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ID3 regulates MDC1-mediated signaling pathway

조선대학교 대학원

의 학 과

김 윤 지

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이 논문을 의학석사학위신청 논문으로 제출함

2011 년 10 월

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2011 년 11 월

조선대학교 대학원

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ID3에 의한 MDC1 조절기전 연구

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ID3는 basic helix-loop-helix 구조를 가진 전사인자의 우성음성 조절인자(dominant negative regulator)로써 세포의 분화, 세포사멸, 성장 등을 조절한다고 알려져 있다. MDC1 (mediator of DNA damage checkpoint 1, DNA 손상 check단백질의 매개 물질1)은 DNA 손상반응에서 주요한 역할을 함이 보고 되여졌다. 본 연구논문에서는 결합단백질을 조사하는 yeast two hybrid screening을 통해 MDC1 단백질에 결합하는 새로운 ID3을 동정하였다. ID3는 MDC1과 세포내에서 결합함을 면역침강(co-immunoprecipitation)을 통해 확인하였고 GST-pull down분석을 통해 MDC1과 ID3가 직접적으로 결합함을 보았다. 이는 ID3 단백질이 MDC1에 의한 DNA 손상반응에 관여할 수 있음을 시사한다. 이를 증명하기 위해 우선 ID3을 억제시킨 세포주에서 DNA 손상반응을 조사하였다. DNA 손상 시 유도되는 MDC1, p-ATM, 53BP1, γH2AX의 foci를 관찰한 결과 ID3가 결핍된 세포주에서 p-ATM과 MDC1의 damage foci가 감소됨을 확인하였다. 또한, ID3가 결핍된 세포

에서 DNA damage signal의 결합을 ATM, MDC1, NBS1의 인산화 western blotting 분석을 통해 확인하였다. 더욱이 ID3결핍된 세포주에서 손상복구가 감소됨을 comet assay와 이중나선절판에 따른 vector을 이용한 system을 통해 확인하였다. 또한 clonal survival assay를 통해 ID3가 결핍된 세포는 정상세포에 비해 IR에 민감함을 확인하였다. 마지막으로, DNA 손상반응으로 세포주기 체크포인트(cell cycle checkpoint)를 조사한 결과 ID3 결핍세포에서 intra-S-phase 체크포인트의 결합을 확인하였다. 반면, G2/M checkpoint 와는 무관함을 보았다. 따라서, 본 연구결과에서는 ID3와 MDC1의 결합을 통해 ID3가 MDC1의 intra-S 체크포인트와 이중나선 절단에 의한 비상동말단결합을 조절함을 확인함으로써 ID3가 DNA 손상반응에 새로운 중요한 조절자임을 제시한다.

I. INTRODUCTION

DNA damage response requires a coordinated nucleo-cytoplasmic cascade of events, depending upon the exogenous nature of the DNA damage signal (Lieberman et al., 2008). DNA double-strand breaks (DSBs) are highly toxic lesions that, if unrepaired or repaired incorrectly, can cause cell death, mutations, and chromosomal translocations and can lead to cancer. Cells react to DSBs by rapidly deploying a host of proteins to the damaged-chromatin regions. Some of these factors engage in DNA repair, while others trigger a signaling pathway (called the DNA-damage checkpoint) that delays cell-cycle progression and coordinates repair processes; together, these events comprise the DNA damage response (DDR). Some DDR factors have intrinsic affinity for free DNA ends, while others, many of which contain BRCA1 carboxy-terminal (BRCT) domains, accumulate in large nuclear aggregates that appear as IR-induced nuclear foci (IRIF) by fluorescence microscopy.

The inhibitor of differentiation (Id) protein are dominant negative basic helix-loop-helix transcription factors that lack the DNA-binding basic domain but have intact HLH domain (Benezra et al., 1990, Murre et al., 1994). ID proteins act as the positive and/or negative regulators on gene expression, through the interactions with other HLH transcription factors in a context-dependent manner. ID proteins affect many of cellular activities including the cell growth, differentiation and apoptosis (Norton et al., 2000). There is overwhelming

evidence in support of the role of ID3 family proteins in both cell cycle progression and S-phase entry. Four Id genes (ID1 to ID4) have been identified in mammals (Riechmann et al., 1994). They directly associate with and regulate the activity of several families of transcriptional regulators (Yates et al., 1999, Lasorella et al., 2000, Norton et al., 2000, Roberts et al., 2001). Remarkably, few connections between this family of proteins and the DNA damage pathway have been shown. Based on these valuable insights we enquired on the possible physiological role of ID3, an important member of the ID3 family in eliciting ionizing radiation (IR) or radiomimetic drug neocarzinostatin (NCS) induced DNA damage response in mammalian cells. Ionizing radiation is an important modality in cancer treatment, it is estimated that more than 50% of cancer patients receive certain types of radiation treatment (Mendelsohn et al., 2002). Although Id3 contains the HLH domain required for protein:protein dimerization, it lacks the basic DNA-binding domain possessed by other members of the family, making it incapable of binding to DNA. They play a crucial role in the coordinated regulation of gene expression during cell growth, differentiation and tumorigenesis (Norton et al., 2000, Benezra et al., 2001). The involvement of Ids was also recently shown in cellular senescence (Alani et al., 2001, Okabe M et al., 2001, Zheng et al., 2004) and in the fate of specialized cells such as lymphocytes, vascular endothelial cells and neurons (Benezra et al., 2001, Lyden et al., 1999, Engel et al., 2001). Such ID-bHLH

heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins (Benezra et al., 1990, Garrell et al., 1990, Ellis et al., 1990). After an initial decline, Id expression is sustained throughout the G1 phase of the cell cycle and is further upregulated as cells enter S phase (Hara et al., 1994, Norton et al., 1998). ID proteins function at multiple stages in cell cycle control by modulating the transcription of several known target genes, in some instances by directly interacting with non bHLH proteins.

MDC1 (mediator of DNA damage checkpoint 1) is a recently identified mammalian DNA damage response factor with an important role in the DNA damage response (Goldberg et al. 2003; Stewart et al. 2003; Lukas et al. 2004; Stucki and Jackson 2006). MDC1 is recruited to chromatin near sites of DNA damage through the interaction of its BRCA1 C-terminal (BRCT) repeats with γ-H2AX, the phosphorylated form of H2AX (Stewart et al. 2003; Lee et al. 2005; Stucki et al. 2005). MDC1 also interacts with the Mre11/Rad50/Nbs1 complex and promotes its stable association with sites of DNA damage (Goldberg et al. 2003), which could explain how MDC1 enhances the local action of the ATM kinase. Thus, MDC1 appears to be part of a feed-forward loop that promotes the stable association of DNA damage response factors in large domains (foci) near the site of damage (Stucki et al. 2005; Lou et al. 2006). However, the function of such DNA damage response foci, the role of the foci in the

regulation of cell cycle progression, and their contribution to DNA repair have remained ill-defined (Stucki and Jackson 2006). DNA damage factors such as 53BP1, γ-H2AX, ATM phosphorylated at S1981 (ATM-S1981-P), and the Mre11/Nbs1/Rad50 complex accumulate in telomere dysfunction-induced foci (TIFs) that extend from the dysfunctional telomere into the subtelomeric regions (d'Adda di Fagagna et al. 2003; Takai et al. 2003; Celli and de Lange 2005).

In this study, we investigate the role of ID3 in MDC1-mediated NDA damage response. We report that the depletion of ID3 results in suppression of MDC1 foci and MDC1 phosporylation in response to IR. The induction of ATM and NBS1 phosphorylation, and p-ATM foci formation after IR is impaired in ID3 depleted cells. Moreover, the knockdown of ID3 sensitizes cells to IR, impairs DNA repair, and causes defects in the intra-S phase checkpoint in response to DNA damage. Thus, we propose that ID3 is critical regulator of DNA damage pathway through a direct physical interaction with MDC1.

II. MATERIALS AND METHODS

1. Cell culture and drug treatment

The human cervix adenocarcinoma cell line HeLa were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA). In both cases, the media was supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Corp., East Rutherford, NJ, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator containing 5% CO2 at 37℃. HeLa cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). To induce DNA breaks, exponentially growing cells were treated with the radiomimetic drug neocarzinostatin (NCS; Sigma, St Louis, MO, USA) at a final concentration of 200 ng/ml in fresh cell media or irradiated at 10 Gray (Gy) from ^{137}Cs source (Gammacell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada) and allowed to recover at 37℃ for various amounts of time.

2. siRNA and transfection

The RNAi procedure was described previously (Brummelkamp et al., 2002). For the knockdown of ID3 expression, two siRNA target sites chosen from the homo sapiens inhibitor of DNA binding3, dominant negative helix-loop-helix protein (ID3) mRNA

sequence (GenBank accession number NM_002167.3), which was extracted from the NCBI Entrez nucleotide database. Hela cells were obtained from the American Type Culture Collection and maintained as recommended. The control siRNA was obtained directly from Ambion. The final concentration for siRNA transfection was 100nM. The siRNA duplexes were transiently transfected into the cells using RNAi MAX (Invitrogen) according to the manufacturer's instructions.

3. Yeast two-hybrid analysis

The full-length ID3 was sub-cloned into pGBT9 vector, which expresses proteins fused to the GAL4-DNAbinding domain (DNA-BD) (Clontech, Mountain View, CA, USA). This vector was used as bait to screen a human prostate cDNA library fused to the GAL4 activation domain according to the manufacturer's (Clontech) instructio ns. Positive clones were verified by ono-on-one transformation and selection on agar plates lacking leucine and tryptophan (-LH) or adenine, histidine, leucine and tryptoph an (-AHLT) and also processes for a β-galactosidase assay.

4. Immunoprecipitation assay and Western Blot analysis

Cells were lysed in ice-cold RIPA lysis buffer [50mM Tris-HCL, pH 7.5, 150mM NaCl,

1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate] containing ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of proteins were then resolved on 6–15% SDS-PAGE gels, followed by electrotransfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1h in TBS-t [10mM Tris–HCl (pH 7.4), 150mM NaCl, 0.1% Tween 20] containing 5% fat-free milk at room temperature and then incubated with the indicated primary antibodies overnight at 4℃. Blots were washed four times for 15min with TBS-t and then incubated for 3h with peroxidase conjugated secondary antibodies (1:4000, 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 the immunoprecipitation assay, the RIPA extracts were pre-cleared with protein A-sepharose bead (GE Healthcare), then incubated at 4℃ overnight with appropriate antibodies. After the addition of fresh protein A-sepharose bead, the reaction was incubated for 3 h at 4℃ with rotation. The beads were washed five times in RIPA buffer without protease inhibitors, resuspended in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

5. GST Pull-Down Assay

Assay was performed as described previously (Choi et al., 2001). Briefly, the cDNAs encoding the ID3 were cloned into the plasmid pGEX4-T-1 and used to transform Escherichia coli strain BL21. The fusion proteins were purified from 10ml bacteria and incubated with glutathione-Sepharose 4B (Amersham-Pharmacia Biotech). Following being incubated for 30min at RT(Room temperaturein) with gentle mixing, the beads were washed four times with 100 volumes of TEN100 buffer [20mM Tris, pH 7.4, 0.1mM EDTA and 100mM NaCl] and mixed with 1mg of cell lysate at 4℃ for 3h. After four times with 100 bed volumes of NETN buffer [0.5% NP40, 0.1mM EDTA, 20mM Tris, pH 7.4, 300mM NaCl], the bound proteins are eluted by boiling in two volumes sample buffer and visualized using Coomassie blue staining or Western blot analysis.

6. Immunostaining assay

To visualize nuclear foci, cells cultured on cover slips coated with poly-L-lysine (Sigma) were treated with IR or NCS, followed by recovery for adequate times. The cells were then washed twice with 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 1 h. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4℃. Next, the cells were washed with PBS and then stained with Alexa Fluor 488 (green, Molecular Probes) or 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 (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken under a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss, Jena, Germany) and analyzed with Zeiss LSM Image Examiner software (Carl Zeiss). For foci quantification experiments, cells with ≥10 foci were counted as MDC1 foci-positive cells and the percentage was calculated among at least 100 cells by dividing the number of MDC1 foci-positive cells by the number of DAPI-stained cells. The error bars represent standard error in three independent experiments.

7. Cell survival assay

After treatment of IR or NCS, 5×10^2 cells were immediately seeded onto a 60mm dish in triplicate and grown for 2–3 weeks at 37℃ to allow colonies to form. Colonies were stained with 2% methylene blue per 50% ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells. The mean

value±s.d. for three independent experiments was determined.

8. Comet assay

Comet assay (single-cell electrophoresis) Single-cell gel electrophoresis assay was carried out as described previously with modification (Chowdhury et al., 2005). Briefly, control and ID3-knockdown cells were treated with 10Gy IR by followed by incubation in culture medium at 37℃ for the indicated times. Aliquots of the cell suspension (20 μl, $2X10^5$ cells) were transferred to 1.5ml tubes and then mixed with 200 ml of low-melting-temperature agarose and distributed onto conventional microscope slides that had been pre-coated with standard agarose (0.5% in DW) and dried at 4℃ for 40min. The slides were then immersed in lysis solution (2.5M NaCl, 100mM Na_2EDTA, 10mM Tris-HCl (pH 10), with freshly added 1% Triton X-100 and 10% DMSO) for 30min at 4℃ and then placed in a horizontal electrophoresis apparatus filled with fresh buffer (1mM Na_2EDTA, 300mM NaOH (pH>13)). After electrophoresis (~30 min at 1 V/cm tank length), air-dried and neutralized slides were stained with 30– 50 ml ethidium bromide (20 mg/ml). Average comet tail moment was scored for 40–50 cells/slide using a computerized image analysis system (Komet 5.5; Andor Technology, South Windsor, CT, USA).

9. Analysis of NHEJ activity

To analyze the role of ID3 in NHEJ in vivo, we used the pEGFP-Pem1-Ad2 system (Kang et al., 2009). The plasmid was digested with HindIII to remove Ad2 and generate different types of ends. Supercoiled pEGFP-Pem1 was used as a positive control for standardization of transfection and analysis conditions. The pCMV-dsRed-express plasmid (Clontech) was co-transfected with either linearized pEGFP-Pem1-Ad2 or supercoiled pEGFP-Pem1 as a control for transfection efficiency. In a typical reaction, 5×10^5 cells were transfected with 0.5 μg of linearized pEGFP-Pem1-Ad2 or 0.5 μg supercoiled pEGFP-Pem1, together with 0.5 μg of pDsRed2-N1 plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended protocol. Twenty-four hours after transfection, green (EGFP) and red (DsRed) fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

10. G2/M checkpoint analysis

G2/M checkpoint assay was performed as described previously (Kolas NK et al., 2007, Reinhardt HC et al., 2007). Control and ID3-depleted cells were treated with 200 ng/ml NCS or 10 J/m2 UV. At 6 or 9 h after NCS or UV treatment, respectively, the cells were harvested and washed with PBS and then fixed with 1% formaldehyde for 10 min at 37℃. The cells

were chilled on ice for 1 min and then permeabilized with 90% methanol at -20℃ overnight. The fixed cells were washed with PBS and blocked with incubation buffer (0.5% BSA in PBS) for 10 min. The cells were stained with anti-phospho-histone H3 (S10)-Alexa Fluor 647-conjugated antibody (Cell Signaling Technology) at 1:10 dilution in incubation buffer for 1 h in darkness at room temperature; they were then washed and resuspended in PBS containing 50 μg/ml propidium iodide. At least 10000 cells were analyzed by fluorescence-activated cell sorting (FACSort, Becton Dickinson, San Jose, CA, USA). The acquired data were analyzed using the CellQuest Pro software (Becton Dickinson).

11. BrdU incorporation assay

To determine the 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.). Control and ID3-depleted cells were seeded in 1 48-well plate and treated with NCS or UV. At 3 or 6h after NCS or UV treatment, respectively, 10μM BrdU was then added to the culture medium for incorporation into freshly synthesized DNA, and the culture was incubated for 2h at 37℃. After fixation of the cells, cellular DNA was partially digested by nuclease treatment. A peroxidase-labeled antibody to BrdU and a peroxidase substrate were added sequentially to yield a colored product were measured in a microplate reader at

405nm with a reference wavelength at approximately 490nm. The relative DNA synthesis was calculated as the percentage of absorbance of cells. The data are presented as the mean± s.d. from triplicate experiments.

12. Antibodies

The ID3 protein was detected by western blotting with a rabbit polyclonal ID3 antibody (H-70, Santa Cruz Biotechnology) at 1:1000 dilution. Anti-MDC1 (Abcam) and anti-53BP1(BD Pharmingen) was detected by immunofluorescence and western blotting at 1:200 and 1:1000 dilutions, respectively. The following antibodies were used for immunofluorescence staining: and γ-H2AX mouse monoclonal antibody (JBW301), anti-ATM pAb(Rockland, 1:200). The following antibodies were used for western blot analysis: anti-Mre11 monoclonal antibody (BD Pharmingen, San Jose, CA, USA, 1:200), anti- Rad50 monoclonal antibody (BD Pharmingen, 1:1000), anti-NBS1 polyclonal antibody (Cell Signaling Technology, Boston, MA, USA, 1:1000), anti-NBS1 pAb (Cell Signaling Technology, 1:1000), anti--BRCA1 pAb (Cell Signaling Technology, 1:1000), anti-BRCA1 Ab (C-20, Santa Cruz, 1:1000), anti-MDC1 pAb (Cell Signaling Technology, 1:1000), anti-Chk2 Ab (Cell Signaling Technology, 1:1000), anti-Chk2 pAb (T68) (Cell Signaling Technology, 1:1000), anti-α-tubulin mAb (TU-02, 1:4000).

13. Statistical analysis

Data are presented as means ± SD. Statistical comparisons were carried out using unpaired *t*- tests, and values of $P<0.01$ were considered to be statistically significant.

III. RESULTS

1. ID3 as a binding partner of MDC1 was identified by yeast two hybrid assasy

We hypothesized that induction of ID3 expression from very low background levels leads to its interaction with pre-existing protein factors. Since Id3 mRNA is induced rapidly after growth factor stimulation, it seemed likely that the ID3 targets would be expressed before growth factor stimulation. In order to specifically seek Id3 associated proteins existing before ID3 induction, yeast two- hybrid screening of a human cDNA library was performed using full length Id3 as bait. One of the positive clones isolated from 2×10^6 transformants turned out to be MDC1. Nucleotide sequence analysis revealed three classes of cDNAs. The interaction between ID3 and MDC1 was verified in yeast by retransformation and growth on reporter selective madia, by liquid β-galactosidase assay (data not shown).

2. ID3 binds to MDC1 in vivo and in vitro

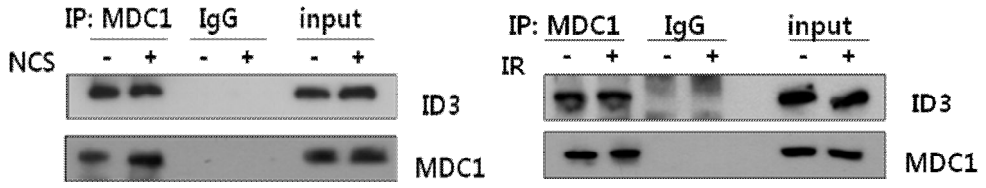
To determine whether ID3 interacts with MDC1 in human cells endogenously expressing both proteins, immunoprecipitation assays was performed. For production of DSBs, HeLa cells were treated with 100 ng/ml of the radiomimetic drug NCS or 5 Gy of IR. The cells were then lysed, and endogenous MDC1 was immunoprecipitat ed with a MDC1-specific antibody. Immunoprecipitates were subjected to western blot ting with an anti-ID3 antibody. Immunoprecipitation with anti-MDC1 antibodies reveal ed that endogenous MDC1 bound ID3 and that treatment with NCS or IR increased the amount of MDC1 bound to Id3 (Figure 1A).

To further confirm this interaction, Hela cells were transiently transfected with an expression construct encoding full-length Id3 and a second construct that expressed full-length MDC1. Co-immunoprecipitation assays were then performed using an Id3-specific antibody for immunoprecipitation and an anti-MDC1 antibody for immunoblot ting. In spite of this, it remains possible that other proteins may mediate the interacti on between MSH6 and Ku70 complex because both complexes possess a variety of interacting partners. In this reciprocal experiments, ID3 antibody was able to co-immunoprecipitate MDC1 (Figure 1B), suggesting that these proteins may interact with each other directly or indirectly in cells. As control, normal rabbit IgG did not co-immunoprecipitate ID3 or MDC1, indicating that the co-immunoprecipitation of

ID3 with MDC1 was not due to non-specific antibody binding.

In addition, we found that bacterially expressed GST-ID3 could retrieve MDC1 from cell lysates in a pull-down assay. We incubated the GST fusion proteins with a protein phosphatase-treated sample of either purified ID3 (Figure 2A). To confirm the interaction by independent methods, we performed GST pull-down assays using the region of Id3 isolated in the pull down screen (GST-Id3) (Figure 2B). The purified fusion protein was incubatd with lysate from Hela cells transiently transfected with an expression plasmid for MDC1. MDC1 was retained on GST-ID3 but not on the GST control (Figure 2C). Together, these results indicate that Id3 interacts with MDC1, and this interaction recruits Id3 to DNA DSBs after DNA damage.

A



B

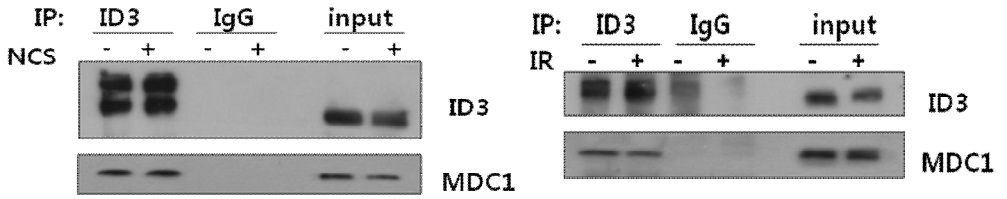


Figure 1. MDC1 interacts with ID3

(A) Hela cells were untreated (UT) or treated with 100ng/ml NCS or 5Gy ionizing radiation (IR). Proteins were immunoprecipitated (IP) from the lysates using an anti-ID3 antibody 3h after treatment. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for ID3 or MDC1. normal rabbit lgG was used for negative control immunoprecipitations. (B) NCS- or IR-treated cell lysates were prepated and Co-immunoprecipitated with anti-ID3 antibody.

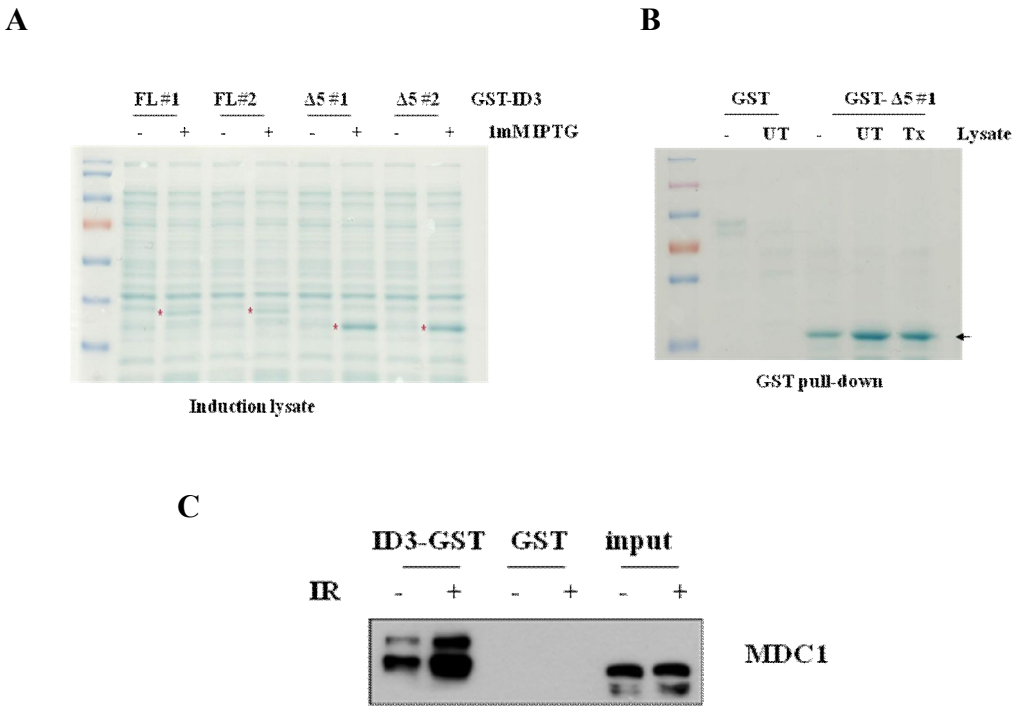


Figure 2. MDC1 directly binds to ID3

(A) Coomassie blue staining of gel after immunoprecipitates from the GST-ID3 pull down.

(B) In vitro binding assay of GST- ID3. Fusion proteins were purified from Escherichia coli.

Proteins were IP and western-blot analysis using antibodies MDC1.

3. ID3 knockdown impairs foci-formation of MDC1 and phospho-ATM after DNA damage

After DNA damage, many DNA-damage-repair proteins are recruited to the DNA-damage sites and form discrete DNA-damage-induced nuclear foci. The order and timing of these events are thought to be critical for DNA damage response and DNA repair (Stewart et al., 2003). To test role of ID3 in MDC1-dependent events, we used siRNA technology (Elbashir et al., 2001) to down-regulated ID3 (Figure 3A) and monitored MDC1-dependent events by immunoprecipitation (Figure 3B). An antibody raised against substrates of MDC1 has been shown to specifically recognize MDC1-dependent events. As shown in Figure 3B in cells transfected with control siRNA ionizing radiation (IR)-induced nuclear foci were present in most irradiated cells, suggesting the accumulation of MDC1 substrates at the sites of DNA breaks. However, down-regulation of ID3 abolished these IR-induced foci. These results suggest that regulates MDC1-dependent events upon DNA damage. Furthermore down-regulation of ID3 resulted in increased sensitivity to IR, implying that ID3 is required for cell survival following DNA double-stranded breaks. Together these data suggest that ID3 is involved in MDC1-dependent DNA damage response pathways.

In agreement with the role of ID3 in ATM-dependent phosphorylation pathways, down-regulation of ID3 also partially decreased phosphor-ATM foci staining after IR. The p-ATM foci became bigger and brighter, as early as about 30min, 1h, 3h and 6h, respectively, after IR (Figure 3C) treatment. Although p-ATM foci formed rapidly after IR treatment in ID3 knockdown cells, the intensity of p-ATM foci was reduced significantly in ID3-depleted cells as compared with control cells. These results suggest that ID3 knockdown regulates

ATM -dependent phosphorylation events upon DNA damage.

Therefore, phosphorylation of H2AX is a reliable indicator of whether DNA damage response pathways are activated in response to DNA damage stimuli (Rogakou et al., 1998; Ward and Chen, 2001). Based on above observations, we investigate the potential localization of γH2AX into the nuclear foci after DNA damage. Thus, after the control and ID3 siRNA-transfected Hela cells were untreated or treated IR, the immnofluorescent staining analysis was conducted (Figure 4A). We next performed to determine whether ID3 would induction of foci formation of 53BP1. The induction of 53BP1 foci by IR raises the possibility that these foci represent actual sites of DNA breaks (Figure4B). In fact, we observed that DNA damage-induced γH2AX foci formation with 53BP1 foci (Figure 4A and B), which have been shown to represent sites of DSBs processing. These results suggest that ID3 depletion activates phosphorylation of H2AX and 53BP1 foci formation after DNA damage, but did not affect γH2AX and 53BP1 foci formation.

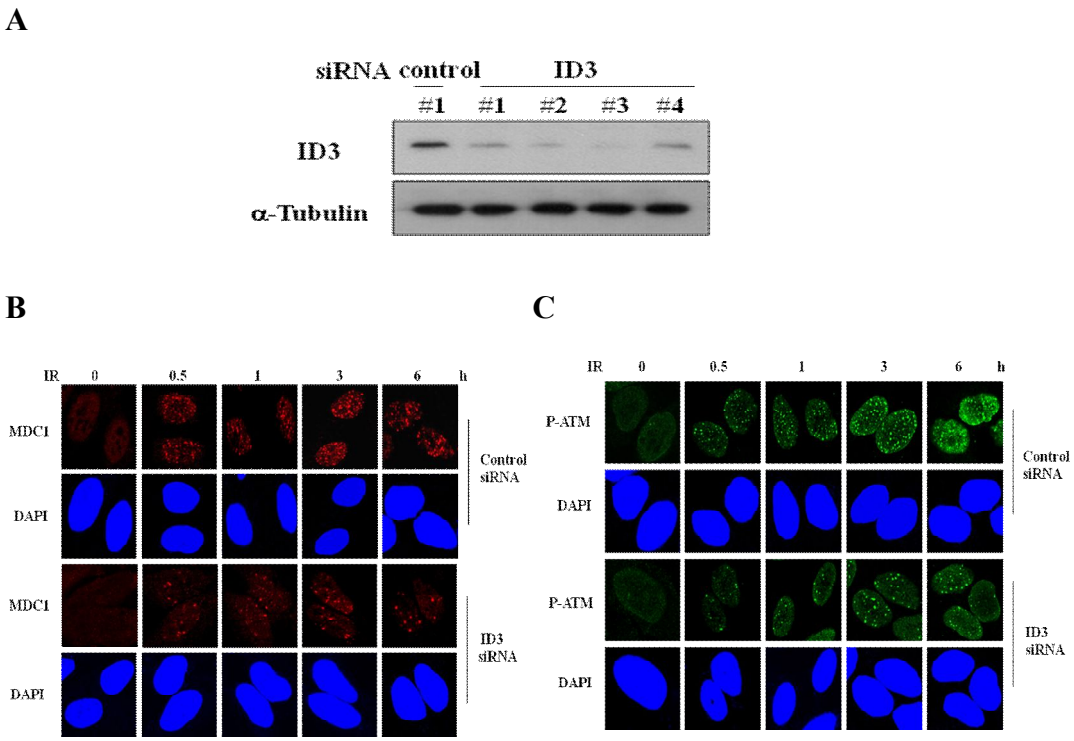
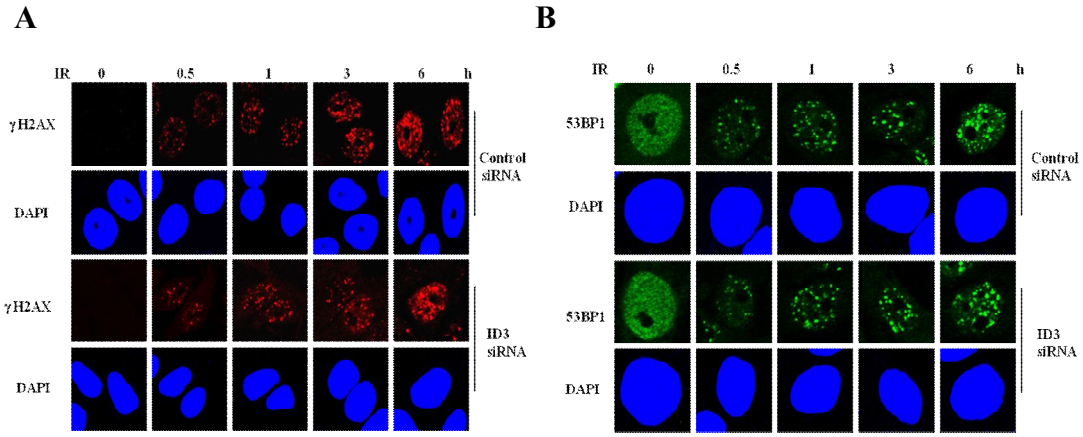


Figure3. ID3 knockdown impairs foci formation of MDC1 and p-ATM after IR

(A) Hela cells were transfected with control or four different ID3 small hairpin RNA (shRNA) expression vectors, and Western blot analysis was performed using specific antibodies against ID3, α-Tubulin.

(B) The control- and ID3-depleted Hela cells were untreated or treated with ionizing radiation (IR). At 0.5, 1, 3, 6h after IR treatment, cells were fixed and immunostained with anti-MDC1 or The nuclei were visualized by 4'-6-Diamidino-2-phenylindole(DAPI) staining was performed to indicate the positions of nuclei.

(C) Control and ID3-depleted localizes with phosphor-ATM following IR. Irradiated Hela cells (5Gy) were fixed 3h later and immunostained with anti-phospho-ataxia telangiectasia mutated (ATM) antibodies.



Figrue 4. ID3 depletion does not suppress the foci formation of γH2AX and 53BP1

(A and B) ID3 small interfering RNAs (siRNA) transfected Hela cells were left untreated or treated with IR, fixed at the indicated time points and immunofluorescence analysis with antibld y aginst ID3 was carried out. 4'-6-Diamidino-2-phenylindole (DAPI) staining was performed to indicate the position of nuclei. Control and ID3 depleted Hela cells were fixed at the times indicated after treatment, respectively, and analyzed by immunofluorescence with anti-γ-H2AX (A) and anti-53BP1 (B) antibodies.

4. ID3 knockdown leads to reduced phosphorylation of DNA-damage signaling proteins

BRCA1 is phosphorylated by ATM, ATR, and chk2 on several serines throughout the length of the protein in response to DNA damage, suggesting that phosphorylation of BRCA1 plays a role in DNA damage response (Cortez et al., 1999; Gatei et al., 2001; Lee et al., 2000). BRCA1 interacts with proteins involved in both the non-homologous end-joining pathway (including the Mre11, Rad50, Nbs1 complex) (Greenberg et al., 2006) and homologous repair (RAD51 and BRCA2, among others) (Chin et al., 1998; Scully et al., 1997b) and forms damage-induced foci irrespective of the cell cycle. The Mre11-Rad50-Nbs1 (MRN) complex is a multifaceted molecular machine, critical for biological processes that detect and repair double strand breaks (DSBs) (K.P Hopner et al., 2002, R.S. Williams et al., 2007). Nbs1 provides the MRN complex with its signaling role through interactions with, and ultimately activation of either ATM (ataxia–telangiectasia mutated) or protein kinases or 53BP1 protein response to DSBs, respectively. Western blot analyses with antibodies Mre11, Rad50, NBS1, BRCA1, p-BRCA1, p-ATM, ATM, 53BP1, p-53BP1 confirmed the existence in DNA damage response. These results explain that ID3 involved in DNA damage response pathways(Figure 5).

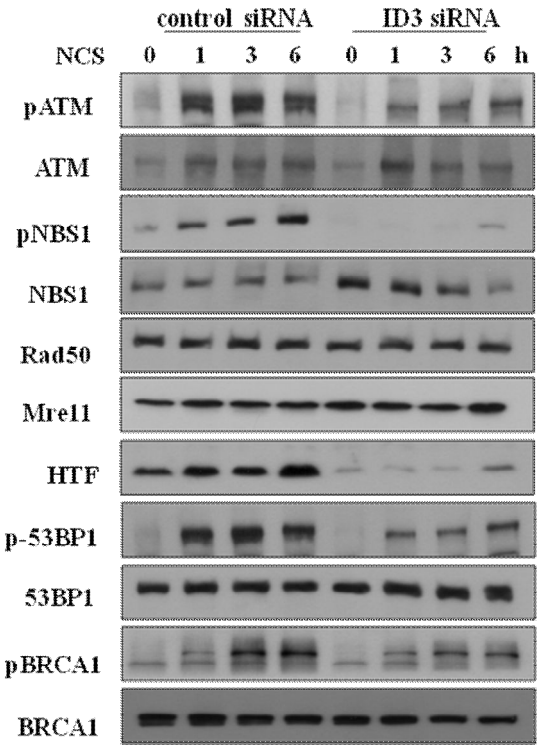


Figure 5. ID3 connect in DNA damage response after NCS drug

Hela cells were transfected with control or ID3 siRNA expression vector, and western blot analysis was performed using specific antibodies against p-ATM, ATM, p-NBS1, NBS1, Rad50, Mre11, ID3, p-53BP1, p-BRCA1, BRCA1, α-Tubulin at 0, 1, 3, 6h after NCS 100ng/ml.

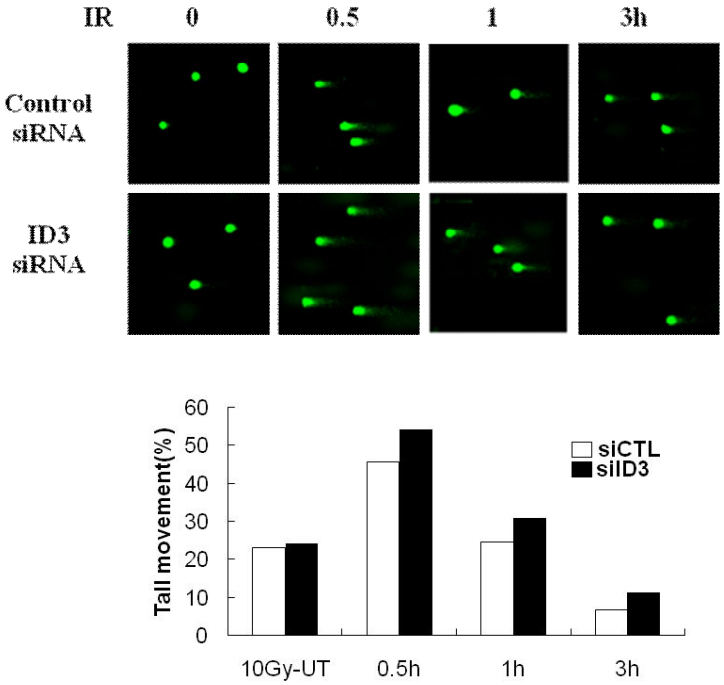
5. ID3 knockdown delays DNA double strand break repair

DSBs are one of the most deleterious types of damage caused by radiation (Jackson and Bartek, 2009). The comet assay, also known as the single-cell gel electrophoresis assay, was developed by Östling and Johanson (O Östling et al.,1984) for the direct visualization of DNA damage. To determine whether ID3 down-regulation affects DNA repair, we measured the persistence of DSBs after IR, as an indicator of unrepaired damaged DNA, by single-cell gel electrophoresis (neutral comet assay) (Figure 6A). At 30min after IR treatment, control and ID3-knockdown cells exhibited comparable levels of DNA damage. However, when the persistence of unrepaired DNA-strand breaks was analyzed at a various times after IR treatment, cells depleted of ID3 exhibited significantly lower repair efficiency than control cells (Figure 6A). Based on the comet tail moments, which quantify the extent of DNA damage, we estimate that ~2-2.5-fold more unresolved DNA damage remains in ID3-deficient cells in comparison to control cells at 1 and 3h after IR treatment (Figure 6A). These results indicate that ID3 deficiency retards DNA repair of IR-induced DNA-strand breaks.

Mammals have evolved at least two genetically discrete ways to mediate DNA DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ). In mammalian cells, most DSBs are preferentially repaired by NHEJ.An established strand break rejoining assay using the plasmid pEGFP-Pem1-Ad2 was used to determine levels of

NHEJ (Seluanov et al., 2004). Figure 6B (left) shows the pEGFP-Pem1-Ad2 plasmid used in the experiments (Seluanov et al., 2004). The plasmid is linearized by HindIII digestion that removes, when complete on both sides, the Ad2 exon and enables upon successful intracellular circularization EGFP expression that can be detected and quantitated by flow cytometry. We combined the pEGFP-Pem1-Ad2 system and short hairpin RNA (shRNA)-mediated depletion to analyze the effects of ID3 knockdown on the efficiency of NHEJ after formation of DSBs. Transfection with the supercoiled pEGFP-Pem1 plasmid is used to evaluate EGFP-signal expression without the requirement for rejoining, and the pCMV-dsRed-express plasmid is used as a control to determine transfection efficiency for this assay. To evaluate the efficiency of NHEJ, ID3 knockdown cells were transfected with either linearized pEGFP-Pem1 together with pDsRed2-N1. The cells were then incubate for 24h to allow expression of EGFP (green) and DsRed (red), followed by flow cytometry analysis. To control for the efficiency of transfection the ratio of GFP+ cells to DsRed+ cells was used as the normalized measure of NHEJ efficiency. In three independent experiments, the average NHEJ efficiency was in the ID3 siRNA cells relative to control siRNA-transfected cells. These results indicate that ID3 knockdown inhibits to DSB repair through regulation of NHEJ.

A



B

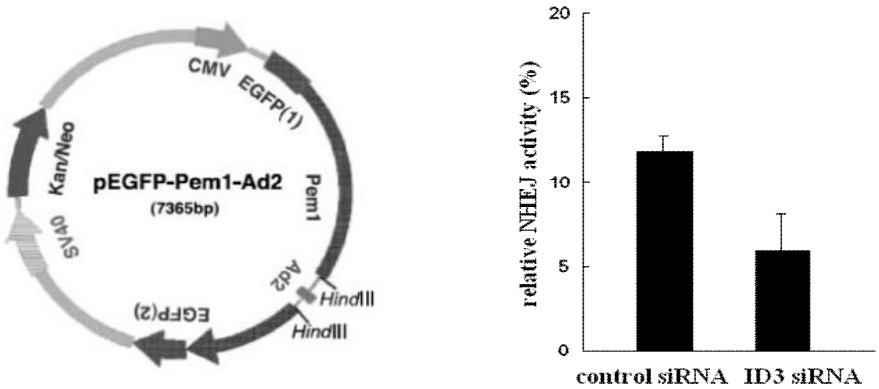


Figure 6. ID3 knockdown results in decreased DSB repair

(A) Control and ID3-depleted Hela cells were untreated or treated with 5Gy ionizing radiation. At the indicated time points, cells were harvested for comet tail formation assays

under alkaline conditions. Comet images were captured using fluorescence microscopy, and tail moment was analyzed in 50-70randomly chosen comets using Komet 5.5 analysis software. Representative comet images observed at different time points after IR treatment are shown.

(B) (left) map of pEGFP-Pem1-Ad2. Note the Pem1 intron, the Ad2 exon and the location of the HindIII cutting sites. (right) Relative levels of plasmid rejoining compared to control shRNA-transfected cells were calculated by dividing the GFP^{+}/ $DsRed^{+}$ ratios of the samples and plotted. Means are representative of at least three independent experiments. Error bars indicate the SD. Asterisk denotes $P<0.01$.

6. ID3 knockdown induces defects at the intra-S, but not G2/M DNA damage checkpoint

Checkpoint activation integrates the signals that regulate DNA damage responses, including DNA damage repair, cell-cycle arrest and apoptosis (Zhou BB et al., 2000). Mutants defective for DNA damage-induced cell cycle arrest have been identified among preexisting collections of mutations that confer radiation sensitivity in budding yeast and fission yeast (Weinert and Hartwell 1988; Al-Khodairy and Carr 1992; Enoch et al. 1992; Rowley et al. 1992; Weinert 1992). Based on above results, knock down of ID3 reduced the number of colonies compared with control cells (Figure 7A), indicating that ID3 function may be required to maintain cell viability. As induced DNA repair and DNA checkpoint activation are generally accepted as critical components of cell survival after exposure to DNA damage, we investigated whether ID3 is involved in the regulation of the intra-S phase and G2/M checkpoints after DNA damage. The intra-S phase checkpoint has a critical role in preventing spontaneous replication-fork collapse and is responsible for inhibiting intra-S phase progression after DNA damage (Bartek et al., 2004). We also examined the effect of ID3 knockdown on the activation of Chk2 and p-Chk2 after IR (Figure 7B), indicating that ID3 has an important role in regulating the Chk2 activity in response to DNA damage. To determine the role deficient cells were treated with NCS, and DNA synthesis inhibition was examined. We found that NCS-treated control Hela (Figure 7C) cells exhibited a significant inhibition of DNA synthesis. In contrast, ID3-depleted cells did not activate the intra-S phase checkpoint efficiently, as shown by the low-level inhibition of DNA synthesis, indicating a role for ID3 in the intra-S phase checkpoint. The G2/M checkpoint is activated

to prevent cells with damaged DNA or incomplete DNA replication from undergoing mitosis. Cells that fail to activate an intra-S phase checkpoint should prevent movement into mitosis by activating the G2/M checkpoint (Sancar et al., 2004). To examine the effects of ID3 knockdown on the G2/M checkpoint, control and ID3-depleted Hela cells were siRNA-treated or exposed to NCS, and then labeled with anti-phospho-histone (Ser10) antibody, a marker for cells in the M phase. Through the detection of the phosphorylated form of H3, we observed that, in contrast to control cells that were not arrested in G2 (figures 7D). Taken together, these results indicates that ID3 knockdown activate only intra-S-phase checkpoint in response of DNA damage.

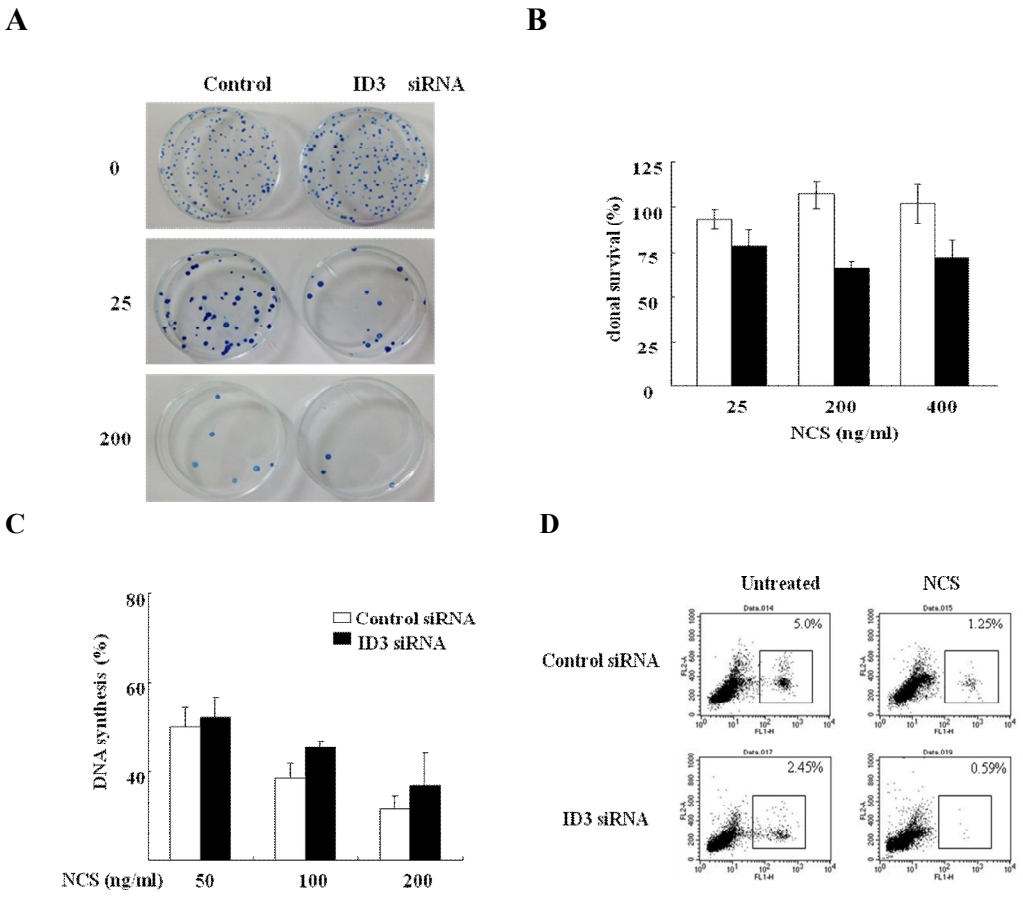


Figure 7. Knockdown of ID3 defects the intra S-phase checkpoint but not G2/M checkpoint

(A) Hela cells were untreated or treated with indicated dose of NCS. Relative cell survival was assed using a clonogenic assay. Data represent the mean ± SD of three independent experiments.

(B) The control- and ID3-depleted Hela cells were untreated or treated with NCS (100ng/ml) for the indicated times. Cell were harvested for western blotting and probed with

antibodies against phospho-Chk2(Thr68), Chk2 and Actin.

(C) The control and ID3 knockdown Hela cells were untreated or treated with various doses of neocarzinostatin(NCS). After 6h of NCS treatment, respectively, DNA synthesis was assessed based on BrdU incorporation. Data are expressed as relative BrdU incorporation of the untreated control cells. Data are presented as mean±s.d.

(D) The control and ID3 knockdown Hela cells were untreated (UT) or treated with 100ng/ml NCS for 6h before fixation. Cells in mitosis were determined by staining with propidium iodide and phospho-histone H3 antibody followed by FITC-conjugated secondary antibody. The percentage of M-phase cells was determined by flow cytometry for phosphor-histone H3.

IV. DISCUSSION

In this study, we identified a direct interaction between the DNA damage mediator MDC1 and the ID3, a major regulator of basic helix-loop-helix transcription factor.

DNA damage in the cell activates the DNA damage response, which includes activation of cell cycle checkpoints, repair of the damage, transcriptional regulation and, if damage is excessive or unrepairable, activation of apoptosis. Proteins involved in the DNA damage response include sensors that detect the damage, transducer kinases that signal to downstream effectors, and mediators that mediate the signal from the transducer kinases to the effectors that execute the response itself (Motoyama et al., 2004).

A Novel Connection between the DNA Damage Response and Cell Cycle Regulation—The connection between MDC1 and the ID3 suggests a new role for the ID3 in the DNA damage response and/or a role for MDC1 in cell cycle regulation. We address the various possibilities that arise and present all relevant existing knowledge that support or contradict these hypotheses.

The interaction between MDC1 and the ID3 is enhanced after DNA damage induction, suggesting a role in regulating the DNA damage response. There are several options for the mechanism by which the interaction regulates the DNA damage response. (a) The ID3 interact MDC1. (b) ID3 knockdown impairs MDC1 signals in response to DNA damage. (c) ID3 knockdown inhibit DNA damage repair. (d) ID3 knockdown regulates intra-S-phase

checkpoint in response to DNA damage.

In summary, our study demonstrates a novel link the DNA damage response. The direct interaction between MDC1 and ID3 suggests that one might regulate the other. Further studies will indicate whether this interaction defines a mechanism of a DNA damage response pathway.

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ABSTRACT

ID3 regulates MDC1-mediated signaling pathway

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ID3 is a dominant negative regulator of basic helix-loop-helix transcription factor, and regulator of differentiation, apoptosis and growth. Especially, the mediator of DNA damage checkpoint protein 1(MDC1) plays a central role in DNA damage response. In this study, we report that the key protein of DNA damage response, MDC1 directly binds to ID3. This interaction of MDC1 and ID3 is confirmed by co-immunoprecipitation and GST-pull down assay. Knockdown of ID3 decreases foci formation of phospho-ATM and MDC1 but not 53BP1 and γ-H2AX, and ID3 depletion also reduces the phosphorylation of ATM, MDC1 and NBS1 after DNA damage. Moreover, ID3 depletion is decreased the clonal survival rate, which may directly contribute DNA repair, especially non homologous end joining. On the other hands, ID3-depleted cells show defect of intra-S phase checkpoint but not G2/M

checkpoint, but IR-induced phosphorylation of chK2 on Thr68 is still observed. Therefore, these results suggest that ID3 is critical regulator of DNA damage signaling pathway.

감사의 글

본 논문이 완성되기까지 많은 도움이 되어주신 여러분들께 감사의 말씀을 드립니다.

반년 남짓한 시간을 거쳐 저의 학위논문이 완성되었습니다. 저는 논문의 완성을 위하여 많은 심혈과 노력을 기울였습니다. 논문자료의 수집부터 힘든 실험과 연구결과를 분석하고 논문 집필단계에서 많은 곤란과 좌절을 겪으면서 한걸음 한걸음씩 오늘까지 오게 되었습니다. 논문을 완성하면서 저는 견강한 의지력의 진정한 의미를 알게 되었으며 지금은 더할 나위 없는 뿌듯함과 보람을 느낍니다.

오늘과 같은 훌륭한 성과를 거두게 된 데는 저 혼자의 힘만 아니라 저의 지도교수님을 비롯한 조선대학교 의학과 교수님들과 대학원 동기들의 공통의 노력이라고 생각합니다. 먼저 2 년 동안 아낌없는 지도와 격려로 이끌어 주신 저의 지도교수님인 유호진 교수님께 깊은 존경과 감사를 드립니다. 특히 저의 석사과정 2 년 동안 조언을 아끼지 않았던 이정희 교수님의 가르침과 지도에 감사를 드립니다. 늘 부족한 저에게 항상 덕담과 격려의 말씀으로 아낌없는 관심과 사랑을 베풀어주셨습니다. 그리고 논문 지도를 위해서도 누구보다 애써주셨습니다. 저는 교수님의 베풀어주신 은혜를 가슴속 깊이 새기면서 기대에 어긋나지 않도록 풍부한 소양과 겸양의 미덕을 갖춘 좋은 인간으로 되기에 최선을 다하겠습니다.

그리고 논문심사를 위해 애써주신 장인엽 교수님과 전제열 교수님에게도 감사의 마음을 전합니다. 그밖에 박춘매 교수님, 윤차경 교수님, 박선주 교수님의 지도아래 2 년 동안의 석사과정에 많은 것을 배우게 되었고 폭 넓은 지식과 그 가치를 더더욱 깨닫게 되었습니다. 또 저의 연구실의 팅팅, 민지, 구루사미 하리하라수단, 성미, 진영, 희진한테도 감사의 인사를 전하고 싶습니다. 유학생활의 희로애락을 같이 겪고 오면서 서로 돕고 부추기면서 두터운 우정을 쌓게 되어서 행복으로 생각합니다. 조선대에서의 2 년의 유학생활이 저의 인생의 소중한 재부로 될 것입니다.

끝으로 저의 가장 소중한 가족들에게 고마운 마음을 전하고 싶습니다. 2 년이라는 유학생활에 든든한 뒤심이 되어 주셔서 공부를 더 잘 할 수 있었습니다. 제가 가끔 힘들고 지치고 포기하고 싶을 때에도 늘 격려해주고 도움을 주셨고 인간으로서 도리를 일깨워 주셨으며 철없는 저를 늘 감싸주시고 이끌어왔던 사랑하는 가족들에게 고맙고 수고하셨다는 말을 전하고 싶습니다.

저작물 이용 허락서

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

- 다 음 -

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

동의 여부: 동의 (0) 반대 ( )

2011 년 12 월 24 일