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The effect of chromatin assembly factor1 in ATM-dependent DNA damage response

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이 논문을 이학박사 학위신청논문으로 제출함.

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CAF1이 ATM pathway에 미치는 영향연구

김 진 우

지도교수 : 유 호 진

조선대학교 대학원 생물신소재학과

유전자 손상반응이란 간략하게 설명하면 세포가 유전자손상을 감지하면 신호 전달 경로를 통해서 checkpoints 을 작동시키는 것이다. 여기서 우선 여러 개의 센서들이 감지되고 다음 transducers 을 통과하고 더 나아가서 effectors 을 일으킨다. 여기서 우리가 연구하자고 하는 Chromatin assembly factor 1 (CAF-1)은 게놈 보전과 유전자 수복에 관여하는 중요한 유전자다. 그렇지만, CAF-l 이 DNA 수복에 어떻게 관여하는지는 많이 연구되어지지 않았다. 본 연구에서는 유전자이중나선 절단에 의한 유전자 손상반응에서 CAF1 은 유전자손상반응의 센서인 ATM 과 결합하는 것을 규명하였다. CAF-1 은 유전자손상을 일으키는 UV, BLM, NCS 에 의해 세포핵에서 foci 가 형성되지만 ATM 과 함께 같은 곳에 보충되지 않는다. 그리고 다른 유전자 손상반응의 센서인 rH2AX, 53BP1, NBS1, BRCA1 등과 역시 함께 집중되지 않는다. RNA interference 를 통한 CAF-1

의 결핍은 유전자손상에 활성화되는 ATM 과 BRCA1 의 인산화를 증가시켰고, foci 형성 또한 증가시켰다. 또한 clonal 생존율을 증가시켰다. 따라서 CAF1 은 유전자 수복에 직접적으로 기여할 것이다. 그리고 또한 CAF1 이 결핍한 세포에서 유전자손상에 활성화되는 NBS1, ChK2, p53 의 인산화는 증가되었고, 세포주기에서 intra-S 기 checkpoint 를 파괴시켰고 G2/M 기 checkpoint 에는 영향을 미치지 않았다. 결론적으로, CAF1 은 ATM 의 신호전달 경로를 통해서 유전자손상 체크시스템을 조절하여 유전자손상을 억제하고 유전자안정성 유지에 매우 중요한 단백질이다.

ABSTRACT

The effect of chromatin assembly factor 1 in ATM-dependent DNA damage response

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DNA damage response is briefly explained as a signal transduction pathway called checkpoints that is detected by several sensors and passed down through transducers and effectors. Here we identify a Chromatin assembly factor 1 (CAF-1) is involved in genome integrity and DNA repair, which binds to DNA damage sensor, ATM (ataxia telangiectasia mutated) using yeast two hybrid screening. CAF-1 forms DNA foci after DNA damages such as UV radiation and radiomimetic drugs, Bleomycin(BLM), Neocarzinostatin(NCS), but it does not colocalize with ATM to sites of DNA damage. Moreover, CAF-1 dose not colocalize with other DNA damage sensors such as rH2AX, 53BP1, NBS1 and BRCA1 to the DNA damage sites. Silencing of CAF-1 p150 via RNA-mediated interference (RNAi) increases foci formation of ATM and BRCA1 following DNA damage, and was also increased the clonal survival rate, which may directly contribute DNA repair. On the other hands, CAF1-depleted cells show reduced NBS1, Chk2 and p53 phosphorylation following DNA damage, and defect of intra-S phase checkpoint but not G2/M checkpoint. Therefore,

our combined results suggest that CAF1 is critical regulator of ATM-dependent DNA damage signaling pathway and plays a direct role in intra-S phage checkpoint machinery.

INTRODUCTION

Eukaryotic cell nuclear genome is assembled into the nucleoprotein structure termed chromatin. The structural subunit of chromatin is the nucleosome, which comprises approximately 147 basepairs of DNA wrapped in just under two superhelical turns around an octamer of histones H3, H4, H2A and H2B. (Luger et al., 1997). 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 and Stillman, 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 et al., 1995; Tyler et al., 1996, 2001; Verreault 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 and Stillman, 1989; Green, C. M., and G. Almouzni. 2002; Krude, T., and C. Keller. 2001). CAF-1 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., and B. Stillman. 1999), Werner syndrome protein (WRN) (Jiao R et al., 2006), 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 DNA DSBs (Linger and Tyler, 2005) and caf1 asf1 double mutants in yeast are 10-fold less competent in repairing DNA 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 DNA DSBs repair by NHEJ pathways in quiescent human cells (Arman Nabatiyan et al., 2006). These findings thus suggest the involvement of a chromatin assembly factor in response to DNA DSBs.

ATM [ataxia telangiectasia (A-T) mutated protein], which is defective in the hereditary disorder ataxia telangiectasia, is a central component of the signal transduction pathway that responds to DNA double-strand breaks (K Savitsky 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 DNA DSBs (Bakkenist CJ and Kastan MB, 2003). When DNA 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 kinase (Wang B et al, 2002; Goldberg M et al 2003; Lou Z et al, 2003; Stewart GS et al, 2003; Yazdi PT et al, 2002; Wynman, C. and Kanaar, R. 2006; Kobayashi J et al., 2008) (Figure.1.). 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. and 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.

Cellular DNA is constantly damaged by the deleterious assaults of both endogenous and environmental DNA damaging agents (C. Bernstein et al., 2002) 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 BB et al., 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 JH, 2001; Sarah A Martin et al., 2008) Defects in these DNA repair mechanisms can therefore result in increased mutation rates and genome instability, leading to diseases such as cancer.

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 MJ et al., 2002; Zhu C et al., 2002; Jackson S. P., 2002; Wynman, C. and 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

In this study, we have now investigated the relationship between CAF-1 and ATM. Our results the largest subunit of chromatin assembly factor 1 (CAF1), physically and functionally associates with ATM. CAF-1 is also through recruited with phosphorylated ATM and BRCA1 in response to DNA DBSs, to involved in DNA repair. Besides, these results indicate that CAF1 p150 involved intra-S phage cell cycle arrest by regulating ATM downstream proteins signaling pathway in DNA DSBs. Collectively, our results suggest that CAF-1 p150 regulates intra-S phase DNA checkpoint and involved in DNA repair through ATM signaling pathway in DNA double strand breaks.

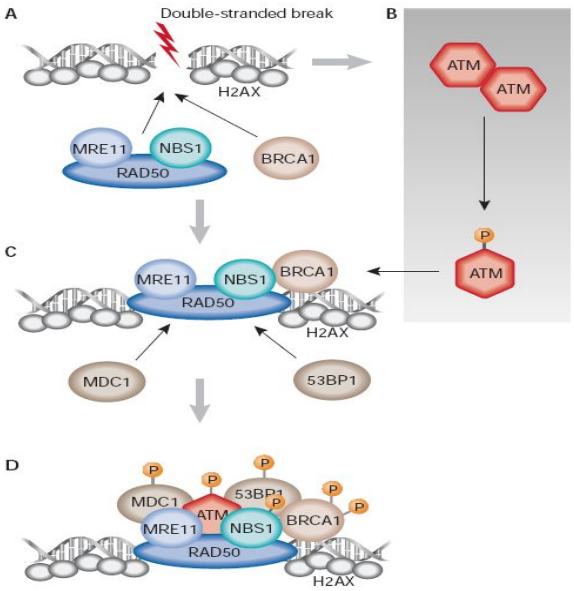


Fig.1. ATM and DNA damage signaling. In response to a DNA double stranded break (**A**) several simultaneous events occur to ultimately activate ATM signal transduction. ATM exists as an inactive multimeric complex that, in response to DNA damage, undergoes autophosphorylation to an active monomer (**B**). 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. 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.

2. Maintenance of Cell Lines

U2OS osteosarcoma cells was cultured in McCoys 5A medium (Cambrex Corp.) 10% fetal bovine serum. 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified incubator in an atmosphere containing 5 % CO_2 at 37 ^{o}C.

3. 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.

4. RNA interference

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) for the p150 gene. These siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. 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.

5. Western blot analysis

The cell 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 13000rpm 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 100μg of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes

were rinsed with TBST and then incubated with appropriate primary antibodies in TBST 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).

6. Immunoprecipitation (IP)

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. clonal survival

Again after selection with G418 for 5-6 days, surviving U2OS cells were replanted 24h before the heat stress in medium without G418. Control and CAF1 depleted cells were seeded in a 6-well plate and treated with NCS 25, 50, 100 and 200ng/ml after 6h, in a concentration 2×10^4 cell in each 30-mm dish representing a cell population. To perform a clonal survival assay, U2OS cells were trypsinzed and replanted directly after the heat shock in appropriate dilution in 30-mm dishes. After 2-3 week incubation at 37 ^{o}C, colonies were stained with 1% methylene blue.

9. Flow cytometry by PI staining

The trypsin-detached U2OS 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 propidium iodide (PI -50 μg/ml), RNase A (50 μg/ml), and 0.05 % Triton X-100. The DNA content of the U2OS 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. 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). Control and CAF1 depleted cells were seeded in a 48-well plate and treated with NCS (200ng/ml) after 24h, 10μM BrdU was added to the culture medium for incorporation into freshly synthesized DNA, and the culture was incubated for 2h at 37 ^{o}C. Following 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 that was produced in proportion to the level of BrdU incorporated into the cellular DNA. Colored products 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 treated with DNA-damaging agents from the absorbance of control cells. The data are presented as the mean ±SD from triplicate experiments.

11. G2/M checkpoint analysis

G2/M checkpoint assay was performed as described previously (Kolas et al. 2007;

Reinhardt et al; 2007). Control and CAF1 depleted U2OS cells were treated with 100ng/ml nocodazole for 3h following the addition of NCS (200ng/ml). after 24h, the cells were harvested and washed with PBS and then fixed with 1% formaldehyde for 10 min at 37 ^{o}C. the cells were chilled on ice for 1 min and then permeabilized with 90% methanol at -20^{o}C 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, Boston, MA) at a 1:10 dilution in incubation buffer for 1h in darkness at room temperature, and the cells were then washed and resuspended in PBS containing 50 μg/ml propidium iodide. At least 10,000 cells were analyzed by fluorescence-activated cell sorting (FACSort, Becton Dickinson, San Jose, CA). The acquired data were analyzed using the Cell Qusest Pro software (Becton Dickinson).

12. Statistical analysis

Results are expressed as mean ± standard deviation (SD). For statistical analysis, ANOVA with P values were performed for both the overall (P) and the pair-wise comparison as indicated by asterisks. Values of $P<0.05$ were considered to be significant.

RESULTS

1. CAF1 physically interacts with ATM, but it does not colocalizes with ATM to sites of DNA damage.

We performed co-immunoprecipitation experiments to investigate whether ATM and CAF-1 formed a complex in vivo. In extracts from untreated and NCS treated U2OS cells, we able to detected a ATM-p150 complex by co-immunoprecipitation using anti-p150 antibodies(Fig.2). Likewise, in a reverse immunoprecipitation experiment, anti-ATM antibodies also co-immunoprecipitation p150 from the treated and untreated U2OS cells. Showed that p150 and ATM formed a complex in NCS treated or untreated U2OS cells.

Based on the physical interaction between CAF-1 and ATM shown above, We used NCS as a chemical means to induce DNA 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 U2OS cells after NCS treatment (Fig.3 A). We did not detect any time point in the colocalization of p150 and phosphorylated ATM. We characterized the indution of DNA DSBs in human cells by using drug BLM, HU, and UV, we examined immuofluorescence microscopy to analyze whether p150 and ATM colocalize in the kinetics of formation in U2OS cells (Fig.3 B). we did not detect any in the colocalization of p150 and phosphorylated ATM.

We used indirect examined immuofluorescence microscopy to analyze whether p150 and ATM downstream proteins BRCA1, 53BP1, rH2AX, NBS1, foci merge in U2OS cells after NCS-24h treated. Among them and BRCA1 weakly foci merge, the rest of CAF-1 did

not foci merge. (Fig.4).

2. CAF1 was required for the phosphorylation and foci formation of ATM and BRCA1 following DNA damage.

We Using the psilencer siRNA expression hygro vector, and G-418 selection marker normal U2OS cancer cell make to p150 RNAi knockdown stable cell lines. we tested p150 siRNA whether efficiently silence p150 expression. As shown in Figure 5, In RNAi efficiently silenced p150 expression.

Given that ATM and CAF1 interacted in vitro and in vivo. However, the between ATM and hp150 is no foci merge. In order to investigate whether CAF1 affected for ATM activity in DNA DSBs repair, we first checked by western blotting for the phosphorylated ATM in Control and CAF1 depleted U2OS cells 0, 3, 6, and 12h after NCS (200ng/ml) treatment. Figure 8 shows the phosphorylated ATM increase in CAF1 depleted U2OS cells after NCS treatment. Moreover, we performed immuofluorescence experiments studies for the phosphorylated ATM in Control and CAF1 depleted U2OS cells 0, 3, 6, 12, and 24h after NCS(200ng/ml) treatment. As shown in Figure 6 A, we observed phosphorylated ATM foci increase in CAF1 depleted U2OS cells after NCS treatment. These results indicated that p150 is required for the phosphorylated ATM in DNA DSBs repair.

In front we evidence CAF-1 and BRCA1 weakly foci merge, we investigate whether CAF1 affected for BRCA1 activity. Likewise, we first checked by western blotting for the phosphorylated BRCA1. Figure 8 shows the phosphorylated BRCA1 increase in CAF1 depleted U2OS cells after NCS treatment. We net used immunofluorescence experiments examined for the BRCA1. We also observed phosphorylated BRACA1 foci increase in CAF1 depleted U2OS cells after NCS treatment (Fig 6. B)

We further studied whether CAF1 affects formation of rH2AX, 53BP1, and which are downstream proteins of ATM in DNA damage response. As shown in Figure 7, the presence p150 did not difference 53BP1, rH2AX affect in Control and CAF1 depleted U2OS cells after NCS treatment. Similarly, p150 did not affect foci formation of 53BP1, rH2AX in p150 depleted U2OS cells after NCS treatment.

3. CAF1 are sensitive to DNA damaging agents.

We investigate whether CAF-1 effects clonal survival, in both untreated or after 25, 50, 100, 200ng/ml treatments p150(RNAi) knockdown and control U2OS cells, followed by a recovery period of 6hours over culture. we next all the same amount seeding after 2-3 weeks observed clonal survival rate. For comparison, asynchronously p150(RNAi) knockdown cells exhibited an approximately twofold lower toxicity to DNA damage for equivalent time points.(Fig. 9). Therefore, we conclude that the CAF-1 through regulate phosphorylated ATM and BRACA1 will be involved repair.

4. CAF1 knockdown causes defects at the intra-S phase DNA damage checkpoints.

It has been certificated that expression of silencing p150 in U2OS cells accelerate DNA repair to DNA damage. Therefore, we tested whether the induction of DNA breaks caused by p150 silencing is preceded by an arrest of the transfected cell in cell cycle distribution. We finally examined the cell cycle effects of the NCS treatment by flow cytometry. more sensitive in CAF1 depleted U2OS cells accumulation in the intra-S phase of the cell cycle afer Neocarzinostatin 12hours treatment. (Fig. 10). For subsequent experiments, we used

bromodeoxyuridine (BrdU) further investigated the cell cycle distribution. (Fig. 11). The halogenated pyrimidine thymidine analog bromodeoxyuridine (BrdU) is selectively incorporated into newly synthesized DNA strands of S-phase cells and is useful for estimating the fraction of cells in S-phase. Additionally, the analysis of the uptake of BrdU is a reliable method to quantitate the degree of DNA synthesis.

5. CAF1 knockdown leads to reduced phosphorylation of checkpoint proteins after DNA damage.

ATM is primarily phosphorylated in response to DNA DSB damage, 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 and Chen J, 2004; Kobayashi J et al., 2008). We next studied whether p150 involved in checkpoint signaling. As shown in Figure 12, phosphorylated NBS1 decreased in p150 depleted cells after NCS treatment. phosphorylated p53 also decreased 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 12). This result indicated that CAF1 is required for cell cycle arrest in response to DNA DSBs.

6. C150 is not altered G2/M phase after DAN damage.

We also tested G2/M phase cell cycle distribution and cellular viability after DNA

damage. Control and CAF1 depleted U2OS cells were untreated or after NCS (200ng/ml) treatment. Next cell cycle profiles were analyzed afer 24hours by flow cytometry using phosphor-histone H3 staining as a mark of mitosis and propidium iodide staining for DNA content. In the lower set of panels, nocodazole (100ng/ml) was added 3hours following NCS (200ng/ml) treatment. As shown in Figure 13, we also tested cell cycle distribution and cellular viability after DNA damage.

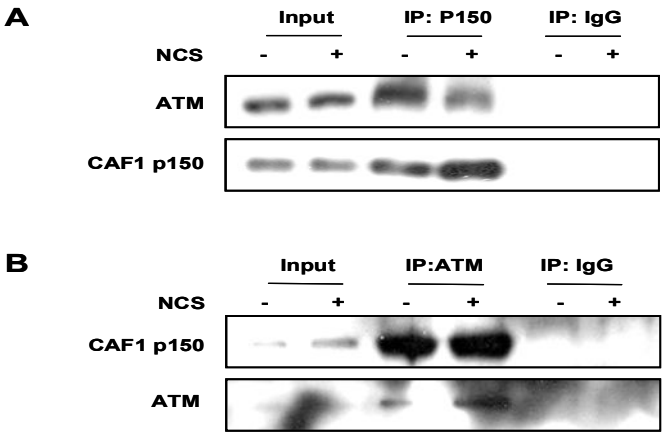
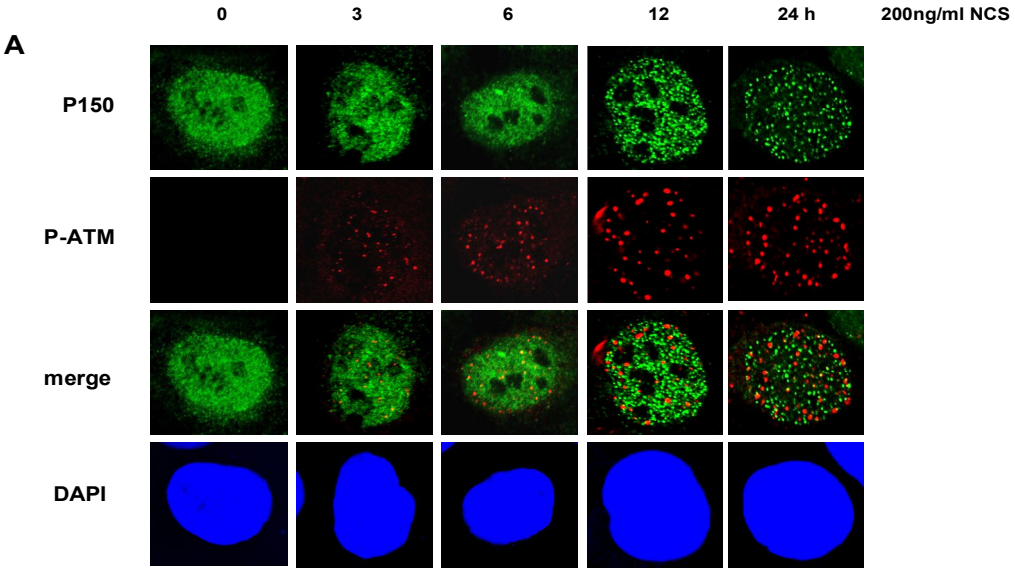


Fig.2. CAF1 physically interacts with ATM. (A) anti-p150 antibodies (lanes 3-4) or control IgG antibodies (lanes 5-6) were used to immunoprecipitate proteins from either untreated (NCS-) or treated (NCS+). (B) anti-ATM antibodies (lanes 3-4) or control IgG antibodies (lanes 5-6) were used to immunoprecipitate proteins from either untreated (NCS-) or treated (NCS+). In both the (A) and (B), U2OS cells were treated with treated 200ng/ml NCS for 1h, total cell extracts were used as input control (lanes 1-2).



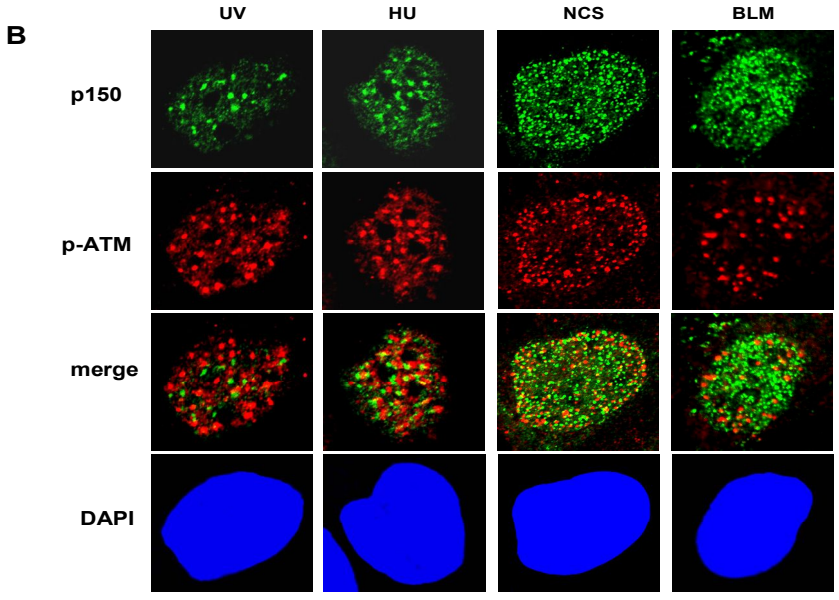


Fig.3. CAF1 colocalizes with p-ATM. (A) kinetics of p150 and p-ATM Colocalization in U2OS cells after NCS treatment. U2OS cells were untreated or treated with 200ng/ml NCS for 3, 6, 12, or 24h. Immunostaining was performed using rabbit anti p150 and mouse anti p-ATM antibodies. In the merged pictures, yellow represents the colocalization of p150 and p-ATM. Nucleus was visualized by DAPI staining. (B) Colocalization of p150 and p-ATM in U2OS cells after UV, HU, BLM, NCS treatment. U2OS cells were treated with 10J UV, 10mM HU, 200ng/ml BLM or NCS for 24h.

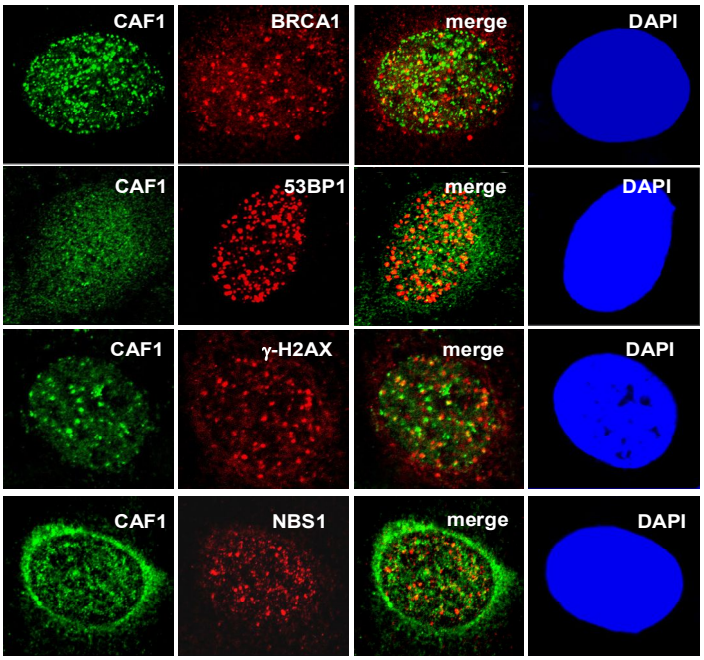


Fig. 4. CAF1 colocalizeas with BRCA1, 53BP1, rH2AX and NBS1. Colocalization of p150 and other DNA damage protein in U2OS cells after NCS treatment. U2OS cells were treated with 200ng/ml NCS for 24h. Following treatment, respectively the cells were then fixed and immunostained with antibodies to rabbit anti-p150 and mouse anti-BRCA1, anti-rH2AX; mouse anti-p150 and rabbit anti-NBS1, anti-53BP1. In the merged pictures, yellow represents the colocalization of p150 and those DNA damage proteins. Nucleus was visualized by DAPI staining.

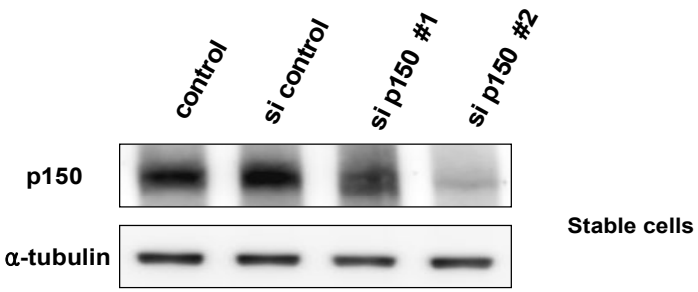
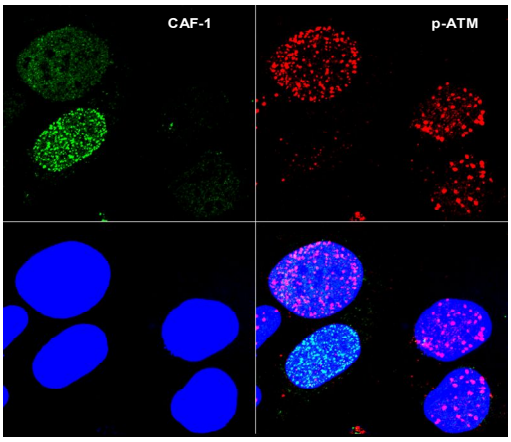


Fig. 5. CAF1 Knockdown stable cell Lines. Western blot to detect presence of p150. Lanes 1 are loading normal U2OS cells extract; Lanes 2 are loading control siRNA U2OS cells extract; Lanes 3 and 4 are loading two different sequences p150 siRNA U2OS cells extract, and blotted for p150 antibody. Detection of α-tubulin served as the loading control, and control from untransfected cells served as a positive control.



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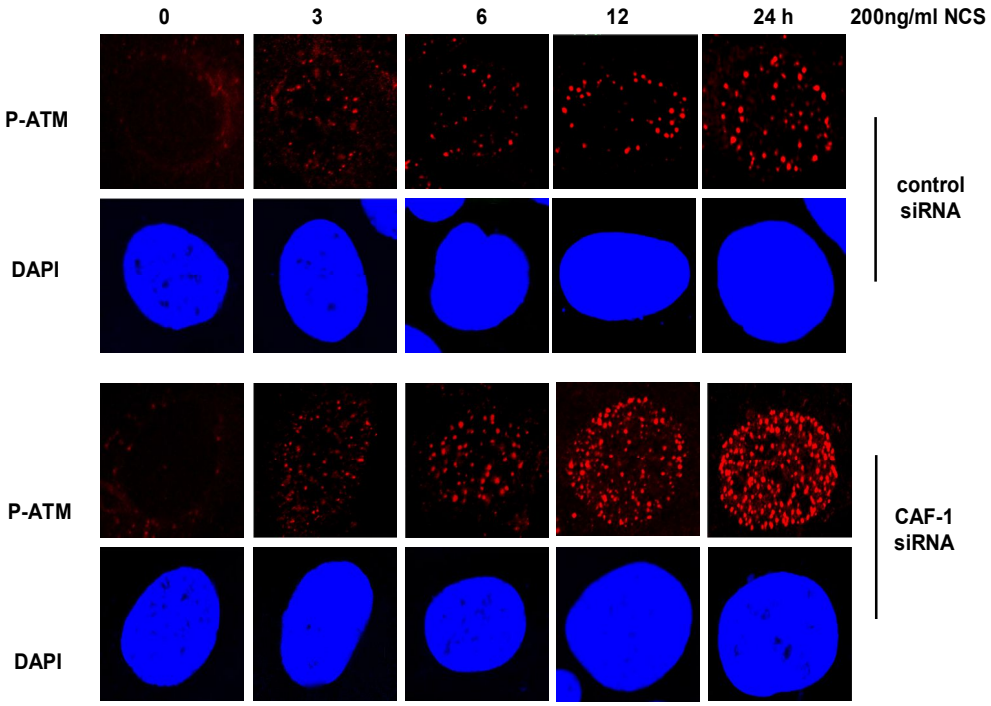

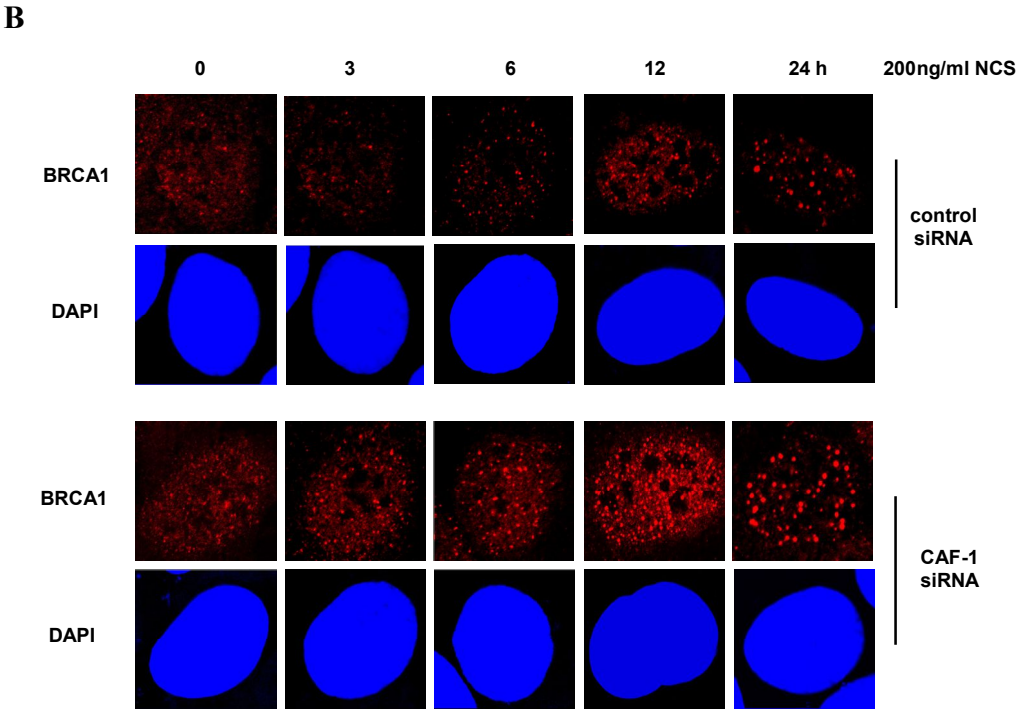

Fig. 6. CAF1 is required for the phosphorylation of ATM and BRCA1 foci formation. CAF1 knockdown U2OS cells increased for the phosphorylated ATM and BRCA1 foci formation. (A) Control and CAF1 depleted U2OS cells were untreated or treated with NCS (200ng/ml) for 3, 6, 12, and 24h, the cells were then fixed and immunostained with rabbit anti-p150 and mouse anti p-ATM antibody. Nucleus was visualized by DAPI staining. (B) Similarly, Control and CAF1 depleted U2OS cells were untreated or treated with NCS (200ng/ml) for indicated times and immunostained with rabbit anti-p150 and mouse anti-BRCA1 antibody. Nucleus was visualized by DAPI staining.

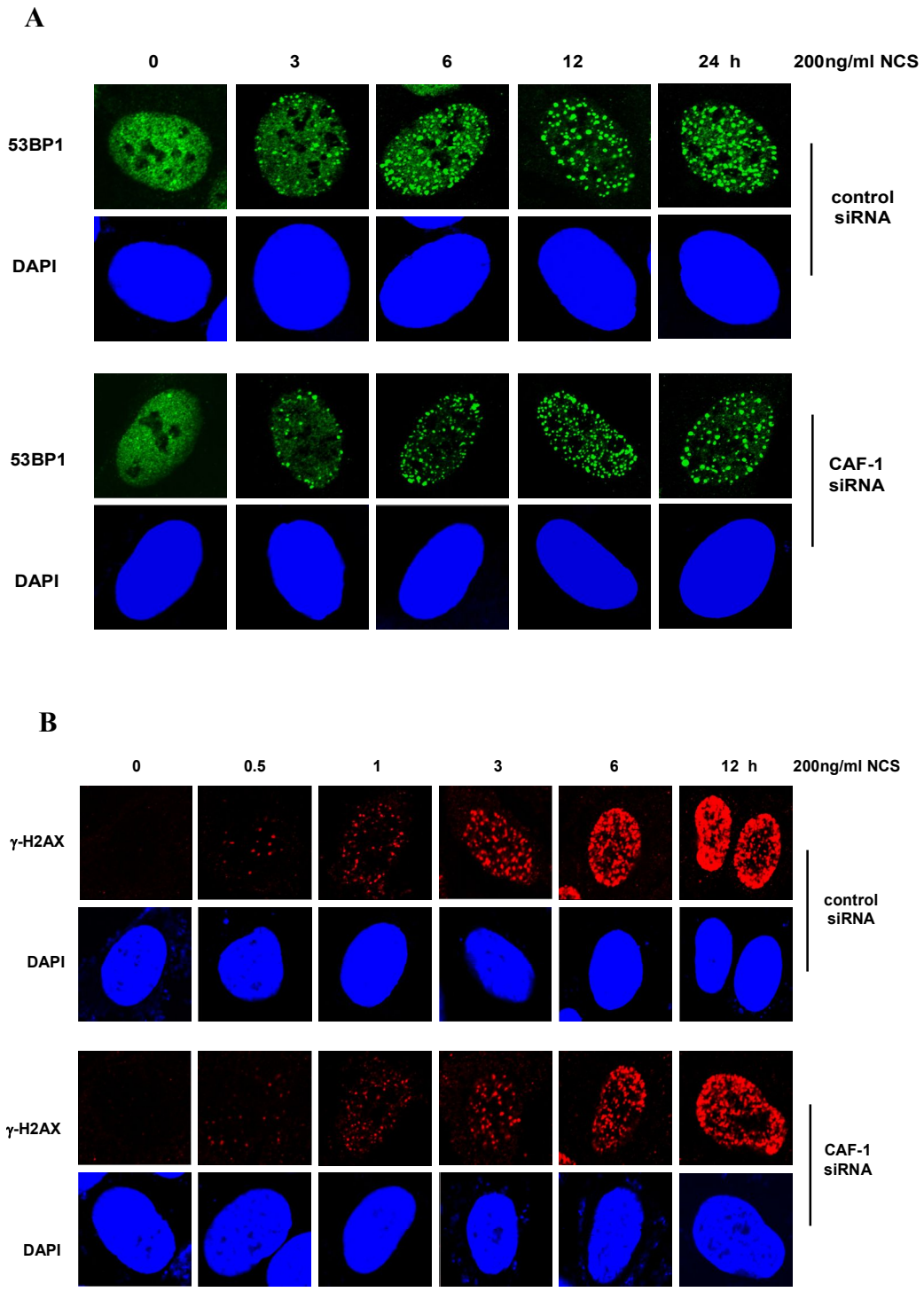

Fig. 7. CAF1 is not affect the 53BP1 and rH2AX foci formation. (A) Control and CAF1 depleted U2OS cells were untreated or treated with NCS (200ng/ml) for 3, 6, 12, and 24h, the cells were then fixed and immunostained with mouse anti-p150 and rabbit anti 53BP1 antibody. Nucleus was visualized by DAPI staining. (B) Similarly, Control and CAF1 depleted U2OS cells were untreated or treated with NCS (200ng/ml), the cells were then fixed and immunostained with rabbit anti-p150 and mouse anti rH2AX antibody. Nucleus was visualized by DAPI staining.

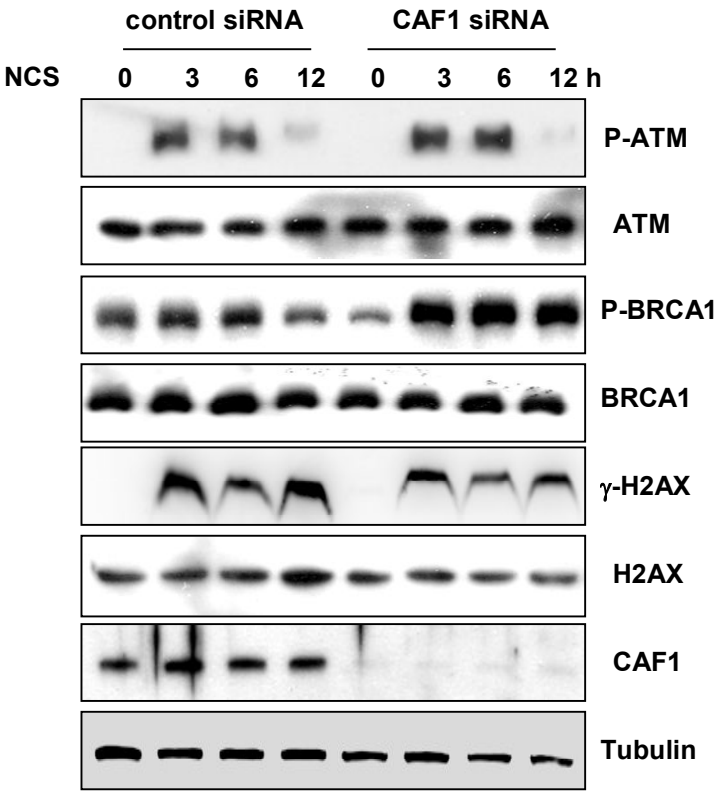

Fig. 8. Defect of CAF1 increased the phosphorylated ATM and BRCA1 in DNA DSBs. Control and CAF1 depleted U2OS cells were untreated or treated with 200ng/ml NCS, then collected at the indicated times. Cell pellets were lysed and sonicated. Equal amounts of protein were run on SDS-PAGE and blotted with indicated antibodies. α-tubulin served as a loading control.

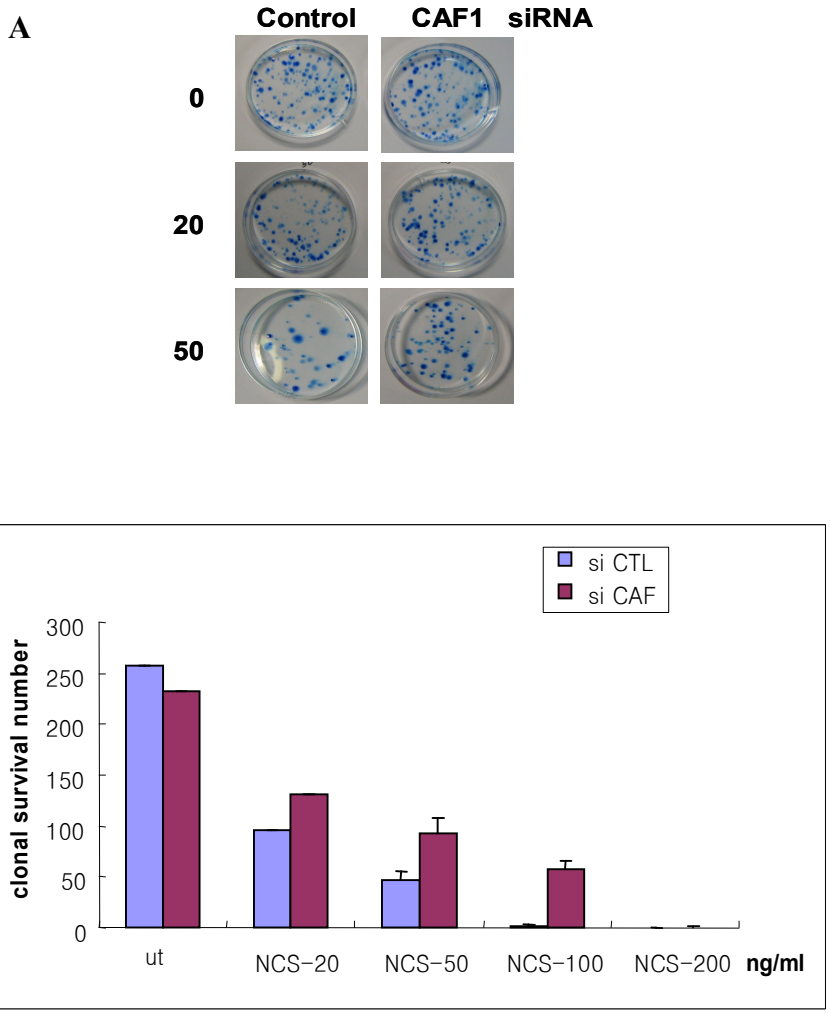

Fig. 9. CAF1 is effect clonal survival after DNA damage. Control and CAF1 depleted U2OS cells were untreated or treated with NCS (20, 50, 100, 200ng/ml). At 6h after treatment, the very little seeding cells 2-3week culture, and using 1% methylene blue staining (A). The control and CAF1 depleted cells clonal survival rate quantitative analysis of results (B).

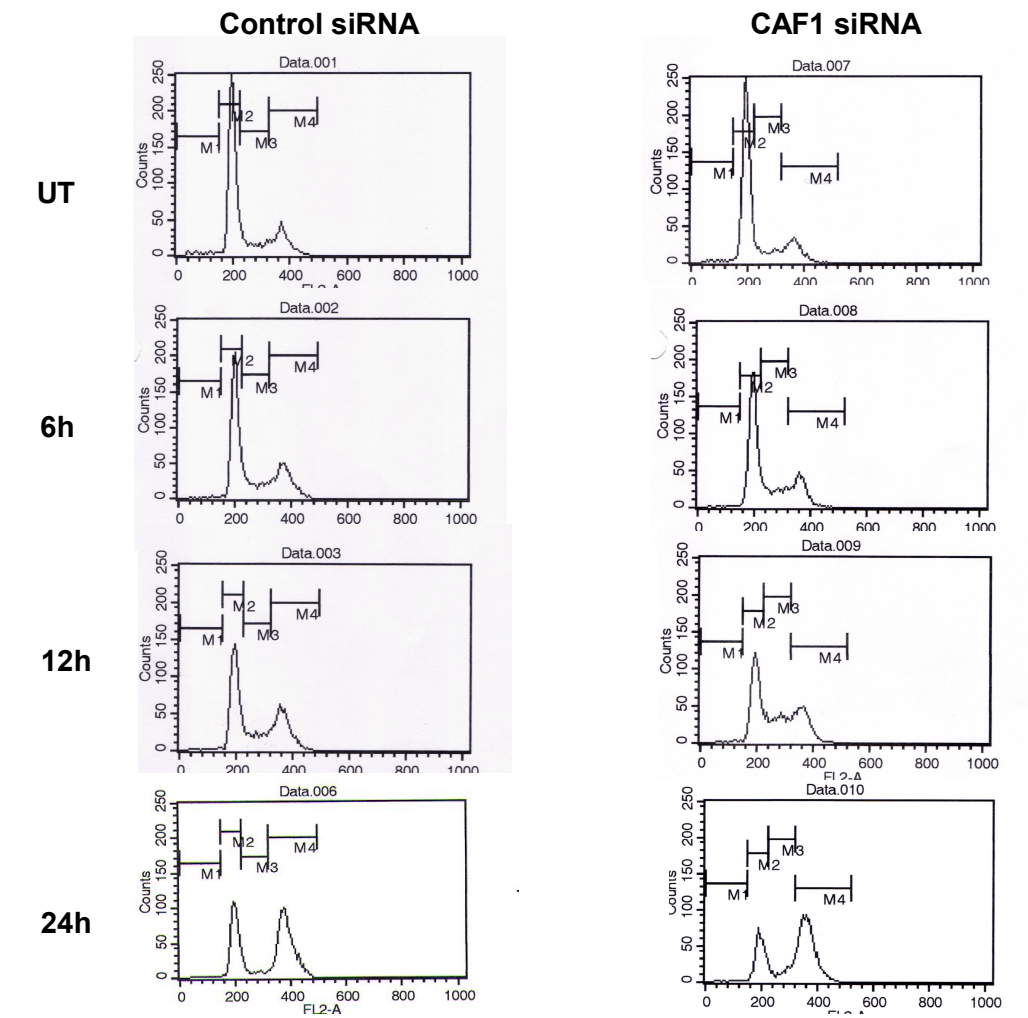

Fig.10. CAF1 defects induced at the intra-S phase arrest. Cell cycle distribution of Control and CAF1 depleted U2OS cells assessed by Flow Cytometry after staning with propidium iodide. Cell were untreated or treated with the indicated times of 200ng/ml NCS.

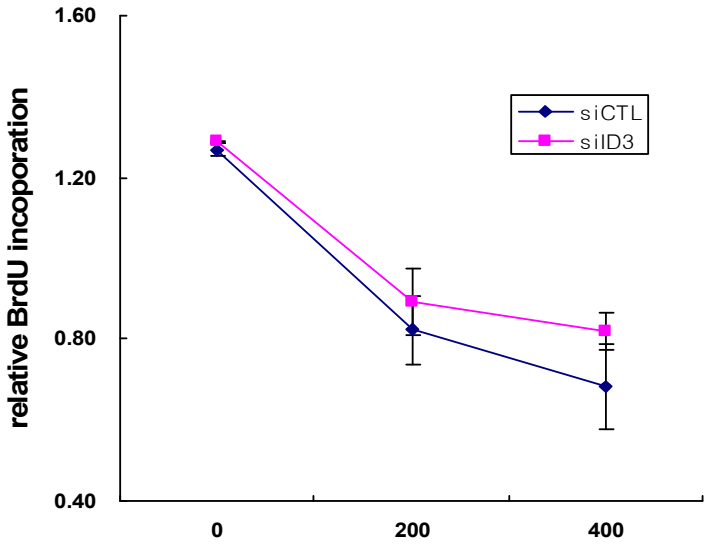

Fig. 11. CAF1 is regulated to the DNA damage induced intra-S phase checkpoints. Control and CAF1 depleted U2OS cells were treated with NCS (0, 200, 400ng/ml) after 24h, and cell cycle profiles analyzed using BrdU. The relative DNA synthesis was calculated as the percentage of absorbance of cells treated NCS form the absorbance of control cells.

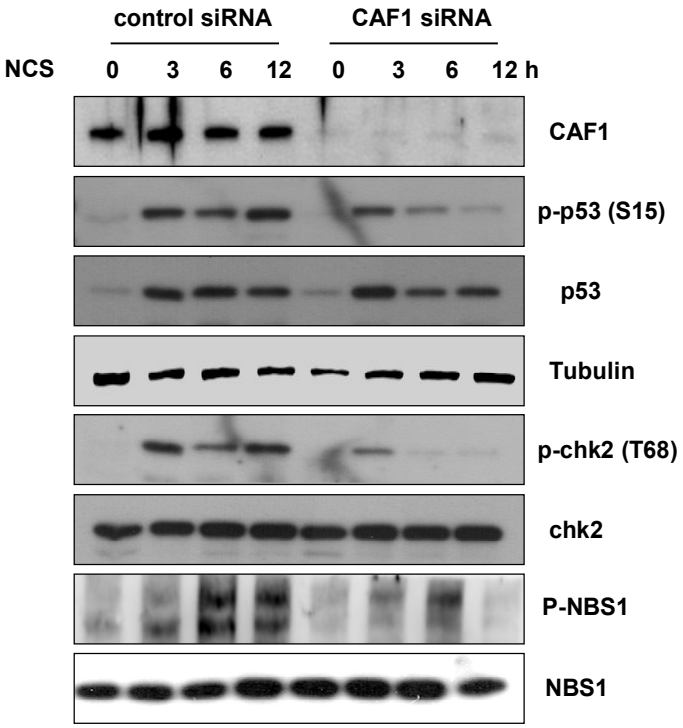

Fig. 12. CAF1 affect checkpoint signal in response to DNA damage. U2OS cells were transiently transfected with control or p150 siRNAi, cells were untreated or treated with 200ng/ml NCS, then collected at the indicated times. Cell pellets were lysed and sonicated. Equal amounts of protein were run on SDS-PAGE and blotted with indicated antibodies. α-tublin served as a loading control

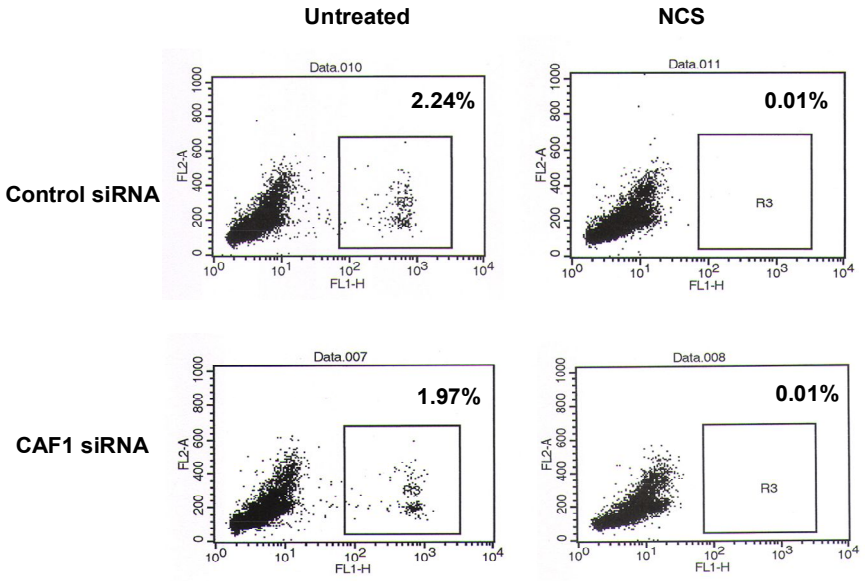

Fig. 13. CAF1 is not required for G2/M phase progression after DNA damage. Control and CAF1 depleted U2OS cells were untreated or treated with NCS (200ng/ml), and cell cycle profiles were analyzed after 24h by flow cytometry using phosphor-histone H3 staining as a marker of mitosis and propidium iodide staining for DNA content.

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, 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 pathway 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 and Kastan, 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. On a cellular level, U2OS cells are hypers ensitive to some, but not all, types of DNA-damaging agents. ATM is clearly involved in DNA repair processes, but more work is needed to establish its in vivo role in DNA transactions.

CAF-1 is a key player for chromatin assembly during DNA replication (Krude, T., and C. Keller. 2001) and NER DNA synthesis through its interaction with PCNA (Green, C. M., and G. Almouzni. 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 and Tyler, 2005; Lewis L. K. et al., 2005; Arman Nabatiyan et

al., 2006). Here we report the interaction of CAF-1 and the ATM. We show that ATM interacts physically with the largest subunit of CAF-1, p150, in vivo through co-immunoprecipitation experiment (Figure 5). We found that CAF1 knockdown U2OS cells increase the phosphorylation of ATM and BRCA1 foci formation. (Figure 9), suggesting that p150 was required for autophosphorylation of ATM, the formation of p-ATM foci (Figure 8) and the ATM downstream signaling (Figure 10). After DSBs, many other proteins including Artemis, MDC1, NBS1, p53, Chk1, Chk2, Rad 17, 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 GS et al, 2003; Yazdi PT et al, 2002; Wynman, C. and 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 13, CAF1 is not affect the 53BP1 and rH2AX foci formation after control and CAF1 depleted U2OS cells were untreated or treated with NCS.

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. 2004; Ye, X., 2003). These effects can be attributed to the failure of efficient chromatin assembly during chromosomal DNA replication. Flow cytometry result showed that CAF1 is not altered G2/M phase in cell cycle. This result indicated that p150 isn't required for G2/M phase repair in DSBs (Figure13).

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논문제목	한글: CAF1이 ATM pathway에 미치는 영향연구 영문: the effect of chromatin assembly factor1 in ATM-dependent DNA damage response				

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.

동의여부 : 동의(0) 반대( )

2009 년 06 월 22 일

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