2010년 2월
석사학위논문

The function of p53 on the DNA damage response

조선대학교 대학원

생물신소재학과

박 지 연

The function of p53 on the DNA damage response

p53의 유전자손상 반응 연구

2010 년 2 월 25일

조선대학교 대학원

생물신소재학과

박 지 연

The function of p53 on the DNA damage response

지도교수 장 인 엽

이 논문을 이학석사학위신청 논문으로 제출함

2009년 10월

조선대학교 대학원

생물신소재학과

박 지 연

박 지 연의 석사학위논문을 인준함

위원장 조선 대학교 교수 장인엽 (인)

위 원 조선 대학교 교수 전제열 (인)

위 원 조선 대학교 교수 유호진 (인)

2009년 11월

조선대학교 대학원

CONTENTS

국문초록

P53의 유전자 손상 반응 연구

박 지 연
지도교수 : 장 인 엽
조선대학교 대학원 생물신소재학과

DNA 손상반응은 checkpoint라 불리는 신호전달 체제로 간략히 설명 할 수 있다. DNA Checkpoint는 sensor에 의해 DNA손상이 인지되고 순차적으로 transducer와 effector에 의해 신호가 전달되어 결국은 유전자의 전사나 세포주기를 조절하거나, 세포사멸이나 DNA 복구 등을 조절하는 일련의 과정을 의미한다.

본 연구에서 우리는 p53에 의해 유도되는 pig3가 DNA checkpoints에 관여하는 여러 sensors와 결합함을 보았다. 또한 pig3가 nuclear foci을 형성함을 관찰하였다. Pig3가 DNA damage의 표시자로 널리 알려진 H2AX와도 결합하며, H2AX 발현을 유도함으로서 G2/M checkpoint와 intra-S checkpoint를 조절함을 확인하였다.

pig3는 p53의 하위유전자로서 p53또한 DNA 손상반응에서 중요한 단백질임이 알려져 있다. 이런 p53과 pig3의 연관관계를 조사한 결과, p53이 결핍된 세포에서는 pig3의 발현양이 현저히 낮았으며, 또한 DNA checkpoint 결함을 보였다. 이런 p53결핍세포에 pig3를 과발현 시키면, DNA checkpoint 반응 역시 회복됨을 관찰하였다. 그러나 p53 돌연변이 세포에서는 pig3가 DNA checkpoint 와는 무관하게 pig3 과발현에 의해 세포사멸을 촉진함을 알 수 있었다.

따라서 본 연구를 통해서 pig3는 p53에 의한 DNA 손상반응을 조절하는 새로운 단백질임을 확인하였다.

INTRODUCTION

In the DNA damage response, phosphatidylinositol 3-kinase-like kinase (PIKK) initiate cellular cascade events that modulate gene transcription, cell cycle checkpoints, apoptosis and DNA repair (Sancar et al., 2004; Zhou and Elledge, 2000). Defect in any of these pathway may cause genomic instability and the development of cancer in multicellular organisms (Motoyama and Naka, 2004). Ataxia-telangiectasia-mutated (ATM), ataxia-telangiectasia, Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PKcs) are members of PIKK family (Falck et al., 2005). ATM and DNA-PKcs response mainly to DNA-double strand breaks caused by ionizing radiation (IR) or radiomimic drugs such as Bleomycin (BLM) or Neocarzinostatin (NCS), whereas ATR is activated by single-stranded DNA, stalled DNA replication fork and other forms of DNA damage such as that caused by ultraviolet light (UV) (Abraham, 2001; Falck et al., 2005). Moreover, PIKK family members are recruited to sites of DNA damage by different factors (Bakkenist and Kastan, 2004). Recruitment of ATR requires ATRIP, MRN complex (Mre11/Rad50/Nbs1) for ATM is required, and DNA-PKcs recruitment is mediated by Ku70-Ku80 heterodimer. After their recruitment to sites of DNA damage, PIKK phosphorylates a number of substrates, including checkpoint kinase Chk1 and Chk2, transcription factor such as p53 and other factors, which in turn target proteins to induce cell cycle transition, DNA repair and apoptosis. Histone H2AX, 53BP1, Mdc1, TopBP1, Claspin and BRCA1 are also targets for PIKK-mediated phoshphorylation, and have been suggested to as scaffold protein to bring together the PIKK and their substrates (Bakkenist and Kastan, 2004; Zhou and Elledge, 2000).

One of the first DNA damage responses is the phosphorylation of H2AX. H2AX is mammalian histone H2A variant, is phosphorylated on serine139 residue at the conserved SQ motif in the C-terminal tail by PIKK to generate H2AX over large chromatin domains surrounding DNA damage. H2AX appear within 1 to 3 minutes

after DNA damage, their numbers increase and hundreds to several thousand H2AX molecules are present per DNA damage sites which can be showed by immunostaining, termed as nuclear foci. H2AX is essential for the recruitment of 53BP1, BRCA1, MDC1, and the MRN complex to the site of DNA damage (Fernandez-Capetillo et al., 2004). H2AX is also critical for DNA repair by interaction with repair protein such as Rad50 and Rad51, so repair-deficient cells reduced ability to increase the phosphorylation of H2AX (Foster and Downs, 2005; Paull et al., 2000). Therefore, loss of H2AX abrogates these function, leading to chromosomal instability and increased susceptibility to cancer (Bassing et al., 2002). H2AX can be detected by Western blotting and visualized as distinct nuclear domains (foci) by immunofluroresecence.

The checkpoint function are mediated by effector proteins termed Chk1, Chk2 and p53. ATR is required for Chk1 phosphorylation after UV exposure, whereas ATM and Nbs1 phosphorylated Chk1 and Chk2 after IR exposure, leading to activation of G1, intra S and G2/M checkpoint (Sancar et al., 2004). A major mechanism of Chk1 and Chk2 is through inactivation of Cdc25 family phosphatases, Cdc25 family are responsible for dephosphorylation of Tyr15 and Thr14 of Cdk/cyclin, which in turn Cdk activation and leads to cell cycle progression (Donzelli and Draetta, 2003). Cdc25 family proteins consist of three related isoform : Cdc25A, Cdc25B and Cdc25C. Cdc25A regulates both early and late cell cycle transition from G1, S and G2 phase to mitosis. DNA damage activated-Chk1 and -Chk2 phosphorylate Cdc25A on Ser 78 and Ser123, leading to ubiquitin-dependent proteosomal degradation of Cdc25A (Donzelli et al., 2002). Cdc25B and Cdc25C more limited roles in transition from G2 phase to mitosis. In DNA damage response, Cdc25B and Cdc25C are phosphorylated on Ser309 and Ser216, respectively, leading to 14-3-3 binding and cytosolic retention (Donzelli and Draetta, 2003; Peng et al., 1997). p38 MAPK as well as Chk1 and Chk2 has a critical role in G2/M and intra S checkpoint-activation after UV radiation (Bulavin et al., 2001). The p38 kinase phosphorylates MAPKAP Kinase-2 on Thr334 after UV radiation, leading to activation of G2/M and intra-S checkpoint through prevention of phosphorylation of Cdc25B on Ser309 and 14-3-3 binding (Bulavin et al., 2001; Manke et al.,

2005; Reinhardt et al., 2007).

The p53 tumor suppressor protein is a critical downstream effector in DNA damage response. The p53 is transcriptional factor that is activated in response to variety of DNA damaging agents, such as IR, UV and hypoxia (Vogelstein et al., 2000). Under normal condition, p53 is expressed at low level by its interaction with MDM2, which mediates both ubiquitination and proteosomal degradation pathway. However, upon DNA damage, p53 protein is accumulated and activated through posttranscriptional modification, and this leads to cell cycle arrest, apoptosis and DNA repair (Agarwal et al., 1998; Giono and Manfredi, 2006; Helton and Chen, 2007; Vogelstein et al., 2000). Like Chk1 and Chk2, p53 protein is activated by PIKK family proteins. ATM and DNA-PK kinases phosphorylate p53 in response to DNA damage, including IR, and activated p53 is participated in cell cycle checkpoint activation, DNA repair and apoptosis through transactivation of downstream genes such as p21, GADD45, 14-3-3, pig3, Bax, Bcl-2 PUMA, NOXA, DR-5/KILLER and several repair proteins (Helton and Chen, 2007; Okorokov, 2003; Vogelstein et al., 2000). The p53 directly phosphorylated on Ser15 by ATM or indirectly activated through Chk2 phosphorylation by ATM (Abraham, 2001). ATM also regulates p53 activity through phosphorylation of MDM2 on Ser395, which is phosphorylation of MDM2 allows the interaction with p53 but inhibits p53 nuclear transport and proteosome-mediated degradation (Maya et al., 2001). ATR directly phosphorylates p53 on Ser15 or Ser20, or through Chk1 activation, leading to normal DNA damage response that help to maintain genomic stability (Abraham, 2001; Hastak et al., 2008). However, it is not exactly know how p53 regulates DNA repair, checkpoint activation and apoptosis.

Pig3 (p53 inducible gene 3) was originally identified through Serial Analysis of Gene Expression study (SAGE) designed to search genes induced by p53 before the onset of apoptosis (Polyak et al., 1997). The gene was localized to chromosome 2p and was highly related to -Crystallin protein in guinea pig3 lens and TED2, plant NADPH oxidoreductase (Demura and Fukuda, 1994; Rao et al., 1992; Rao and Zigler, 1992).The p53 tumor suppressor directly bound to pentanucleotide microsatellite sequence $(TGYCC)_n$ (Y=C or T) within pig3 promotor. Polymorphic

microsatellite of the pig3 promotor have been reported to correlate with activation by p53, and pig3 activity was related to the number of repeats within it (Contente et al., 2002). The p53 proline-rich functional domain (between amino acids 64 and 92) was required for ROS generation-related apoptosis, was also correlated with pig3 gene transactivation (Venot et al., 1998). Moreover, it was related with proliferation, reversible growth arrest as well as p53-depentent apoptosis (Campomenosi et al., 2001). Pig3 shared significant homology with oxidoreductase, which involved in cellular response to oxidative stress. It was reported that NADH quinine oxidoreductase 1(NQO1) regulated stability of p53 protein and p53 dependent apoptosis against -irradiation (Asher et al., 2001). On the other hand, Pig3 alternative splice protein (PIG3 AS) inducing by UV light identified, it was rapidly degraded through proteasome degradation pathway (Nicholls et al., 2004). Recent reports showed that human cellular apoptosis susceptibility protein (hCAS/CSE1L) interacted with pig3 promoter and regulated its expression leading to p53-dependent apoptosis (Tanaka et al., 2007). Nevertheless, the exact role of Pig3 in DNA damage response is not yet clear.

Here, we report a role of pig3 in the DNA damage response. In contrast with previous reports, which pig3 cause p53-dependent apoptosis through ROS generation, knockdown of pig3 induced cell death and impaired DNA repair. We find that pig3 is associated with DNA damage sensors, such as MRN complex (Mre11/Rad50.Nbs1), 53BP1, ATM, DNA-PK and H2AX, after DNA damage through forming of nuclear foci, and also regulates recruitments of DNA damage sensors to foci, and is required for cell cycle checkpoint function. We go on to show that pig3 is essential for maintenance of genetic stability through p53-mediated normal DNA damage response using cancer cells
having different p53 status.

MATERIALS AND METHODS

Cell culture and treatment

HCT116 p53+/+ and p53-/- colorectal carcinoma cells were cultured in Iscove's modified Dulbeco's medium (IMDM, Gibco-BRL, Grand island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Camblex, Walkersville, MD), 100units/ml penicillin and 100 g/ml streptomycin sulfate (Invitrogen, Carlsbad, CA) in a 5% CO_2 humidified incubator at 37C. For UV treatment, cells were rinsed in culture media and exposed to 254nm UVC lamp (UVP; Model UVGL-25) in a minimal volume of serum free culture media at a dose of 10J. After exposure of UV, changed complete culture media followed by continued culture at 37C.

Small interfering RNAs (siRNA) and transfection

For knockdown of pig3 expression, the target site of siRNA was chosen from the human tumor protein p53 inducible protein 3 (TP53I3, pig3)mRNA sequence (Gene Bank accession number NM_004881), which was extracted from the NCBI Entrez nucleotide database. This target site was also searched with National Center for Biotechnology Information BLAST to confirm the specificity only to the human pig3. The sequences of the 21-nucleotide sense and antisense RNA are as follows : 5`-AAAUGUUCAGGCUGGAGACUAdTdT-3` and 5`-UAGUCUCCAGCCUGAACAUUUdT dT-3`. Negative control siRNA duplex was purchased from Bioneer. The sequences are follows : 5`-CCUACGCCAAUUUCGUdTdT-3` and 5`-ACGAAAUUGGUGGCGUAGGdT dT-3`. siRNA duplexes were transiently transfected into the cells using RNAimax according to the manufacturer's instructions (invitrogen). These siRNAs were prepared by a transcription-based methods using the Silencer siRNA construction kits according to manufacturer's instructions (Ambion, Austin, TX). Cells were transfected with the constructed siRNA expression plasmid based on pSilencer-hygro vector (Ambion), which includes a human U6 promoter and a hygromycin resistance gene, using LipofectAMINE2000 (Invitrogen). For stably knockdown of pig3, after transfection with *psilencer*-empty or -pig3 vector, we selected several resistant

colonies against 300 g/ml hygromycin in the culture medium. ATM, ATR and DNA-PK siRNA duplex was purchased from Santa cruz biotechnology.

Transfection of plasmid

For manufacture of pig3 expression vector, the entire coding region of human pig3 cDNA was amplified by RT-PCR using the pig3 oligo primer : sense 5`-ATGTTAG CCGTGCACTTTGACAA-3` and antisense 5`-TCACTGGGGCAGTTCCAGGAC-3` from human fibroblast GM00637 cells. The amplified pig3 PCR products were inserted into a mammalian expression *pcDNA3.1* neo vector or *pcDNA3.1* zeo vector containing a CMV promoter and neomycin resistance gene or zeocin resistance gene, respectively (Invitrogen). Pig3 sequences and orientation were confirmed by automated DNA sequencing. If required, we selected antibiotics resistant colonies after transfection. To produce $p53^{R248W}$ and $p53^{R273H}$ mutants, pcDNA-myc3-wtp53 (a gift from JH Jeong, National University of Seoul) was used as template DNA. Site directed mutagenesis of p53 was performed using Muta-Direct™ Site-Directed Mutagenesis kits according to manufacturer's instructions (iNtRON Biotech, Seoul, Korea). The sequences of mutagenesis-primer are as follows : forward primer 5`-ATGGGCGG CATGAACTGGAGGCCCATCCTC-3` and reverse primer 5'-GAGGATGGGCCTCC AGTTCATGCCGCCCAT-3' for $p53^{R248W}$ construct, and forward primer 5'-AACAGCTTT GAGGTGCATGTTTGTGCCTGT-3` and reverse primer 5`-ACAGGCACAAACATGCA CCTCAAAGCTGTT-3' for $p53^{R273H}$ construct. Cells were transfected with appropriate plasmid using LipofectAMINE 2000 (Invitrogen) according to manufacturer's instructions.

Immunoprecipitation assay and Western Blot analysis

The cells were lysed in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecy sulfate) or M-PER buffer (Mammalian Protein Extraction Reagent, Pierce, Rockford, IL) with protease inhibitor (Roche Diagnostic corp., Indianapolis, IN). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedfore, MA). The

membranes were blocked with TBS-t (10 mM Tris-HCl, pH 7.4, 150 mM NaCl)containing 5% non-fat milk for 1h and then incubated at room temperature with primary antibodies. Blots were washed four times for 15 min with 0.5% Tween 20-containing TBS-t and then incubated for 1h with peroxidase conjugated secondary antibodies (1:5000, Jackson immunoResearch Inc. West Grove, PA). The membranes were washed again four times and developed using an enhanced chemiluminesence detection system (ECL, Amersham Corp., Cardiff, UK). For immunoprecipitation assay, the RIPA extracts were pre-cleared with protein A-agarose bead (Amersham Corp., Cardiff, UK), and then incubated at 4C overnight with fresh protein A-bead in the presence of appropriate antibodies. The beads were washed five times in RIPA buffer without protease inhibitor, resuspended in SDS sample buffer, and boiled for 5 min. Samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression.

Antibodies

Antibodies against of pig3 used in this study are pig3 H300 (Santa Cruz Biotechnology, Santa Cruz, CA), pig3 C-20 (Santa cruz) and pig3 Ab-1(Calbiochem). Pig3 protein was detected by Western blotting (WB)with a rabbit polyclonal pig3 H300 antibody at 1:1000 dilution and pig3 foci was also detected by immunofluorescence staining (IF) using pig3 H300 antibody at 1:50 dilution. H2AX was detected by IF and WB with mouse monoclonal antibody, clone JBW301 (Upstate Biotechnology, Temecula, CA) at 1:200 and 1:1000 dilution, respectively. H2AX antibody was purchased from Upstate. Following antibodies were used for immunofluorescence staining : anti-53BP1 polyclonal antibody (Santa cruz, 1:50), anti-Mre11 monoclonal antibody (BD Phamingen, San Jose, CA, 1:200), anti-Nbs1 polyclonal antibody (Cell Signaling Technology, Danvers, MA, 1:200), anti-Rad50 monoclonal antibody (BD Phamingen, 1:200), anti-ATM protein kinase pS1981 monoclonal antibody (Rockland, Immunochemicals Ins., Philadelphia, PA, 1:500) anti-DNA-PK polyclonal antibody (Santa cruz, 1:50), anti-ATR-p(S428) polyclonal antibody (Cell Signaling Technology, a:200) and anti-p53 DO-1 monoclonal antibody (Santa cruz, 1:50). Following antibodies were used for

Western blot analysis : anti-ATM 5C2 mAb (1:500), anti-ATR N-19 pAb (1:300), anti-DNA-PKcs G4 mAb (1:500) anti-p53 DO-1 mAb (1:2000), anti-Tubulin TU-02 mAb (1:5000) and anti-53BP1 pAb (1:500, Santa cruz) ; anti-p53-p(S15) pAb (1:1000, Cell Signaling Technology); anti-Rad50 mAb (1:750), anti-Mre11 mAb (1:1000) and anti-Nbs1 mAb (1:1000, BD Phamingen).

Immunofluorescence microscope

Cells culturedon coverslips coated with poly-L-lysine (Sigma) were treated UV, NCS or BLM followed by recovery for adequate times. Cells were then washed with PBS twice, fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.3% Triton X-100 for 10 min at room temperature. After permeabilization, coverslip were washed PBS three times and then blocked with 5% BSA in PBS for 1h. Cells were single or double immunostained with primary antibodies against various proteins for overnight at 4C. Cells were washed with PBS and then stained appropriate Alexa Fluor 488-(green, Molecular Probe), Alexa Fluor 594-(red, Molecular Probe) conjugated secondary antibodies. After washing, cells were mounted using Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence images were taken using Zeiss Axioplan 2 imaging epifluorescent microscope equipped with a charge-coupled device camera and ISIS software (MetaSystems, Altlussheim, Germany).

BrdU incorporation assay

To determine cell populations in the S phase, the incorporation of BrdU was monitored as a parameter for DNA synthesis according to the instructions of the manufacturer (Roche Diagnostic corp.). Mock- and sipig3 cells were plated in 48-well plate and treated with indicated dose of NCS, BLM or UV. After 24h, 10 M BrdU was added to the culture medium for 2 h at 37° C for incorporation into freshly synthesized DNA. Following fixation of the cells, cellular DNA was partially digested by nuclease treatment. A peroxidase-labeled antibody to BrdU and a peroxidase substrate were sequentially added to yield a colored reaction

product, which is proportional to the level of BrdU incorporated into cellular DNA. Colored products were measured at microplate reader at 405nm with a reference wavelength at approx. 490nm. The relative DNA synthesis was calculated as the percentage of absorbance of cells treated with DNA damaging agents from the absorbance of control cells. The data are presented as the average SD value of representative triplicate experiments.

G2/M checkpoint analysis

Cells were treated with 100ng/ml nocodazolefor 3h following addition of 200ng/ml NCS or 10J UV. After 24h, cells were harvested and washed with PBS and then fixed with 1% formaldehyde for 10min at 37C. Cells were immediately chilled on ice for 1min and then cells were permeabilized with 90% methanol at -20C overnight. Fixed cells were washed with PBS and blocked with incubation buffer (0.5% BSA in PBS) for 10 min. Cells were stained with anti-phospho-Histone H3(S10)-Alexa Fluor 647 conjugate antibody (Cell signaling Technology) at 1:10 dilutionin incubation buffer for 1h in the dark at room temperature. Cells were washed and then resuspended in PBS containing 50 g/ml propidium iodide. At least 10,000 cells were analyzed by fluorescent-activated cell sorting (FACSort, Becton Dickinson, San Jose, CA). Acquired data were analyzed by cell Quest Pro software. (Becton Dickinson).

Flow cytometry by Propidium Iodide staining

The trypsin-detached cells were collected and washed with cold PBS, followed by fixing in 70% cold-ethanol for 30min at 4C. The cells were then stained with PBS containing 50 g/ml propidium iodide. Fluorescence emitted from the PI-DNA complex in each cell nucleus was measured after laser excitation at 488nm with a flow cytometry (FACSort, Becton Dickinson). For measurement of cell death, the percentage of cells in sub-G1 population was calculated. Aggregates of cell debris at the origin of histogram were excluded from the sub G1 cells.

RESULTS

Pig3 is associated with several DNA damage sensors after DNA damage.

Cellular response to genotoxic stress is very complex process. It can be briefly explained as a signal transduction that is detected by sensors and passed down through transducers and effectors. The large protein kinase Ataxia-Telangiectasia Mutated (ATM), ATM-and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) initiate this cascade. These three phospatidylinositol-3 kinases (PIKKs) were known to initiators of the cellular genotoxic stress response (Yang et al., 2003). Moreover, H2AX is phosphorylated at Ser-139 at the conserved SQ motif by these three kinases. Phosphorylated H2AX termed H2AX. Mre11-Rad50-Nbs1 (MRN) complex recruited ATM to DNA damage site (Falck et al., 2005; Paull and Lee, 2005). 53BP1 is also a sensor of DNA damage (Zgheib et al., 2005). First, to investigate whether pig3 interacts with DNA damage sensors after exposure of cells to UV radiation, co-immunoprecipitation assay of pig3 was performed. Endogenous pig3 coimmunoprecipitation with endogenous MRN complex (Mre11, Rad50 and Nbs1), 53BP1 and PIKK in HeLa cells. These interactions increase with DNA damage (Fig. 1A). To confirm the interaction between pig3 and DNA damage sensors, we carried out immunofluorescence studies. Whereas untreated cells showed small diffuse nuclear staining of pig3, in the exposing cells to UV, pig3 became localized to nuclear foci (Fig. 1B). As shown in Fig. 1B, pig3 foci colocalized with 53BP1 foci, known to early sensor on cellular response to DNA DSBs. 53BP1 also colocalized with MRN complex, which have been reported to localize to sites of DNA DSBs. We further examined whether pig3 colocalized with MRN complex and PIKKs. Alike 53BP1, pig3 foci colocalized with Mre11, Rad50, Nbs1, ATM, DNA-PK at site of UV-induced DNA damage (Fig. 1B). However, unlike 53BP1 and PIKKs, foci of MRN complex less colocalized with Pig3 foci only at same time points after exposure of cells to UV radiation. These data indicate that pig3 is formed nuclear foci in response to DNA damage, and pig3 foci show colocalization with foci formed by

the several DNA damage sensors such as 53BP1, MRN complex, ATM and DNA-PK.

Pig3 forms nuclear foci.

To investigate further the foci formation of pig3 in DNA damage response, we carried out immunofluorescence assay of pig3 at the various time points against UV (Fig. 2A). Untreated cells disappeared diffuse nuclear staining of pig3, within 6h of treatment of UV, pig3 formed nuclear foci. pig3 foci were still present up to 24h later, and foci size gradually increased at HCT116 cells. We further investigated whether foci formation of pig3 depend on PIKK. Pretreatment of Wortmannin at a concentration (Sarkaria et al., 1998) known to inhibit these PIKK, remarkably inhibited the formation of pig3 and H2AX (Fig. 2B). These data demonstrated that pig3 foci can be constituted by more than one member of the PIKK family. Furthermore, because pig3 is downstream target gene of p53, we investigated that whether formation of pig3 foci is dependent to p53. As shown in Fig. 2C, pig3 foci was formed in p53+/+ and p53-/- HCT116cells by treatment of 10J-UV. Therefore, formation of Pig3 foci is independent to presence of p53. Taken together, Pig3 can be recruited to DNA damage sites through interaction between pig3 and DNA damage sensors, pig3 foci is formed by PIKK family members and it's independent to p53.

Pig3 interacts with H2AX and regulates recruitment of the DNA damage sensors to foci.

One of the primary events in the DNA damage response is phosphorylation of H2AX at Ser139 at conserved SQ motif on the C-terminal tail by members of the PIKK family to create H2AX. H2AX foci forms within minutes in response DNA damage, followed by the formation of 53BP1, BRCA1 and MRN complex foci that colocalize with H2AX (Celeste et al., 2003; Fernandez-Capetillo et al., 2004; Motoyama and Naka, 2004; Paull et al., 2000). Therefore, H2AX plays an important role in recruiting DNA damage sensor and initial recognition of DNA breaks. First, to investigate whetherpig3 which binds to several DNA damage sensors, also interacts with H2AX, we performed co-immunoprecipitation and

immunofluorescence assay confirmingthe association between Pig3 and H2AX. Although Pig3 does not associate with H2AX or H2AX in untreated cells, pig3 coimmunoprecipitate with H2AXbut not H2AX in cell lysates extracted 12h after UV-induced DNA damage (Fig. 3A). After exposure of cells to UV, pig3 foci colocalized with H2AX (Fig. 3B). These results raised the possibility that the pig3 may play a direct role in DNA damage sensing.

Next, we examined whether the pig3 would affect the formation of H2AX foci after DNA damage, the HCT116-sihygro and HCT116-sipig3 cells were treated with UV radiation, and then carried out immunofluorescence assay with antibodies against pig3 and H2AX. This revealed that cells in which pig3 downregulated are markedly impaired formation of H2AX foci after DNA damage (Fig. 3C). We also confirmed decrease of H2AX expression in pig3 knockdown HCT116 cells using Western blot analysis (Fig. 3D).

Pig3 is required for G2/M and intra-S phase checkpoints after DNA damage.

Based on above results, knock down of pig3 reduced the number of colonies compared with control cells (Fig. 4A), indicating that Pig3 function may be required to maintain cell viability. The increased DNA damage sensitivity of pig3-knockdown cells could be owing to defect of DNA damage checkpoints as well as DNA damage repair. DNA damage checkpoints have evolved to maintain genomic stability by preventing cells with damaged DNA from entering mitosis. Therefore, we investigated that whether pig3 affects cell cycle checkpoints after DNA damage. To check the direct effects of the pig3 in the intra S-phase checkpoints, control or pig3-knockdown HCT116 cells were treated with UV, allowed to recover for 6h, and then added to BrdU for 2h. The sipig3 cells were showed a increased S phase population after UV radiation compared with control cells (Fig. 4B). We next examined the integrity of the G2/M checkpoints. In one set of experiments, the spindle fiber disassembly agent, nocodazole was added 3h followed UV treatment, to cause any cells progressing through the cell cycle arrest in mitosis. DNA content was monitored by PI staining and phospho-histone H3 staining, which is used as an indicator of mitotic entry. The reduction in M

phase cells was showed at the control cells after UV, whereas a significant increase in M phase cells was observed at the pig3-knockdown cells (Fig. 4C), which indicates that pig3 enforces the G2/M checkpoint. Taken together, these results indicated that Pig3 is critical mediator of DNA damage check point pathway.

The role of p53 in activation of DNA damage checkpoint machinery

Based on above results, pig3 is a novel regulator (mediator) of the DNA damage checkpoints. Although function of pig3 as checkpoint regulator is independent to p53, pig3 is downstream target of p53 (Polyak et al., 1997). DNA damage checkpoints are an important self-defense mechanism for the maintenance of genomic stability. Defects in normal DNA damage checkpoint and repair lead to genomic instability and tumorigenesis in humans. Many proteins were involved in DNA damage checkpoint signaling. Particularly, the tumor suppressor p53 is essential for the normal responses to DNA damage that help to maintain genomic stability. PIKK such as ATM, ATR and DNA-PK was primarily activated in response to DNA damage, and they amplify the damage signal by phosphorylation of downstream substrates. The tumor suppressor protein p53 is major downstream effector of these DNA-damage kinase pathways (Harris and Levine, 2005). Phosphorylated p53 on Ser15 induced expression of many downstream targets, including $p21^{waf1/CIP1}$, 14-3-3 and bax, and leads to cell cycle arrest, DNA repair and apoptosis. Loss of p53 function induced defect of cell cycle checkpoints and cell death, and this leads to genomic instability that promotes tumor progression (Adamsen et al., 2007; Xue et al., 2007). However, p53-deficient cells underwent normal DNA repair, cell cycle arrest and apoptosis through p53-independent mechanism or p38 MAPK/MK2 pathway (Lips and Kaina, 2001; Prost et al., 1998; Reinhardt et al., 2007). Until now, the exact mechanism of p53-mediated DNA damage response is poorly understood.

Excitingly, we observed that the expression level of endogenous pig3 is low in p53-null cells whereas expression level of pig3 is high in wild type p53 cells such as HCT116 and U20S cells (Fig. 5A). The pig3 expression may be low due to

low level of p53 expression in p53 null cells, therefore p53 null cells present defect of DNA damage checkpoints leading to chromosomal instability. According to this hypothesis, pig3 may be contributed to genomic stability through normal p53-mediated DNA damage checkpoint activation. To begin to address this hypothesis, effect of pig3 was investigated in DNA damage response in both wild type and null p53 cells. As above mentioned, phosphorylation of H2AX is essential for DNA damage checkpoint activation and genomic stability (Bassing et al., 2002; Fernandez-Capetillo et al., 2004). The intensity of H2AX phosphorylation on Ser-139 varies in cells with different tumor protein p53 status (Tanaka et al., 2006). In background experiments, we observed phosphorylation of H2AX in both wild type and null p53 cells. The degree of H2AX phosphorylation is distinctly lower in HCT116 p53-/- cells compared to HCT116 p53+/+ cells, respectively, after DNA damage (data not shown). We also showed that formation of H2AX foci after DNA damage was decreased in p53 null cells compared with p53 wild type cells. Therefore, p53 plays a role in facilitating H2AX phosphorylation, an important step in activation of DNA damage checkpoint machinery

Pig3 is essential for p53-mediated DNA damage response.

Further experiments were done to investigate whether pig3 retrieves defect of DNA damage checkpoints resulting in genomic stability in p53 null cells. Checkpoint activation was also monitored as induction of H2AX. Knockdown of pig3 in p53 wild type HCT116 cells decreased H2AX expression after UV radiation (Fig. 5A). We also observed decrease of p53 phosphorylation on S15 in pig-knockdown cells compared to control cells. Although p53 is present, knockdown of pig3 was shown defect of phosphorylation of H2AX and p53. These results indicated that pig3 is essential for p53-mediated DNA damage checkpoint activation. In contrast, overexpression of pig3 in p53 null-HCT116 cells increased H2AX expression against DNA damage as p53 wild type cells (Fig. 5B). We also showed that formation of H2AX foci after DNA damage was decreased by pig3-knockdown, increased by pig3-overexpression (Fig. 5C). Therefore, pig3 status regulated

formation of H2AX foci and induction of H2AX against DNA damage. Moreover, pig3 supplements checkpoint activation of p53 through regulation of phosphorylation on Ser15 and expression of p53. Therefore, pig3 is essential for DNA damage response through p53 activation.

Pig3 is not necessary for activation of DNA damage checkpoints in p53 gain-of-function mutants.

The p53 gene is most frequently target of genetic alteration in human cancer. Wild type p53 plays multiple cellular roles, including cell cycle arrest, apoptosis, differentiation, senescence and DNA repair through transcriptional activation of a large number of p53-target genes (Vogelstein et al., 2000). These function of p53 protect the genome from accumulating DNA damage and mutating genes, so p53 has function as the guardian of the genome (Efeyan and Serrano, 2007). The p53 gene is the most frequently mutated gene in human cancer. About half of all human cancers have lost p53 or express an inactive or mutant p53 (Hollstein et al., 1991). The majority of the p53 gene mutation are missense mutations within DNA-binding core domain. About 25% of these mutations, there are four hotspot mutations at 175, 248, 249 and 273 amino acids (Roemer, 1999). Both R248W and R274H p53 mutation induced conformational change and disrupt the tumor suppressor activity of p53, and then p53 mutants also gain function as oncogenes (Sigal and Rotter, 2000). Recently, it was reported that p53 gain-of-function mutants interact with Mre11 and suppress the binding of MRN complex to DNA damage sites, leading to the impaired recruitment of ATM to the site of DNA damage. Therefore, p53 gain-of-function mutants can induce genetic instability by disrupting ATM-mediated DNA damage response (Song et al., 2007; Song and Xu, 2007; Xu, 2008). Genomic instability is a major force driving human cancer development. These p53 mutants are transcriptional activation or repression of gene, including BFGH, EGFR, HSP70, c-myc and multi-drug resistance gene (MDR-1) and VEGFG, leading to promote transformation, proliferation and amgiogenesis, thus contributing to gain of function in promoting cancer (Roemer, 1999).

In opposition to expectation, we observed that pig3 was still highly expressed in p53 gain-of-function mutant cells (Fig. 6A), although mutant p53 had no normal transcriptional activity. However, pig3 expression was not increased against DNA damage in a time-dependent manner due to mutant p53 (Fig. 6A). First, to test the effect of pig3 in DNA damage response at p53 gain-of-function mutants, SW480 mutant p53 colon cancer cells, we were transiently transfected with pig3 siRNA, and then investigated checkpoint activation. Expression of H2AX after DNA damage such as UV (Fig. 6A) in pig3 knockdown SW480 cells was not distinguishable from H2AX expression of control cells. Therefore, pig3 is not required for DNA damage checkpoints in p53 gain-of-function mutant cells. We also observed that expression of H2AX was not increased in pig3-overexpressing SW480 cells compared with control cells (Fig. 6B). Phosphorylation of p53 on S15 also did not be changed by knockdown or overexpression of pig3. Moreover, foci formation of H2AX when pig3-knockdown SW480 cells were treated with DNA damage was not different from H2AX foci of pig3-overespressing cells (Fig. 6C). Taken together, it was indicated that pig3 did not function as regulator DNA damage checkpoint in p53 mutant cells although pig3 is highly expressed.

Pig3 induces the cell death after DNA damage in p53 gain-of-function mutants.

Previous studies reported that pig3 was induced by p53 transactivation, leading to ROS generation and apoptosis (Polyak et al., 1997). However, our data presented that knockdown of pig3 repressed activation of DNA damage sensing and checkpoints after DNA damage, increased cytotoxicity and impaired DNA repair. In contrast to our results, we observed that overexpression of pig3 increased DNA damage-induced cell death in gain-of-function p53 mutant SW480 cells. It was confirmed by PI staining (Fig. 6D).

Therefore, checkpoint-unrelated-pig3 induced cell death after DNA damage. Little is known of detailed mechanism in pig3-induced apoptosis at p53 mutant cells. Taken together, this led us to conclusion that pig3 is necessary to maintenance of chromosomal stability through normal p53-mediated DNA damage checkpoint activation.

DICUSSION

In this study, we identified that the role of pig3 in DNA damage response. Pig3 is formed nuclear foci against DNA damage in the time dependent manner, and pig3 foci colocalized with MRN complex, 53BP1, ATM, DNA-PK and H2AX foci. Pig3 also regulated induction of H2AX and recruitment of 53BP1, MRN complex and DNA-PK to sites of DNA damage. Moreover, pig3 regulated G2/M and intra-S phase checkpoints after DNA damage. Our present data showed that pig3 as downstream target of p53, supplemented p53 activity via regulation of p53 expression and phosphorylation, suggested that pig3 was essential for p53 mediated DNA damage response. Our findings are first report, demonstrating novel function of pig3 as a mediator of DNA damage checkpoints.

Nuclear pig3 may be novel DNA damage sensor on the DNA damage response.

Although previous works have suggested that pig3 might be induced p53-dependent apoptosis through ROS generation at the cytoplasm (Flatt et al., 2000), our results clearly demonstrate its role in the DNA damage checkpoints at the nucleus. A recent study reported that pig3 was localized to the cytoplasm in Hlp53 cells, an H1299 cell line containing an ecdysone-inducible p53 expression vector (Flatt et al., 2000). However, our findings revealed that pig3 was expressed in nucleus about 30% of total pig3 proteinat HeLa and HCT116 cells, although pig3 mainly localized in cytoplasm like previous study. In addition, Flatt et al. reported that pig3 locates to cytoplasm in MCF7 cells using laboratory manufactured full length pig3 antibody, but a little nuclear localization was still observed (Flatt et al., 2000). However, many questions remains for future investigation.

Pig3 is critical factor in the p53-mediated DNA damage checkpoint activation.

Tumor suppressor p53 integrates signals from many different pathways that were activated by diverse stimuli such as DNA damage, hypoxia and oncogene

activation. In DNA damage condition, p53 triggers various cellular response through stabilization and modification of p53that can leads to cell cycle arrest, DNA repair, apoptosis, senescence, differentiation and inhibition of angiogenesis (Vogelstein et al., 2000). ATM and ATR phosphorylate p53 on Ser15, and also regulates p53 activity via phosphorylation of Mdm2 on Ser395. Moreover, ATM and ATR phosphorylate their downstream kinase Chk2 on Thr68 and Chk1 on Ser317/345, respectively, which turn phosphorylates p53 on Ser20 (Helton and Chen, 2007). The phosphorylated p53 results in upregulation of p53 downstream genes involved in the DNA damage response. p53 regulates cell cycle checkpoints by inducing the expression of p21, 14-3-3, Cdc25C, GADD45 (Giono and Manfredi, 2006). p53 induces Bcl-2, Bax, Puma, Noxa, APAF1 and p53AIP1, which promote cell death through mitochontrial pathway, and p53 also promotes the extrinsic cell death pathway by upregulating the expression of TRAIL receptor, Hardeath receptor-4 (DR4), Fas/CD95 receptor and DR5/KILLER (Harms et al., 2004). p53 target genes including XPC, XPE and GADD45 have been implicated in nucleotide excision repair, and p53 also upregulates two mismatch repair proteins : MutS homologue 2 (MSH2) and proliferating cell nuclear antigen (PCNA) (Harms et al., 2004). Furthermore, several p53 target genes play a role in the regulation of p53 through negative feedback-loop (Mdm2, Pirh2, COP1, p73 delta N, cyclin G, Wip-1 and Siah-1) and positive feedback-loop (PTEN-AKT, p14/ p19 ARF and Rb) (Harris and Levine, 2005). As mentioned above, p53 tumor suppressoris a critical downstream effector in DNA damage response.

Several previous reports were mentioned that DNA damage checkpoint pathway is mediated by another kinase pathway in cells lacking p53, for instance p38 MAPK/MK2 pathway (Reinhardt et al., 2007). Moreover, downstream effects such as apoptosis and cell cycle progression were defected in cells lacking p53 (Adamsen et al., 2007). On the contrary, hypersensitivity was appeared after treatment with -irradiation, UV light and alkylating agent both in primary p53-knockdown fibroblast and various established p53-deficient cell lines (Lackinger and Kaina, 2000; Lips and Kaina, 2001). However, a physiological role of p53 in regulating DNA damage sensing at the upstream of DNA damage has been questioned.

Importantly, the various responses of p53 are frequently stimuli and cell type specific. In our experimental setting, H2AX phosphorylation (H2AX), an important step in activation of DNA damage checkpoint machinery, as well as foci formation of MRN complex and 53BP1, was decreased in p53 null cells compared with p53 wild type, even though p53 is downstream effector. Moreover, we observed that p53 phosphorylated on Ser15 against DNA damage, whereas not showed phosphorylation of p53 at Ser20 (data not shown), suggesting that p53 may function in upstream level of DNA damage and as direct target of PIKK in our system. Overexpression of pig3 in p53 null cells retrieved defect of DNA damage sensing as shown by induction of H2AX and increased foci formation of MRN complex, suggesting that pig3 may be critical factor in the p53-mediated DNA damage checkpoint activation. Consistent with this hypothesis, transiently knockdown of p53 did not affect H2AX induction , because pig3 expression was not changed.

The tumor suppressor p53 is essential for the normal responses to DNA damage that help to maintain genomic stability. Defects in normal DNA damage checkpoint-signaling and repair lead to various disorders and genetic instability, and induce tumorigenesis in humans. The p53 gene is the most frequently mutated gene in human cancer. Two common p53 mutants, $p53^{R248W}$ and $p53^{R273H}$, were shown to disrupt DNA-damage check-points (Sigal and Rotter, 2000; Song et al., 2007). It suggested that the gain of function p53 mutants could contribute to tumor development or progression through enhanced genomic instability. Our data showed that pig3 knockdown cells were detected decrease of DNA damage foci compared with control cells, and overexpression of pig3 rescued its decrease. Moreover, expression level of endogenous pig3 was consistent with expression of p53. These data provide definitive evidence that pig3 may be contributed to genomic stability through normal p53-mediated DNA damage checkpoint activation. However, in opposition to above hypothesis pig3 was still highly expressed in p53 gain-of-function mutant cells and checkpoint activation in pig3 knockdown or overexpressing SW480 cells was not different from control cells. Therefore, mutant p53 could play a role regulator of DNA damage checkpoint at the parallel or upstream of pig3 in gain-of-function p53 mutant

cells. Moreover, pig3 did not affect DNA damage checkpoints in gain-of-function mutant cells, because of association of mutant p53 and Mre11. Therefore, defining how pig3 regulates DNA damage checkpoints, will be subject of further investigation to get conclusion that pig3 is necessary to maintenance of chromosomal stability.

Pig3 induced cell survival as well as cell death.

Although exact role of pig3 in apoptosis is not yet elucidated, there are several reports that pig3 is involved in p53-dependent apoptosis. In previous studies, it was reported that pig3 modulated the levels of intracellular reactive oxygen species (ROS), leading to p53-induced apoptosis (Asher et al., 2001; Polyak et al., 1997; Venot et al., 1998). Flatt et al. noted that pig3 was required for proliferation, genotoxic stress and reversible growth arrest (Flatt et al., 2000). Pig3 also was responsible for increased generation of ROS as well as disruption of mitochondrial integrity, resulted in induction of apoptosis (Ostrakhovitch and Cherian, 2005). However, our studies suggested reverse function of pig3 in DNA damage response. Using comet assay, pulsed-field gel electrophoresis and clonal survival assay, we showed that knockdown of pig3 led to reduced DNA repair after DNA damage, guiding to cell death. To explain this discrepancy, many further investigations will be needed.

In contrary, using PI staining and MTT assay, we observed that pig3 induced cell death against DNA damage rather than DNA repair and activation of checkpoints in SW480 cells, p53 gain-of-function mutant cells. In p53 gain-of-function mutant cells, pig3 may be play a role in cell death, because pig3 did not affect in DNA damage sensing. Therefore, our data are represented that pig3 induces cell survival through p53-mediated DNA damage checkpoint activation in cells presenting p53 wild type, whereas pig3 induces cell death in gain-of-function mutant cells. Maybe, deciding whether pig3 induces cell death or cell survival will be dependent to p53 status. Moreover, cell fate by pig3 overexpression is decided by type of cell and damage as well as p53 status. But, this phenomenon, which p53 is regulated for pig3 expression and cell death, does

not depend certainly in p53 status. We showed that the expression level of endogenous pig3 was high, even though H1299 cells are colon cancer cells lacking p53 protein. Our data showed that in this H1299 cells, overexpression of pig3 induced DNA repair and DNA damage checkpoints activation instead of cell death against DNA damage. Previous report showed that pig3 owns p53-responsiveness pentanucleotide microsatellite sequence $(TGYCC)_n$ (Y=C or T) (Contente et al., 2002). They suggest that the microsatellite is found to be polymorphic, and the number of pentanucleotide repeats correlate with transcriptional activation by p53. Moreover, they indicated the possibility that inheritance of microsatellite of the pig3 promotor may affect susceptibility of an individual in cancer. Therefore, based on the previous report, we suppose that pig3 may have a different function or a various expression pattern (variants of pig3 protein), due to polymorphic microsatellite of the pig3 promotor. Taken together, these finding suggest a wider role for pig3 in the cellular response to DNA damage than has been previously reported. Further experiments will be done to determine detailed mechanism of regulation of pig3 induced cell death.

In summary, pig3 is formed nuclear foci against DNA damage, and regulates recruitment of damage foci and checkpoint of G2/M and intra-S through binding of several DNA damage sensors. It suggests that pig3 has function as novel regulator of DNA damage checkpoints. Pig3 also mediates DNA damage checkpoints by p53 activation. In p53 gain-of-function mutant cells, pig3 possesses another function, which induces cell death against DNA damage. Further studies for clarification of detailed mechanism of pig3-induced cell death will be required. Therefore, our study implies a essential role of pig3in the p53-mediated DNA damage checkpoint activation leading to genomic stability.

REFERENCES

1. Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev *15*, 2177-2196.

2. Adamsen, B.L., Kravik, K.L., Clausen, O.P., and De Angelis, P.M. (2007). Apoptosis, cell cycle progression and gene expression in TP53-depleted HCT116 colon cancer cells in response to short-term 5-fluorouracil treatment. Int J Oncol *31*, 1491-1500.

3. Agarwal, M.L., Taylor, W.R., Chernov, M.V., Chernova, O.B., and Stark, G.R. (1998). The p53 network. J Biol Chem *273*, 1-4.

4. Asher, G., Lotem, J., Cohen, B., Sachs, L., and Shaul, Y. (2001). Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. Proc Natl Acad Sci U S A *98*, 1188-1193.

5. Bakkenist, C.J., and Kastan, M.B. (2004). Initiating cellular stress responses. Cell *118*, 9-17.

6. Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421-429.

7. Bassing, C.H., Chua, K.F., Sekiguchi, J., Suh, H., Whitlow, S.R., Fleming, J.C., Monroe, B.C., Ciccone, D.N., Yan, C., Vlasakova, K., *et al.* (2002). Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. Proc Natl Acad Sci U S A *99*, 8173-8178.

8. Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E., and Fornace, A.J., Jr. (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature *411*, 102-107.

9. Campomenosi, P., Monti, P., Aprile, A., Abbondandolo, A., Frebourg, T., Gold, B., Crook, T., Inga, A., Resnick, M.A., Iggo, R., *et al.* (2001). p53 mutants can often transactivate promoters containing a p21 but not Bax or PIG3 responsive elements. Oncogene *20*, 3573-3579.

10. Celeste, A., Fernandez-Capetillo, O., Kruhlak, M.J., Pilch, D.R., Staudt, D.W., Lee, A., Bonner, R.F., Bonner, W.M., and Nussenzweig, A. (2003). Histone

H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol *5*, 675-679.

11. Contente, A., Dittmer, A., Koch, M.C., Roth, J., and Dobbelstein, M. (2002). A polymorphic microsatellite that mediates induction of PIG3 by p53. Nat Genet*30*, 315-320.

12. Demura, T., and Fukuda, H. (1994). Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. Plant Cell *6*, 967-981.

13. Donzelli, M., and Draetta, G.F. (2003). Regulating mammalian checkpoints through Cdc25 inactivation. EMBO Rep *4*, 671-677.

14. Donzelli, M., Squatrito, M., Ganoth, D., Hershko, A., Pagano, M., and Draetta, G.F. (2002). Dual mode of degradation of Cdc25 A phosphatase. EMBO J *21*, 4875-4884.

15. Efeyan, A., and Serrano, M. (2007). p53: guardian of the genome and policeman of the oncogenes. Cell Cycle *6*, 1006-1010.

16. Falck, J., Coates, J., and Jackson, S.P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature *434*, 605-611.

17. Fernandez-Capetillo, O., Lee, A., Nussenzweig, M., and Nussenzweig, A. (2004). H2AX: the histone guardian of the genome. DNA Repair (Amst) *3*, 959-967.

18. Flatt, P.M., Polyak, K., Tang, L.J., Scatena, C.D., Westfall, M.D., Rubinstein, L.A., Yu, J., Kinzler, K.W., Vogelstein, B., Hill, D.E., *et al.* (2000). p53-dependent expression of PIG3 during proliferation, genotoxic stress, and reversible growth arrest. Cancer Lett *156*, 63-72.

19. Foster, E.R., and Downs, J.A. (2005). Histone H2A phosphorylation in DNA double-strand break repair. FEBS J *272*, 3231-3240.

20. Giono, L.E., and Manfredi, J.J. (2006). The p53 tumor suppressor participates in multiple cell cycle checkpoints. J Cell Physiol*209*, 13-20.

21. Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S.P. (2003). MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature *421*, 952-956.

22. Harms, K., Nozell, S., and Chen, X. (2004). The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci *61*, 822-842.
23. Harris, S.L., and Levine, A.J. (2005). The p53 pathway: positive and negative feedback loops. Oncogene *24*, 2899-2908.
24. Hastak, K., Paul, R.K., Agarwal, M.K., Thakur, V.S., Amin, A.R., Agrawal, S., Sramkoski, R.M., Jacobberger, J.W., Jackson, M.W., Stark, G.R., *et al.* (2008). DNA synthesis from unbalanced nucleotide pools causes limited DNA damage that triggers ATR-CHK1-dependent p53 activation. Proc Natl Acad Sci U S A *105*, 6314-6319.
25. Helton, E.S., and Chen, X. (2007). p53 modulation of the DNA damage response. J Cell Biochem *100*, 883-896.
26. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science *253*, 49-53.
27. Kastan, M.B., and Lim, D.S. (2000). The many substrates and functions of ATM. Nat Rev Mol Cell Biol *1*, 179-186.
28. Kim, S.T., Lim, D.S., Canman, C.E., and Kastan, M.B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem *274*, 37538-37543.
29. Kurz, E.U., and Lees-Miller, S.P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) *3*, 889-900.
30. Lackinger, D., and Kaina, B. (2000). Primary mouse fibroblasts deficient for c-Fos, p53 or for both proteins are hypersensitive to UV light and alkylating agent-induced chromosomal breakage and apoptosis. Mutat Res *457*, 113-123.
31. Lips, J., and Kaina, B. (2001). DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts. Carcinogenesis *22*, 579-585.
32. Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., *et al.* (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev *14*, 1448-1459.
33. Long, X.H., Zhao, Z.Q., He, X.P., Wang, H.P., Xu, Q.Z., An, J., Bai, B.,

Sui, J.L., and Zhou, P.K. (2007). Dose-dependent expression changes of early response genes to ionizing radiation in human lymphoblastoid cells. Int J Mol Med *19*, 607-615.

34. Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. (2003). MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways. Nature *421*, 957-961.

35. Manke, I.A., Nguyen, A., Lim, D., Stewart, M.Q., Elia, A.E., and Yaffe, M.B. (2005). MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. Mol Cell *17*, 37-48.

36. Matsuoka, S., Huang, M., and Elledge, S.J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

37. Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S.J. (2000). Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A *97*, 10389-10394.

38. Maya, R., Balass, M., Kim, S.T., Shkedy, D., Leal, J.F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y., *et al.* (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev *15*, 1067-1077.

39. Meek, D.W. (2004). The p53 response to DNA damage. DNA Repair (Amst) *3*, 1049-1056.

40. Motoyama, N., and Naka, K. (2004). DNA damage tumor suppressor genes and genomic instability. Curr Opin Genet Dev *14*, 11-16.

41. Nicholls, C.D., Shields, M.A., Lee, P.W., Robbins, S.M., and Beattie, T.L. (2004). UV-dependent alternative splicing uncouples p53 activity and PIG3 gene function through rapid proteolytic degradation. J Biol Chem *279*, 24171-24178.

42. Okorokov, A.L. (2003). p53 in a crosstalk between DNA repair and cell cycle checkpoints. Cell Cycle *2*, 233-235.

43. Ostrakhovitch, E.A., and Cherian, M.G. (2005). Role of p53 and reactive oxygen species in apoptotic response to copper and zinc in epithelial breast cancer cells. Apoptosis *10*, 111-121.

44. Pabla, N., Huang, S., Mi, Q.S., Daniel, R., and Dong, Z. (2008). ATR-Chk2 signaling in p53 activation and DNA damage response during cisplatin-induced apoptosis. J Biol Chem *283*, 6572-6583.

45. Paull, T.T., and Lee, J.H. (2005). The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM. Cell Cycle *4*, 737-740.

46. Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M., and Bonner, W.M. (2000). A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol *10*, 886-895.

47. Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.

48. Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. Nature *389*, 300-305.

49. Prost, S., Bellamy, C.O., Clarke, A.R., Wyllie, A.H., and Harrison, D.J. (1998). p53-independent DNA repair and cell cycle arrest in embryonic stem cells. FEBS Lett *425*, 499-504.

50. Rao, P.V., Krishna, C.M., and Zigler, J.S., Jr. (1992). Identification and characterization of the enzymatic activity of zeta-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. J Biol Chem *267*, 96-102.

51. Rao, P.V., and Zigler, J.S., Jr. (1992). Purification and characterization of zeta-crystallin/quinone reductase from guinea pig liver. Biochim Biophys Acta*1117*, 315-320.

52. Reinhardt, H.C., Aslanian, A.S., Lees, J.A., and Yaffe, M.B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell *11*, 175-189.

53. Roemer, K. (1999). Mutant p53: gain-of-function oncoproteins and wild-type p53 inactivators. Biol Chem *380*, 879-887.

54. Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem *73*, 39-85.

55. Sarkaria, J.N., Tibbetts, R.S., Busby, E.C., Kennedy, A.P., Hill, D.E., and Abraham, R.T. (1998). Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res *58*, 4375-4382.

56. Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res *60*, 6788-6793.

57. Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol *9*, 573-580.

58. Song, H., and Xu, Y. (2007). Gain of function of p53 cancer mutants in disrupting critical DNA damage response pathways. Cell Cycle *6*, 1570-1573.

59. Stewart, G.S., Wang, B., Bignell, C.R., Taylor, A.M., and Elledge, S.J. (2003). MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature *421*, 961-966.

60. Tanaka, T., Kurose, A., Huang, X., Traganos, F., Dai, W., and Darzynkiewicz, Z. (2006). Extent of constitutive histone H2AX phosphorylation on Ser-139 varies in cells with different TP53 status. Cell Prolif *39*, 313-323.

61. Tanaka, T., Ohkubo, S., Tatsuno, I., and Prives, C. (2007). hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. Cell*130*, 638-650.

62. Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., and Debussche, L. (1998). The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. EMBO J *17*, 4668-4679.

63. Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

64. Xu, Y. (2008). Induction of genetic instability by gain-of-function p53 cancer mutants. Oncogene *27*, 3501-3507.

65. Xue, C., Haber, M., Flemming, C., Marshall, G.M., Lock, R.B., MacKenzie, K.L., Gurova, K.V., Norris, M.D., and Gudkov, A.V. (2007). p53 determines multidrug sensitivity of childhood neuroblastoma. Cancer Res *67*, 10351-10360.

66. Yang, G., Zhang, G., Pittelkow, M.R., Ramoni, M., and Tsao, H. (2006).

Expression profiling of UVB response in melanocytes identifies a set of p53-target genes. J Invest Dermatol *126*, 2490-2506.

67. Yang, J., Yu, Y., Hamrick, H.E., and Duerksen-Hughes, P.J. (2003). ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. Carcinogenesis *24*, 1571-1580.

68. Zgheib, O., Huyen, Y., DiTullio, R.A., Jr., Snyder, A., Venere, M., Stavridi, E.S., and Halazonetis, T.D. (2005). ATM signaling and 53BP1. Radiother Oncol *76*, 119-122.

69. Zhao, H., Watkins, J.L., and Piwnica-Worms, H. (2002). Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci U S A *99*, 14795-14800.

70. Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

FIGURE LEGENDS

Figure 1. Pig3 is associated with several DNA damage sensors in the DNA damage response.

(A) Interaction of endogenous pig3 with DNA damage sensors was determined in the HCT116 cells were treated with UV. Cells were used for binding of MRN complex. Total cell lysates were prepared and immunoprecipitated with anti-pig3 antibody or rabbit IgG as negative control. Immunoprecipitates were analyzed with the indicated antibodies.

(B) Colocalization of pig3 with 53BP1, MRN complex, ATM and DNA-PK at UV-induced foci. Cells were untreated or treated with 10J UV for 6h, fixed and immunostained with antibodies to pig3 and 53BP1, Mre11, Rad50, Nbs1, ATM or DNA-PK followed by cognate Alex 488-conjugated and Alex 647-conjugated secondary antibodies. The nuclei were visualized by DAPI staining.

Figure 2. Pig3 forms nuclear foci in the DNA damage response.

(A) Pig3 foci is formed in the time dependent manner. HCT116 cells were treated with 10J UV for indicated times. cells were fixed and immunostained with polyclonal antibody to pig3. DAPI staining was included to indicated the positions of nuclei.

(B) Wortmannin inhibits pig3 foci formation. HCT116 cells were pretreated with 10 M wortmannin for 2h and analyzed for immunostaining after DNA damage.

(C) Pig3 foci formation is independent to p53. HCT116 p53+/+ or p53-/- cells were treated with 10J UV for 6h and stained with pig3 antibody.

Figure 3. Pig3 is associated with H2AX and affected to recruitment of the DNA damage sensors to foci.

(A) Pig3 interacts with H2AXbut not H2AX after UV radiation. HCT116 cells were treated with 10J UV for 12h. Lysates from untreated or treated cells were

immunoprecipitated with control IgG or Pig3-specific antibody followed by immunoblotting with antibodies to H2AX, H2AX or pig3.

(B) Colocalization of Pig3 and H2AX in HCT116 cells. Cells were treated with 10J UV for 6h and double stained with antibodies for pig3 and H2AX. In the merged pictures, yellow color represents the colocalization of pig3 and H2AX. DAPI staining was included to indicated the positions of nuclei.

(C) Abrogation of H2AX foci formation in pig3-knockdown cells. (upper-left) HCT116-sihygro cells were treated with UV , and after 6h, the cells were double stained with antibodies for pig3 and H2AX.

(D) Immunoblot analysis for the expression of pig3,H2AX and -tubulin from cells used above. DNA damage-induced HCT116 sihygro and sipig3 cells were lysed using sonication for dissociation of chromatin-associated proteins. Whole cells lysates were analyzed by Western blotting using specific antibodies against pig3 and H2AX. Data were normalized with -tubulin expression.

Figure 4. Pig3 function in DNA damage checkpoints

(A) DNA damage sensitivity of the cells lacking pig3. HCT116-sihygro and HCT116-sipig3 cells were irradiated with various dose of UV, and seeded at low density (cell number of 100). After two weeks, percentage of survival colonies was determined as described in Materials and Methods. These results are shown as means SD of three independent experiments.

(B) Pig3 is required for intra-S phase checkpoints following DNA damage. Cells were treated with indicated dose of UV, and incubated for 24h. Percentage of cells in showing BrdU incorporation at 2h following BrdU addition were measured. The number of BrdU-positive untreated cells was set to 100%, and an average percentage from five independent experiments was plotted as graph. Columns, mean; bars, SD. $P<0.01$, $*P<0.05$, compared to the value of control.

(C) Pig3 mediates activation of G2/M checkpoint following DNA damage. A 100ng/ml Nocodazole was added to media 3h following treatment of 10J UV for 24h in HCT116 sihygro and sipig3 cells. Cells were double stained with PI and phospho-histone H3 antibody as marker of mitotic entry followed by FACS

analysis. Percentage mitotic cell is indicated. Columns, mean; bars, SD. P<0.01, *P<0.05, compared to the value of control

Figure 5. Pig3 is required for p53-mediated DNA damage response.

(A) Impairment of H2AX induction by pig3-knockdown. HCT116 sihygro and sipig3cells were treated with 10J UV, whole cell lysates were prepared after incubation for indicated time points. Western blot analysis was carried out using specific antibodies against pig3, p53, phospho-p53(S15), H2AX and -tubulin.

(B) Overexpression of pig3 retrieves reduction of H2AX. HCT116 p53-/- mock and pig3 cells were treated as above. The H2AX expression was detected using specific antibody of phospho-H2AX (S139). Data were normalized with -tubulin expression.

(C) Representative confocal images of H2AX foci. HCT116 sihygro and sipig3 cells or HCT116 p53-/- mock and pig3 cells were treated with 10J UV for 6h. After incubation, cells were stained with antibody for H2AX. DAPI staining was included to indicated the positions of nuclei.

Figure 6. Pig3 induces the cell death after DNA damage in p53 gain-of-function mutants.

(A) pig3 is not required for DNA damage checkpoints in SW480 cells. SW480 cells were transfected with control or pig3 siRNA using RNAimax as described in materials and methods. After 48h, cells were treated with 200ng/ml NCS (left) or 10J UV (right) for indicated times. Cells were lysed by sonication, and Western blot analysis was done using specific antibodies against pig3, p53, phospho-p53(S15) and H2AX. Data were normalized with -tubulin expression. Results are representative of three independent experiments.

(B) Activation of DNA damage checkpoints is not increased by pig3 overexpression. SW480 cells were transiently transfected with either empty vector or pig3 overexpression vector for 48h and were treated with DNA damaging agents as above, and then whole cells lysates were prepared. Western blot

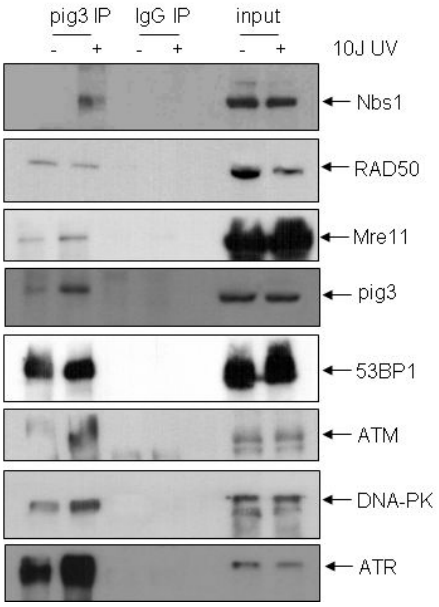
analysis was carried out using specific antibodies against pig3, p53, phospho-p53(S15) and H2AX. -tubulin was used as the loading control.

(C) Representative confocal images of H2AX foci.

(D) Pig3 induced cell death. The cells in above were treated with 20J UV for indicated times. Apoptotic subG1 DNA contents were estimated by FACS analysis using PI staining. Data are representative of three independent experiments. Points, mean; bars, SD.

Figure 1

A


pig3 IP
IgG IP
input
- + - + - +
10J UV
Nbs1
RAD50
Mre11
pig3
53BP1
ATM
DNA-PK
ATR

B

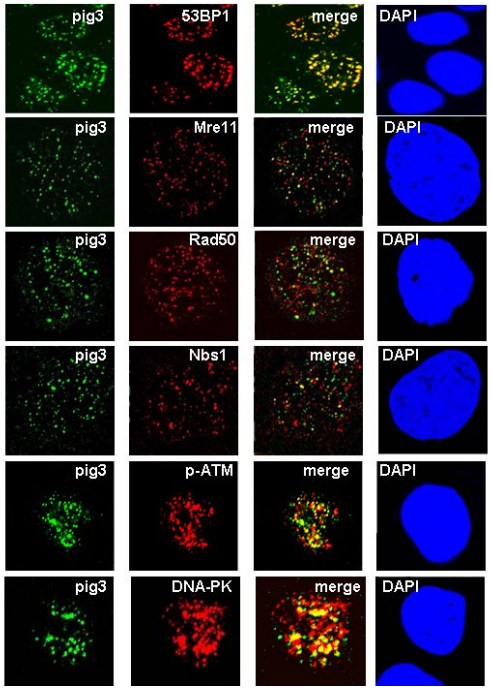
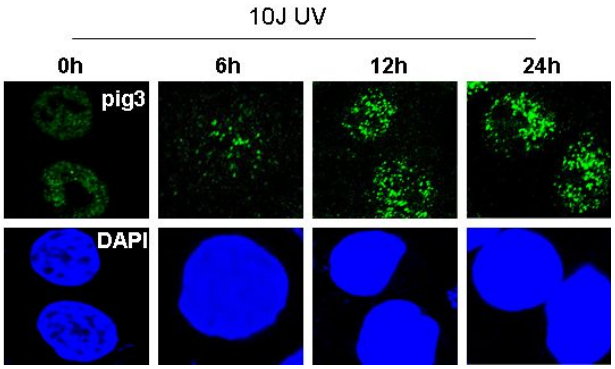
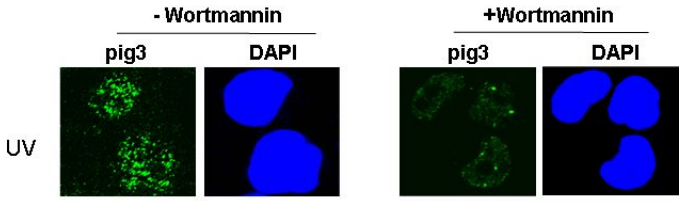

pig3
53BP1
merge
DAPI
pig3
Mre11
merge
DAPI
pig3
Rad50
merge
DAPI
pig3
Nbs1
merge
DAPI
pig3
p-ATM
merge
DAPI
pig3
DNA-PK
merge
DAPI

Figure 2

A


10J UV
0h
6h
12h
24h
pig3
DAPI

B


- Wortmannin
+Wortmannin
pig3
DAPI
pig3
DAPI
UV

C

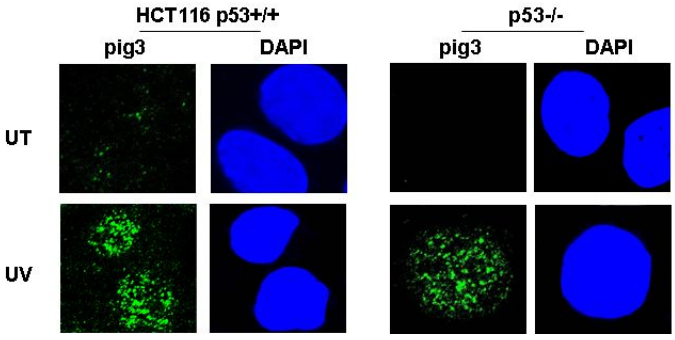
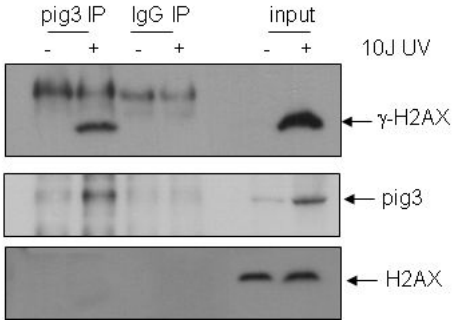
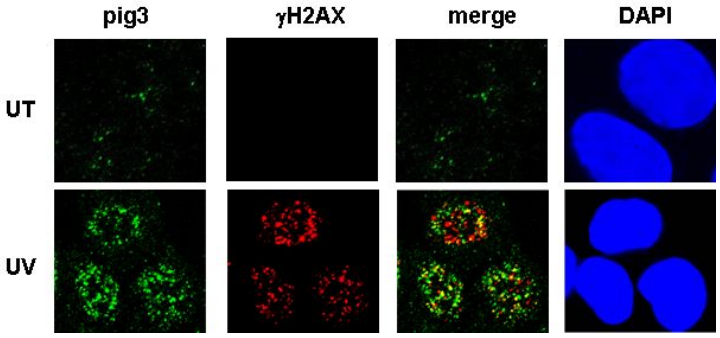

HCT116 p53+/+
p53-/-
pig3
DAPI
pig3
DAPI
UT
UV

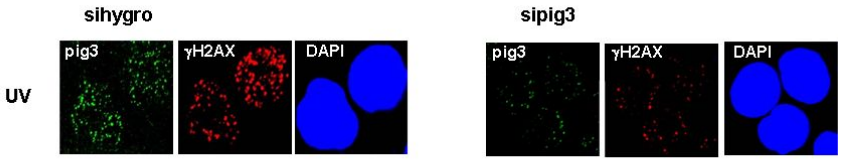
Figure 3 A



B



C



D

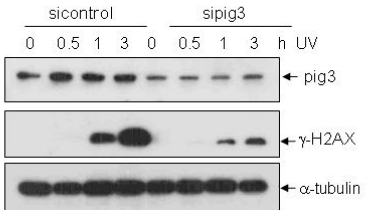
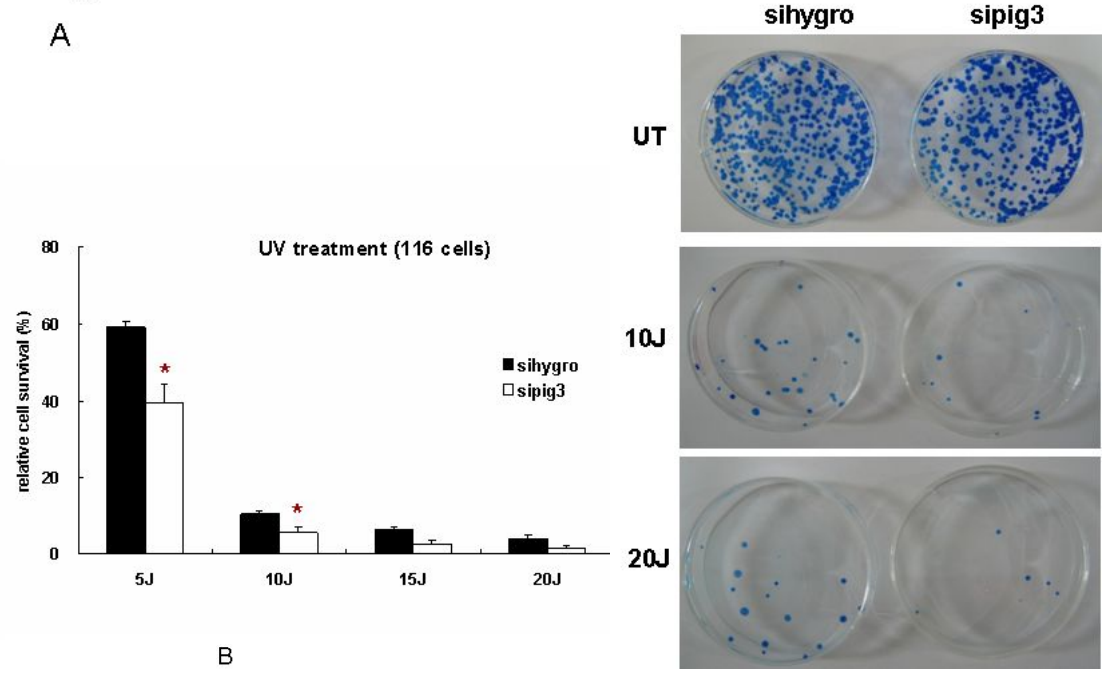
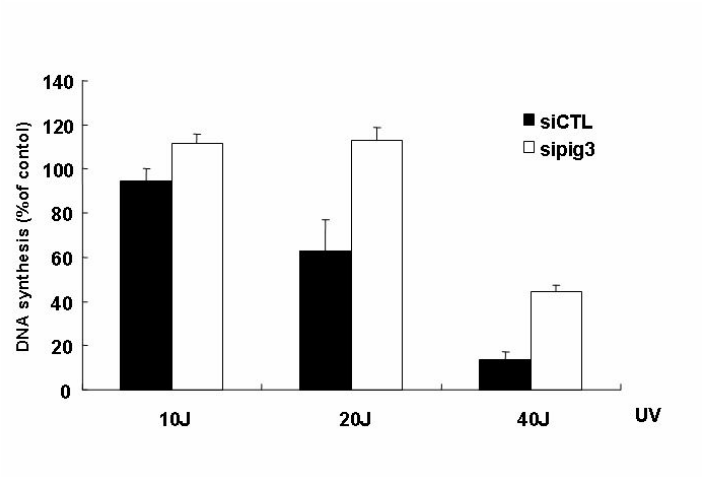


Figure 4

A


sihygro
sipig3
UT
10J
20J
UV treatment (116 cells)
relative cell survival (%)
80
60
40
20
0
5J
10J
15J
20J
sihygro
sipig3
*
*

B


DNA synthesis (%of contol)
140
120
100
80
60
40
20
0
10J
20J
40J
UV
siCTL
sipig3

C

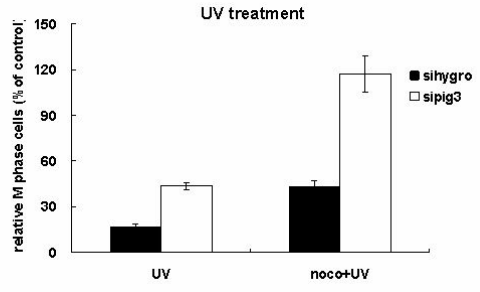
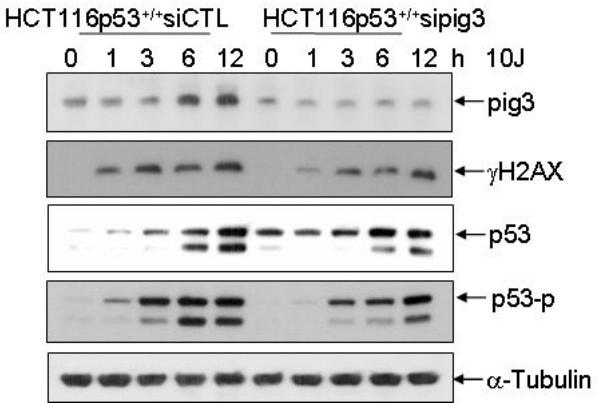
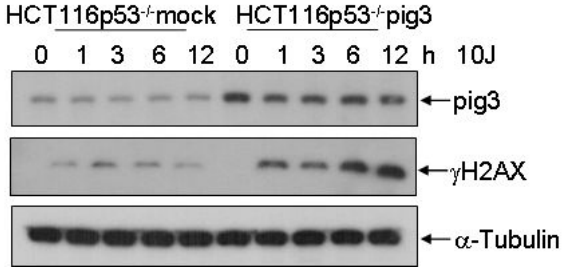

UV treatment
relative M phase cells (% of control)
150
120
90
60
30
0
UV
noco+UV
sihygro
sipig3

Figure 5

A


HCT116p53+/+siCTL
HCT116p53+/+sipig3
0 1 3 6 12 0 1 3 6 12 h 10J
pig3
γH2AX
p53
p53-p
α-Tubulin

B


HCT116p53-/-mock
HCT116p53-/-pig3
0 1 3 6 12 0 1 3 6 12 h 10J
pig3
γH2AX
α-Tubulin

C

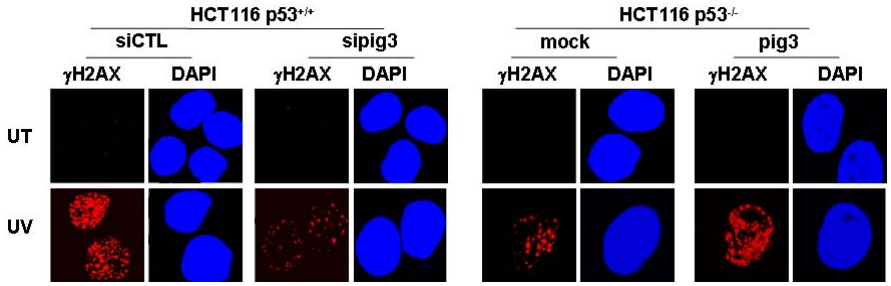
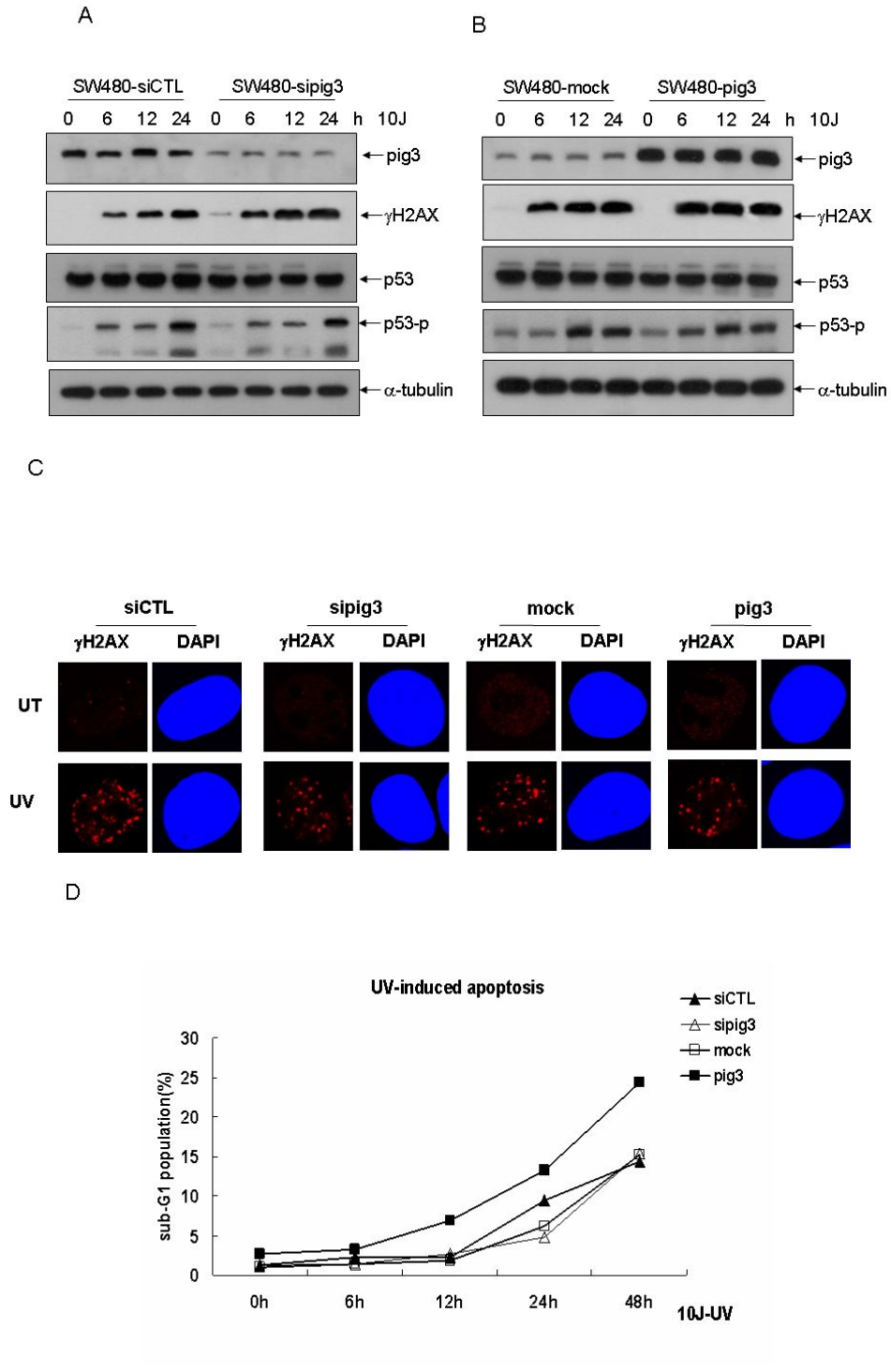

HCT116 p53+/+
siCTL
sipig3
HCT116 p53-/-
mock
pig3
γH2AX
DAPI
UT
UV

Figure 6


A
SW480-siCTL
SW480-sipig3
0 6 12 24 0 6 12 24 h 10J
pig3
γH2AX
p53
p53-p
α-tubulin
B
SW480-mock
SW480-pig3
0 6 12 24 0 6 12 24 h 10J
pig3
γH2AX
p53
p53-p
α-tubulin
C
siCTL
sipig3
mock
pig3
γH2AX
DAPI
UT
UV
D
UV-induced apoptosis
siCTL
sipig3
mock
pig3
sub-G1 population(%)
30
25
20
15
10
5
0
0h
6h
12h
24h
48h
10J-UV

저작물 이용 허락서

학 과	생물신소재	학 번	20087283	과 정	석사
성 명	한글 박 지 연	한문 朴 志 娟	영문 Park Ji Yeon		
주 소	광주광역시 북국 각화동 금호타운 9				
연락처	E-mail : baby_babu@naver.com				
논문제목	한글 p53의 유전자손상 반응 연구				
	영문 The function of p53 on the DNA damage response				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 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 편집과 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 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2010년 2월

저작자: 박 지 연 (인)

조선대학교 총장 귀하