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SERTAD1 is a novel regulator of DNA damage response

조선대학교 대학원

의과학과

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SERTAD1에 의한 DNA손상 반응 조절 기전 연구

최 은 지

지도교수 : 이 정 희

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의과학과

DNA 손상반응 단백질 중 하나인 53BP1은 DNA손상시 손상인식, 복구 및 세포주기 조절에 관여하고, 특히 비 상동 말단 결합 활성에 중요한 단백질로 알려져 있다. 그러나 이런 DNA 손상 반응에서의 53BP1의 자세한 기전은 아직 밝혀지지 않았다. 본 연구에서는 Yeast two-hybrid screening을 통해 DNA손상반응에 중요한 역할을 하는 53BP1과 결합하는 SERTAD1을 동정하였다.

P34로 알려진 단백질 Sertad1은 Cdk4활성을 조절하고, 암 세포에서 항 세포 사멸 활성을 나타내는 것으로 보고 되어있다. 그러나 본 연구에서 우리는 SERTAD1이 53BP1과의 결합을 통해 DNA손상반응에 관여하는 새로운 기전을 밝히고자 한다. 먼저 세포 내(in vivo)에서 SERTAD1과 53BP1이 결합함을 확인하였고, SERTAD1이 53BP1의 downstream인 DNA손상반응 단백질 Rif1과 PTIP와도 세포 내에서 결합함을 확인하였다. 이는 53BP1이 매개된 DNA손상 반응에 SERTAD1이 기능적으로 연관됨을 시사한다. SERTAD1이 53BP1 활성 조절에 미치는 영향을 조사하기 위해 먼저 SERTAD1이 결핍된 세포에서 53BP1의 기능을 조사한 결과 53BP1의 DNA손상 foci와 인산화가 감소됨을 관찰하였다. 또한 SERTAD1이 결핍된 세포에서는 G1 단계에서 Rif1과 PTIP foci가 감소하고 BRCA1과 RPA foci가 증가되는 것을 방사선 조사로 확인하였다. 또한, SERTAD1이 결핍된 세포에서 DNA손상 복구 활성이 떨어짐을 clonal survival assay, comet assay, late γ-H2AX 염색법 등을 통해 관찰하였다. 특히 SERTAD1결손에 의한 53BP1의 기능저하에 따른 비 상동 말단 결합 활

성의 저하에 관여함을 확인하였다. 또한, SERTAD1 결핍에 의한 DNA 손상복구 저하가 53BP1의 상동재결합 활성을 증진시킴을 SA-GFP 벡터 시스템과 DR-GFP 벡터 시스템을 통해 확인하였다. 따라서, 본 연구 결과는 SERTAD1이 53BP1과 상호작용을 통해 DNA손상반응, 특히 비 상동 말단 결합 활성을 조절하는 새로운 기전임을 제안한다.

INTRODUCTION

DNA Double-strand breaks (DSBs), which are generated through ionizing radiation (IR) and through various DNA-damaging chemicals, are the most dangerous DNA lesions, because if they are not efficiently and accurately repaired, they can result in mutations, genomic instability, and cell death, which can lead to cancer [1]. DSBs must be repaired correctly to ensure genomic stability. To ensure proper maintenance of genomic integrity, opportune signaling to identify damage and initiate cellular repair of DSBs with suitable fidelity is critical for genome maintenance as unrepaired DSBs can lead to cancer, increased aging and immune deficiency [2, 3].

DSB repair involves two major pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). DNA repair mechanisms differ at different stages of the cell cycle. NHEJ is the major pathway for DSB repair as the sister chromatid is absent in G1 phase. Cells use HR repair to rejoin DNA

breaks when homologous sequences on the sister chromatid are present in S and G2 phase [4].

The HR repair pathway need a homology template and is initiated by DNA end resection, which is carried out by the MRE11/RAD50/NBS1 (MRN) complex and facilitated by CtIP[5]. CtIP plays a critical regulatory role in ssDNA resection, along with the MRN complex. A more substantial end resection is carried out by EXO1, Dna2 and BLM to produce longer ssDNA stretch [6, 7]. Replication protein A (RPA) then binds to ssDNA, protects ssDNA from nuclease cleavage, and inhibits hairpin formation. Afterward, the BRCA2/PALB2 complex eliminate RPA and loads the recombinase RAD51 onto ssDNA to form a nucleoprotein filament that catalyzes homologous search and strand invasion, which leads to strand exchange[8, 9]. Contrastively DSB repair by NHEJ does not need any homologous template; instead, it advances a direct ligation of two broken ends. Furthermore, the different requirement for homologous template,

HR and NHEJ also differ at the initiation steps. The choice of DSB repair by HR or NHEJ is also dictated by the 5′-3′ DNA end resection at break sites; substantial end degradation is an necessary step in HR, whereas NHEJ can only occur at DNA ends with limited or no progressing[10, 11].

Different DNA damage response proteins control these two repair pathways. For example, p53- binding protein 1 (53BP1) is well known key DNA repair factor that plays a critical role in defining DSB repair pathway choice in G1 and S/G2 phases cell-cycle[12]. Breast cancer 1 (BRCA1) is a well-known tumor suppressor gene frequently mutated in familial breast and ovarian cancers that has a critical role in HR repair. Depletion of BRCA1 leads to impaired HR[13], which may arise from inefficient DNA end resection[5, 14] and the impaired loading of BRCA2-PALB2 to DSBs[8]. BRCA1 and 53BP1 were previously believed to work independently. But, recent studies that BRCA1 and 53BP1 are involved in a competition between HR and NHEJ repair pathways.

Loss of 53BP1 rescued embryonic lethality, HR deficiency, and genome instability associated with BRCA1 deficiency [15-17].

53BP1 suppresses HR and is a positive regulator of NHEJ by protecting DSB from BRCA1-mediated end processing. Thus, the physical presence of 53BP1 at DSB ends is required for HR-suppressive activity. It certainly several studies have shed light on the involvement of other factors that work together with 53BP1 in protecting DSB ends. Among these factors, RAP1-interacting factor 1 (RIF1) and Pax transactivation domain-interacting protein (PTIP) were shown to be recruited to DSB sites in a 53BP1-dependent manner [18-22]. This suggests that 53BP1 acts as a scaffold protein to facilitate the recruitment of the end protection factors RIF1 and PTIP to the DSB site and hence committing the repair to NHEJ. Interestingly, the recruitment of RIF1 and PTIP was found to depend on the ataxia telangiectasia mutated (ATM)-mediated 53BP1 phosphorylation [19, 21, 22], putting ATM at the center of

the end protection process. This raises a paradox because it is already known that ATM is critical for the end resection process and that ATM-deficient cells are deficient in end resection [23-28].

In this study, we found that 53BP1 interacts with SERTAD1 by yeast-two hybrid screening. Also, we identified SERTAD1 interacts with PTIP and Rif1 downstream of 53BP1 by endogenous co-Immunoprecipitation. Our results indicated that SERTAD1 deficient cells were hypersensitive to IR-induced colony formation and have a major DSB-repair defect, shown by presence of late γ-H2AX foci. Moreover, SERTAD1 depletion diminished the recruitment of 53BP1 and their downstream proteins, RIF1 and PTIP, to site of DNA damage after DNA damage. In contrast, knockdown of SERTAD1 leads to increase of BRCA1 and RPA foci formation in G1 phase cells. Furthermore, SERTAD1 deficient cells showed impaired Non-homologous repair activity and enhanced Homologous recombination activity. Based on the results, we propose a critical

role of SERTAD1 in cellular DNA damage response, especially choice of DSB repair by HR or NHEJ, through association with 53BP1.

MATERIALS AND METHODS

1. Cell culture and treatment

HeLa, HEK293T and U2OS cells were purchased from ATCC. They were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin (0.1 mg/ml), penicillin (100units/ml) at 37℃ in a 5% CO_2 incubator. Cell growth was monitored under an inverted microscope. Upon reaching 70-80 % confluency, cells were digested with 0.5 % trypsin-EDTA before being passaged. Cells in exponential growth were harvested for subsequent experiments. To induce DNA double strand breaks, exponentially growing cells were irradiated at 10 Gy from ^{137}Cs source (Gamma cell 3000 Elan irradiator, Best Theratronics) and allowed to recover at 37 °C incubator for various times.

2. siRNA transfection

HeLa and U2OS cells were transfected with siRNA oligonucleotide duplexes against SERTAD1 using RNA IMAX (Invitrogen) according to the manufacture's instruction. The siRNA sequences targeting SERTAD1 (SERTAD1 siRNA #1: 5'-TGACACCTCTATGTATGACAATGAA-3', SERTAD1 siRNA #2: 5'-TGAGGATATTGACACCTCTATGTAT-3', SERTAD1 siRNA #4: 5'-GCAAGGGUCUGAAGCGGAA-3', SERTAD1 siRNA #5: 5'-GGAAACGGGAGGAGGAGGA-3') designed and synthesized for transient transfection.

3. Immunoprecipitation assay

The whole cell lysates prepared by extracting with NP-40 buffer (50mM Tris (PH 8.0), 150mM NaCl, 1% NP-40, and 5mM EDTA) with protease inhibitors (Roche Diagnostic Corp.) The lysates were added to anti-SERTAD1 antibody (Abcam) at 4℃ for 24hours. And then, protein A/G plus-agarose

beads(Santa Cruz Biotechnology), G-sepharose and A sepharose(GE Healthcare) were added to the lysates, and beads mixtures were incubated at 4℃ for 4hours with shaking. The beads were washed five times in NP-40 buffer without protease inhibitors, resuspended in equal volume 2X SDS sample buffer. The samples were extracted from the bead by boiling at 95℃ for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

4. Western blot analysis

Cells were lysed in RIPA buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate(NADOC), 0.1% sodium dodecyl sulfate(SDS)) with protease inhibitors (Roche Diagnostic Corp.). Cell lysates were collected by centrifugation at 13,200rpm for 30min. Protein concentrations were measured using the Bradford assay (Bio-Rad). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electro transfer onto a polyvinylidene difluo-

ride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hours with TBS-t (10mM Tris-HCl (pH 7.4), 150mM NaCl and 0.1% Tween-20) containing 5% skim milk and then incubated at 4℃ with primary antibodies(1:1000). The blots were washed six times for 10min with 0.1% Tween 20 containing TBS-t and then incubated for 2 hours with peroxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were washed six times for 10min, and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

5. Antibodies

We used the following primary antibodies: Rabbit polyclonal anti-53BP1 (sc-22760, Santa Cruz Biotechnology), Rabbit monoclonal anti-SERTAD1 (ab65446, Abcam), Rabbit monoclonal anti-Rif1 (A300-567A, Bethyl), Rabbit monoclonal anti-PTIP (A300-370A, Bethyl), Mouse monoclonal anti-α- Tu-

bulin (MS-581-P0, Neomarkers), Mouse monoclonal anti-ß-actin (sc-47778, Santa Cruz), Rabbit polyclonal anti-CENP/F antibody (ab5, Abcam) , Mouse polyclonal anti-BRCA1 (sc-6954, Santa Cruz), Mouse polyclonal anti-RPA (NA18, Calbiochem), Goat polyclonal anti-Lamin B (sc-6216, Santa Cruz), Rabbit polyclonal anti- γ -H2AX(05-636, Millpore).

6. Clonal survival assay

After treatment with IR, $1x10^3$ cells were immediately seeded onto a 60mm dish in duplicate and grown for 2-3weeks at 37℃ to allow colony formation. Colonies were fixed with 95% methanol for 10min and stained with 1% methylene blue in 20% ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells.

7. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were irradiated with 10 Gy of ionizing radiation (IR). Cells were then washed twice with 0.01M PBS, fixed with 4% paraformaldehyde for 10 min and ice-cold 98% methanol for 5 min, followed by permeabilization with 0.5% Triton X-100 for 15 min at room temperature. Next, the cover slips were washed three times with 0.01M PBS and then blocked with 5% BSA in 0.01M PBS for 1hrs. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4° C. Next, the cells were washed with 0.01M 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 (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken using a confocal microscopy (Zeiss LSM510 Meta:

Carl Zeiss) analyzed with ZEN software.

8. Non-homologous end joining assay

To measure the NHEJ repair, stable cells lines expressing HeLa and U2OS EJ5-GFP reports were generated by transfection using turbofectamine. EJ5-GFP contains a promoter that is separated from a GFP coding region by puromycin resistance gene, which is flanked by two I-SecI sites that are in the same orientation. When the I-SecI-induced DSBs are repaired by NHEJ in HeLa EJ5-GFP cells, the puro gene is removed, and the promoter is rejoined to the rest of the GFP expression cassette, leading GFP expression. After 43hours, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

9. Homologous recombination assay

DR-GFP assay : To measure the HR repair, stable cell lines expressing DR-GFP reports were generated by transfection using turbofectamine.

DR-GFP is shown along with the HDR product that uses *iGFP* as the template for nascent DNA synthesis, which results in restoration of a GFP expression cassette. HeLa DR-GFP cells were transfected with Control, 53BP1 siRNA and SERTAD1 siRNA, after 3hrs transfected with 2□g of I-SceI-expressing vector. After 48hrs, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

hprtSA-GFP assay : To measure the HR repair, stable cell lines expressing hprtSA-GFP reports were generated by transfection using turbofectamine.

The SA-GFP reporter consists of two tandem GFP gene fragments: 5'GFP and SceGFP3'. Repair of the I-SceI-generated DSB in SceGFP 3' by SSA

results in a functional GFP gene when a DNA strand from SceGFP3' is annealed to the complementary strand of 5' GFP, followed by suitable DNA-processing steps. As a result, SSA between the homologous sequences in the GFP gene fragments produces a 2.7-kb deletion in the chromosome. The SA-GFP reporter can also be repaired by HDR, but this repair does not restore a functional GFP gene. The hprtSAGFP construct has homology to the hypoxanthine phosphoribosyl transferase (hprt) locus and contains the selectable puromycin resistance gene (puroR) as well as the SSA reporter substrate SA-GFP. hprtSA-GFP is shown along with the SSA repair product that utilizes 266 nt of homology between the tandem GFP segments, thereby restoring a GFP ex-pression cassette. HeLa and U2OS hprtSA-GFP cells were transfected with Control, 53BP1 siRNA and SERTAD1 siRNA, after 3hrs transfected with 2□g of I-SceI-expressing vector. After 48hrs, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cy-

tometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

10. Statistical analysis

Data in all of the experiments are presented as the mean ± standard deviation (SD). Analyses were performed using software (Image J) and Excel (Microsoft).

RESULT

1. SERTAD1 interacts with 53BP1, Rif1 and PTIP.

Although the role of mammalian 53BP1 in the DNA damage response (DDR) is well documented, its regulation and underlying mechanism of action are only partially understood. In order to better characterize the regulatory network relevant to 53BP1 and to gain further insight into the molecular mechanism of action of 53BP1 in the DDR, a yeast two-hybrid screen was performed using a HeLa cDNA plasmid library with the N-terminal fragment (amino acid 1-699) of human 53BP1 as the bait. Out of the 1 x 10^7 transformants that were screened, 24 independent positive clones were isolated. One of the positive clones isolated from this transformants was identified as human SERTAD1.

To verify that an interaction between 53BP1 and SERTAD1 occurs in human cells, we used co-immunoprecipitation assays followed by Western blotting to assess protein-protein interactions. As shown in Figure 1A, endoge-

nous 53BP1 and SERTAD1 co-immunoprecipitation and although the association occurred in non-irradiated cells, it was enhanced in response to DNA damage. Also, we determined SERTAD1 interacts with PTIP and Rif1, downstream of 53BP1. To confirm that SERTAD1 interacts 53BP1, Rif1, PTIP in human cells endogenous level expressing both proteins, we performed reciprocal co-Immunoprecipitation assay with anti-SERTAD1 antibody (Fig 1B). In this reciprocal experiment, SERTAD1 antibody was able to co-immunoprecipitation of 53BP1, Rif1 and PTIP. The Rabbit IgG was used for negative control of immunoprecipitations. Reciprocally, SERTAD1 was associated together with Rif1 and PTIP, confirmed by immunoprecipitation assay of Rif1 and PTIP specific antibodies (Fig 1C and 1D). These results suggest that SERTAD1 interacts 53BP1, Rif1 and PTIP with each other and SERTAD1 may play a role in DNA damage response.

Figure 1

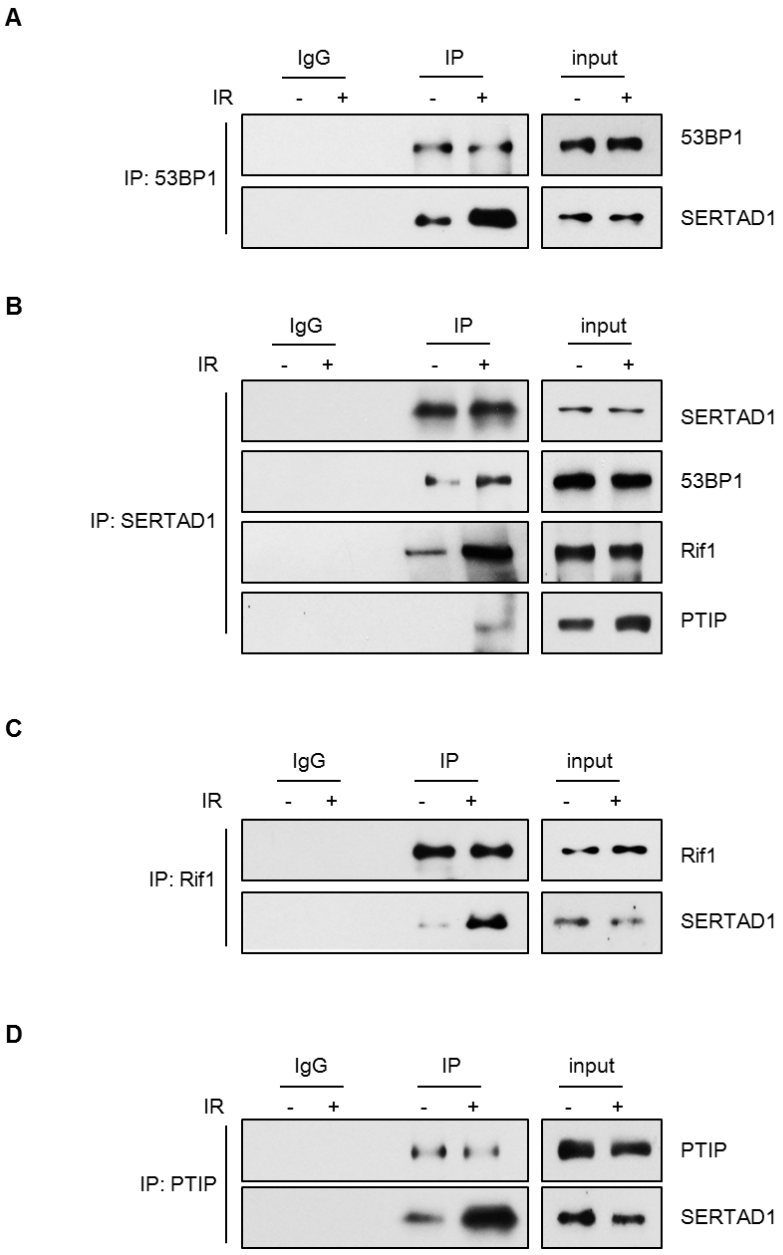


Figure 1. SERTAD1 interacts with 53BP1, Rif1 and PTIP.

Hela cells were untreated (UT) or treated with ionizing radiation (IR) 10Gy for 3hrs. (A) Whole cell lysates were subjected to immunoprecipitation (IP) using a 53BP1 antibody followed by Western blotting using the 53BP1 and SERTAD1 antibodies. Normal rabbit IgG was used for negative control immunoprecipitations. (B) HeLa cells were prepared as in (A), and lysates were subjected to immunoprecipitation using an anti-SERTAD1 antibody followed by Western blotting using the antibodies indicated to the right of the blot. (C), (D) The same experiments Fig 1A, were performed using SERTAD1 antibody instead of 53BP1, Rif1 and PTIP antibodies.

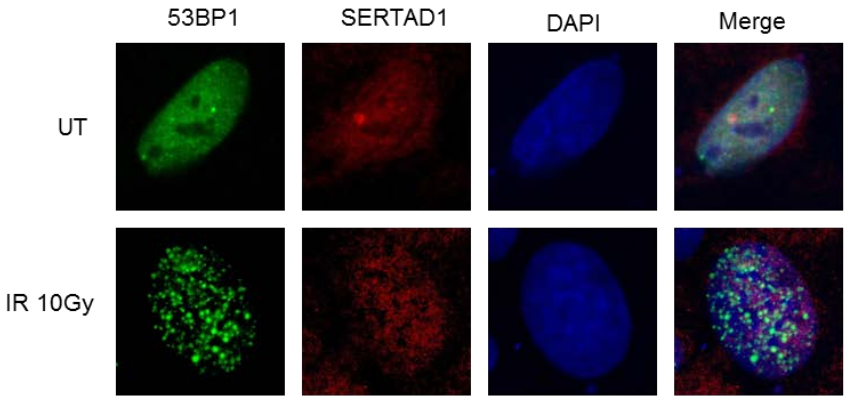
2. Subcellular localization of SERTAD1 protein

The DNA damage induced by ionizing radiations (IR) or other damaging agent's localize/recruit/interact many DNA repair proteins in the nucleus and form distinct structures called as foci. It was previously shown that 53BP1, a protein proposed to be involved in the repair of DSB, has also been shown to localize to sites of ionizing radiations induced DNA DSBs. To identify the physiological relevance of the interactions, we first investigated localization of SERTAD1 and 53BP1 using immunofluorescence staining. In untreated HeLa cells, SERTAD1 forms diffuse nuclear staining, and SERTAD1 foci formation was not detected in irradiated cells (Fig 2A). Moreover, many DNA damage-related proteins have been localized in nucleus. We examined the subcellular localization of SERTAD1 before and after DNA damage. Nuclear and cytosolic fractions were prepared from HeLa cells treated with or without IR and performed western blot analysis of SERTAD1 as well as its binding partner,

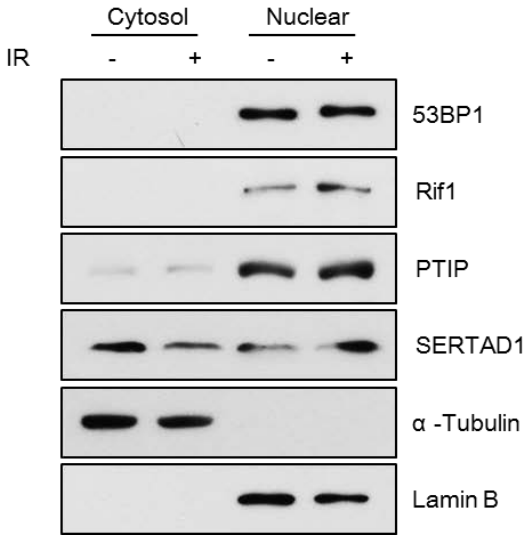
53BP1, PTIP and Rif1. Fractionation was confirmed by presence of α-Tubulin and LaminB in cytosol and nuclear fractions, respectively. We showed that SERTAD1 was existed in both the cytoplasm and the nucleus, 20% and 46%, respectively (Fig 2B). These results suggest that SERTAD1 is located in both cytosol and Nucleus, and that nuclear fraction of SERTAD1 may be involved in the 53BP1-related DNA damage response.

Figure 2

A



B



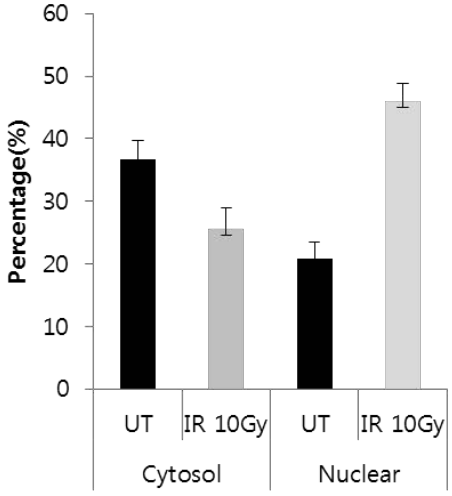


Figure 2. SERTAD1 is localized in Nucleus.

(A) HeLa cells were untreated or treated with 10 Gy IR for 3hrs, and then fixed. Cells were stained with anti-53BP1 and anti-SERTAD1 antibodies. DAPI staining was performed to indicate the position of nuclei.

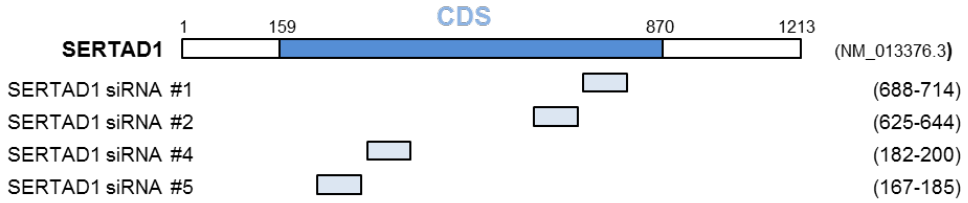
(B) HeLa cells were untreated or treated with IR of 10Gy. After 3hrs the cells were fractionated into cytosol and nuclear extracts, and were subjected to western blotting with antibodies anti-53BP1, anti-Rif1, anti-PTIP, anti-SERTAD1, anti-α-tubulin and anti-Lamin B. Quantitative densitometry of SERTAD1 protein analyzed using Image J software (right). Results are shown as the mean ± SD.

3. Knockdown of SERTAD1 by siRNA

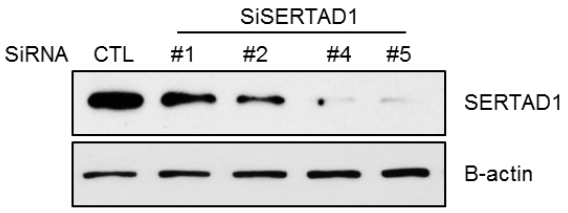
Next, to determine whether SERTAD1 is involved in the DNA damage response, we created the SERTAD1-knockdown cells using siRNA of SERTAD1. First, we designed SERTAD1 siRNA by the targeting prediction methods of the several algorithms. Figure 3A is indicated that schematic diagram of SERTAD1 siRNA sequences. These four candidate siRNA were further screened for knockdown effect on SERTAD1 expression in HeLa cells after transiently transfecting each siRNA. Western blot analysis showed that SERTAD siRNA #4 and #5 sequences strongly suppressed SERTAD1 expression (Fig 3B). Especially, the expression of SERTAD1 was reduced by more than 92% in cell line transfecting with SERTAD1 siRNA#5, compared with that of control siRNA-transfected cells (Fig 3C). We used SERTAD1 siRNA#5 further studies.

Figure 3

A



B



C

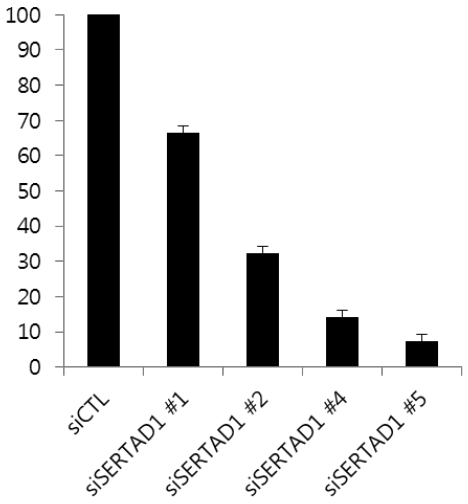


Figure 3. Knockdown of SERTAD1 using siRNA

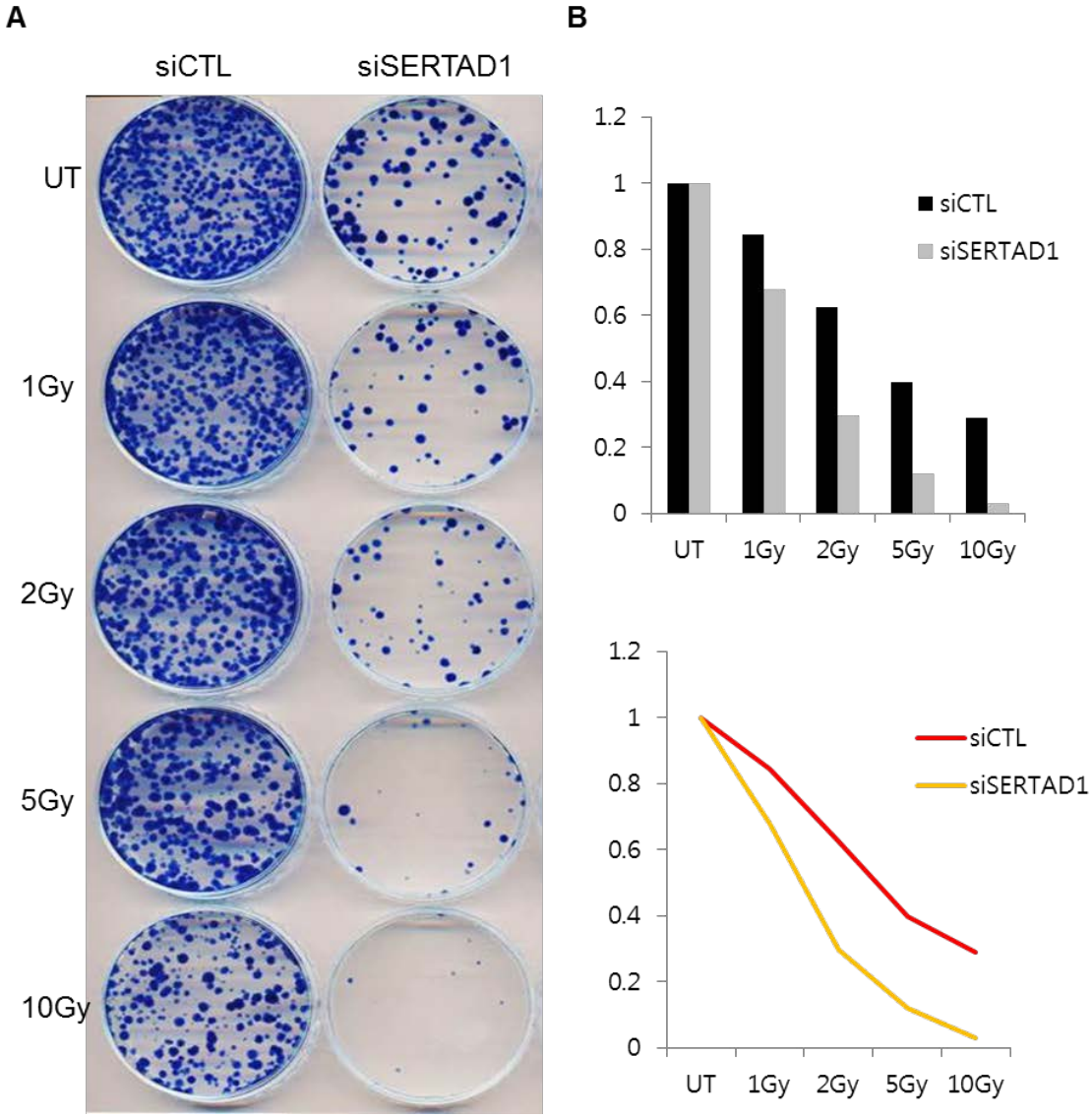
(A) Schematic diagram of human SERTAD1 siRNA sequences. The position of CDS (dark blue), we designed siRNA#1, 2, 4, 5 (sky blue). (B) HeLa cells were transfected with siRNA-control and siRNA-SERTAD1#1, 2, 4, 5. After 48hrs, the expression level of SERTAD1 was confirmed by western blot analysis using anti-SERTAD1 antibody. ß-actin was used as loading control. (C) Quantification of SERTAD1 western blot signals from three independent experiments as performed in (B) using Image J software. SERTAD1 protein levels were normalized using β-Actin as a loading control. Data represent as the mean ±SD (n=3).

4. Depletion of SERTAD1 sensitive to IR and are defective DNA repair.

To define a possible role for SERTAD1 in 53BP1-related DNA damage response, we first investigated. Whether cells lacking SERTAD1 would be more sensitive to DNA damage. Control and SERTAD1 siRNA #5-transfected HeLa cells were treated with indicated does of IR, and clonal cell survival as-say was performed. We found that SERTAD1 depleted cells exhibited decline of colony numbers after treatment with IR, compare to control cells. These re-sults indicated that SERTAD1 knockdown cells showed increased sensitivity to DNA damage (Fig 4A and 4B). Next, a role for SERTAD1 in clearance of IR-induced γ-H2AX foci was explored, the formation of γ-H2AX foci was ana-lyzed by immunofluorescence microscopy. γ-H2AX is used as the indicator for identifying of DSB induction and DNA repair, suggesting that the cells with unrepaired DNA damage still remains a formation of γ-H2AX foci. Control cells rapidly formed γ-H2AX foci following exposure to IR, and these foci

were almost completely resolved 24hours after the exposure, indicating efficient DNA repair. Cells were lacking SERTAD1 also rapidly formed γ-H2AX foci after IR exposure, but a little decrease of foci as compared to the control cells 30minutes following exposure. 16hours later, large amount of foci still remained in SERTAD1-depleted cells, indicating defective DNA repair (Fig 4C and 4D). These results suggest that SERTAD1 promotes DSB repair.

Figure 4



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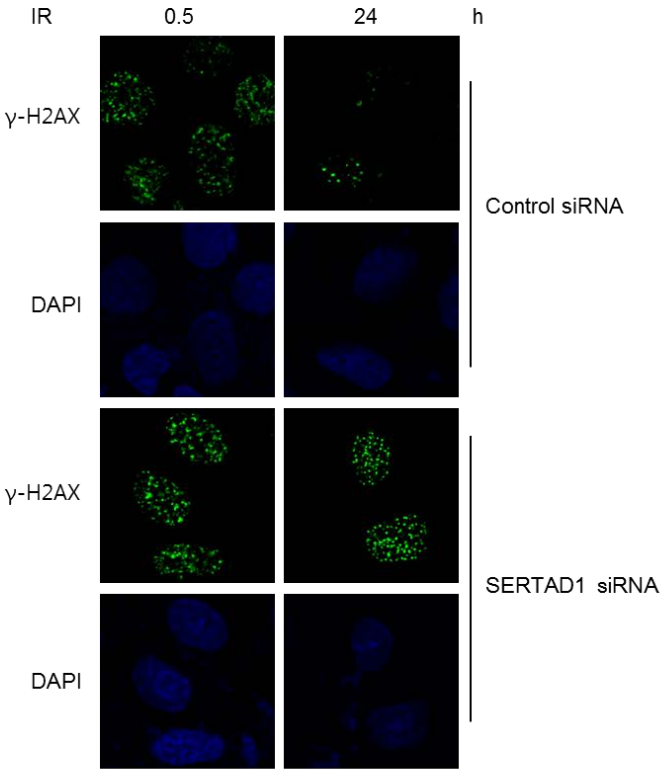

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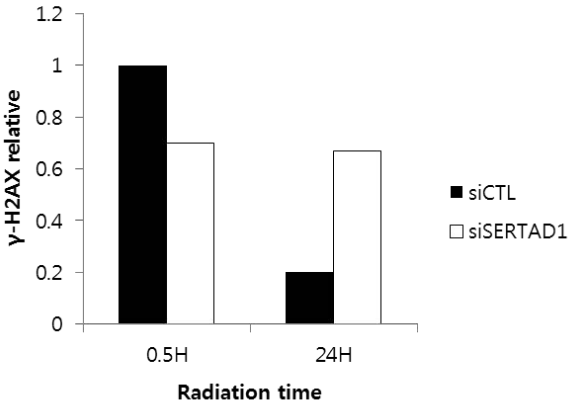

Figure 4. SERTAD1-Depleted cells are sensitive to IR and are defective via-bility.

(A), (B) SERTAD1 affects sensitivity of cells following exposure to IR in Hela cells. Control and SERTAD1 depleted Hela cells were untreated or treated with 1, 2, 5 and 10Gy ionizing radiations. After 2 weeks, cells were stained with methylene blue, and the number of surviving colonies was counted. (C) Control and SERTAD1-depleted Hela cells were untreated or treated with 10Gy γ-irradiation and were then fixed at the indicated times. Cells were stained with an anti-r-H2AX antibody and the DNA was counterstained using DAPI. (D) Quantification of Fig 1C.

5. SERTAD1 is required for the recruitment of 53BP1/ Rif1/ PTIP to DSBs.

Many studies have previously reported that 53BP1 requires both its localization to DSB sites and its phosphorylation by ATM in response to DNA damage [29, 30]; but, how 53BP1 phosphorylation affects DNA repair is unknown. Rif1 and PTIP have been reported as downstream of 53BP1. 53BP1, Rif1 and PTIP limit the resection of DSB ends, suppress HR and facilitate the repair to NHEJ. The above results, pointing to a biochemical interaction between SERTAD1, and 53BP1/Rif1/PTIP, promoted the prediction that SERTAD1 is required for recruitment of 53BP1/ Rif1/ PTIP to DSB. To test this hypothesis, we examined 53BP1 foci formation after DSB in control and SERTAD1-depleted HeLa cells. Control and SERTAD1-depleted cells were treated with 10Gy IR to make DSB and harvested in different time intervals. Cells were fixed with 4%paraformaldehyde and immunofluorescence assay performed. Our observation showed that depletion of SERTAD1 decreased the 53BP1 foci for-

mation as compared with control cells (Figure 5A and 5B). We also observed that HeLa cells with a knockdown of SERTAD1 had dramatically less recruitment of Rif1 to DNA damage sites (Fig5C and 5D). Other downstream protein of 53BP1, PTIP foci also decreased in SERTAD1-depleted HeLa cells (Fig 5E and 5F). This improved level of 53BP1/Rif1/PTIP recruitment was not due to decrease in the total amount of their proteins, because we detected no difference in 53BP1/Rif1/PTIP protein levels between the knockdown cells and wild type cells (Fig 5G). Together, these data showed that SERTAD1 is required for 53BP1 localization at DSBs thereby facilitating recruitment of downstream factors.

Figure 5

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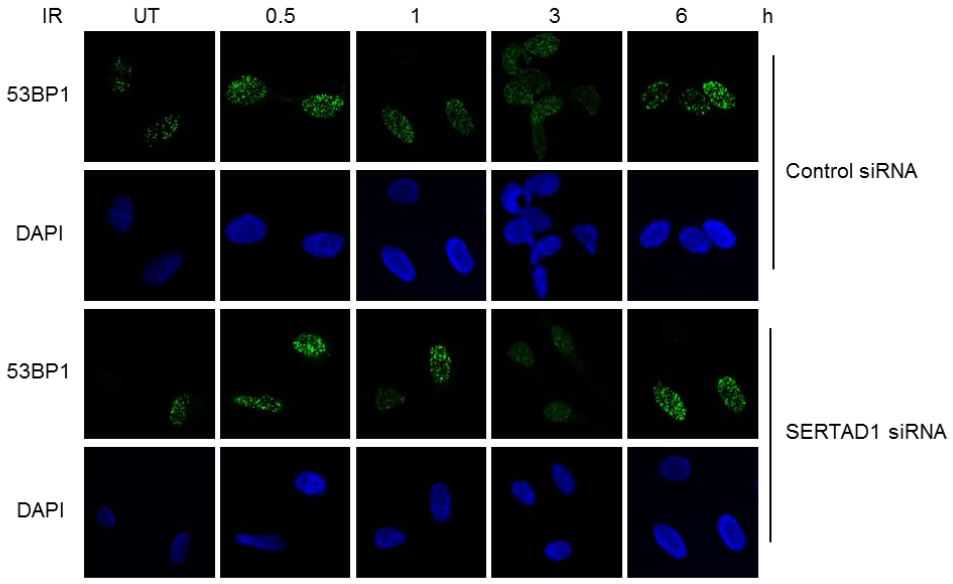

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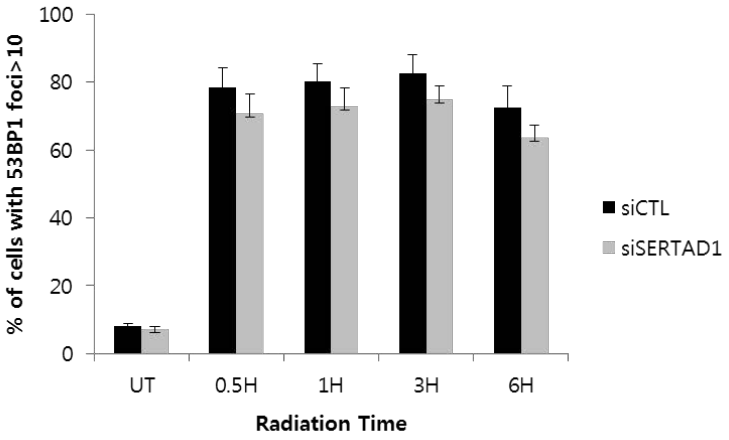

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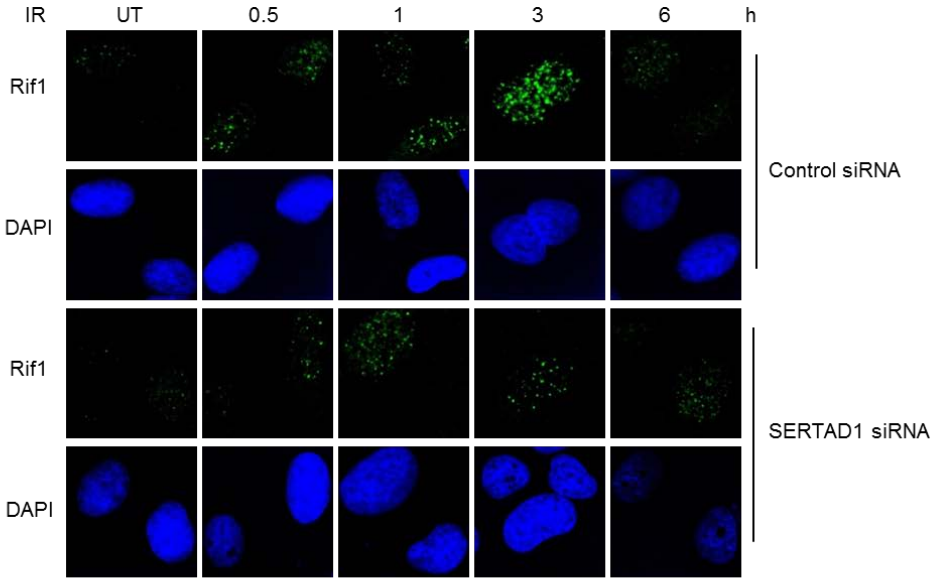

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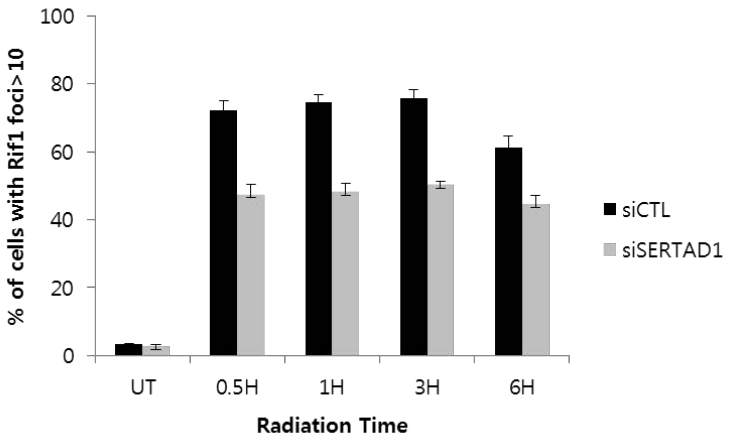

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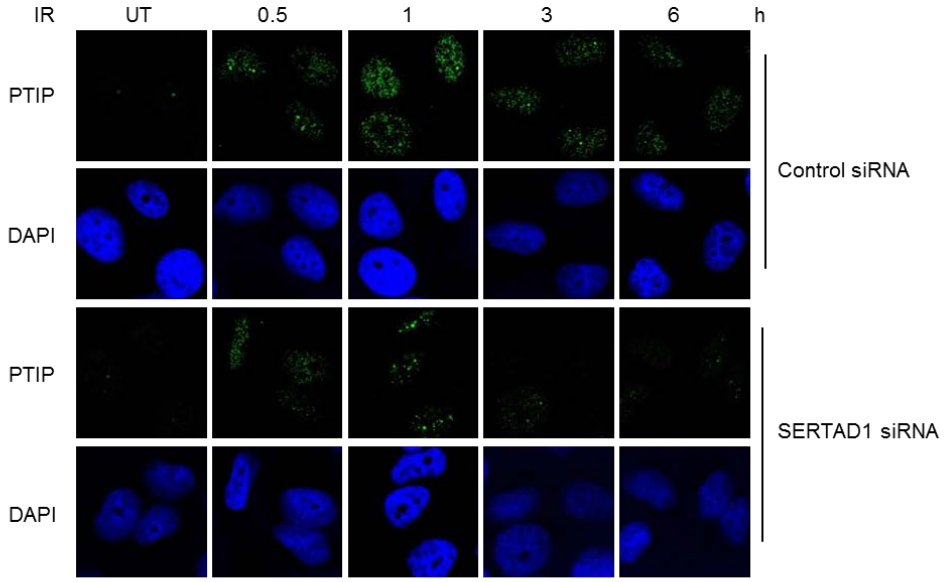

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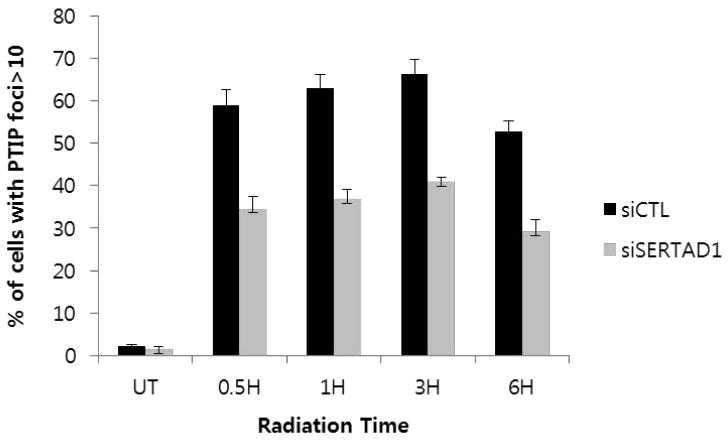

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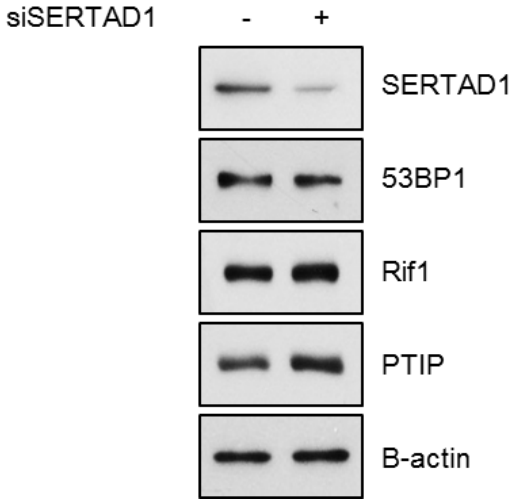

Figure 5. SERTAD1 affects 53BP1, Rif1 and PTIP foci formation after DNA damage.

Control and SERTAD1-depleted HeLa cells were untreated or treated with 10Gy irradiation and were then fixed at the indicated times. Cells were stained with an anti-53BP1 **(A)**, anti-Rif1 **(C)** and anti-PTIP **(E)** antibodies. The histogram shows the number of cells with foci. Results are shown as mean ±SD (n=3). **(G)** HeLa cells were transiently transfected with either Control or SERTAD1 siRNA. Western blotting to the indicated proteins shows the expression levels of each.

6. Increased BRCA1 and RPA foci formation in G1 phase cells by knockdown of SERTAD1.

DNA repair-related protein, BRCA1 plays essential, yet enigmatic role in homologous recombination and nucleotide excision repair. BRCA1 performed in HR by promoting DNA end resection[14], but this activity is not generally watched. A major function of BRCA1 appeared when it was found that the lethality, tumorigenesis, and genome instability associated with BRCA1 depletion can be rescued by the attendant deficiency of 53BP1[17]. Deficiency of 53BP1 facilitate end resection and HR, which has led to a model conjecturing that the function of BRCA1 in DSB repair is to antagonize 53BP1-dependent end protecting. BRCA1 is mostly absent in the G0/G1 phase[31] of connection-inhibited cells it had been presumed that the regulatory of BRCA1 protein levels describes the absence of BRCA1 IR-induced foci in this phase of the cell cycle[22].

We further investigated BRCA1 and RPA foci formation after DSB in control

and SERTAD1-depleted Hela cells in G1 phase. Control and SERTAD1-depleted cells were treated with 10Gy IR to make DSB and harvested in different time intervals. Cells were fixed with 4%paraformaldehyde and immunofluorescence assay performed. Cells were co-stained with G2 phase marker, CENP-F. BRCA1 and RPA foci were similarly detected in G2 phase cells. Interestingly, after depletion of SERTAD1, we observed significant increases in the formation of BRCA1 (Fig 6A and 6B), and RPA (Fig 6C and 6D) foci in the CENP-F negative cells, G1 cells. Taken together, we found that SERTAD1 inhibits the recruitment of 53BP1/Rif1/PTIP, and therefore increase the subsequent accumulation of BRCA1/RPA at DSBs in G1 phase of cell cycle.

Figure 6

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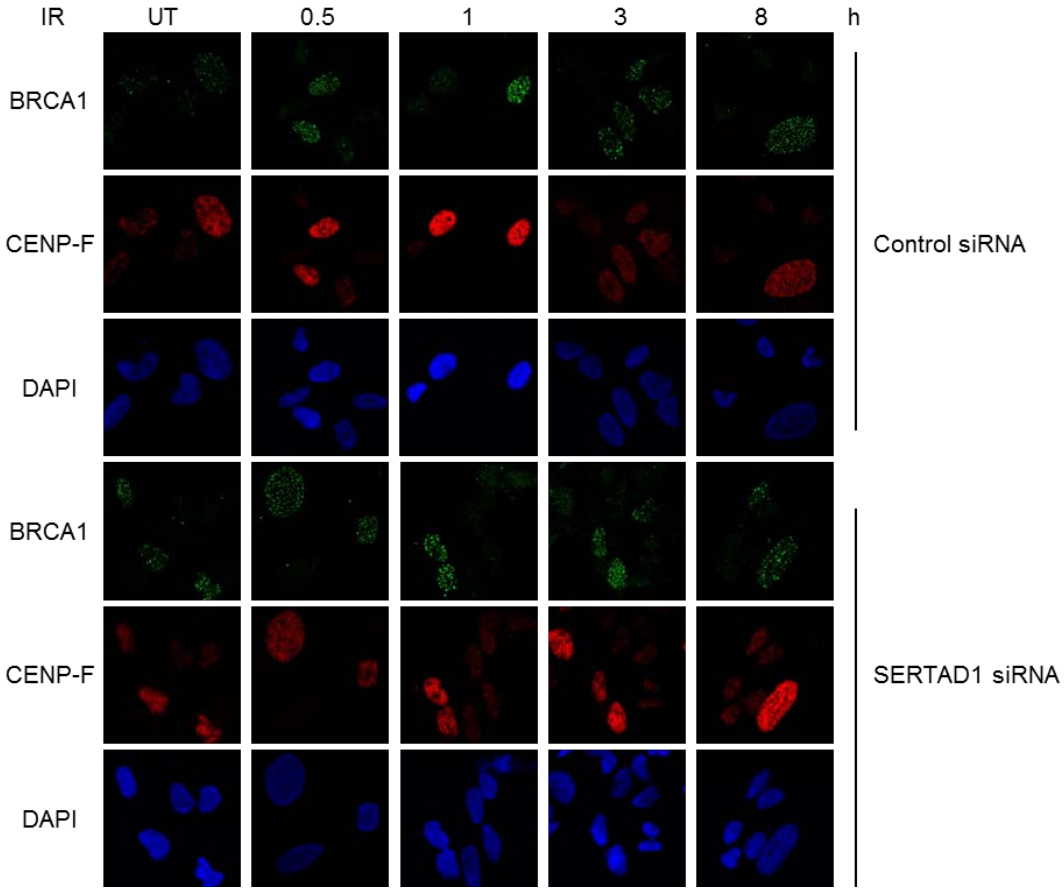

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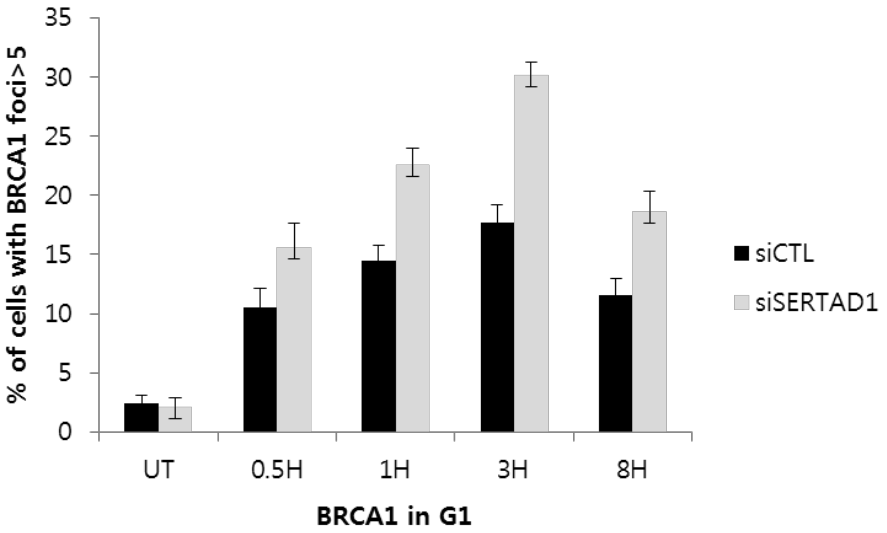

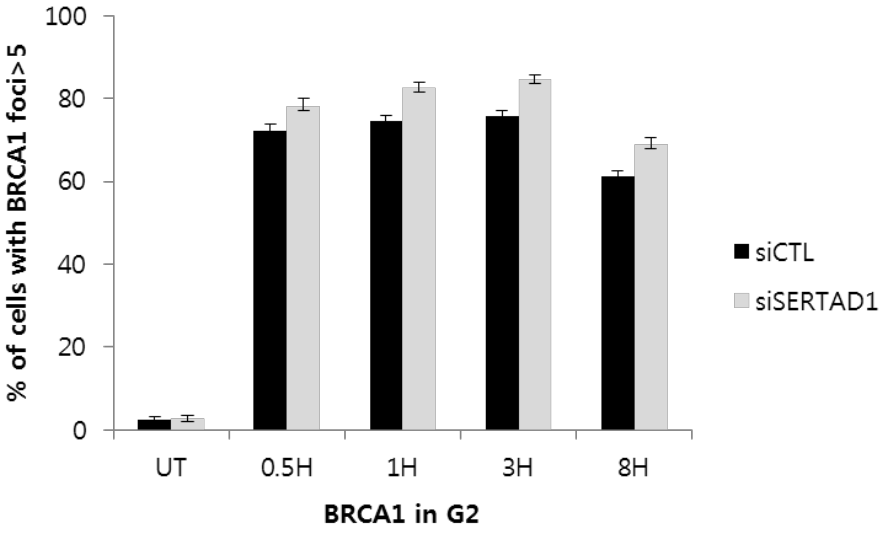

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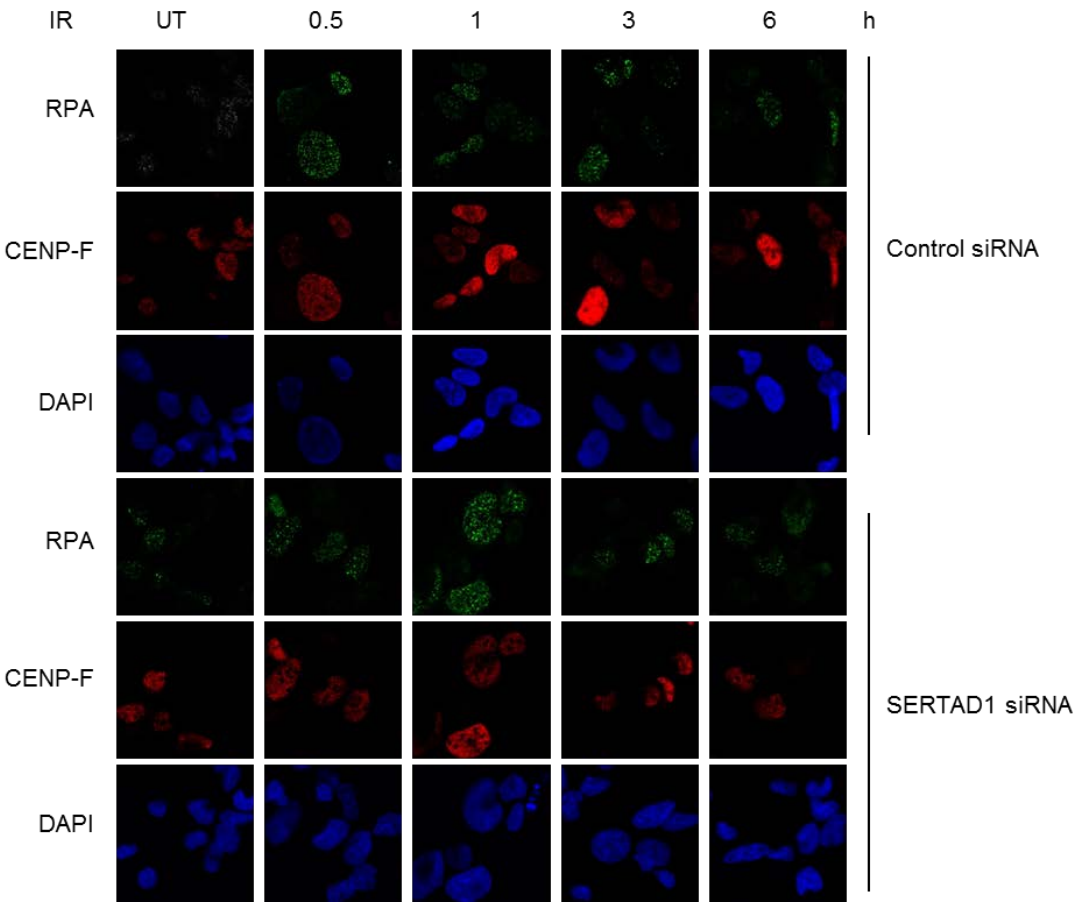

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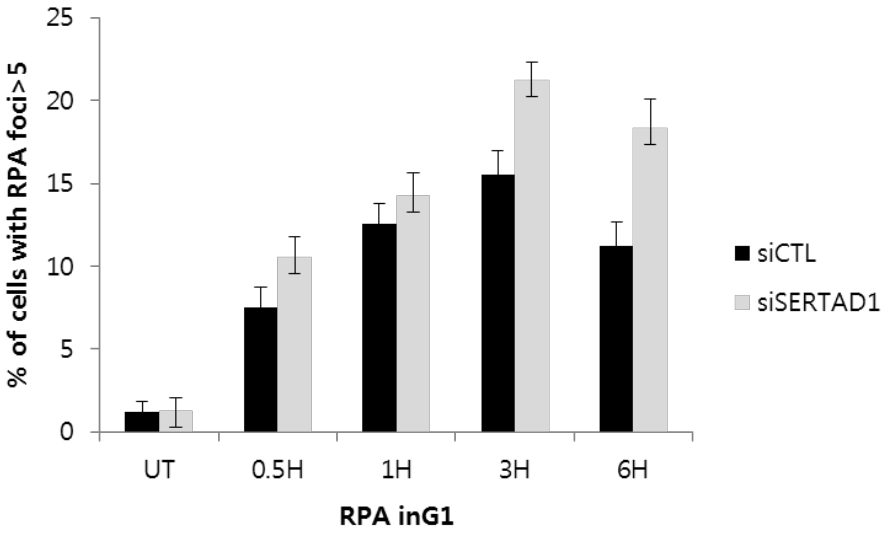

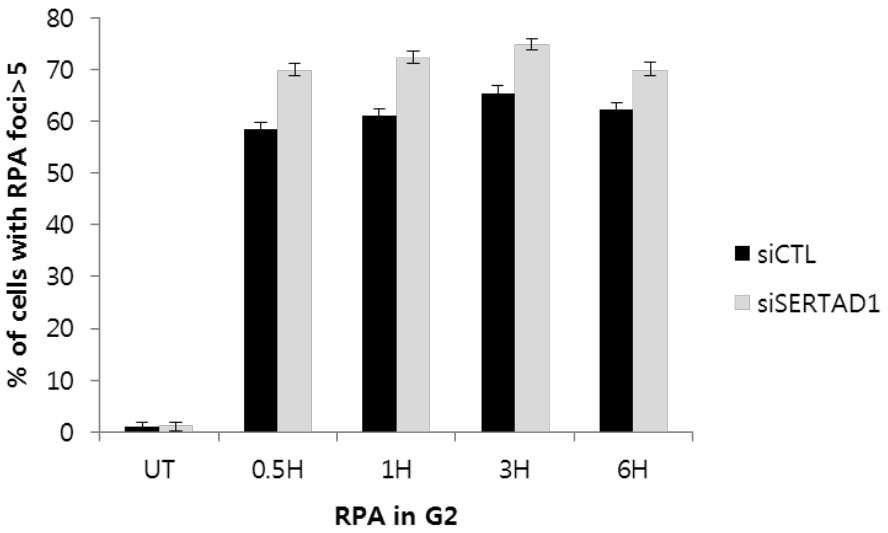

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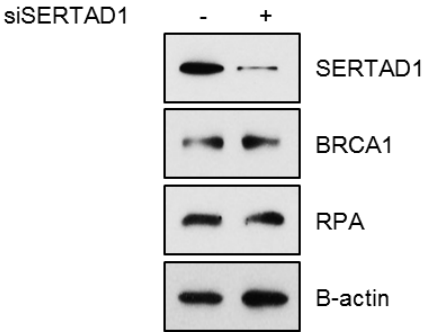


Figure 6. SERTAD1-depleted cells enhance BRCA1 and RPA foci formation of DSBs in G1 cells.

Control and SERTAD1-depleted HeLa cells were untreated or treated with 10Gy IR and fixed at the indicated times. Cells were stained with an anti-BRCA1 (A) and anti-RPA antibody (C). CENP-F was co-stained with as G2 phase marker. Nuclei were stained with DAPI (B, D). Representative images (Fig 4C) were quantified. At least 150 cells were counted each time points. Data are reported as mean ±SD (n=3). (E) Western blot analysis was carried out using specific antibodies against SERTAD1, BRCA1, RPA and β-actin.

7. Effect of SERTAD1 in DSB repair pathway.

Reporters for DSB repair by EJ5-, DR-, SA-GFP pathways.

DNA double-strand break (DSB) repair can take place through two major pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ has two types of end-joining systems are defined in the NHEJ: the major one is the conservative-NHEJ (C-NHEJ), which is principally connected with exact joining of DSB ends without altering the DNA sequence. The alternative pathway for NHEJ (Alt-NHEJ) is highly mutagenic since it catalyzes DNA resection and utilizes defective micro homology for end-joining partners and thus resulting in elimination at repair junctions. HR has a major pathway homology-directed repair (HDR) pathway, which is a comparatively exact form of repair and a minor sub pathway called single-strand annealing (SSA), which causes DNA resection until homology at repair junctions is revealed. The NHEJ and HR mediated DSB repair systems were

established to study the effect of SERTAD1 in DSB repair. The reporter system that was stably integrated in the GFP-based chromosomal reporter EJ5-GFP in the HeLa and U2OS cells was used to measure the total NHEJ repair efficiency. The DR-GFP in the U2OS cells and the SA-GFP in the HeLa and U2OS cells were used to measure the HR-mediated DSB repair efficiency. The majorities of these assays are fluorescence based and use the rare cutting endonuclease, I-SceI, to induce a single site specific DSB in cells. GFP-based chromosomal reporter assays in three stable cell lines, EJ5-GFP, SA-GFP and DR-GFP, were used to measure DSB repair.

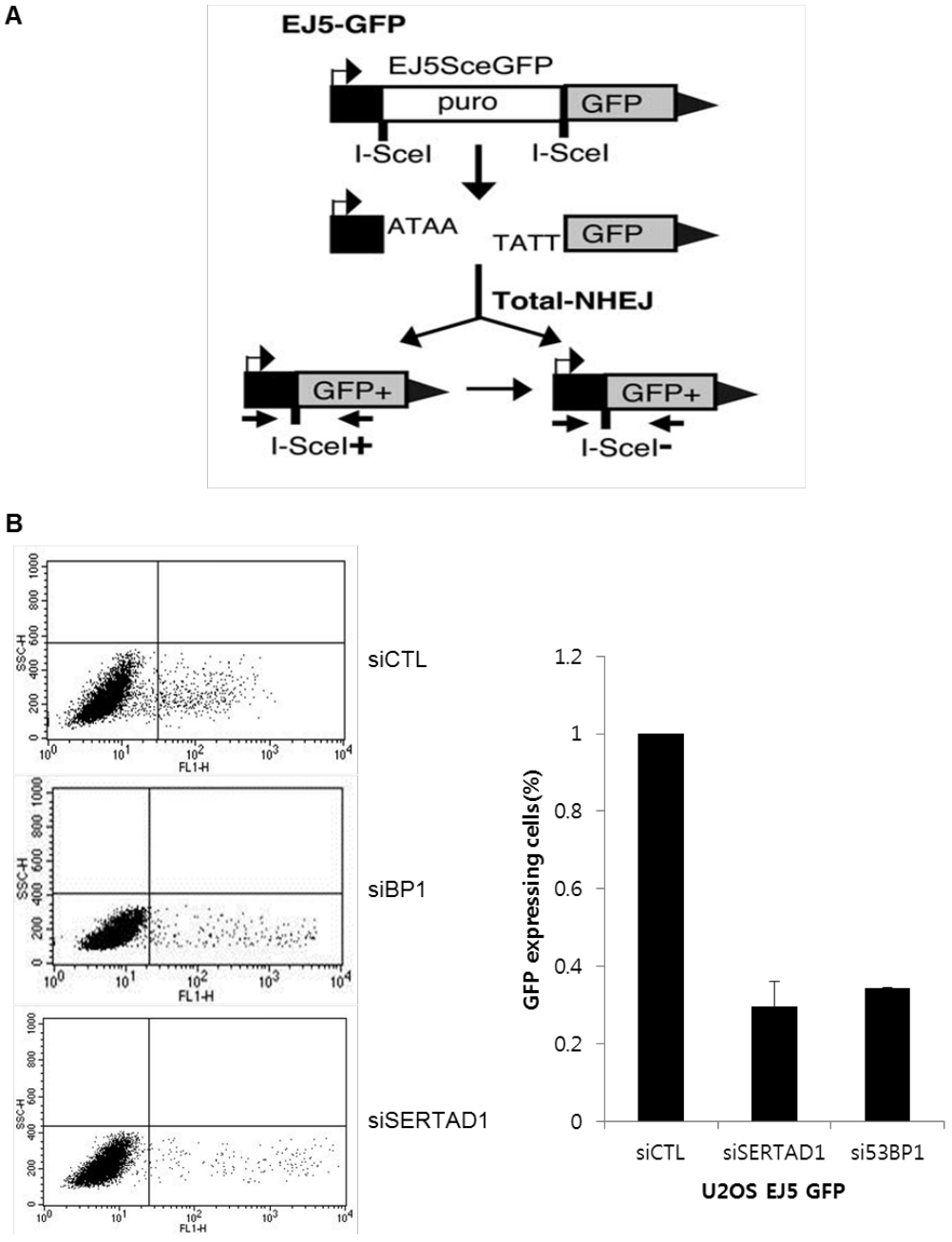
SERTAD1- depleted cells impaired NHEJ and improved HR.

To estimate the involvement of SERTAD1 in NHEJ-mediated repair, we used EJ5-GFP cells, which contain a promoter that is separated from a GFP coding cassette by a puro gene that is flanked by two I-SceI sites in the same orientation. Once the puromycin gene is flanked by the two I-SceI-induced

DSBs, the promoter is joined to the rest of the expression cassette by NHEJ repair, leading to restoration of the GFP+ gene. In this system, repair via NHEJ is monitor using flow cytometry to measure the percentage of cells expressing GFP (Fig 7A). We found that an SERTAD1 knockdown lowered NHEJ by 29%, which is comparable to the 34% reduction obtained upon depletion of 53BP1 in U2OS EJ5-GFP cells (Fig 7B). Similar results were obtained in HeLa EJ5-GFP cells (Fig 7C). Moreover, to prove the role of SERTAD1 in the HR, we performed HDR and SSA assay. In the HR reporters strain, DR-GFP was constructed using the homology-directed repair (HDR) product that uses intense GFP (iGFP) as the template for nascent DNA synthesis, which results in the restoration of a GFP expression cassette (Fig 8A). Notably, we found that in an SERTAD1 knockdown, the level of HR repair was increased to that observed when 53BP1 was depleted (Fig 8B). In addition, SA-GFP reporter consists of the *GFP* gene fragments *5′ GFP* and *SceGFP3′* , which have 266

bp of homology. Repair of the I-SceI-generated DSB in *SceGFP3′* by SSA results in a functional *GFP* gene when a DNA strand from *SceGFP3′* is annealed to the complementary strand of *5′ GFP*, followed by appropriate DNA-processing steps. As a result, SSA between the homologous sequences in the *GFP* gene fragments produces a 2.7-kb deletion in the chromosome. The SA-GFP reporter can also be repaired by HDR, but this repair does not restore a functional *GFP* gene [32-34]. These reporters, the presence of a functional *GFP* gene was scored in individual cells by green fluorescence using flow cytometric analysis. The analysis of SSA in hprtSA-GFP-U2OS cells showed a 1.5 fold increase in the SERTAD1 Knockdown cells compare with control cells (Fig 8D). Also, when SERTAD1 was depleted, we found that the percentage GFP-positive cells were 1.5 fold higher than control HeLa cells (Fig 8E). Taken together, these data identify SERTAD1 as a regulator of DNA repair pathway choice that promotes NHEJ and inhibits HR.

Figure 7



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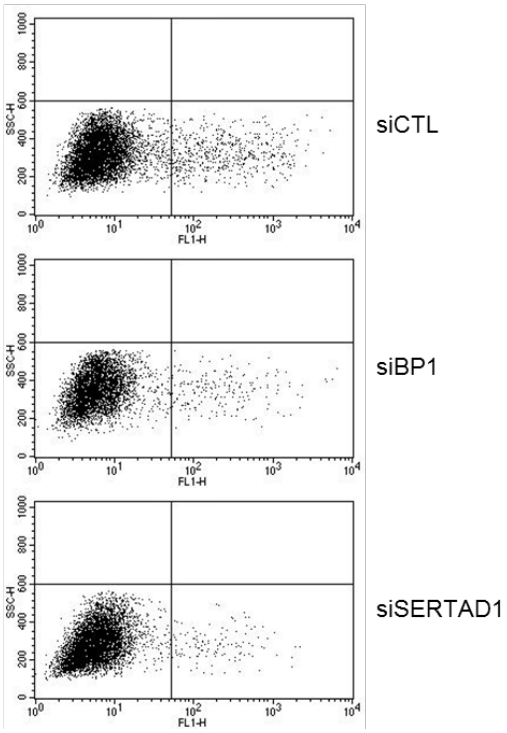


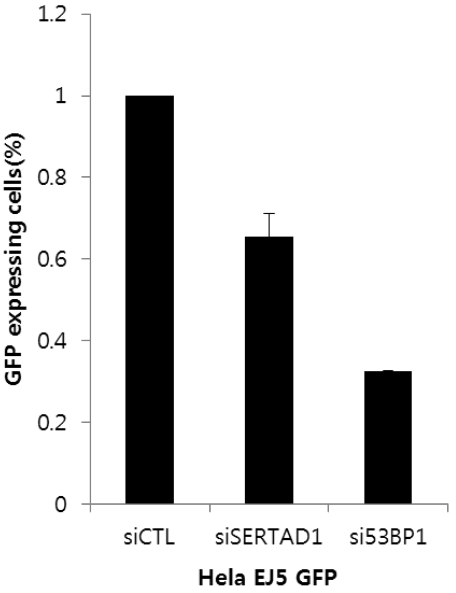
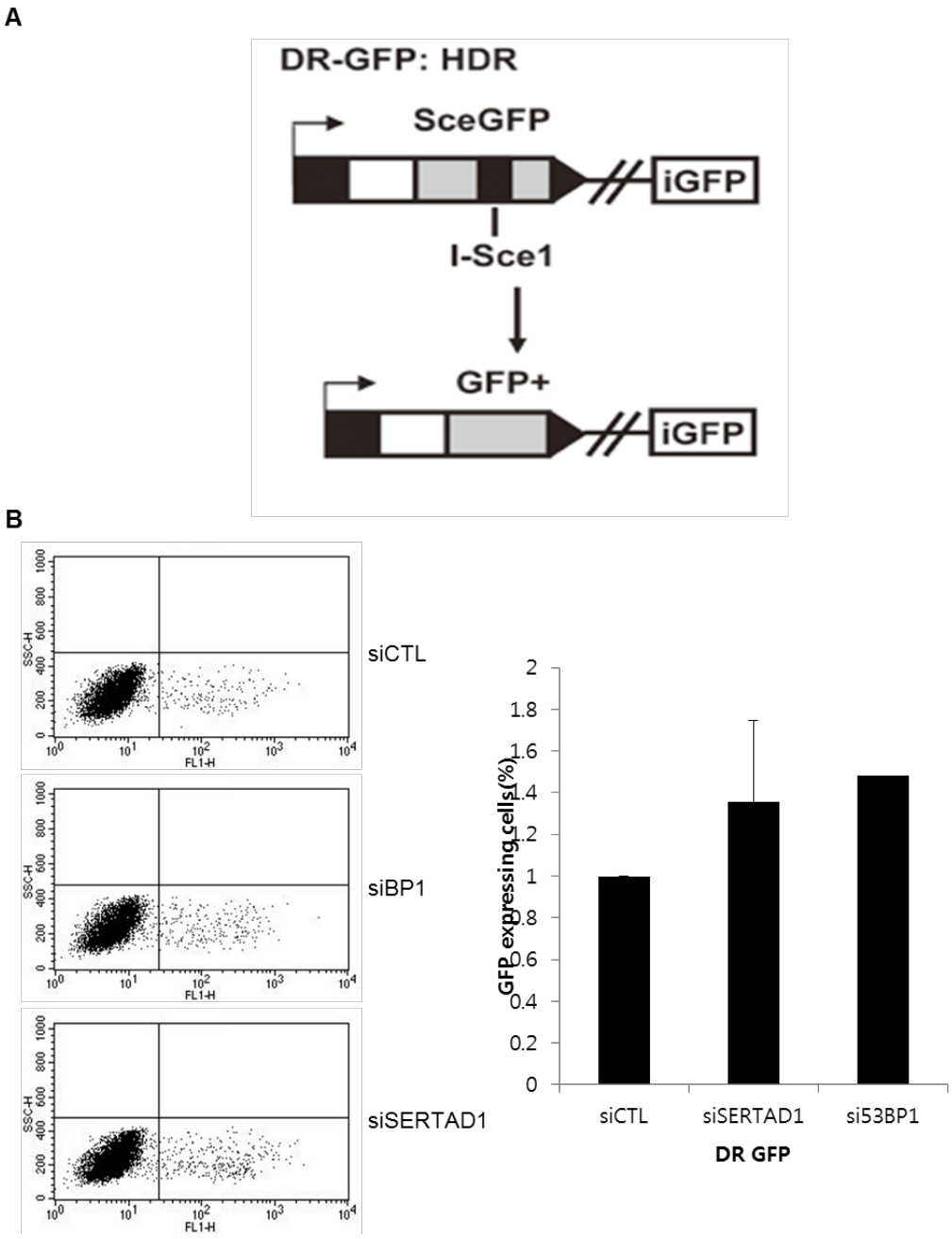


Figure 7. SERTAD1 depleted cells decrease on NHEJ activity.

(A) A diagram for the NHEJ assay based on the EJ5-GFP reporter, which contains two tandem endonuclease cut sites for the I-SceI. EJ5-GFP contains a promoter that is separated from a GFP coding cassette by a puro gene that is flanked by two I-SceI sites in the same orientation. Once the puromycin gene is excised by the two I-SceI-induced DSBs, the promoter is joined to the rest of the expression cassette by NHEJ repair, leading to restoration of the GFP+ gene. HeLa EJ5-GFP cells (B) or U2OS EJ5-GFP cells (C) were transfected with Control, 53BP1 and SERTAD1 siRNA for 4hours and then transfected with an I-SceI expression vector. After 48hours, the population of the GFP-positive cells was measured by flow cytometry (left panel). Quantification of cells with GFP expression in Control, 53BP1 and SERTAD1 siRNA cells. The percentage of GFP expressing cells determined (right panel).

Figure 8



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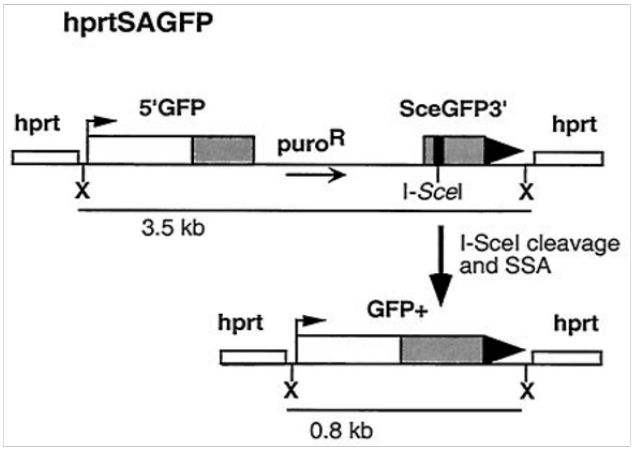

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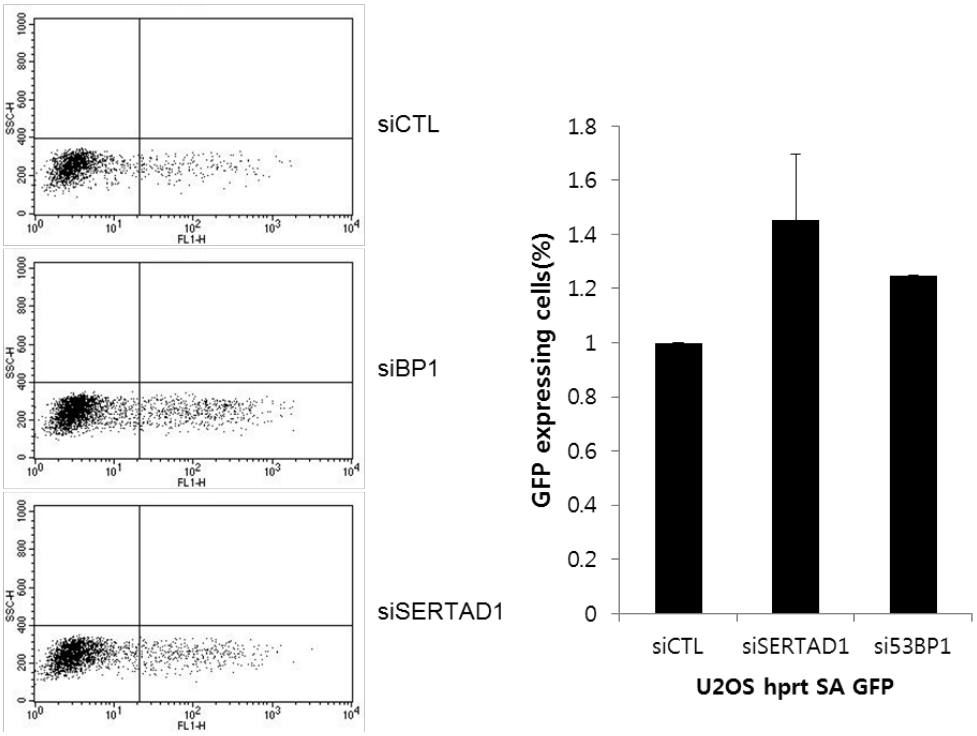

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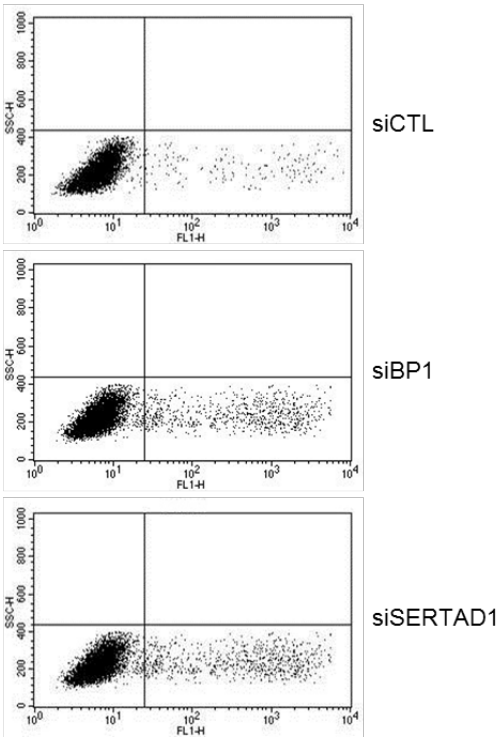

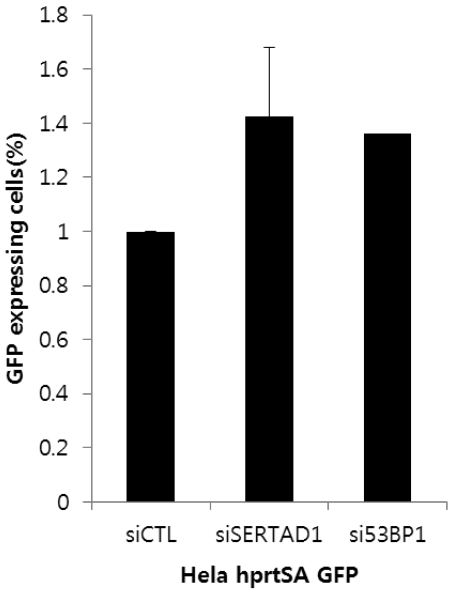

Figure 8. SERTAD1 depleted cells increase on HR and SSA activity.

Establishment of the hprtSA-GFP and DR-GFP systems. (A) DR-GFP is shown along with the HDR product that uses iGFP as the template for nascent DNA synthesis, which results in the restoration of a GFP expression cassette. (B) The efficiency of HR was measured in HeLa cells that contained DR-GFP and had been transfected with either Control, 53BP1 or SERTAD1 siRNA. When the DSB is repaired, the reporter construct will then express GFP that can measured by flow cytometry (left panel). Quantification of cells with GFP expression in Control, 53BP1 or SERTAD1 siRNA-transfected cells. The % of GFP expressing cells determined (right panel). Results are shown as means ±SD (n=3). (C) A diagram of the assay for measuring SSA repair using an hprtSA-GFP report is shown. SA-GFP is shown along with the SSA repair product that utilizes 266 nt of homology between the tandem GFP segments, thereby restoring a GFP expression cassette. The hprtSA-GFP-U2OS (E) and Hela (F) cells were trans-

fected with Control, 53BP1 and SERTAD1 siRNA for 4hours and then infected with an I-SceI expression vector. After 48hours, the population of the cells for GFP expression was measured by flow cytometry (left panel). Quantification of cells with GFP expression in Control, 53BP1 or SERTAD1 siRNA-transfected cells. The % of GFP expressing cells determined (right panel). Results are shown as means ±SD (n=3).

Figure 9

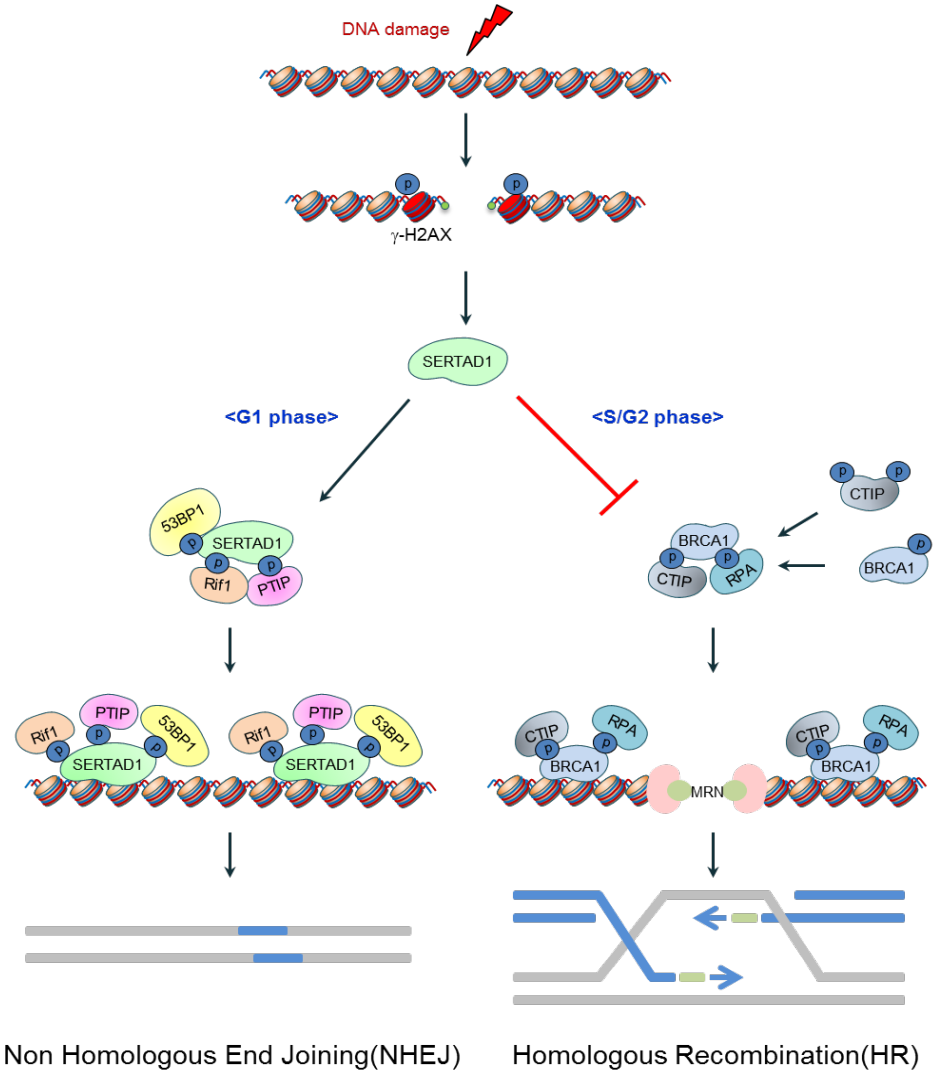

Figure 9. A schematic representation of the role of SERTAD1 in regulating the DSB repair is shown.

See Discussion for Details.

DISCUSSION

In compliance with DNA Double strand breaks, elements of DDR signaling drives two major pathways NHEJ and HR. NHEJ is an effective DSB repair mechanism that does not require excessive processing of the broken DNA ends and homology-directed base pairing with a DNA template and, is functional in all phases of cell cycle despite the risk for deleterious consequences. Contrastively, HR is limited to S/G2 phase of the cell cycle, as HR requires an undamaged tem-plate DNA strand for base pairing and repair synthesis[35].

53BP1 mediated NHEJ and BRCA1 mediated HR promotes the DSBs repair during different phages of the cell cycle. In compliance with DNA double strand break, ATM dependent phosphorylation of checkpoint signaling molecules pro-mote DNA double strand break repair by two major pathway, NHEJ (arise in G1 cells) and HR (arise in S/G2 cells). Due to deficiency of a sister chromatid, 5′ end resection is repressed and HR is inhibited in G1. BRCA1 promotes the break

resection. In BRCA1 depleted cells, Rif1 and PTIP elimination the continuous re-section required to rescue the HR. During S/G2 phase of the cell cycle, BRCA1 is recruited at the sites of DNA DSBs, and then promotes HR. CDK phosphorylation of CtIP facilitates its interaction with BRCA1 and it also binds to the MRN com-plex to promote the nucleolytic resection of the 5′ end to generate the homolo-gy ends required for HR-mediated DNA DSBs repair (Fig 9) [36].

In this study, we report that SERTAD1 is a novel regulator of DNA damage response identified by yeast two-hybrid screening. We showed SERTAD1 in-teracts 53BP1, Rif1 and PTIP in endogenous co-immunoprecipitation assay and association of SERTAD1 with 53BP1, Rif1 and PTIP are enhanced in response to DNA damage, such as IR. We also identified SERTAD1-depleted cells shown hypersensitive to IR induced less colony formation which is proved by a clonal survival assay, and we found that SERTAD1-depleted cells accumulate high level of persistent DSBs, as detected by formation of late γ-H2AX foci. These

studies suggest that SERTAD1 is required for DSB repair. We report that SERTAD1-depleted cells showed decrease of 53BP1, Rif1 and PTIP foci formation and increase of BRCA1 and RPA foci in G1 phases after γ-irradiation. In addition, SERTAD1 deficient cells also showed impaired NHEJ and improved HR after DSB (Figure 9).

Previously report showed the protein SERTAD1, also known as $p34^{SEI-1}$ or Trip-Br1, has been shown to indicate multiple biological functions. Sertad1 identified an antagonist of p16 INK4a that promotes the formation and activation of cyclin D-Cdk4 complexes. Furthermore studies revealed that it directly binds and activates Cdk4 in a concentration dependent manner [37]. Functions in addition to regulation of Cdk4 have been described for SERTAD1including stimulation of the transcriptional activities of p53 [38]. Also, SERTAD1 was reported to exhibit anti-apoptotic activity by stabilizing XIAP in cancer cells [39]. Here, we have identified SERTAD1 as a novel interaction partner of 53BP1 Rif1 and PTIP,

additionally SERTAD1 involved in DSB repair. In conclusion, our results illuminate SERTAD1 as a novel regulator of DNA repair pathway choice.

However, the exactly contribution of SERTAD1 to DNA damage response is not yet clear. Therefore, we must have done to further demonstrate with detailed mechanism of SERTAD1. Furthermore, effect of SERTAD1 in function of 53BP1 must be investigated.

ABSTRACT

SERTAD1 is a novel regulator of DNA damage response

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Tumor suppressor p53-binding protein 1 (53BP1) -plays a role in DNA damage response (DDR) to repair DNA damage, especially non-homologous end-joining repair. However, the detailed mechanism of 53BP1 in DDR remains elusive. Here, we report that SERTAD1 is a novel 53BP1-binding protein identified by yeast two-hybrid screening. We also show that SERTAD1 is interacted with Rif1 and PTIP, which is downstream protein of 53BP1 in DDR. Depletion of SERTAD1 results in cellular hypersensitivity and impaired DNA damage repair to

IR, as detected by clonal survival assay and late γ-H2AX foci staining. SERTAD1-depleted cells decrease the Rif1 and PTIP damage foci to IR, and increase BRCA1 and RPA damage foci in G1 cells. However, we show that a 53BP1 damage focus is similar in SERTAD1 absence or presence condition. Furthermore, depletion of SERTAD1 shows a decrease non-homologous end-joining (NH) and an increase single-strand annealing (SSA) and homologous recombination repair (HR). Thus, our combined results suggest that SERTAD1 is a novel regulator of 53BP1-mediated DNA damage repair.

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