSB repair using two different assays that measure

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the levels of DSBs, the γ−H2AX and comet assays. After DNA damage, many DNA damage repair proteins are recruited to the DNA damage sites and form discrete DNA damage induced nuclear foci. The γ−H2AX focus formation on the DSB sites is one of the earliest events in response to forms of DNA damage that induce DSBs. Using γ−H2AX foci analysis as an approach to measure DSB re−pair, we compared repair in PIG3−knockdown cells and control cells treated with the IR. We results showed that PIG3−deficient HCT116 cells have a significant repair defect that was detected both by γ−H2AX foci and comet tail analyses. Comet assay data shown in also indicate that rejoining of IR induced DSBs was significantly delayed by PIG3 knockdown, similar to γ−H2AX foci measure−ments. These findings support a role for PIG3 in DSB repair in human cells. As described above, we demonstrated that PIG3 interacts with Ku70 and that HCT116 cells stably knockdown PIG3 shRNAs exhibit impaired ability to repair DSBs. Thus, it seems reasonable to expect that PIG3 contributes directly to

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NHEJ activity. PIG3-deficient HeLa cells were shown to exhibit an 20% reduction in EJ5-GFP induced NHEJ.

The Ku heterodimer (Ku70/Ku80) is a main component of the nonhomologous end-joining (NHEJ) pathway that repairs DNA double-strand breaks (DSBs). Ku binds the broken DNA end and recruits other proteins to facilitate the processing and ligation of the broken end[23]. Thus, the PIG3 could further increase the binding affinity of Ku for DNA, modulate the amount of Ku70 or facilitate the recruitment of other important NHEJ proteins to bind the broken-DNA ends. Furthermore, studies aimed at determining the detailed mechanisms underlying PIG3 modulation of NHEJ efficiency are currently under way.

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ABSTRACT

PIG3 is involved in the Non-Homologous End Joining through interaction with Ku70

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The Tumor suppressor protein p53-Inducing gene3 (PIG3) recently has been reported to be a new player in DNA damage signaling and response, but it was not clear that the mechanism involved in DNA repair pathway. In this study, we sought to elucidate molecular mechanism of PIG3-related DNA double strand breaks repair. We show that depletion of endogenous PIG3 sensitizes cells to DNA damage agents such as IR, bleomycin and UV. Comet assay and remaining γ-H2AX show that knockdown of PIG inhibits DNA repair. In addition, PIG3-

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depleted cells show defect of non-homologous end joining (NHEJ) but not homologous recombination (HR). Moreover, PIG3 is associated with non-homologous end joining related protein including Ku70, Ku80 and DNA-PK in vivo. Especially, Ku70 directly interacts with PIG, suggesting a possible link between a cancer and DNA repair, co-immunoprecipitation analyses revealed that the PIG segment comprising amino acids 201-250 is critical for PIG-Ku70 interaction. Therefore, these results suggest that PIG contributes to non-homologous end joining of the DNA damage response through interaction with Ku70.

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