2008년 8월
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조선대학교 대학원

의학과

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ATM pathway 조절 연구

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지도교수 유 호 진

이 논문을 의학박사 학위신청논문으로 제출함.

2008 년 4 월 일

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의학과

조 병 옥

조 병 옥의 박사학위논문을 인준함

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DNA DSB repair에서 CAF1 p150에 의한 ATM pathway 조절 연구

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지도교수 : 유 호 진
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Chromatin assembly factor 1 (CAF-1)은 게놈 보전과 DNA 수복에 관여하는 중요한 유전자다. 그렇지만, CAF-l 이 DNA 수복에 어떻게 관여하는지는 많이 연구되어지지 않았다. 본 연구에서는 DNA double strand breaks (DSBs) 수복에서 CAF-1 p150 의 생물학적인 역할을 연구했다. CAF-1 p150 은 DSBs 수복 반응에서 우선적으로 활성화 되어지는 ATM (ataxia telangiectasia mutated) 유전자와 직접 상호작용을 한다. 또한, CAF-1 p150 은 DSBs 수복이 일어나는 곳에 보충되고, 동시에 ATM 과 함께 집중된다. RNA interference 를 통한 CAF-1 p150 의 silencing 은 ATM 과 H2AX 의 인산화를 감소시켰고, foci 형성 또한 약간 감소시켰다. 그러나, 53BP1, MDC1, BRCA1, Chk2 and MRN complex 같은 DSBs 수복에 관여하는 DNA 수복 단백질의 보충에는 영향을 미치지 않았다. 또한, CAF-1 p150 이 knockdown 된 세포에서 Chk2 의 인산화는 지연되었고 감소되었다. 그렇지만, BRCA1 과 p53 의 인산화는 증가되었다. DSBs 를 일으키는 물질인 Neocasinostatin (NCS)를 처치한 후에 DNA 가 손상된 세포는 G2/M 기에서 세포주기가 정지하였다. 이러한 결과는 DSBs 수복에서 CAF-1

p150 이 ATM 신호전달 경로를 조절함으로써 세포주기 정지에 관여한다는 것을 나타낸다. 결론적으로, DNA double strand breaks 수복에서 CAF-1 p150 은 G2/M 기 DNA checkpoint 를 조절하고 ATM 신호전달 경로를 통해서 DNA 수복에 관여할 것으로 사료된다.

INTRODUCTION

Cellular DNA is constantly damaged by the deleterious assaults of both endogenous and environmental DNA damaging agents (Bernstein, C. *et al.*, 2002) (Fig.1). Cellular DNA damage responses, ensuing from breaks in the DNA structure, involve numerous checkpoint and repair proteins that coordinate a complex signaling cascade responsible for damage detection, checkpoint activation, DNA repair, cell cycle arrest and/or apoptosis (Zhou, B.B. & Elledge, S.J., 2000). In normal cells, to protect the integrity of their DNA, cells have evolved a genome surveillance network that initiate repair and carefully coordinate it with DNA transcription, replication, and cell cycle progression. There is several DNA repair system to remove these lesions to maintain the genomic integrity. The main repair strategies are direct repair to remove photoreactivation, nucleotide excision repair (NER) able to repair bulky DNA adducts, base excision repair (BER) able to repair non-bulky base modifications, mismatch repair (MMR) able to repair mispaired bases and insertion/deletion, homologous recombination (HR) and non-homologous end joining (NHEJ) able to repair double strand breaks (DSBs) (Hoeijmakers, J.H., 2001; Martin, S.A. *et al.*, 2008) (Fig.2). Defects in these DNA repair mechanisms can therefore result in increased mutation rates and genome instability, leading to diseases such as cancer.

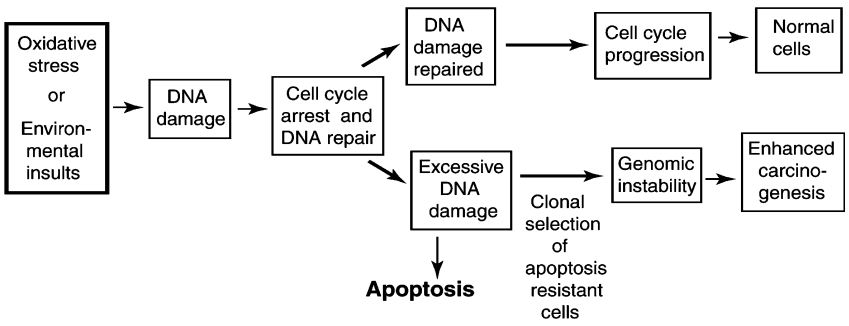

Fig.1. Roles of DNA damage, DNA repair, cell cycle arrest and apoptosis in carcinogenesis.

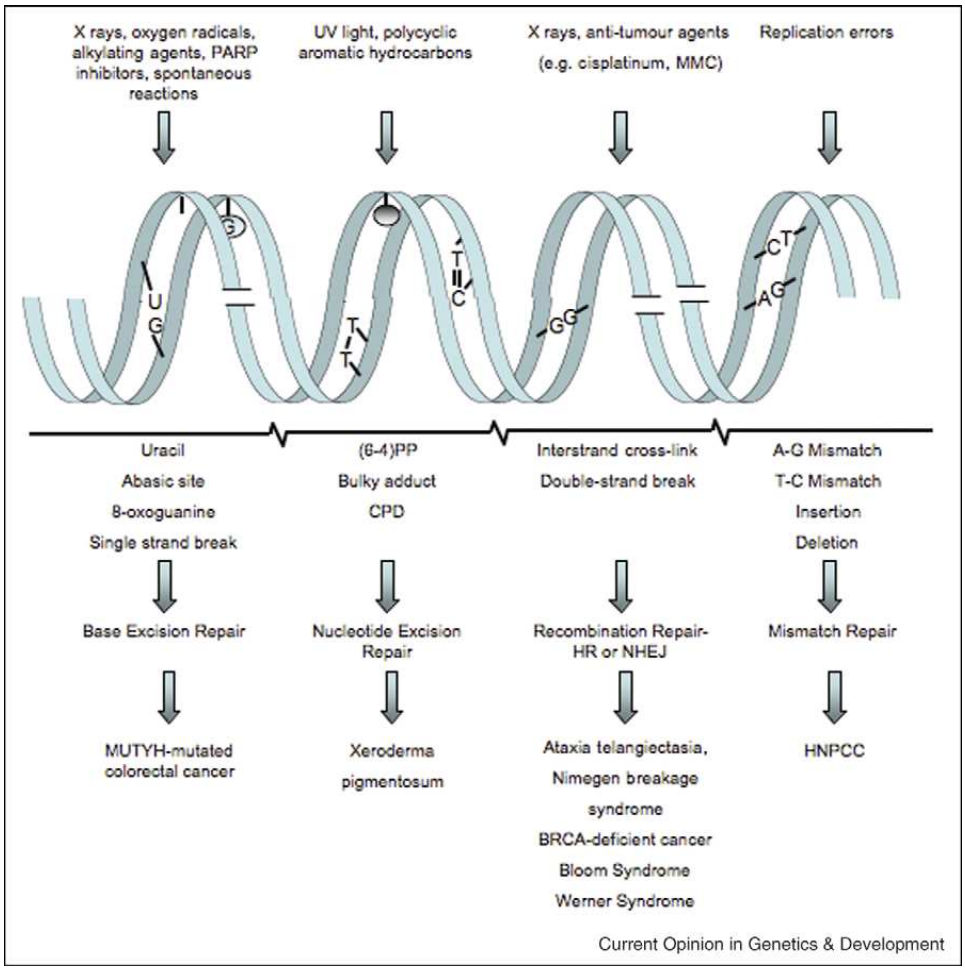

Fig.2. DNA damage, DNA repair pathways and mutation-associated disease. Common DNA damaging agents (top); examples of DNA lesions induced by these agents (top middle); most predominant DNA repair mechanism responsible for the removal of these lesions (lower middle) and cancer resulting from deficiency in particular DNA repair pathway (bottom). Abbreviations: cis-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light).

DSBs are considered the most toxic type of DNA damage. DNA DSBs can be generated globally by exposure to ionizing radiation or radiomimetic chemicals such as bleomycin and neocasinostatin (NCS). They can also arise as by-products of oxidative metabolism or by replication of damaged DNA. If left unrepaired or repaired improperly, they cause chromosomal aberrations (such as translocation, amplifications or deletions), genomic instability, cancer predisposition and cell death. There are at least two repair pathways which can repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (Difilippantonio, M.J. *et al.*, 2002; Zhu, C. *et al.*, 2002; Jackson, S.P., 2002; Wyman, C. & Kanaar, R., 2006). These damage responding repair pathways are thought to be regulated by several major steps. First, a sensor protein (probably, ATM or MRN complex) recognizes damage induction by radiation. Second, mediator proteins receive a structural modification by the sensor proteins, and this modification is converted to a compatible form for signal amplification by transducer proteins. These transducers amplify the signal, and finally, effecter proteins accomplish enzymatic reactions of DNA end processing, rejoining, or cell cycle regulation (Fig.3.).

ATM [ataxia telangiectasia (A-T) mutated protein], which is defective in the hereditary disorder A-T, is a central component of the signal transduction pathway that responds to DNA double-strand breaks (Savitsky, K. *et al.*, 1995). The ATM gene encodes a 370kDa protein that belongs to the phosphoinositide 3-kinase (PI3K) suprefamily, but which phosphorylates proteins rather than lipids. The 350 amino acid kinase domain at the carboxy-terminus of this large protein is the only segment of ATM with an assigned function. ATM protein is now shown to be present in undamaged cells as an inactive dimer or multimer. ATM is dissociated and autophosphorylated on Ser 1981 after DSBs (Bakkenist, C.J. & Kastan, M.B., 2003). When DSBs are generated, ATM protein kinase is activated and relocates through an interaction with MRN complex. Then ATM phosphorylates histone H2AX and many other substrate proteins including Artemis, MDC1, NBS1, p53, Chk1, Chk2, Rad17, BRCA1, BLM, SMC1, 53BP1 and DNA-PKcs

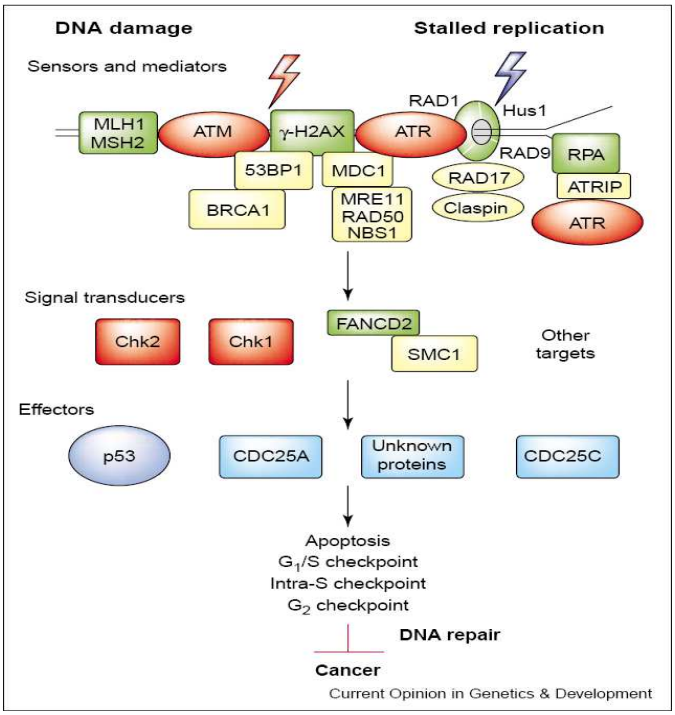

Fig.3. DNA damage checkpoint machinery. In response to DNA damage, ATM and ATR phosphorylate histone H2AX and thereby facilitate the recruitment and phosphorylation of mediators such as MDC1, 53BP1, BRCA1, and the MRE11–RAD50–NBS1 complex. Stalling of the DNA replication fork results in the recruitment of the ATR–ATRIP complex by RPA. In turn, the formation of nuclear foci of mediator complexes promotes transmission of the DNA damage signal to downstream targets such as Chk1, Chk2, FANCD2, and SMC1. The PCNA-like RAD1–RAD9–Hus1 complex, the RFC-like RAD17, and Claspin may collaborate in checkpoint regulation by detecting different aspects of a DNA replication fork. The mismatch repair proteins MLH1 and MSH also implicate in the activation of ATM-Chk2 pathway. The kinases Chk1 and Chk2 phosphorylate effectors such as p53, CDC25A, and CDC25C and thereby delay cell cycle progression or induce senescence or apoptosis via activation of the G1–S, intra-S, or G2 cell cycle checkpoints. Thus, these DNA damage checkpoint mechanisms cooperate with DNA repair machinery to suppress genomic instability and cancer.

kinase (Wang, B. *et al*., 2002; Goldberg, M. *et al*., 2003; Lou, Z. *et al*., 2003; Stewart, G.S. *et al*., 2003; Yazdi, P.T. *et al*., 2002; Wyman, C. & Kanaar, R., 2006; Kobayashi, J. *et al*., 2008) (Fig.4.). Low doses of IR, which induce only a few DNA breaks, activate at least half of the total ATM protein present, possibly in response to changes in chromatin structure. Exposure of cells to IR triggers ATM kinase activity, and this function is required for arrests in G1, S and G2/M phases of the cell cycle. Several substrates of the ATM kinase participate in these IR-induced cell cycle arrests. These include p53, MDM2 and Chk2 in the G1 checkpoint; NBS1, BRCA1, FancD2 and SMC1 in the transient IR-induced S phase arrest; and BRCA1 and Rad17 in the G2/M checkpoint. ATM-phosphorylated proteins activate cell cycle checkpoints, NHEJ repair pathway, and HR repair related pathways (Kitagawa, R. *et al*., 2004; Falck, J. *et al*., 2005; Bartkova, J. *et al*., 2005; Lavin, M.F. & Kozlov, S., 2007; Kobayashi, J. *et al*., 2008). However, the factors that mediate chromatin dynamics during the NHEJ and HR pathways of repairing DNA double strand breaks (DSBs) have not yet been identified.

For an efficient DNA damage response, not only must the DNA damage be repaired, but the surrounding chromatin must be accessed, remodeled, and restored as well (Gontijo, A. M. *et al*., 2003; Peterson, C.L. & Cote, J., 2004). The eukaryotic nuclear genome is assembled into the nucleoprotein structure termed chromatin. The basic repeating unit of chromatin is the nucleosome, which comprises approximately 146 basepairs of DNA wrapped around an octamer of histone proteins (two molecules each of histones H3, H4, H2A and H2B) (Luger, K. *et al*., 1997). An efficient DNA damage response in eukaryotic cells therefore relies on DNA damage recognition, access, and repair within chromatin as well as on the successful reestablishment of chromatin structure and information following DNA repair. These requirements are contextualized in the access-repair-restore (ARR) model (Green, C.M. & Almouzni, G., 2002). The majority of chromatin is assembled immediately following DNA replication. This is mediated in part by the histone chaperone chromatin assembly factor 1 (CAF-1) that deposits histones H3 and H4 onto newly

replicated DNA in vitro (Smith, S. & Stillman, B., 1989). CAF-1 is a heterotrimeric protein complex consisting of three subunits, p150, p60, and p48, which are required for replication-dependent nucleosome assembly in human cell extracts (Kaufman, P.D. *et al*., 1995; Tyler, J.K. *et al*., 1996, 2001; Verreault, A. *et al*., 1996). CAF-1 targets acetylated histones H3 and H4 to sites of DNA synthesis during DNA replication and NER as the first step of nucleosome assembly (Smith, S. & Stillman, B., 1989; Green, C.M. & Almouzni, G., 2002; Krude, T. & Keller, C., 2001). CAF1 has been reported to interact with several proteins including, proliferating cell nuclear antigen (PCNA) in an ATP-dependent manner (Moggs, J.G. *et al.*, 2000; Shibahara, K. & Stillman, B., 1999), Werner syndrome protein (WRN) (Jiao, R. *et al*., 2007), Bloom's syndrome protein (BLM) (Jiao, R. *et al*., 2004) able to repair single strand breaks through NER pathway. It has also been reported that CAF-1 has a role in the assembly of chromatin following or during the repair of DSBs (Linger, J. & Tyler, J.K., 2005) and *caf1 asf1* double mutants in yeast are 10-fold less competent in repairing DSBs via recombination and also exhibit 3- to 4-fold decreased repair proficiency and accuracy in NHEJ-mediated repair (Lewis, L.K. *et al*., 2005). Moreover, CAF-1 participated in DSBs repair by NHEJ pathways in quiescent human cells (Nabatiyan, A. *et al*., 2006). These findings thus suggest the involvement of a chromatin assembly factor in response to DSBs.

In this study, we report that p150, the largest subunit of chromatin assembly factor 1 (CAF1), physically and functionally associates with ATM. CAF-1 p150 is also recruited and colocalizes with ATM and γ-H2AX in response to DSBs. DNA damaged cells after necocasinostatin (NCS) treatment causing DSBs arrested G2/M phase in cell cycle. These results indicate that CAF1 p150 involved in cell cycle arrest by regulating ATM signaling pathway in DSBs. Collectively, our results suggest that CAF-1 p150 regulates G2/M phase DNA checkpoint and involved in DNA repair through ATM signaling pathway in DNA double strand breaks.

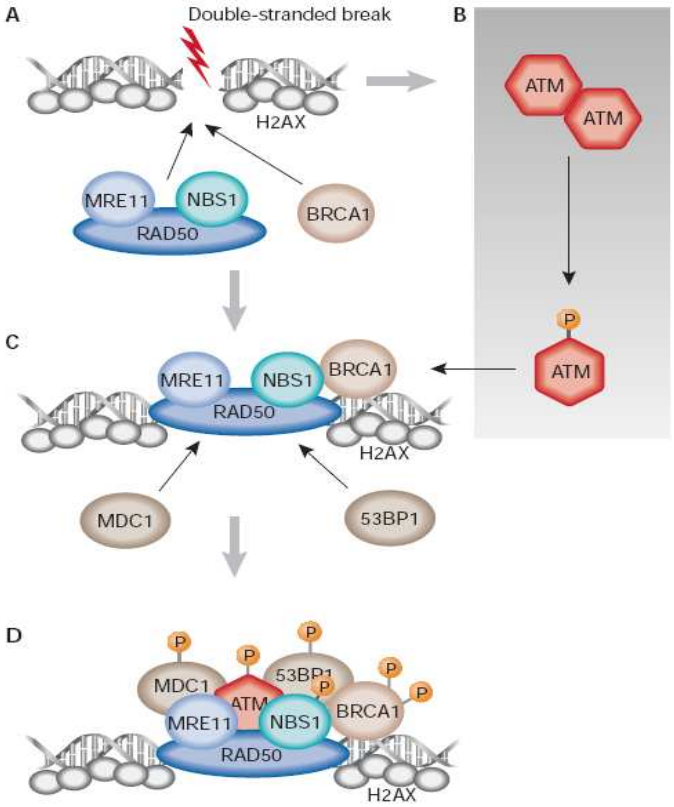

Fig.4. ATM and DNA damage signaling. In response to a DNA double stranded break (**A**) several simultaneous events occur to ultimately activate ATM signal transduction. ATM exists as an inactive multimeric complex that, in response to DNA damage, undergoes autophosphorylation to an active monomer (**B**). A histone variant, histone H2AX, present within chromatin, becomes phosphorylated and serves as a tethering platform for repair factors. The MRE11–RAD50–NBS1 complex locates to the DNA lesion together with BRCA1 (**C**). Assembly of this complex facilitates coordinated co-localization of active ATM together with other factors including MDC1/NFBD1 and 53BP1. BRCA1,MDC1 and 53BP1 are also phosphorylated in an ATM dependent manner (**D**). The assembly of this multiprotein complex facilitates the cellular response to a DNA double-stranded break. 53BP1, p53-binding protein 1; ATM, ataxia telangiectasia mutated; BRCA1, breast-cancer associated 1; MDC1, mediator of damage checkpoint 1.

MATERIALS AND METHODS

1. Plasmids construction

The human ATM-PI3Kc region cDNA was amplified by RT-PCR following oligo primer; sense 5'-CCC GGG GAA TTT ACC AAA AAT AAT AGA TTG-3' with SmaI site and antisense 5'-GTC GAC CTC TTG TAG TCT CAT TAA GAC ACG-3' with SalI site from human HeLa cells. To produce pGBT9-ATM-PI3Kc, a bait plasmid for yeast two hybrid screening, the amplified carboxy-terminal region (residues from 8089 to 9045) of ATM was inserted into pGBT9 vector (Clontech, Cat. No. K1605-A). After confirming the DNA sequence, the pGBT9-ATM-PI3Kc construct was transformed into yeast strain AH109 by lithium acetate method.

2. Yeast Two Hybrid screening using mating method

Yeast two-hybrid screening was performed using pre-transformed Matchmaker human HeLa library (Clontech, Cat. No. 638862) as prey, according to the manufacturer's instructions. Briefly, pGBT9-ATM-PI3Kc was transformed into yeast strain AH109 by lithium acetate method to produce a bait strain. The prey and bait strains were grown in SD medium lacking leucine (SD/-Leu) and SD medium lacking tryptophan (SD/-Trp), respectively, until the $OD_{600\ nm}$ reached 0.8. The strains were then gently incubated together in YPDA/kanamycin at 30°C for 20-24 h, and the resulting diploid yeast cells were spread on SD medium lacking histidine, tryptophan and leucine (SD//-His-/Trp/-Leu, TDO) plates containing 400 μg/ml X-α-gal for selection of positive clones. After 3–8 days, colonies were streaked on SD/-Ade/-His-Trp/-Leu (QDO) plates containing 400 μg/ml X-α-gal (Clontech). Positive (blue) clones were selected and isolated plasmids from yeast. PCR was performed using primers with sequences of 5′- CTA TTC GAT GAT GAA GAT ACC CCA CCA A -3′ (Sense) and 5′- GTG AAC TTG CGG GGT TTT TCA GTA TCT A -3′

(Antisense). The PCR DNA fragments were analyzed by direct sequencing using a primer with a sequence of 5′- TAA TAC GAC TCA CTA TAG GG -3′. To confirm the interaction between ATM and p150 in yeast, yeast strains AH109 and Y187 were transformed with indicated bait and prey plasmids, respectively. Yeast diploids that contain both bait and prey plasmids were generated through yeast mating and selected on SD medium lacking tryptophan and leucine (SD/-Trp/-Leu). Yeast colonies were streaked on SD/-Ade/-His/-Trp/-Leu (QDO) plates and incubated for 3 days at 30°C to evaluate the interaction.

3. Maintenance of Cell Lines

Human cervix carcinoma HeLa cell lines (Korean Cell Line Bank, KCLB No. 10002) were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 ug/ml of streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified atmosphere of 95% air and 5 % CO_2 at 37 ^oC.

4. Reagents and DNA damage

Neocarzinostation (NCS, N9162) and Hydroxyurea (HU, H8627) were purchased from Sigma-Aldrich. Cells were treated with NCS (indicated dose) or 10 mM HU or 100 J/m^2 UV using UV radiometer (Upland, CA91786, USA) for indicated times.

5. RNA interference

For small interfering RNA experiments, two target sites within the large subunit p150 of CAF1 genes were chosen from the human p150 mRNA sequence (Gene Bank accession number NM_005483), which was extracted from the NCBI Entrez nucleotide database. After selection, each target site was searched with NCBI BLAST to confirm the specificity

only to the p150. Control siRNA and p150 siRNA oligos were purchased from Bioneer (Daejeon, South Korea). The sequences of the 21-nucleotide sense and antisense RNA are as follows: p150 siRNA, 5'- GAAGUUAAUACAAGCCCGUtt -3' (sense) and 5'- ACGGGCUUGUAUUAACUUCtt -3' (antisense) for the p150 gene (nt 188-208); Control siRNA was used as the negative control. Cells were transiently transfected with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

6. Co-immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS) and lysed on ice for 20 minutes in RIPA buffer (25mM Tris, pH 7.2, 150mM NaCl, 5mM $MgCl_2$, 0.5%NP-40, 1mM DTT, and 5% glycerol) added protease Inhibitor Cocktail tablet (Roch). After incubation, total cell extracts were sonicated and centrifuged at 13,000rpm at 4℃ for 20min. 2mg of extract from HeLa cells were incubated with either 2ug anti-CAF-1 p150 pAb and anti-ATM mAb (Santa Cruz Biotechnology, Santa Cruz, CA) or 2ug rabbit control IgG (R&D) and mouse control IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in RIPA buffer at 4℃ for 6 hours in a total reaction volume of 500ul. 50ul protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were then added, and the mixture was incubated at 4℃ overnight. The beads were washed five times with RIPA buffer before the protein complexes bound to the beads were eluted and split into two portions for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). 40ug of total cell extract were used as the input. Western blot analysis with an anti-ATM mAb and an anti-p150 CAF1 mAb (Abcam, Cambridge, England) were used to detect proteins immunoprecipitated by anti-CAF-1 p150 pAb. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

7. Immunofluorescence microscopy

Cells grown on cover glass were fixed in 4% paraformaldehyde for 15 min at room temperature (RT), washed in PBS for 10 min, and permeabilized for 15 min at RT in 0.3% Triton X-100. Samples were blocked in PBS with 0.5% bovine serum albumin (BSA) for 1 hour at RT. After blocking, samples were incubated with a mixture of different rabbit and mouse antibodies for 2 hours at RT, washed in PBS for 10 min 3 times. Rabbit antibodies were detected with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories, Newmarket, Suffold, England; 1:200 in PBS with 0.5% BSA), and mouse antibodies were detected with FITC-conjugated goat anti-mouse IgG (Jackson Laboratories 1:100 in PBS with 0.5% BSA) for 1 hour at RT, washed in PBS for 20 min 3 times. To visualize nuclear DNA, samples were incubated with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 0.4 ug/ml). Cells were washed in PBS for 10 min 3 times and mounted using Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Zeiss Axioplan 2 imaging epifluorescent microscope equipped with a charge-coupled device camera and ISIS software (MetaSystems, Altlussheim, Germany). All antibodies used in this study are anti-ATM P(Ser1981) polyclonal antibody (pAb), anti-p150 CAF1 monoclonal antibody (mAb) (Abcam, Cambridge, England); anti-H2AX-P(Ser139) mAb (Upstate, Walthers, MA); anti-CAF-1 p150 pAb, anti-BRCA1 pAb, anti-53BP1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MDC1 pAb (Novus Biologicals, Littleton, USA); anti-Chk2 pAb (Cell Signaling Technology, Danvers, MA); anti-Mre11 pAb, anti-NBS1 mAb (BD Phamingen, San Jose, CA), followed manufacturer’s protocol for dilution of all primary antibodies.

8. Western blotting

The cells were washed with phosphate-buffered saline (PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added protease Inhibitor Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13,000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 30ug of protein per lane were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto PVDF membranes (Pall Corporation). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.1% Tween-20 (TBS-T, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 1 hour. The membranes were rinsed with TBS-T and then incubated with appropriate primary antibodies in TBS-T at 4℃ overnight. All antibodies used in this study are anti-ATM protein kinase P(Ser1981) (clone 10H11.E12) monoclonal antibody (mAb) (Rockland, Gilbertsville, PA); anti-H2A.X. polyclonal antibody (pAb), anti-H2AX-P(Ser139) mAb (Upstate, Walthers, MA); anti-p53-P(Ser15) pAb, anti-BRCA1-P(Ser1524) anti-Chk2 pAb, anti-Chk2-P(Thr68) pAb, anti-NBS1-P(Ser343) pAb (Cell Signaling Technology, Danvers, MA); anti-NBS1 monoclonal antibody (mAb) (BD Phamingen, San Jose, CA); anti-ATM mAb, anti-CAF-1 p150 pAb, anti-a-tublin mAb, anti-BRCA1 pAb, anti-p53 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 1.5 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

9. Flow cytometry by PI staining

The trypsin-detached HeLa cells were collected and washed once with ice-cold PBS, followed by fixing in 70% cold ethanol for 30minutes at 4℃. The cells were then stained in PBS with PI (50 μg/ml), RNase A (50 μg/ml), and 0.05 % Triton X-100. The DNA content of the HeLa cells was analyzed by fluorescent-activated cell sorting (FACSort, Becton Dickinson, Franklin Lakes, NJ). At least 10,000 events were analyzed, and the percentage of cells in sub-G_1 population was calculated. Aggregates of cell debris at the origin of histogram were excluded from the sub-G_1 cells.

10. Cytotoxicity assay

The cell cytotoxicity was also assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. Cells were seeded on well plate. Next day, Cells were transiently transfected with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 48 hours later, cells were treated with Neocarzinostation (NCS) (SIGMA, Saint Louis, USA). After 24 hours, MTT(10mg/ml) was incubated with cells in a plate for 4 h at 37℃. The medium containing MTT was removed, and dimethyl sulfoxide (DMSO) was added. Cells were incubated for 15min at room temperature with gently shaking. The absorbance was read on a scanning elisa reader (BIO-TEK INSTRUMENTS, INC.) using a 540nm filter. Cell viability was calculated from relative dye intensity compared with untreated samples.

RESULTS

1. p150 interacts with ATM

To demonstrate a directly interaction between p150 and ATM, we performed co-immunoprecipitation (Fig.5). Immunoprecipitation experiments showed that p150 and ATM formed a complex in NCS treated or untreated HeLa cells.

2. Colocalization of p150 and pATM on S1981 following NCS treatment

CAF1 p150 is recruited to chromatin in DSBs induced cells (Nabatiyan, A. *et al*., 2006) and ATM also recruited. We used NCS as a chemical means to induce DSBs. In order to investigate for a indirectly interaction between p150 and ATM, we examined immuofluorescence microscopy to analyze whether p150 and ATM colocalize in the kinetics of formation in HeLa cells after NCS treatment (Fig.6). In untreated or 3h, 6h treated cells, we did not detect any colocalization of p150 and pATM, although these results were different to co-immunoprecipitation experiments. However, p150 and pATM were colocalized at 12 h and 24 h.

3. CAF1 p150 was required for the phosphorylation of ATM in DNA DSBs repair following NCS treatment

In order to investigate whether p150 affected for ATM activity in DNA DSBs repair, we examined for the phosphorylation of ATM in HeLa cells treated with 200ng/ml NCS after p150 siRNA transfection. The phosphorylation of ATM reduced in p150 siRNA transfected HeLa cells after NCS treatment. Moreover, γ-H2AX which is downstream protein of ATM and DSBs marker also reduced. However, the level of p150 not changed at NCS damage (Fig.7). These results indicated that p150 is required for the phosphorylation of ATM in DNA DSBs repair.

4. Colocalization of pATM on S1981 and γ-H2AX in 200ng/ml NCS treated HeLa cells after siRNA transfection

When DNA is damaged in cells, many DNA damage-signaling proteins are recruited to the damaged loci and form discrete nuclear foci (Paull, T.T. *et al.*, 2000; Rouse, J. & Jackson, S.P., 2002). The order and timing of these events are thought to be critical for checkpoint response and DNA repair (Stewart, G.S. *et al*., 2003). In early step of DNA damage response, pATM on S1981 and γ-H2AX appears at discrete nuclear foci (Rogakou, E.P. *et al*., 1999). In order to investigate whether p150 affected the kinetics of pATM on S1981 and γ-H2AX colocalization in DNA DSBs repair, we examined immunofluorescence studies in time dependent manner. As shown in Figure 8, pATM on S1981 and γ-H2AX colocalized. However, depletion of p150 by small interfering RNA reduced the colocalization of pATM on S1981 and γ-H2AX after NCS treatment. This result indicated that p150 play an important role of recruiting pATM on S1981 and γ-H2AX in DNA DSBs repair and may function in upstream of ATM.

5. The involvement of p150 of recruitment of DNA damage response proteins at the double strand breaks site

We further studied whether p150 affects foci formation of MDC1, 53BP1, pNBS1, BRCA1, Chk2 and Mre11, which are downstream proteins of ATM in DNA damage response. As shown in Figure 9, we did not detect any foci formation of pNBS1 and Mre11 in p150 siRNA treated or control siRNA treated HeLa cells after NCS treatment. p150 did not affect foci formation of 53BP1, Chk2 and MDC1 in p150 depleted HeLa cells after NCS treatment. However, BRCA1 foci formation increased p150 siRNA treated HeLa cells, is thought involving cell cycle arrest. These results indicated that recruitment of MDC1, 53BP1, pNBS1, BRCA1, Chk2 and Mre11 is p150 independent in DSBs repair.

6. p150 affect checkpoint signal in response to DNA damage

ATM is primarily phosphorylated in response to DSBs, while ATR reacts to a wider range of lesions, including stalled replication forks. Once activated, ATM and ATR amplify the damage signal by phosphorylating various substrates including histone H2AX, MDC1, NBS1, p53, Chk1, Chk2, Rad17, BRCA1, SMC1, 53BP1 and DNA-PKcs kinase (Ward, I. & Chen, J., 2004; Kobayashi, J. *et al.*, 2008). We next studied whether p150 involved in checkpoint signaling. As shown in Figure 10, BRCA1 phosphorylation surprisingly increased in p150 depleted cells after NCS treatment, consistent with Figure 9 result. p53 phosphorylation also increased in p150 depleted cells after NCS treatment. These two results is thought that p150 play an important role in cell cycle arrest or cell survival in response to DNA damage. Chk2 phosphorylation decreased in p150 depleted cells, involved in ATM down-regulation (indicated Figure 7). This result indicated that p150 is required for cell cycle arrest/repair in response to DSBs. NBS1 and Mre11 (data not shown) phosphorylation is not changed, consistent with Figure 9. This result supposed that p150 regulated ATM activity in MRN complex independent manner in DSBs repair.

7. Silencing of p150 by RNAi induced G2/M arrest and decreased cell viability after DNA damage

We tested p150 siRNA whether efficiently silence p150 expression. As shown in Figure 11A, B, RNAi efficiently silenced p150 expression. We also tested cell cycle distribution and cellular viability after DNA damage. NCS-treated HeLa cells resulted in the accumulation of cells in G2/M phase, more sensitive in p150 siRNA transfected cells (Fig. 11C). Figure 11D indicated cell viability after NCS treatment. p150 siRNA transfected cells were more sensitive than control siRNA transfected cells after NCS treatment.

8. Recruitment of p150 and γ-H2AX in DNA double strand breaks site

We finally studied colocalization of p150 and γ-H2AX in HeLa cells after NCS treatment. An early step of DSBs response is the local phosphorylation of histone variant H2AX by ATM/ATR protein kinases, which can extend megabases away from the break site (Rogakou, E.P. *et al*., 1999). CAF1 p150 forms foci in single and double strand breaks site during DNA repair (Nabatiyan, A. *et al*., 2006). As shown in Figure 12, p150 and γ-H2AX colocalized in HU or UV damaged HeLa cells, also in NCS damaged HeLa cells. These results supposed that p150 served not only in single strand breaks repair, but also double strand breaks repair.

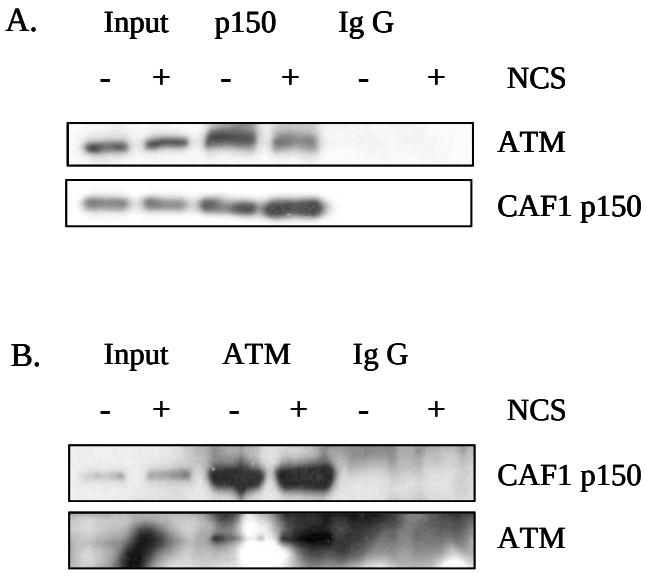

Fig.5. p150 interacts with ATM. HeLa cells were treated with 200ng/ml NCS for 1 h, and then lysate from untreated (NCS -) or treated (NCS +) was incubated with p150 (A) and ATM (B) antibody or control Ig G. The immunoprecipitates were separated on SDS-PAGE gels and blotted with ATM and p150. Input (total cell lysate) served as a positive control for the immunoblots.

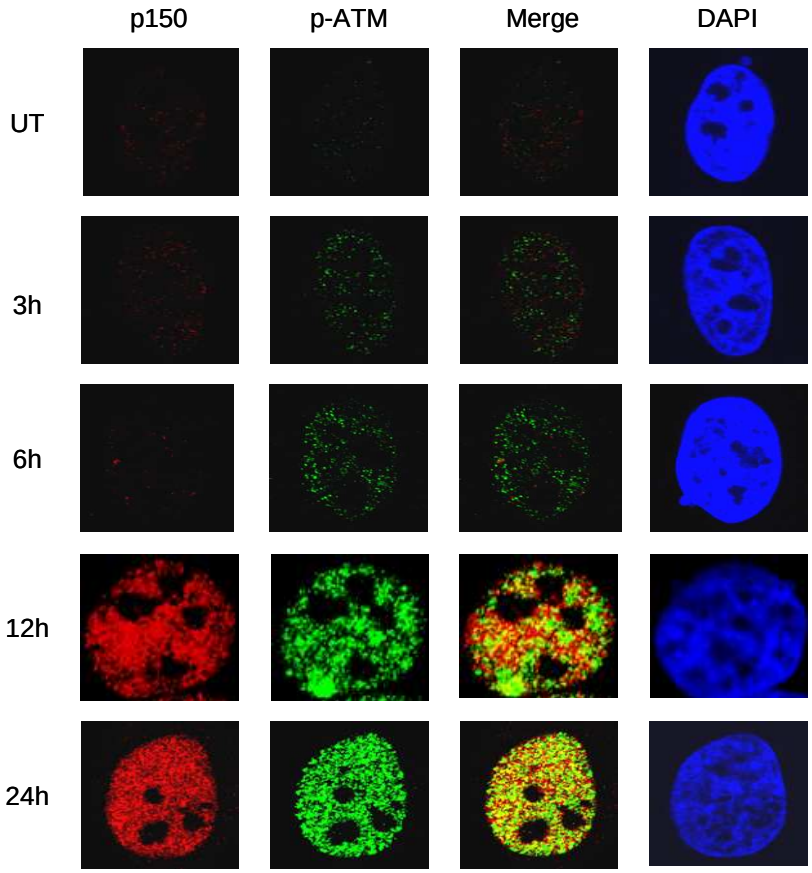

Fig.6. Colocalization of p150 and pATM on S1981 in NCS treated HeLa cells. p150 colocalizes with pATM in DNA repair foci. HeLa cells grown on slides were untreated or treated with 200ng/ml NCS for indicated times and costained with mouse anti-p150 and rabbit anti-pATM S1981. In the merged pictures, yellow represents the colocalization of p150 and pATM S1981. Nucleus was visualized by DAPI staining.

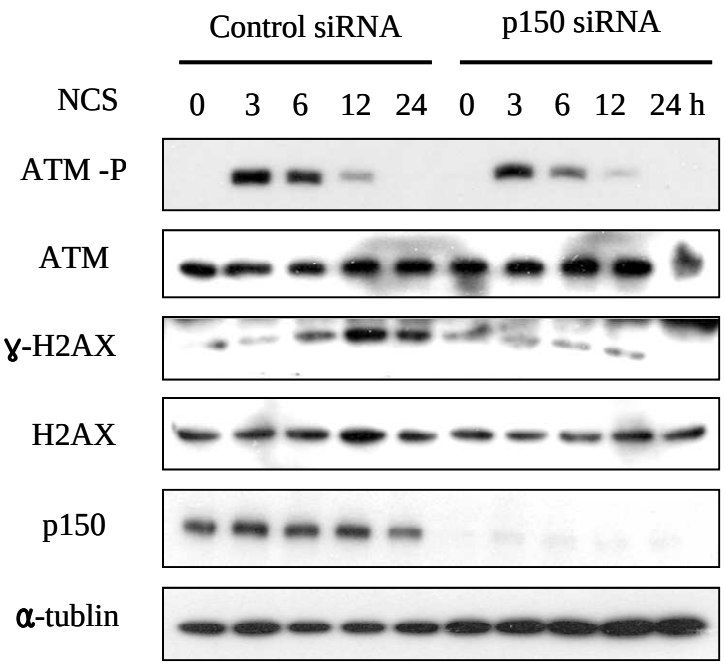

Fig.7. Silencing of p150 by RNAi decreased the phosphorylation of ATM and H2AX in double strand breaks by NCS. HeLa cells were transiently transfected with control or p150 siRNA. Transfection 36 h later, cells were untreated or treated with 200ng/ml NCS, then collected at the indicated times. Cell pellets were lysed and sonicated. Equal amounts of protein were run on SDS-PAGE and blotted with indicated antibodies. α-tublin served as a loading control.

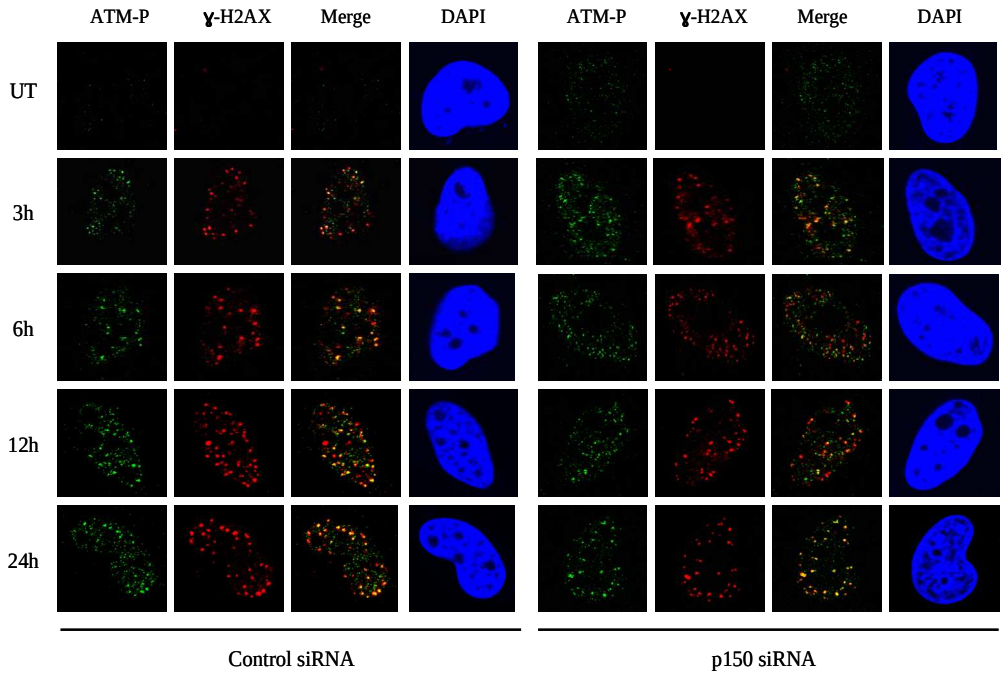

Fig.8. Colocalization of ATM-P and γ-H2AX in 200ng/ml NCS treated HeLa cells after siRNA transfection. HeLa cells grown on slides were transiently transfected with control or p150 siRNA. Transfection 36 h later, cells were untreated or treated with 200ng/ml NCS for indicated times and co-stained with mouse anti-p150 and rabbit anti-pATM on S1981. In the merged pictures, yellow represents the colocalization of p150 and pATM on S1981. Nucleus was visualized by DAPI staining.

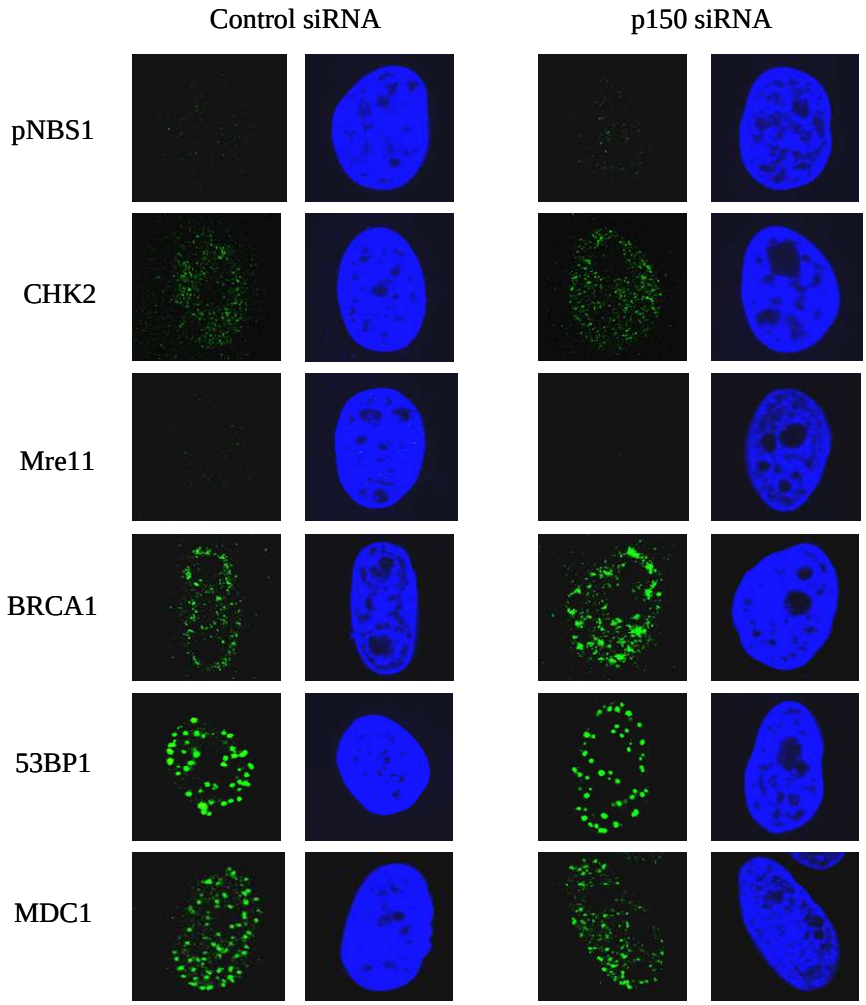

Fig.9. p150 not affects recruitment of DNA damage response proteins at the double strand breaks site. HeLa cells grown on slides were transiently transfected with control or p150 siRNA. Transfection 36 h later, cells were treated with 200ng/ml NCS for 12 h and co-stained with indicated antibody. Nucleus was visualized by DAPI staining.

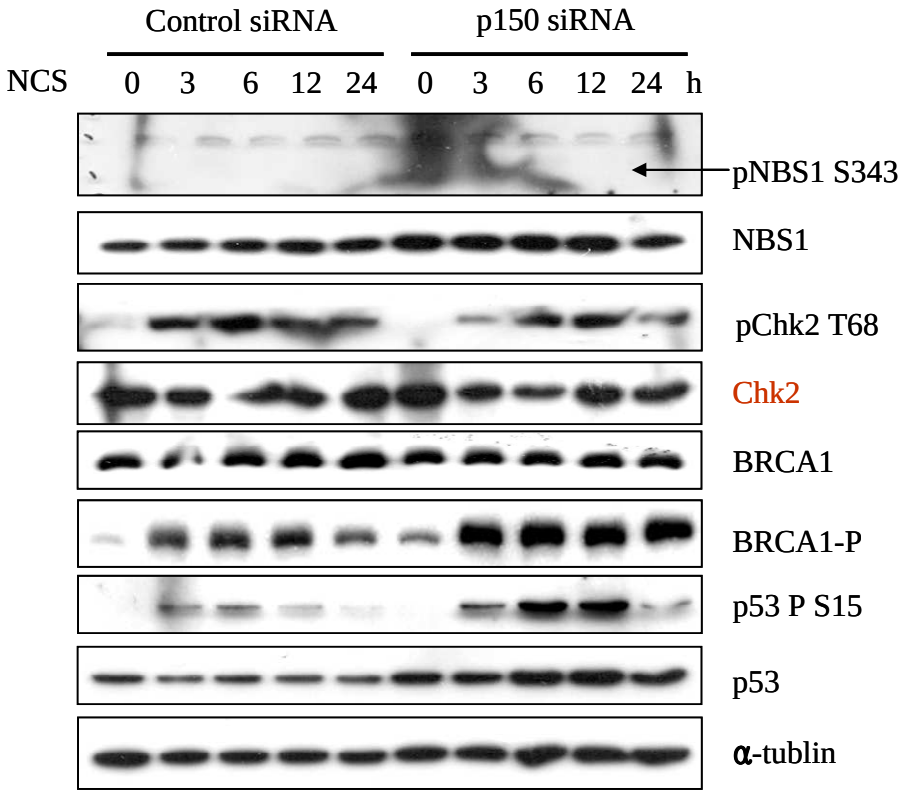

Fig.10. p150 affect checkpoint signal in response to DNA damage. HeLa cells were transiently transfected with control or p150 siRNA. Transfection 36 h later, cells were untreated or treated with 200ng/ml NCS, then collected at the indicated times. Cell pellets were lysed and sonicated. Equal amounts of protein were run on SDS-PAGE and blotted with indicated antibodies. α-tublin served as a loading control.

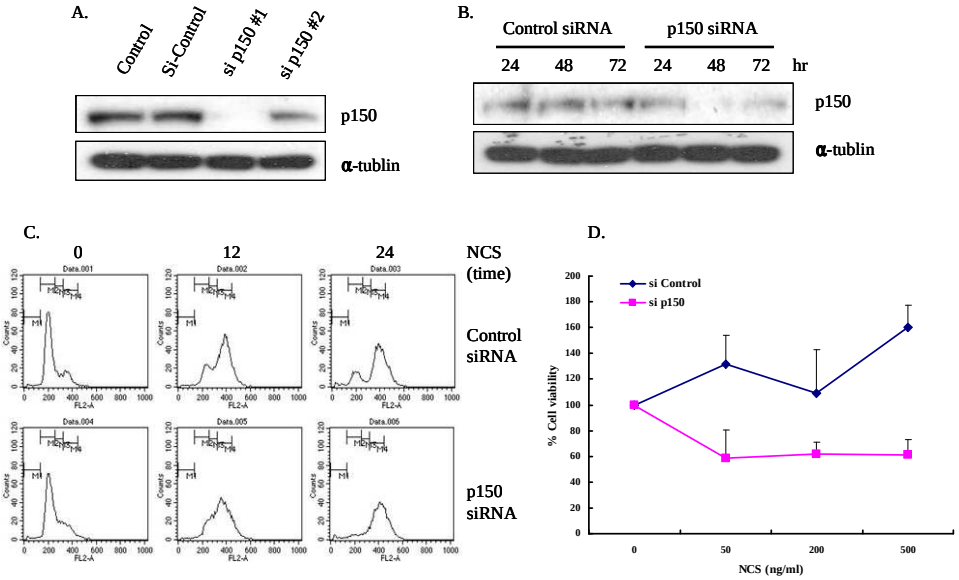

Fig.11. Silencing of p150 by RNAi induced G2/M arrest and decreased cell viability. (A) HeLa cells were transiently transfected with control or two different sequences p150 siRNA, harvested 48 h later, and blotted for p150 antibody. α-tublin is a loading control, and control from untransfected cells served as a positive control. (B) Time course of p150 siRNA-mediated silencing of protein expression. Hela cells were transfected with control or p150 siRNA and harvested at the indicated times. (C) Cell cycle effects. Control or p150 siRNA-transfected HeLa cells were untreated or treated with the indicated times of 200ng/ml NCS and determined using flow cytometry. (D) Cell viability assay. SiRNA-transfected HeLa cells were treated with the indicated dosage of NCS for 24 h and assessed using MTT.

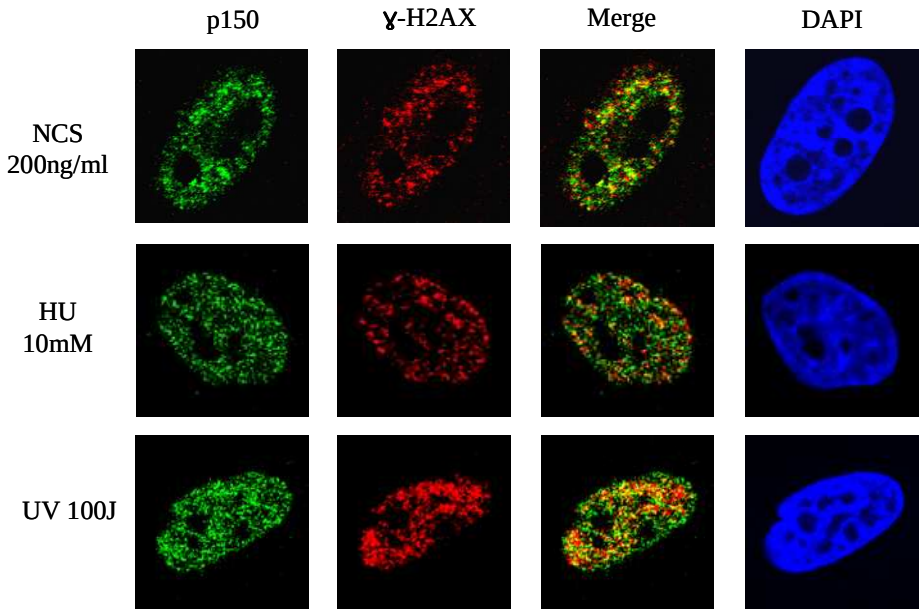

Fig.12. Recruitment of p150 and γ-H2AX in DNA double strand breaks site. HeLa cells grown on slides were treated with 200ng/ml NCS for 12 h (upper panels) or 10mM HU for 6 h (middle panels) or 100J/m^2 UV (lower panels, 6 h recovery). Following treatment, cells costained with rabbit anti-p150 and mouse anti-γ-H2AX. In the merged pictures, yellow represents the colocalization of p150 and γ-H2AX. Nucleus was visualized by DAPI staining.

DISCUSSION

In vivo, DNA is wrapped with histones and other proteins into chromatin, a structural organization that protects DNA from being damaged and inhibits DNA-dependent reactions such as replication, repair and recombination (Wolffe, A.P., 1998). The human genome is constantly challenged by endogenous and environmental factors that can alter its structure and corrupt its encoded message. A signaling network of checkpoint pathways has evolved to respond to these challenges to maintain genomic integrity. CAF-1 is a key player for chromatin assembly during DNA replication (Krude, T. & Keller, C., 2001) and NER DNA synthesis through its interaction with PCNA (Green, C.M. & Almouzni, G., 2002). CAF-1 is thereby recruited to sites of replication- and UV damage repair-associated DNA synthesis, apparently via the same mechanism. CAF1 participate in DNA repair by NHEJ and BER pathways through its interacting proteins such as XRCC4, XRCC1. Recently, it was reported that the p150 is related to double strand breaks repair (Linger, J. & Tyler, J.K., 2005; Lewis, L.K. *et al*., 2005; Nabatiyan, A. *et al*., 2006).

Our studies have revealed a new role for p150 in the DNA damage response pathway. Here, we first used a yeast two hybrid screening method to identify CAF1 p150 as a ATM interacting protein (data not shown). This interaction was confirmed inside mammalian cells through co-immunoprecipitation experiment. Here, we report the interaction of p150 and ATM. We show that p150 interacts physically with ATM in vivo (Figure 5). In response to double strand breaks, p150 formed foci that are coincident with the DNA damage response protein ATM (Figure 6). Because p150 directly and indirectly interacts with ATM, we hypothesized that p150 might participate in the cell cycle arrest and repair in DSBs.

ATM is the major kinase involved in immediate responses to DSBs upon ionizing irradiation. In nonirradiated cells, ATM is not phosphorylated and exists as an inactive dimer. Following irradiation, DSBs trigger autophosphorylation of ATM and formation of

phosphorylated monomers (Bakkenist, C.J. & Kastan, M.B., 2003). The phosphorylated ATM monomers then relocate to the DSBs and form the IRIF. γ-H2AX, a marker of DNA DSBs is very important protein in response to DSBs. In fact, we found that depletion of p150 reduced ATM phosphorylation (Figure 7), suggesting that p150 was required for autophosphorylation of ATM, the formation of p-ATM foci (Figure 8) and the ATM downstream signaling (Figure 10). Furthermore, we also show here that p150 depletion interfere with the formation of NCS induced γ-H2AX and pATM foci, indicating that p150 functions in parallel with or upstream of γ-H2AX and ATM in the signal pathways (Figure 8, 9). To confirm whether p150 related to DSBs repair, we further studied that p150 colocalized with γ-H2AX in DNA damaged cells. γ-H2AX forms the complex with p150 in response to DSBs damage. Not only HU or UV treated cells but also NCS treated cells, p150 colocalized with γ-H2AX (Figure 12). Our result is consistent with previous report (Nabatiyan, A. *et al*., 2006). The functional interaction between p150 and ATM is important for the regulation of cell cycle checkpoints. These facts suggest that the p150 may function for DSBs repair together with ATM and γ-H2AX.

After DSBs, many other proteins including Artemis, MDC1, NBS1, p53, Chk1, Chk2, Rad17, BRCA1, BLM, SMC1, 53BP1 and DNA-PKcs kinase were recruited at DSBs site (Wang, B. *et al.*, 2002; Goldberg, M. *et al.*, 2003; Lou, Z. *et al.*, 2003; Stewart, G.S. *et al*., 2003; Yazdi, P.T. *et al.*, 2002; Wyman, C. & Kanaar, R., 2006; Kobayashi, J. *et al.*, 2008). Hence, we examined whether p150 affect recruitment of other proteins and checkpoint signaling. As shown in Figure 9, we did not detect any foci formation of pNBS1 and Mre11 in p150 siRNA treated or control siRNA treated HeLa cells after NCS treatment, and western blot also not detect (Figure 10). This result indicated that p150 is related to NHEJ and S or G2/M phase repair because of NBS1 involved in HR and G1 phase repair (Kobayashi, J. *et al.*, 2008). p150 did not affect foci formation of 53BP1, Chk2 and MDC1 in p150 depleted HeLa cells after NCS treatment. However, BRCA1 foci formation increased p150 siRNA treated HeLa cells, is thought involving cell cycle arrest (Figure 9).

Since ATM signaling is defective when p150 is depleted, increase of NCS-induced BRCA1 and p53 phosphorylation in p150 depletion cells is likely regulated by other kinases, such as ATR (Figure 10). These results are thought that ATM induced cell cycle arrest and repair was regulated by p150.

In a previous study, CAF-1 is essential in proliferating cells, and its functional or physical depletion leads to phenotypes ranging from DNA damage to S-phase arrest, checkpoint activation, and programmed cell death (Nabatiyan, A. & Krude, T., 2004; Ye, X. *et al.*, 2003). These effects can be attributed to the failure of efficient chromatin assembly during chromosomal DNA replication. These reports are consistent with our result that p150 is required for cell growth, repair, arrest (Figure 11C, D). Flow cytometry result showed that p150 depletion cells were more far accumulated in G2/M phase than control cells in NCS treated cells (Figure 11C). This result indicated that p150 is required for G2/M phase repair in DSBs.

In summary, we demonstrate in this study that p150 participate in DSBs repair through interaction of ATM and cell cycle arrest. DNA damaged cells after necocasinostatin (NCS) treatment causing DSBs arrested G2/M phase in cell cycle. CAF1 p150 involved in cell cycle arrest by regulating ATM signaling pathway in DSBs. Collectively, our results suggest that CAF-1 p150 regulates G2/M phase DNA checkpoint and involved in DNA repair through ATM signaling pathway in DNA double strand breaks.

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ABSTRACT

The p150 large subunit of chromatin assembly factor 1 regulate ATM pathway in double strand breaks repair.

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Chromatin assembly factor 1 (CAF-1) is involved in genome integrity and DNA repair. Here we investigated the biological role of CAF-1 p150 to DNA double strand breaks (DSBs) in HeLa cell. CAF-1 p150 directly interacted with ATM (ataxia telangiectasia mutated), which is primarily activated in response to DSBs. CAF-1 p150 is also recruited to foci with kinetics to, and colocalizes with ATM in response to DSBs. Silencing of CAF-1 p150 via RNA interference decreased phosphorylation levels of ATM and H2AX and foci formation a little, but didn't affect to recruit another DNA repair protein including 53BP1, MDC1, BRCA1, Chk2 and MRN complex in response to DSBs, although BRCA1 foci slightly induced. Chk2 phosphorylation were delayed and decreased in CAF-1 p150 depleted cells. However, BRCA1 and p53 phosphorylation surprisingly increased in CAF-

1 p150 depleted cells. DNA damaged cells after necocasinostatin (NCS) treatment causing DSBs arrested G2/M phase in cell cycle. These results indicate that CAF1 p150 involved in cell cycle arrest by regulating ATM signaling pathway in DSBs. Collectively, our results suggest that CAF-1 p150 regulates G2/M phase DNA checkpoint and involved in DNA repair through ATM signaling pathway in DNA double strand breaks.

저작물 이용 허락서

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논문제목	한글: DNA DSB repair에서 CAF1 p150에 의한 ATM pathway 조절 연구				
	영문: The p150 large subunit of chromatin assembly factor 1 regulate ATM pathway in double strand breaks repair.				

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 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 의사표시가 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. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2008년 8 월 일

저작자: 조 병 옥 (서명 또는 인)

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