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Effect of TMPO in the DNA double-strand break end resection and repair

조선대학교 대학원

의과학과

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TMPO에 의한 DNA 이중나선절단 절제 및 복구 조절기전 연구

김 혜 림

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의과학과

LAP2α(TMPO)는 염색질과 연관되어 있는 핵단백질로 대부분의 연구가 세포주기 조절에 치우쳐 있으며 DNA 손상 복구 조절에 대한 연구는 미흡한 실정이다. 따라서 본 연구에서 우리는 DNA 이중 가닥 손상 복구에 관여하는 LAP2α(TMPO)의 분자적 메커니즘에 대해 밝히고자 한다. 먼저 yeast two hybrid 분석을 통해 LAP2α가 상동재조합 관련 단백질 NBS1, 비상동말단결합 연관단백질 Rev7과 결합함을 확인하

고 LAP2α(TMPO)의DNA손상복구 연관성을 조사하였다. LAP2α(TMPO)의 기능 상실은 방사선조사에 의한 DNA 손상에 따른 세포 민감도를 증가시키고 DNA 손상 복구를 결핍 시킨다. 또한 세포 내 LAP2α(TMPO)의 결핍은 comet 분석과 γ-H2AX 염색을 통해 LAP2α(TMPO)의 감소가 DNA 손상 복구의 결함을 나타냄을 확인 하였다. LAP2α(TMPO) 의 결핍은 방사선조사에 의한 DNA 손상에 의한 MRE11 과 CtIP 손상foci 를 감소 시켰다. 이러한 연구결과를 종합해 볼 때 LAP2α(TMPO)는 NBS1 과의 결합을 통하여 상동 재조합 활성을 조절하는 단백질로서 DNA 말단 절제와 DNA 손상 반응에 관여하는 중요한 물질임을 제시한다. 다음으로 우리는 LAP2α(TMPO)와 REV7과의 관련성을 조사하였다. REV7은 DNA 절제를 억제하고 DNA 손상에 따른 복구를 조절하지만 명확한 기전이 알려져 있지 않다. REV7의 결핍은 5’ 절단 절제와 상동 재조합 매개 복구를 증가시킨다. REV7은 DNA 손상에 의한 MRE11, CtIP 그리고 EXO1의 foci 를 억제시킨다. 또한 REV7 이 결핍된 세포에서는 G1 단계에서 BRCA1 과 RPA foci 가 증가되는 것을 확일 할 수 있다. 우리의 연구 결과는 REV7 이 말단부 절제의 핵심 조절 인자임을 확인하였다. 따라서 본

연구 논문은 LAP2α(TMPO)가 NBS1과 REV7과의 결합을 통해 DNA손상복구와 DNA 말단부 절개를 조절하는 중요한 인자임을 시사한다.

INTRODUCTION

Double-strand breaks (DSBs) arise from many sources, including exposure to ionizing radiation, reactive oxygen species and collapse of replication forks[1]. Double-strand break (DSB) repair is essential for the maintenance of DNA integrity. Deregulation of this process leads to significant genetic instability, which can result in the development of cancer[2]. A conserved and intricate signaling network of responses to DSB damage is critical for the maintenance of genomic integrity.

DNA repair mechanisms differ at different stages of the cell cycle. Two distinct pathways, homologous recombination (HR) and non-homologous end joining (NHEJ), are responsible for repair of DSBs[3]. It has been established that HR precisely repairs the broken DNA ends by utilizing and intact sister chromatid as a template, whereas NHEJ directly re-ligates the DNA ends resulting in a poten-

tially error-prone repair[4]. DNA double strand break repair is a fascinating process that involves a plethora of proteins in a complex choreography of dynamic events in which the MRE11-RAD50-NBS1 (MRN) complex plays critical roles[5]. The MRE11/RAD50/NBS1 (MRN) complex is a major sensor of DNA double strand breaks (DSBs) that exerts essential roles in DNA repair processes and in the DNA damage response (DDR)[6]. The role of the MRN complex in HR repair is largely dependent on resection of DSBs that occurs. In the first step of HR repair, MRN complex and C-terminal binding protein (CtBP)-interacting protein (CtIP) recognize DNA breaks, resect single stranded DNA (ssDNA) together with BLM/Dna2 and Exo1, and thus generate a long stretch of 3′-overhanging ssDNA[7, 8]. Following DNA resection, replication protein A (RPA) proteins are recruited to the ssDNA to stabilize the structure, and mediator protein, including Rad51, Rad52 and BRCA2, promote the formation of Rad51 filaments[9]. Nijmegen breakage syndrome (NBS1) is one component of the MRE11-RAD50-NBS1 (MRN) complex,

which plays important roles in signal transduction related to DNA repair and cell cycle checkpoints[10]. NBS1 contains several functional domains, mainly in the N-terminus and the C-terminus. NBS1 includes a forkhead-associated (FHA) domain and BRCA1 C-terminus (BRCT) domains. The FHA and BRCT domains are widely conserved in eukaryotic nuclear proteins related to various cellular processes such as cell cycle checkpoint and DNA repair[11]. The C-terminus of NBS1 is essential for interaction domains MRE11, a homologous recombination repair nuclease, and ATM, a key player in signal transduction after the generation of DSBs, which is induced by IR. NBS1 regulates chromatin remodeling during DSB repair by histone H2B ubiquitination through binding to RNF20 at the C-terminus[12]. Furthermore, NBS1 is considered to be involved in the maintenance of genomic stability and the prevention of cancer development.

In this study, we found that NBS1 interacts with LAP2α (TMPO) by yeast-two hybrid screening. LAP2α (TMPO) is a member of the LAP2 family of nuclear

proteins and encoded by the LAP2 gene[13]. Most LAP2 isoforms and other LEM-domain proteins are integral proteins of the inner nuclear membrane and bind to the peripheral nuclear lamin network, which organizes chromatin and regulates gene expression[14]. The mammalian LAP2 (TMPO) gene, encodes six splice isoforms (α, β, γ, δ, ε, ζ), all sharing a common~180 aa long N-terminal domain including the LEM-motif and an additional LEM-like motif in the very N-terminus, which interacts with DNA directly[15]. The non-membrane-bound lamina associated polypeptide 2 isoform, LAP2α, forms nucleoskeletal structures with A-type lamins and interacts with chromosomes in a cell cycle dependent manner[16]. LAP2α (TMPO) specifically interacts with A-type lamins within the nuclear interior as part of a detergent/salt-resistant nucleoskeletal structure[17, 18]. LAP2α (TMPO) interacts with chromosomes via its α-specific C-terminal domain in a phosphorylation dependent manner[19]. LAP2α (TMPO) fragment containing the C terminus could either interfere with cell proliferation or cell viability,

or the stability of these fragments in the cells could be lower than those of the N-terminal fragment[20]. LAP2α (TMPO) associates with chromosomes very early during nuclear reassembly after sister chromatid separation[16]. LAP2α (TMPO) plays a role in maintaining nuclear structure, in nuclear assembly/disassembly, and in transcriptional regulation. LAP2α (TMPO) forms a stable complex with lamin-A/C in the nucleoplasm in interphase cells in order to maintain the cell in a proliferative state by controlling localization and phosphorylation of the retinoblastoma-associated protein[17, 21]. The nucleoplasmic lamin A/C- LAP2α (TMPO) complexes exist in G1 and early S-phase of proliferating cells but are absent during mitosis[22]. The lamin-A/C- LAP2α-BAF1 protein complex regulates mitotic spindle assembly and positioning[23]. LAP2α (TMPO) and BAF transiently localize to telomeres and specific regions on chromatin during nuclear assembly[24]. Our result indicated that LAP2α (TMPO) deficient cells were hypersensitive to IR induced colony formation and have a major DSB repair defect.

We show that LAP2α (TMPO) is essential for DNA resection by the MRN complex at DSBs, and is required for proper localization of the MRN complex to the DSBs. Here we identify LAP2α (TMPO) as a cofactor of the MRN complex required for efficient DNA end resection, recruitment of CtIP and RPA.

Recently studies uncovered a function of REV7 as a DNA resection inhibitor, limiting genomic repair by an unknown mechanism. We report here the first analysis of a previously uncharacterized LAP2α (TMPO)-interacting protein, REV7. REV7 was first identified as the small subunit of DNA polymerase ζ, an enzyme involved in the replication of damaged DNA[25]. REV7, a protein of 211 amino acids, has emerged as an important regulator of the cell cycle by acting as an APC/C inhibitor[26]. REV7 interacts with chromosome alignment maintaining phosphoprotein, a kinetochore microtubule attachment protein in mitotic cells[27]. REV7 is involved in translesion DNA synthesis, one of the damage tolerance processes, which completes DNA synthesis through DNA lesions to prevent DNA

damage induced cell death. REV7 plays a crucial role in proper mitotic progression and faithful DNA replication[28, 29]. REV7 is required for anaphase promoting complex dependent ubiquitination and degradation of translesion DNA polymerase REV1[30]. REV7 is essential for DNA damage tolerance via two REV3L binding sites in mammalian DNA polymerase ζ [31]. REV7 is recruited to DSBs in a manner dependent on the H2AX-MDC1-RNF8-53BP1 chromatin pathway, and seems to block HR and promote end joining in addition to its regulatory role in DNA damage tolerance[32]. Like 53BP1 together with RIF1 and PTIP, REV7 function as a NHEJ factor that performs a regulatory role in DSB repair pathway choice[33]. REV7 accumulates at uncapped telomeres and promotes NHEJ mediated fusion of depro-tected chromosome ends and genomic instability. REV7 depletion causes elongated 3′ telomeric overhangs, indicating that REV7 inhibits 5′ end resection[34]. REV7 is recruited to DSBs in H4K20 dimethylated chromatin by forming a protein com-plex with 53BP1 and RIF1 and that REV7, similar to 53BP1 and RIF1, suppresses

DSB accumulation of BRCA1[35]. REV7 acts downstream of 53BP1/RIF1 in a REV1/REV3 independent manner to promote NHEJ by inhibiting resection, mostly in G1. In addition, REV7, as part of Pol ζ, might promote HDR by facilitating replication of damaged DNA formed after resection in G2[34].

In this study, we found that LAP2α (TMPO) interact with REV7 by immunoprecipitation. Similar to 53BP1 and RIF1, REV7 is needed to inhibit DNA 5′end resection.

MATERIALS AND METHODS

1. Cell culture

The human osteosarcoma bone morphogenetic cell line U2OS, human cervix adenocarcinoma cell line HeLa, ER-AsiSI U2OS cells were cultured in Dulbecco's modified Eagles' s medium. In all cases, the media was supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 unit/ml), and streptomycin sulfate (100mg/ml). All cells were maintained in a humidified incubator containing 5% CO2 at 37℃. Upon reaching 80-90% confluency, cells were digested with 0.5% trypsin-EDTA before being passaged. Cells in exponential growth were harvested for subsequent experiments.

2. Clonal survival assay

After treatment with IR, 5 × 10^2 cells were immediately seeded onto a 60mm

dish in duplicate and grown for 2-3 weeks at 37℃ to allow colony formation. Col-onies were fixed with 95% methanol for 10min and stained with 2% methylene blue in 50% ethanol and counted. the fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated dells to untreated cells,

3. comet assay

DSB repair was assayed by alkaline single-cell agarose-gel electrophoresis as described previously. HeLa cells, control and siLAP2α cells were treated with 10Gy OF IR followed by incubation in culture medium at 37℃. Cells were then harvested, mixed with low-melting temperature agarose, and layered onto aga-rose-coated glass slides. Slide were submerged in lysis solution 10mM Tris-HCl(pH 10), 2.5M NACL, 01M EDTA, 1% Triton X-100, 10% dimethyl sulfoxide incubated for 30min in alkaline electrophoresis solution (300mM NaOH, 200mM EDTA at pH>13). The electrophoresis ran for 30min (1V/cm tank length), after

which the slides were and fixed in 70% ethanol for 5 min, air-dried and neutralized slides were stained with 30-50 μl ethidium bromide (20 mg/ml). Cells were screened per sample in a fluorescent microscope. Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system (Komet 5.5; Andor Thechnology, South Windsor, CT, USA)

4. Immunoprecipitation assay and Western blot

Cells were lysed in RIPA buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors (Roche Diagnostic Corp.). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hr 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° C with primary antibodies(1:1000).

The blots were washed four times for 15 min with 0.1% Tween 20 containing TBS-t and then incubated for 1 hr with peroxidase-conjugated secondary anti-bodies (1:4000). The membranes were washed four more times and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

For the immunoprecipitation assays, aliquots of soluble cell lysates were pre-cleared with protein A/G plus-agarose beads (Santa Cruz Biotechnology), G se-pharose and A sepharose (GE Healthcare) as indicated and then incubated at 4℃ for 4h. Next, the appropriate antibody was added, and incubated at 4℃ for 12h. After the addition of fresh protein A/G plus-agarose bead, G sepharose and A sepharose, the reaction was incubated overnight at 4℃ with rotation. The beads were washed three times in RIPA buffer without protease inhibitors, resuspended in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodie

5. Chromatin Fractionation

Cells were first lysed with buffer A (150mM NaCl, 50mM Hepes pH7.5, 1mM EDTA, 0.1% Triton x-100, protease inhibitor, phosphatase inhibitor) on ice for 15min. Following centrifugation at 14,000g for 3min, the supernatant was collected, and the pellet was further extracted for 10min on ice with 200ul of fresh lysis buffer. The pellet was further incubated in 200ul of extraction buffer without Triton but supplemented with 200ug/ml RNaseA (sigma) for 30min at 25° C. Following centrifugation at 14,000g for 3min, the supernatant was separated from the pellet. When necessary, the pellet was incubated for 20min at 25° C in the micrococcal nuclease buffer. Following centrifugation at 15000 rpm for 30 min, the supernatant was separated from the pellet.

6. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were ir-radiated with 5Gy of ionizing radiation. cells were then washed twice with PBS, fixed with 4% paraformaldehyde for 15 min and ice-cold 100% methanol for 5 min, followed by permeabilization with 0.3% Triton X-100 for 15 min at room temper-ature. Next, the cover slips were washed three times with PBS and then blocked with 5% BSA in PBS for 1hr. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4° C. Next, the cells were washed with PBS and then stained with 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 Zeiss Axioplan 2 imagingepifluorescent microscope equipped with a charge-coupled device camera and ISIS software (MetaSystems, Altlussheim, Germany).

7. DR-GFP assay (HR assay)

To measure the HR repair, stable cells 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, LAP2α siRNA and REV7 siRNA, after 4hrs transfected with 1 μg of I-SceI-expressing vector. After 48hrs, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SceI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

8. Non Homologous end joining assay

To measure the NHEJ repair, stable cells lines expressing HeLa EJ5-GFP reports were generated by transfection using turbofectamine. EJ5-GFP contains

a promoter that is separated from a GFP coding region by puromycine resistance gene, which is flanked by two I-SceI sites that are in the same orientation. When the I-SceI 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 48hours, that percentage of GFP-positive cells which had repaired the DSBs generated by I-SceI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

9. End resection assay

In vivo 5′ end resection was measured in AID-DIVA U2OS cells (from Dr. Gaelle Legube). AID-DIVA U2OS cells were transfected with Control, LAP2α siRNA, 53BP1 siRNA, CtIP siRNA and REV7 siRNA, 24hrs later, cells were treated with 700nM 4-OHT (4-Hydroxytamoxifen) for 4hrs, and then processed for the

resection assay. Genomic DNA was isolated using kit (Bio Rad). Three ug of genomic DNA sample was digested or mock digested with 20units of restriction enzymes (BsrGI, BamHI or HindIII : New England Biolabs) at 37° C overnight. This assay is based on the assumption that ssDNA grnerated by resection events surrounding the DSB site is resistant to digestion by restriction enzymes that by definition require duplex DNA. To quantify the extent of resection, the availability of selected DNA sites. Three microliters of digested or mock digested samples (~20ng) were used as templates in 25ul of qPCR reaction. For each sample, a △Ct was calculated by subtracting the Ct value of the mock-digested sample from the Ct value of the digested sample. The ssDNA% was calculated with the following equation : ssDNA% = 1/(2^(△Ct-1)+0.5)*100.

RESULT

1. LAP2α interacts with NBS1

The MRN complex binds to DSB ends and plays important roles in initiating HR mediated DSB repair[36]. We examined the interaction between LAP2α and members of the MRN complex and found that endogenous LAP2α co-immuno-precipiates with NBS1 and MRE11 (Fig 1A). For production of DSBs, HeLa cells were treated with 5Gy of IR. The cells were then lysed, and endogenous LAP2α was immunoprecipitated with a LAP2α-specific antibody. Immunoprecipitates were subjected to western blotting with an anti-NBS1 and anti-MRE11 antibodies. In this reciprocal experiments, NBS1 antibody was able to co-immunoprecipitate LAP2α, suggesting that these proteins may interact with each other directly in cells. As control, normal rabbit IgG did not co-immunoprecipitate NBS1 or LAP2α, indicating that the co-immunoprecipitation of NBS1 with LAP2α was not due to non-specific antibody binding (Fig 1A). To further confirm this interaction, HeLa

cells were transiently transfected with an expression constructs encoding full length NBS1 tagged with Flag and a second construct that expressed full length LAP2α tagged with HA. These results showed us interaction between Flag-NBS1 and HA- LAP2α might be direct exogenously (Fig 1B).

2. Deletion of LAP2α leads to decrease DNA double strand break (DSBs) repair

To determine whether LAP2α is involved in the DNA damage response, we created the LAP2α knockdown cells using siRNA of LAP2α. Three candidate siRNA were further screened for knockdown effect of LAP2α expression in HeLa cells after transiently transfecting each siRNA. Western blot analysis showed that LAP2α siRNA #1 and #2 work efficiently, we used LAP2α siRNA#1 to next ex-periment (Fig. 2A).

We analyzed control siRNA and LAP2α siRNA expressing cells for their responses to DNA-damaging reagents. Control and LAP2α knockdown HeLa cells were treated with 1Gy, 2Gy and 5Gy IR. LAP2α-depleted cells showed significantly compromised clonogenic cell survival after exposure to IR. LAP2α knockdown cells formed less colony numbers compare to negative control cells. These results indicated that LAP2α knockdown cells showed increased sensitivity to DNA damage (Fig 2B).

The γ-H2AX is often used as a marker for exploring the spatial distribution and the DNA repair kinetics of cells following ionizing radiation exposure[37]. At DSB sites, γ-H2AX foci persist if DSBs are not repaired[38]. We measured the number of DSBs remaining after IR exposure as an indicator of now much of the damaged DNA remained unrepaired. The formation γ-H2AX foci was analyzed by immunofluorescence microscopy. As shown in Figure 3A, the depletion of LAP2α with siRNA led to increased level of unrepaired DSBs 16 h, 24h after treatment

with IR, as evidenced by the number of γ-H2AX foci remaining. These results suggest that LAP2α is required for DSB repair.

Further evidence of an overt repair defect was shown using the comet assay, which monitors the presence of DSBs in single cells. This very sensitive method can be used to detect low levels of DNA breaks IR treatment induces DSBs, visible as increased DNA mobility or 'comet tails'[39]. For this, control and both LAP2α knockdown siRNA HeLa cells were treated 10Gy IR to make DSB and harvested in different time points. In the initial time point's tail movement will be similar between control and LAP2α knockdown cells. But time persist, the DSB repair was measured based on tail movement. The unrepaired DNA strand breaks per-centage of control cells decreased in different time (1, 3 h) whereas LAP2α knockdown cells showed long comet tails. The observation of long comet tails in LAP2α deficient cells indicates impaired DNA DSB repair (Fig 3B). The direct association between NBS1 and LAP2α suggested the involvement of the LAP2α

in the DSB repair. Together, these result suggest that LAP2α is critical for its function in DSB repair.

3. LAP2α promotes Homologous recombination repair

DSBs in which both strands in the double helix are severed, are the most dangerous type of DNA lesion[40]. DSBs are repaired by two major mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR). Plasmid rejoining assays in transfected cells and protein extracts were used initially, and they have yielded enormous insights into DSB repair mechanisms. The majority of these assays are fluorescence based and use the rare cutting endonuclease, I-SceI, to induce a single site specific DSB in cells. DR-GFP-HeLa and EJ5-GFP-HeLa cells were used for measurement of HR and NHEJ, respectively.

To investigate the biological significance of the LAP2α/NBS1 interaction, we will assess the role of LAP2α in homologous recombination. To demonstrate the

role of LAP2α in the HR, we determined whether depletion of LAP2α is related with HR. In the HR reporter strain, DR-GFP, DSBs are generated through the expression of I-SceI endonuclease, which cleaves a specific recognition site located in the GFP gene. In this system, repair efficiency via HR is monitored by measuring the percentage of cells expressing GFP using flow cytometry (Fig 4A). We examined the levels of HR when LAP2α was depleted and found that the percentage of GFP-positive cells was ~2 fold lower than in control cells (Fig 4B). We found that LAP2α depletion led to decreased HR frequency to a level similar to that achieved by depleting the key HR factor NBS1. Knockdown of LAP2α did cause additional reduction of HR in NBS1-depleted cells (Figure 4B), suggesting that LAP2α regulates HR repair through NBS1-dependent and independent manner.

To assess the involvement of LAP2α in NHEJ-mediated repair, we used EJ5-GFP-HeLa cells, which contain a promoter region separated from a GFP

coding cassette by a puromycin (puro) gene flanked by two similarly oriented I-SceI site. Transfection of cells with I-SceI encoding constructs leads to the excision of the puro gene, and restoration of GFP expression relies on efficient NHEJ repair (Fig 5A). The analysis of NHEJ in EJ5-GFP-HeLa cells showed no difference in the LAP2α knockdown cells compare with control cells (Fig 5B).

We show that HR activity decreased significantly in the LAP2α-depleted cells in comparison to control cells, but NHEJ activity not decreased. Taken together, these results indicate that LAP2α contributes to DSB repair through regulation of HR but not contributes to NHEJ.

4. LAP2α depletion suppresses recruitment of DNA end resection related proteins to DSBs

Repair of DSBs by homologous recombination requires 5′-3′ resection of the DSB ends[41]. The end resection is essential for HR repair because the resulting

3′-ended single-strand DNA (ssDNA) invades a homologous template to initiate HR repair[42]. It is known that following DSB generation, the highly conserved MRN protein complex consisting of MRE11, Rad50, and NBS1 is the early group of proteins recruited to DSB ends in a process dependent on Rad17 and MDC1[43, 44]. The MRN complex, and the nuclear protein CtIP have been suggested to operate together in the DNA end resection and DNA damage checkpoint activation[45]. NBS1 is a key component of the MRN complex. The above results, pointing to a biochemical interaction between LAP2α and NBS1, prompted the prediction that LAP2α is required for recruitment of NBS1 to DSBs. To test this hypothesis, we exposed control and LAP2α-depleted U2OS cells to IR, and used immunofluorescence staining to detect NBS1. Aa shown (Fig 6A) in control and LAP2α siRNA transfected cells, NBS1 foci formed after IR. LAP2α-depleted cells formed less foci numbers of NBS1 compare to control cells. Consistent with the knowledge the NBS1 is necessary for recruitment of MRE11 and RAD50 to the

DSB site. We also found that percentage of the cells positive for MRE11 foci were significantly reduced in LAP2α-depleted cells compared with control cells (Fig 6B), confirming that LAP2α is important for recruitment of the MRN complex to DSBs. MDC1 is required for the retention of additional DDR proteins and is thus considered to be an upstream regulator of this process. MDC1 is another binding partner NBS1. We predicted that depletion of LAP2α might also affect the localization of several DDR factors to nuclear DSB sites. We found that depletion of LAP2α severely reduced recruitment of MDC1 to IR-induced DNA damage foci (Fig 6C). LAP2α is required for localization of MDC1 at DSBs. CtIP is essential for efficient DNA end processing during DSB repair, we checked CtIP foci formation in LAP2α knockdown cells. As expected, depletion of LAP2α dramatically inhibited CtIP foci formation (Fig 6D). These result indicate that LAP2α is a putative component of the resection machinery required for the efficient processing of DSBs.

5. LAP2α promotes DNA end resection during HR dependent DSB repair

AID-DIVA U2OS cells have been used to directly quantify ssDNA generated by 5′ end resection at two AsiSI-induced DSBs. With addition of 4-OHT, the AsiSI endonuclease fused to an estrogen receptor ligand binding domain translocates from the cytoplasm to the nucleus to induce DSBs[46]. The formation of ssDNA makes BsrGI sites in the adjoining DNA resistant to digestion by that enzyme, thus allowing us to measure whether the end resection complex has digested past each of the three BsrGI site. DSB induced by 4-OHT treatment resulted in more ssDNA at the BsrGI site closer to AsiSI cut site[47]. Consistent with a previous report, we observed notably lower ssDNA in cells treated with CtIP siRNA because CtIP stimulates DNA end resection[48]. To investigate the role of LAP2α in 5′ end resection, ssDNA content was measured in LAP2α siRNA transfected AID-DIVA U2OS cells. The amount of ssDNA generated at position from DSB1 was lower

than that in control siRNA treated cells, and to the reduction caused by CtIP depletion (Fig 7A). 'No DSB' was used as a negative control. Reconstitution of LAP2α-depleted cells with a LAP2α siRNA resistant-LAP2α expression vector rescued amount of ssDNA in LAP2α depleted cells.

The abundance of phosphorylated PRA32 on serine 4 and 8, a maker of ssDNA bound RPA, knockdown of LAP2α resulted in significantly decreased PRA Ser4/8 phosphorylation (Fig 7B). LAP2α-depleted cells upon IR correlated with the reduction of phosphorylation of NBS1 (Fig 7B). HR is initiated by DNA end resection, which generates 3′ ssDNA tails that are coated by RPA. Subsequently, RAD51 displaces RPA-ssDNA complex to form a helical nucleoprotein filament. To test this, we analyzed RPA and RAD51 focus formation in response to IR in LAP2α depleted cells. We observed that LAP2α depletion resulted in decreased RPA recruitment and RAD51 loading to DSBs(Fig 7 C and D).

6. REV7 interacts with LAP2α

The REV7 is known to cooperate functionally with 53BP1 to limit resection at DNA breaks, REV7 was not detected in 53BP1 immunocomplexes, and it is unknown how REV7 connects with 53BP1 *in vivo*[49]. However, the 53BP1 is required for the recruitment of REV7 at DSBs. Recently, multiple groups reported the function of Rev7 in the DNA end resection. Interestingly, we searched for novel interacting partner of Rev7 by yeast two-hybrid screenings using human Rev7 as a bait and identified LAP2, a new Rev7 interacting protein (data not shown).

First, we examined the interaction between LAP2α and REV7, found that endogenous LAP2α co-immunoprecipiates. (Fig 8A). For production of DSBs, HeLa cells were treated with 5Gy of IR. The cells were then lysed, and endogenous LAP2α was immunoprecipitated with a LAP2α-specific antibody. Immunoprecipitates were subjected to western blotting with an anti-REV7 antibody. In this reciprocal experiments, REV7 antibody was able to co-immunoprecipitate LAP2α,

suggesting that these proteins may interact with each other directly in cells. As control, normal rabbit IgG did not co-immunoprecipitate REV7 or LAP2α, indicating that the co-immunoprecipitation of REV7 with LAP2α was not due to non-specific antibody binding (Fig 8).

In the previous results, LAP2α is involved in the repair of DSBs through a direct interaction with NBS1. The association between Rev7 and LAP2α suggested the involvement of the Rev7 in LAP2-related DSB repair. To investigated the biological significance of the LAP2α/REV7 interaction, we further assessed the role of LAP2α in HR.

7. Knockdown of REV7 is hypersensitive to DNA damage

To determine whether REV7 is involved in the DNA damage response, we created the REV7 depleted cells using siRNA of REV7. Two candidate siRNA were further screened for knockdown effect of REV7 expression in HeLa cells after

transiently transfecting each siRNA. Western blot analysis showed that REV7 siRNA #1 and #2 sequence reduced rev7 expression at the protein levels (Fig. 9A). Next, to investigate the potential role of in DDR, we hypothesize Rev7-depleted cells would be more sensitive to DNA damage. Control and Rev7 siRNA transfected HeLa cells were treated with indicated does of IR, and then monitored for 14 days. REV7-depleted cells showed significantly less colony numbers compare to negative control cells. These results indicated that REV7 knockdown cells showed increased sensitivity to DNA damage (Fig 9A).

8. Efficiency of DSB repair in REV7 depleted cells

DSBs can be repaired by either HR or NHEJ. We examined whether REV7 affects the repair of DSB by HR or NHEJ using cell lines bearing DR-GFP or EJ5-GFP reporter cassettes, respectively. The majority of these assays are fluorescence based and use the rare cutting endonuclease, I-SceI, to induce a single site

specific DSB in cells. To demonstrate the role of REV7 in the HR, we determined whether depletion of REV7 is related with HR. In the HR reporter strain, DR-GFP, DSBs are generated through the expression of I-SceI endonuclease, which cleaves a specific recognition site located in the GFP gene. In this system, repair efficiency via HR is monitored by measuring the percentage of cells expressing GFP using flow cytometry. We examined the levels of HR when REV7 was depleted and found that the percentage of GFP-positive cells was ~2 fold higher than in control cells (Fig 10B).

Next, to assess the involvement of REV7 in NHEJ-mediated repair, we used EJ5-GFP-HeLa cells, which contain a promoter region separated from a GFP coding cassette by a puromycin (puro) gene flanked by two similarly oriented I-SceI site. Transfection of cells with I-SceI encoding constructs leads to the excision of the puro gene, and restoration of GFP expression relies on efficient NHEJ repair. The analysis of NHEJ in EJ5-GFP-HeLa cells showed decrease in the

REV7 knockdown cells compare with control cells (Fig 10C). We show that HR activity was increased significantly in the REV7-depleted cells in comparison to control cells, but NHEJ activity decreased. Taken together, these data identify REV7 as a regulator of DNA repair pathway choice that promotes NHEJ and inhibits HR (Fig 10B and C).

9. REV7 is required for the recruitment of end resection related protein to DSB

DNA end resection is orchestrated by several nucleases. The MRE11 subunit possesses the catalytic function of MRN complex in resection and has both 5′-flap endonuclease activity and 3′-5′ exonuclease activity[50]. MRE11 endonuclease activity initiates resection and the exonuclease activities of MRE11, EXD2, and EXO1/BLM digest DNA for extensive resection[51]. We found that percentage of the cells positive for MRE11 foci were significantly increased in REV7-depleted

cells compared with control cells (Fig 11A). These results suggest that REV7 inhibits DNA 5' end resection through regulation of Mre11 foci formation. CtIP is required for catalyzation of 5' end resection at DSBs following MRN recruitment. CtIP is also essential for efficient DNA end processing during DSB repair. Next, we checked CtIP foci formation in REV7 knockdown cells. As expected, REV7 depletion resulted in enhanced CtIP foci formation (Fig 11B). REV7 depleted cells had increased levels of EXO1 focus formation compared with the control cells (Fig 11C). These results indicate that REV7 suppresses the recruitment of MRE11, CtIP and EXO1 to DSBs, with potential inhibitory effects on DNA end resection.

10. REV7 inhibits DNA end resection and the generation of ssDNA

CtIP in association with MRN regulates the resection during HR, and thus we quantified 5′ end resection using AID-DIVA U2OS cells. To quantitate ssDNA at sites of DSBs, we used the ER-AsiSI system in which the restriction enzyme AsiSI

is fused to the estrogen receptor hormone binding domain. The AsiSI enzyme can be induced to enter the nucleus and generate DSBs at sequence specific sites (5′-GCGATCGC-3′) on treatment of the cells with 4-OHT[52]. The ssDNA% generated by resection at various sites was measured by qPCR as described. Quantitative PCR (qPCR) primers were designed across BsrGI or BamHI restriction sites at various distances from each AsiSI site. Consistent with a previous report, 53BP1 knockdown increased the resection whereas CtIP knockdown reduced the resection.

To investigate the role of REV7 in 5′ end resection, ssDNA content was measured in REV7#1 and #2 siRNA transfected AID-DIVA U2OS cells. REV7 knockdown increased DNA resection by 335, 1618, 3500nt, respectively from the DSB1. The amount of ssDNA generated at position from DSB2 was higher than that in control siRNA treated cells (Fig 12A). The abundance of phosphorylated PRA32 on serine 4 and 8, a maker of ssDNA bound RPA, knockdown of REV7 resulted in

significantly increased PRA Ser4/8 phosphorylation (Fig 12B). Together these results suggest that REV7 protects the DSBs by limiting resection. HR is initiated by DNA end resection, which generates 3′ ssDNA tails that are coated by RPA. Accumulation of the single strand binding protein, RPA, was used as a marker for the generation of ssDNA. Subsequently, RAD51 displaces RPA-ssDNA complex to form a helical nucleoprotein filament. Then, RPA facilitates the recruitment of RAD51 to initiate homologous DNA recombination and complete the homologous recombination repair process. To test this phenomenon, we examined RPA and RAD51 focus formation in response to IR in REV7-depleted cells. We found that REV7 depletion significantly increased foci formation by RPA and RAD51 (Fig 12 C and D). Moreover, subcellular fractionation analysis showed that following DSB induction, the abundance of HR-related proteins in the chromatin fraction is mark-edly increased as a result of REV7 knockdown. Together these results suggest that REV7 protects the DSBs by limiting resection.

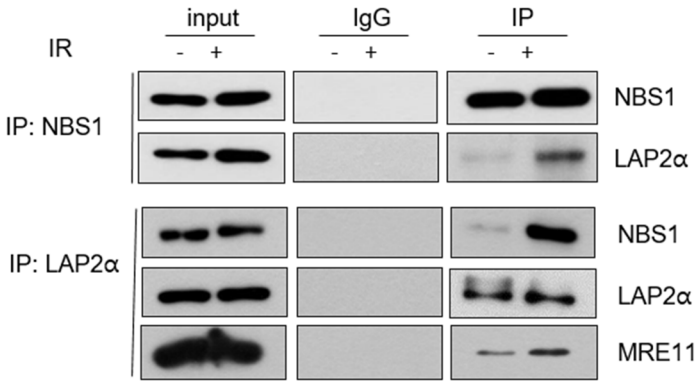
11. REV7-depleted cells enhance BRCA1 and RPA foci formation in G1 phase cells

BRCA1 plays multiple roles that include controlling DNA repair, signaling, chromatin organization, and transcription. Among these functions, its role in HR is critically important for maintaining genomic stability and suppressing tumorigen-esis[53]. BRCA1 is mostly absent in the G0/G1 phase 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. BRCA1 promotes HR by activating DNA end resection. RPA is the main eukaryotic ssDNA binding protein that is essential for a variety of DNA metabolic pathways including DNA replication, recombination, DNA damage checkpoint, as well as DNA repair. We further investigated BRCA1 and RPA foci formation after DSB in control and REV7-depleted U2OS cells in G1 phase. Control and REV7-depleted cells were

treated with 5Gy IR to make DSB and harvested in different time intervals. 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 REV7, we observed significant increases in the formation of BRAC1 (Fig 13A), and RPA (Fig 13B) foci in the CENP-F negative cells, G1cells. These result that increase the subsequent accumulation of BRCA1/RPA at DSBs in G1 phase of cell cycle.

Figure 1

A



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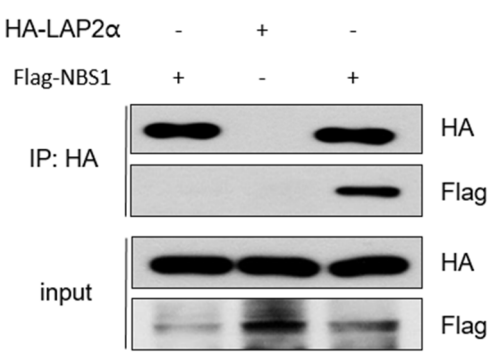

Figure 1. LAP2α interacts with NBS1 and MRE11

A. HeLa cells were untreated or treated with 5Gy γ-irradiation for 3h. Proteins were immunoprecipitated from the lysates using an anti-NBS1 and anti-LAP2α antibody. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for LAP2α NBS1 and MRE11. Normal rabbit IgG was used for negative control immunoprecipitation. B. HeLa cells were transfected with full-length HA-LAP2α and Flag-NBS1 expression vectors. Proteins were immuno-precipitated from the lysates using an anti-HA antibody. Immunoprecipitates were then subjected to western blot analysis using antibodies specific for Flag-NBS1 or HA-LAP2α. Normal rabbit IgG was used for negative control immunoprecipi-tation.

Figure 2

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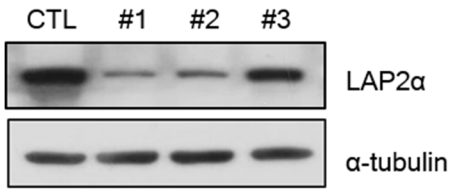

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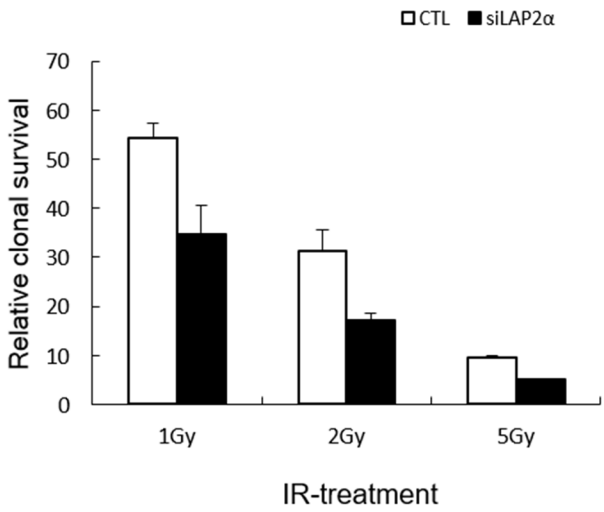
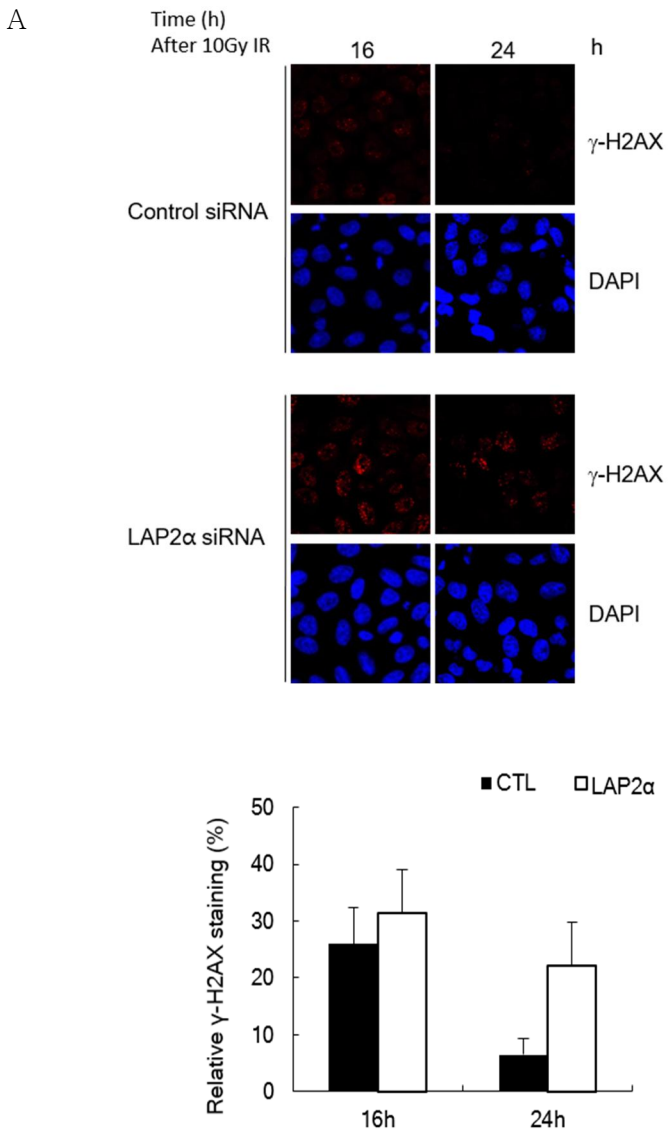


Figure 2. Knockdown of LAP2α sensitized cells to DNA damage

A. HeLa cells were transfected with siRNA#1, 2, 3. After 48hrs, expression level of LAP2α was confirmed by western blot analyzing using anti-LAP2α antibody. α-tubulin was used as loading control.

B. LAP2α depletion affects cell viability following exposure to γ-irradiation in HeLa cell. Control and LAP2α depleted cells were untreated of treated with 1, 2 and 5Gy ionizing radiations, and the number of surviving colonies was counted using staining of methylene blue. Data are presented as mean $\pm$SD.

Figure 3



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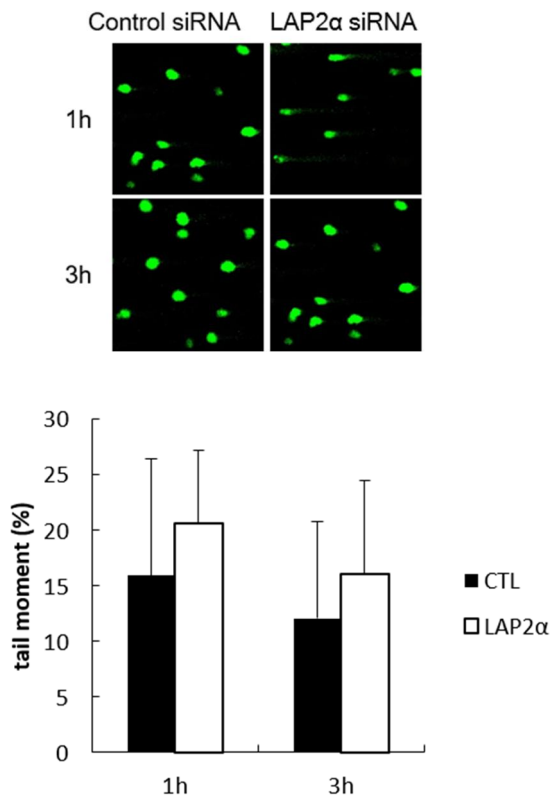

Figure 3. Depletion of LAP2α leads to impaired DSB repair

A. The control and LAP2α depleted HeLa cells were untreated (UT) or treated with 10Gy ionizing radiation(IR) for 16h and 24h, and then fixed and immunofluorescence analysis with antibody against γ-H2AX was carried out. 4′-6-Diamidino-2-phenylindole (DAPI) staining was performed to indicate the positions of nuclei. The percentage of cells and the respective foci are indicated. Representative images (left panel) and quantification (right panel) are shown. Results are shown as means ±SD (n=3) B. Control and LAP2α-depleted HeLa cells were untreated or treated with 10Gy ionizing radiation. At the indicated time points, cells were harvested for comet tail formation assay under alkaline conditions. Comet images were captured using fluorescence microscopy. The length and intensity of DNA tails relative to heads is shown as % of the relative tail moment (n=100. Results are shown as means ±SD (n=3).

Figure 4

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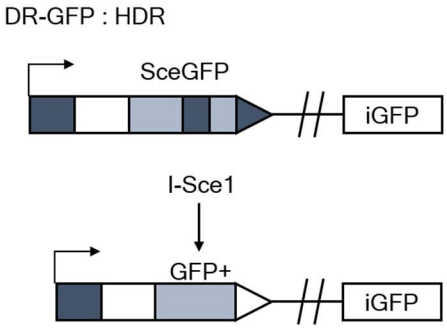

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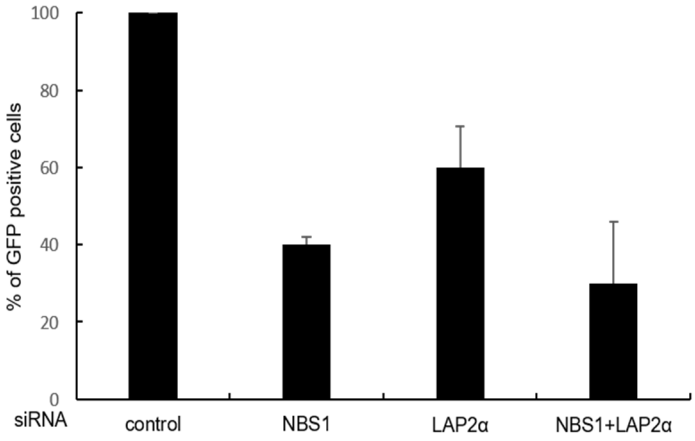

Figure 4. LAP2α is important for DNA damage-induced activation of HR

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, NBS1 and LAP2α siRNA. When the DSB is repaired, the reporter construct will then express GFP that can measured by flow cytometry. Quantification of cells with GFP expression in Control, NBS1 and LAP2α siRNA transfected cells. The % of GFP expressing cells determined. Results are shown as means ±SD (n=3).

Figure 5

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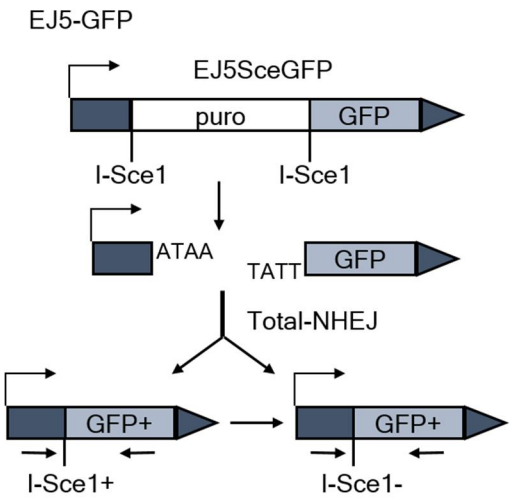

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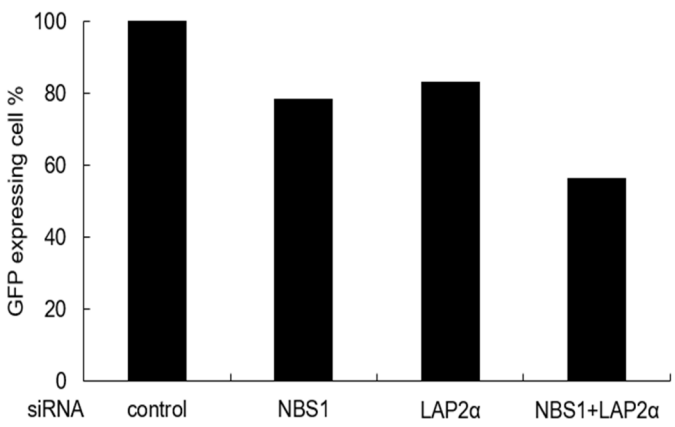

Figure 5. LAP2α does not affect the NHEJ

A. A diagram for the NHEJ assay based on the EJ5-GFP reporter, which contains two tandem recognition site for the I-SceI endonuclease. Once DSBs are generated by I-SceI and the puro gene is excised by NHEJ repair, the promoter is joined to the rest of the expression region, leading to restoration of functional GFP. B. EJ5-GFP-HeLa cells were transfected with the control, NBS1 and LAP2 α siRNA for 4hours and then transfected with the I-SceI expression vector. After 48hours, the GFP-positive cells were measured by flow cytometry. The percentage of GFP expressing cells determined. Results are shown as means.

Figure 6

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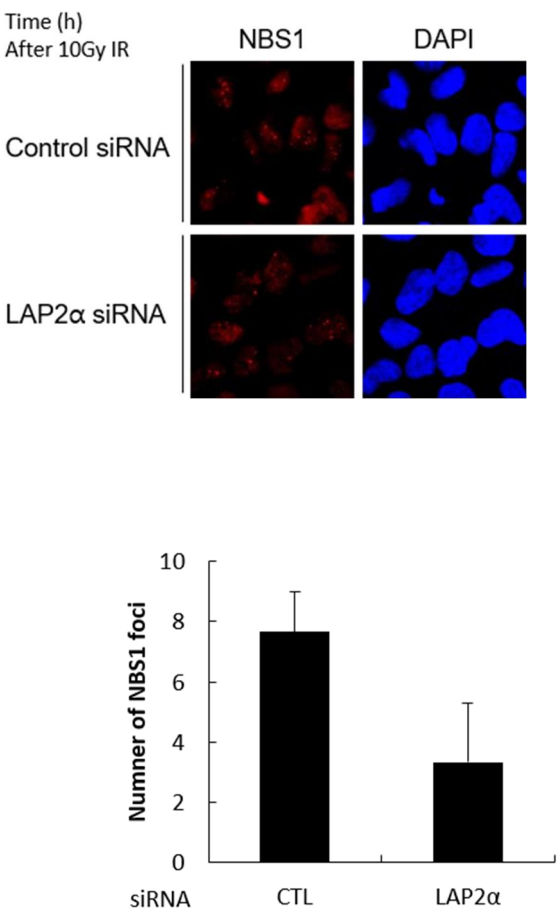

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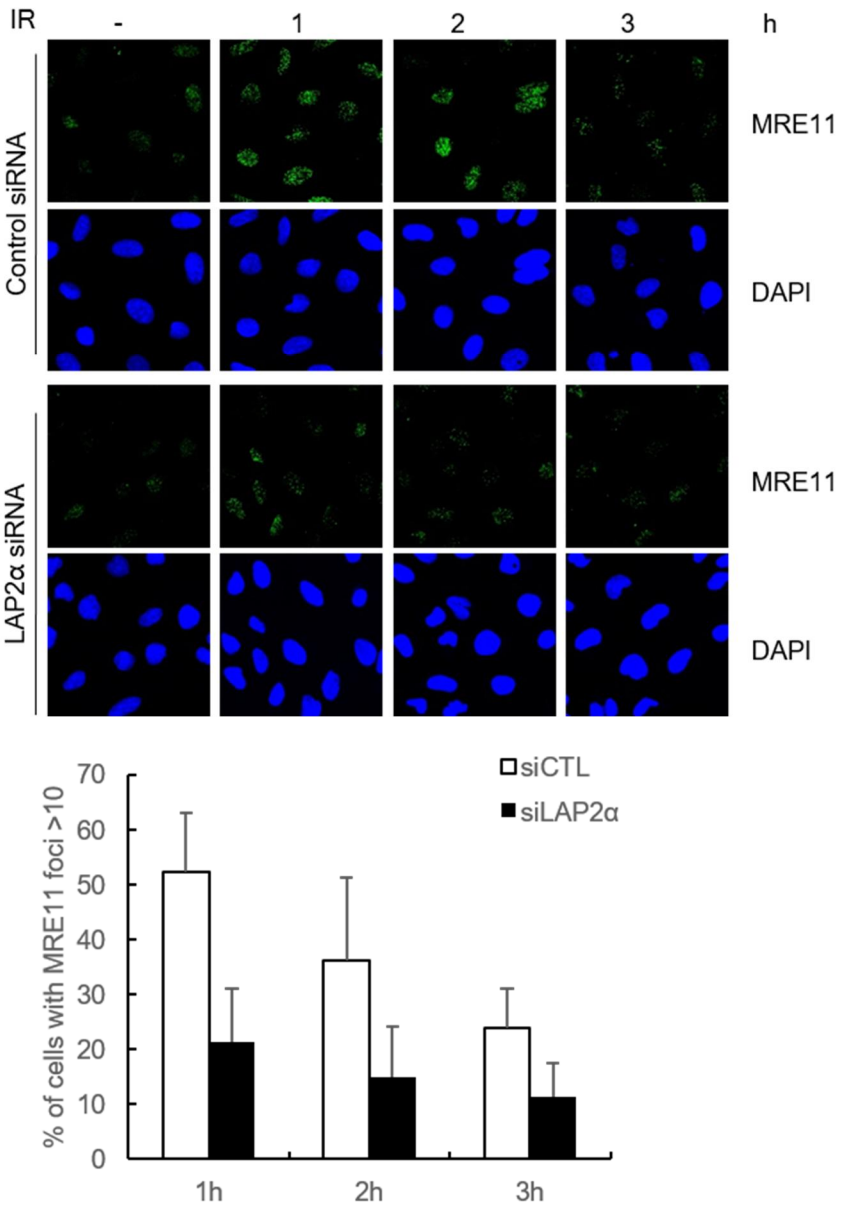

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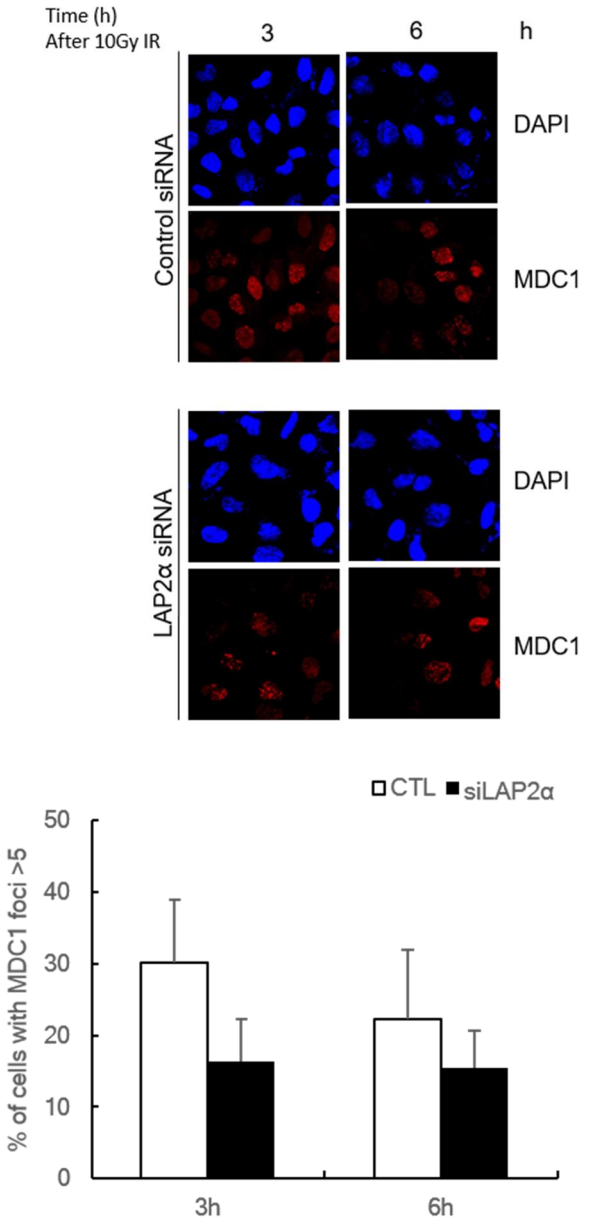

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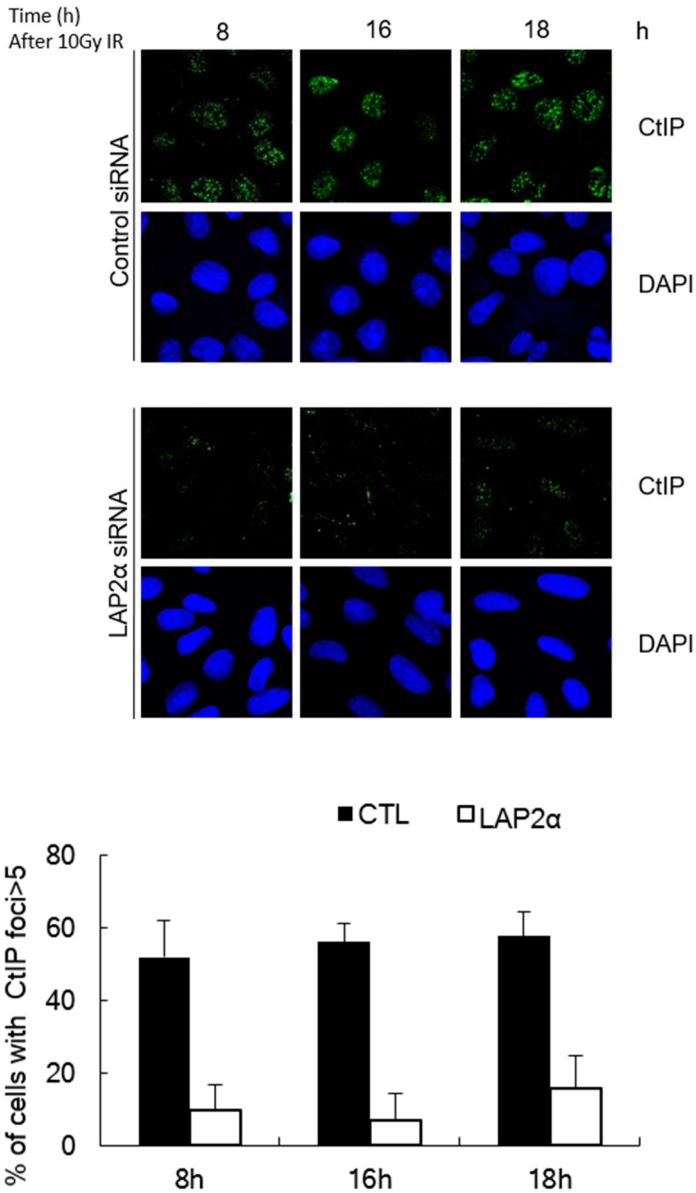
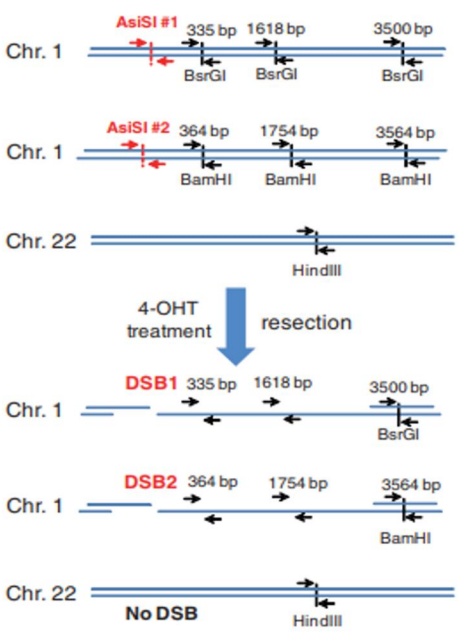


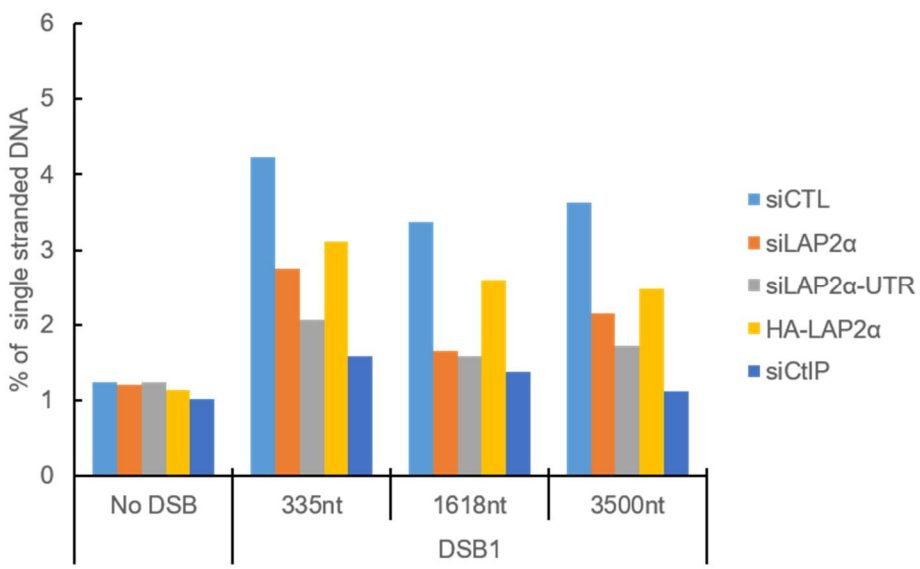
Figure 6. LAP2α regulates DNA end resection.

A. U2OS cells were transiently transfected with either Control siRNA or LAP2α siRNA. Control or LAP2α-depleted U2OS cells were exposed to 10 Gy of IR and fixed. Immunostaining experiments were performed using an anti-NBS1 antibody. Nuclei were stained with DAPI. B. U2OS cells transfected with the indicated siRNA for 2 days were either exposed to 5Gy of IR. Immunofluorescence was performed with anti-MRE11. Error bars represent mean ±SD (n=3) C. U2OS cells transfected with either Control siRNA or LAP2α siRNA were exposed 5Gy of IR and fixed at the indicated time points. Immunofluorescence was performed using antibodies MDC1. D. LAP2α is required for CtIP foci formation. LAP2α-depleted U2OS cells were treated with IR(5Gy) and fixed at the indicated time points. Quantification results were presented as the means ±SD of three independent experiments.

Figure 7

A





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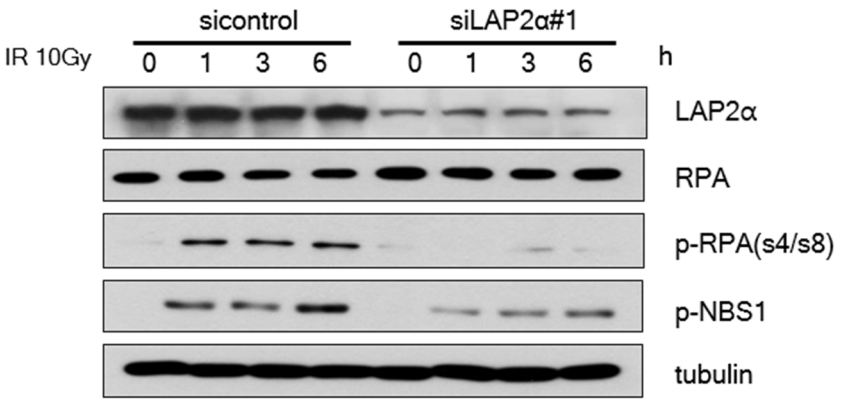

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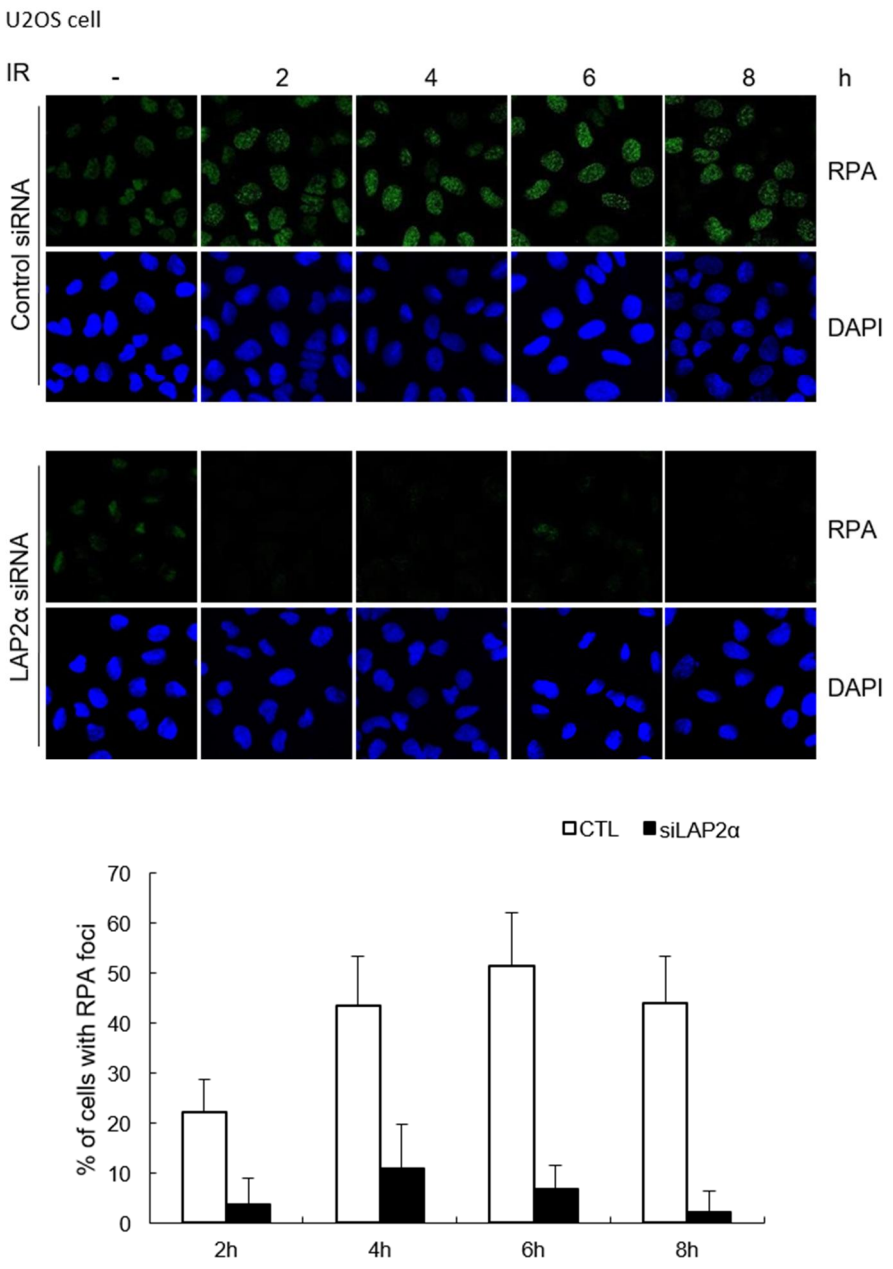

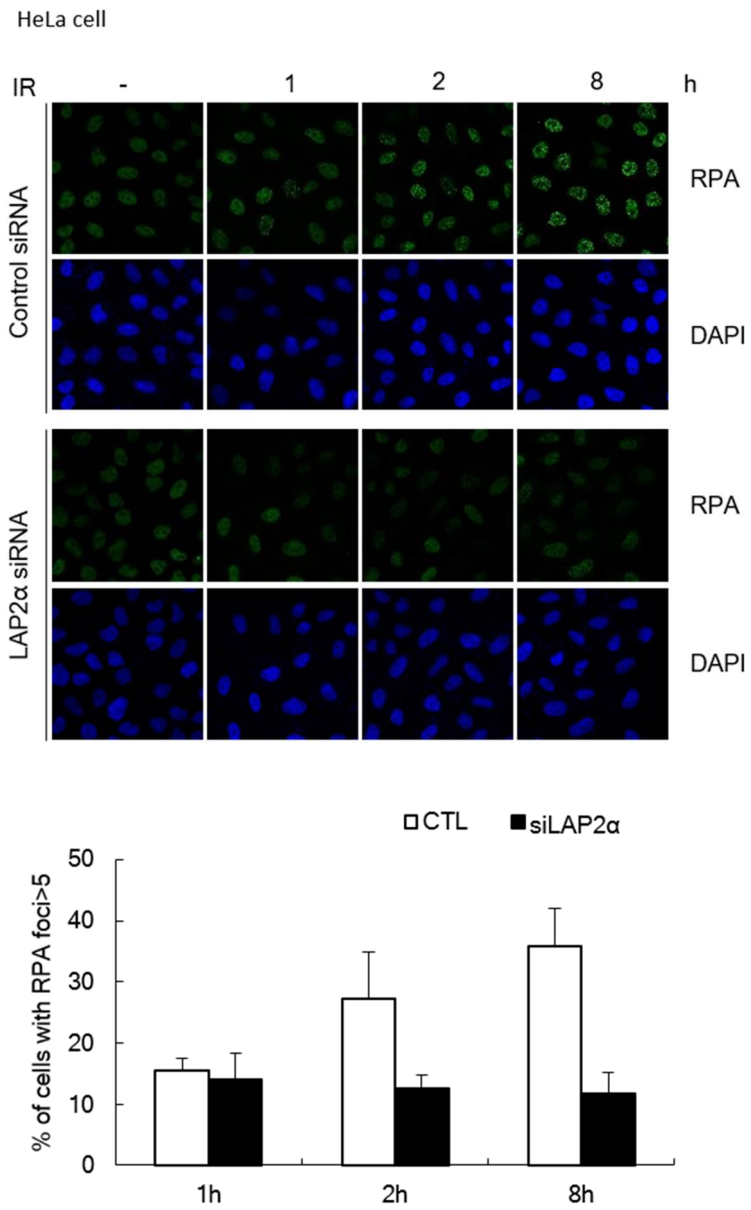

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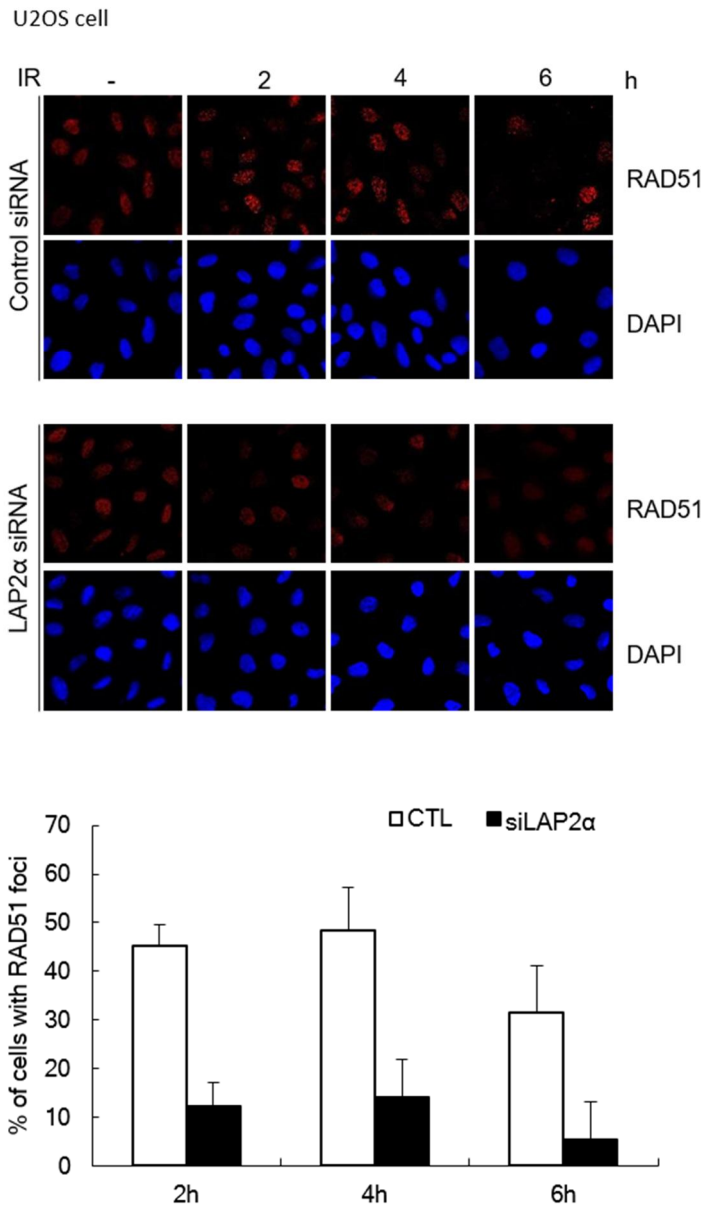
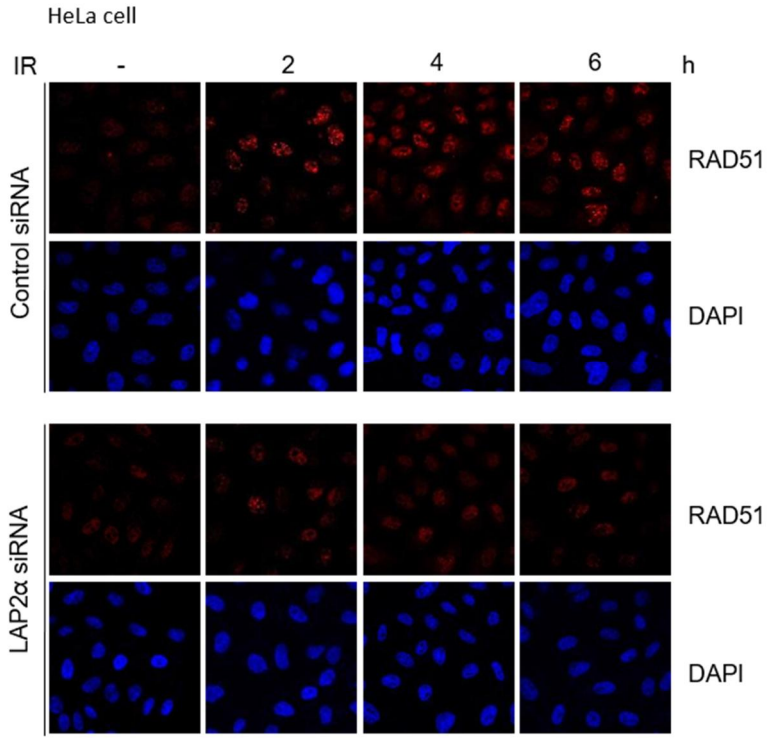




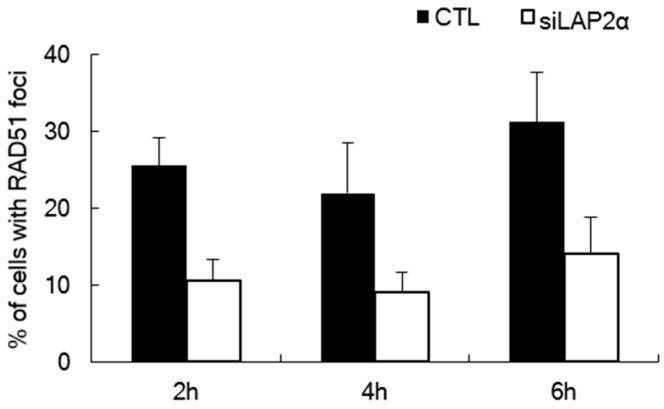


Figure 7. LAP2α is required for HR-mediated DNA end resection and repair. A. The ssDNA generated by 5' end resection at AsiSI-induced DSB in AID-DIVA U2OS cells. LAP2α regulate resection at DSBs. Quantification of the ssDNA generated from resection at DSB1 in cells with knockdown for LAP2α and CtIP. B. LAP2α depletion significantly altered DNA end resection after IR. HeLa cells transfected with indicated siRNA were either untreated or exposed to 10Gy of IR. Cell lysates were prepared at indicated times, and western blotting was performed. C and D. Control and LAP2α-depleted HeLa and U2OS cells were treated with 5Gy irradiation and were then fixed at the indicated times. Cells were stained with an anti-RPA (C), anti-RAD51 (C) antibodies. The histogram shows the number of cells with foci. Results are shown as mean ±SD (n=3)

Figure 8

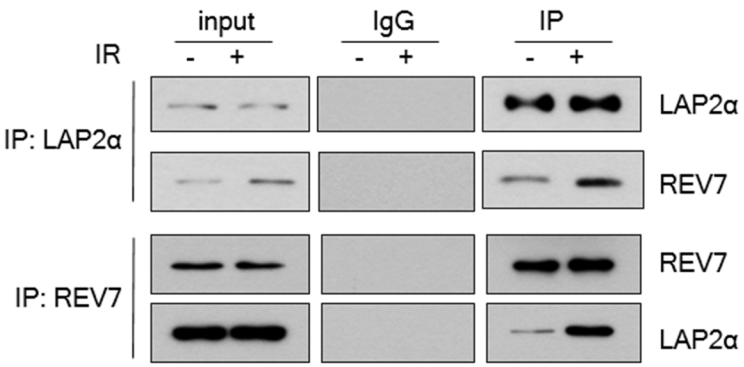
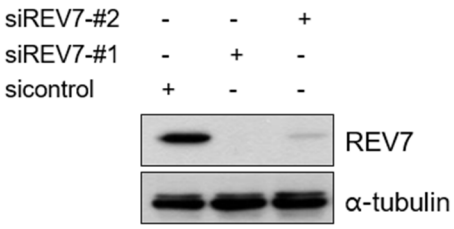


Figure 8. LAP2α interacts with REV7

HeLa cells were untreated or treated with 5Gy irradiation for 3h. Proteins were immunoprecipitated from the lysates using an anti-REV7 and anti-LAP2α anti-body. Immunoprecipitates were then subjected to western-blot analysis using an-tibodies specific for LAP2α and REV7. Normal rabbit IgG was used for negative control immunoprecipitation.

Figure 9

A



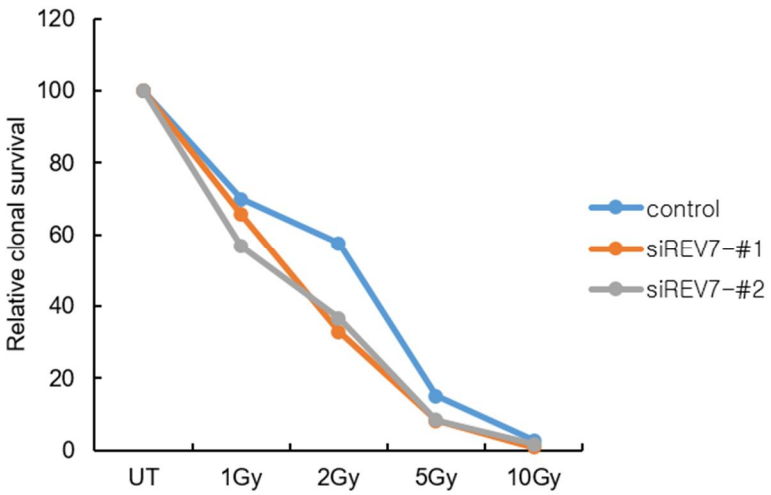
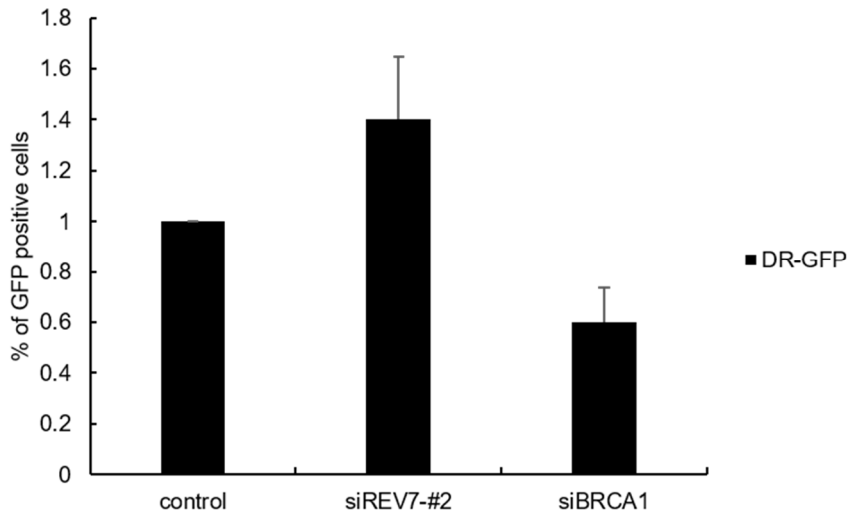


Figure 9. REV7 leads to impaired DSB repair

A. HeLa cells were transfected with siRNA#1, 2. After 48hrs, expression level of REV7 was confirmed by western blot analyzing using anti-REV7 antibody. α-tubulin was used as loading control. REV7 depletion affects cell viability following exposure to γ-irradiation in HeLa cell. Control and REV7 depleted cells were untreated of treated with 1, 2,5 and 10Gy ionizing radiations, and the number of surviving colonies was counted using staining of methylene blue. Data are presented as mean $\pm$SD.

Figure 10

A



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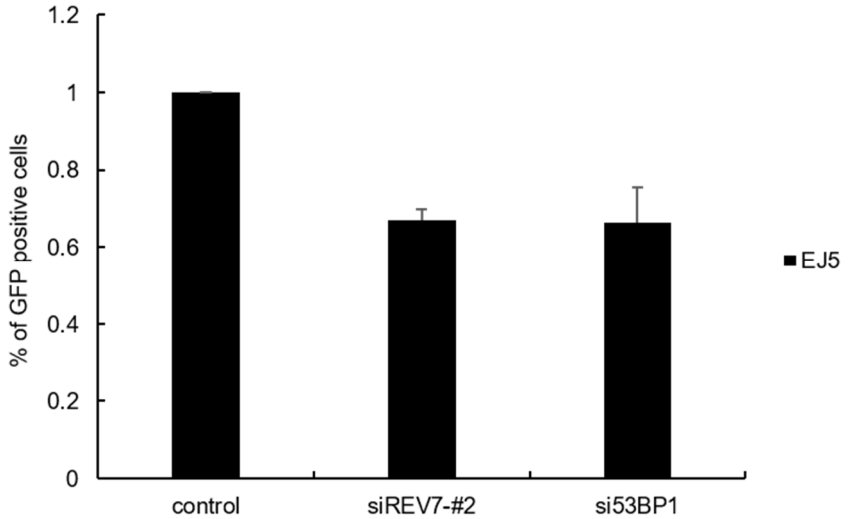
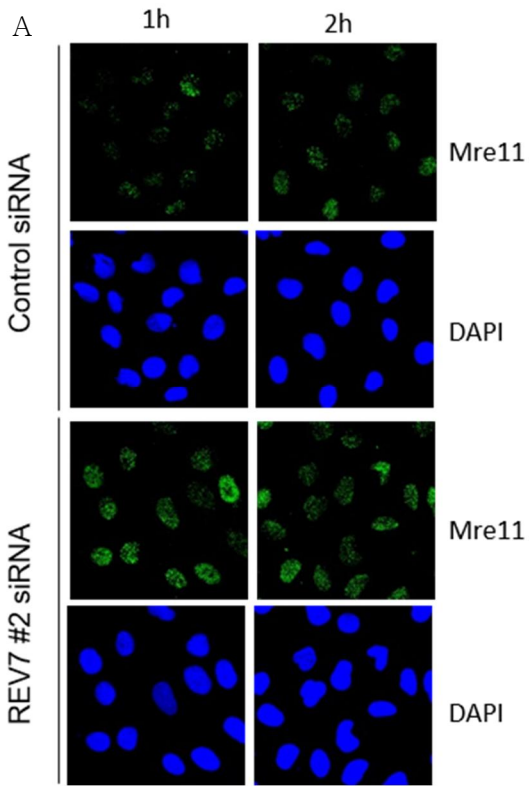
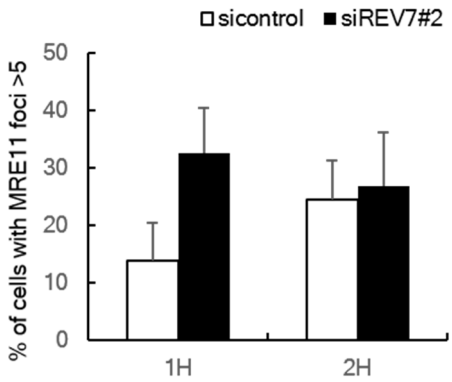


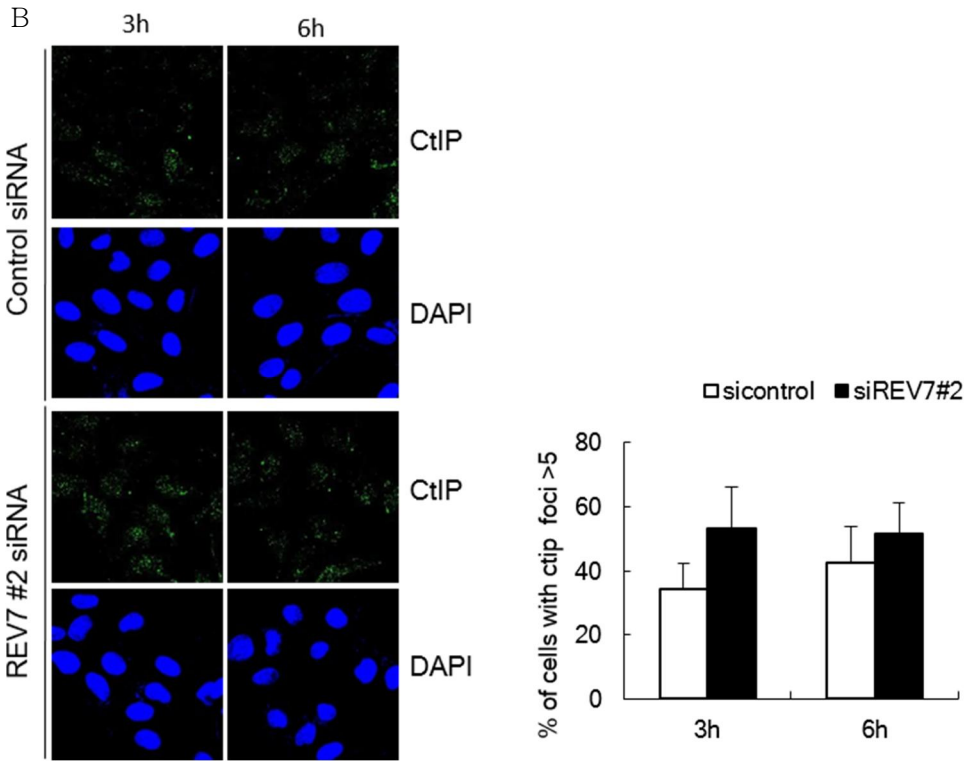
Figure10. Knockdown REV7 functions in DNA repair facilitating HR

A. The efficiency of HR was measured in HeLa cells that contained DR-GFP and had been transfected with either Control, REV7 #2 and BRCA1 siRNA . When the DSB is repaired, the reporter construct will then express GFP that can measured by flow cytometry. Quantification of cells with GFP expression in Control, BRCA1 and REV7 #2 siRNA transfected cells. The % of GFP expressing cells determined. Results are shown as means ±SD (n=3). B. EJ5-GFP-HeLa cells were transfected with the Control, 53BP1 and REV7 #2 siRNA for 4hours and then transfected with the I-SceI expression vector. After 48hours, the GFP-positive cells were measured by flow cytometry. The percentage of GFP expressing cells determined. Results are shown as means ±SD(n=3).

Figure 11







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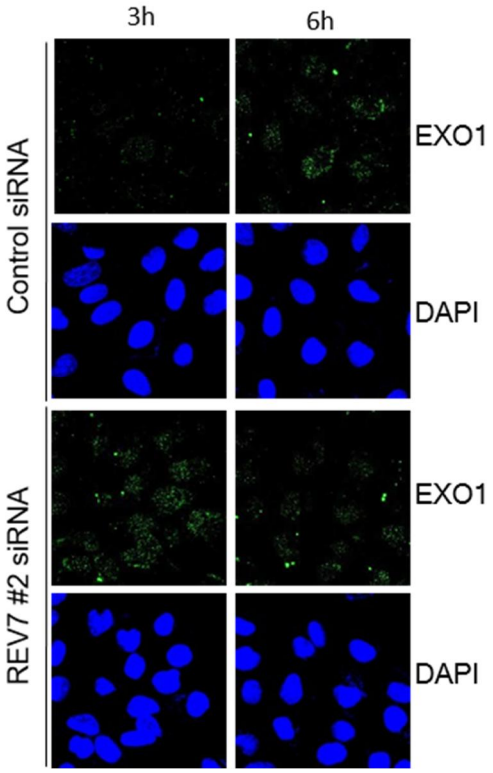


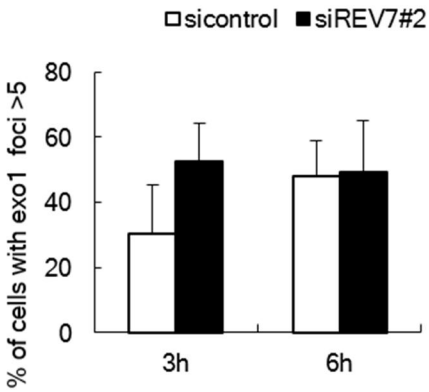
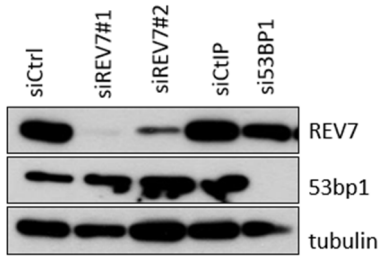


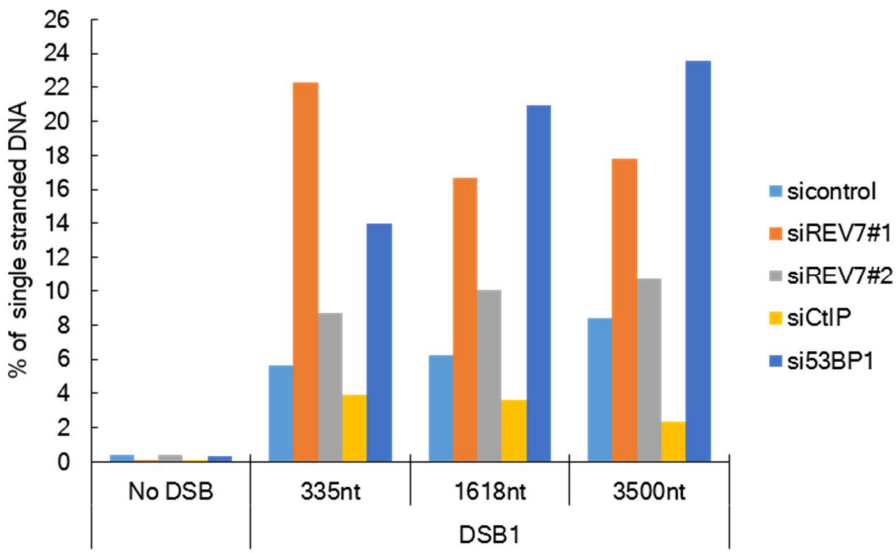
Figure11. REV7 regulates DNA end resection

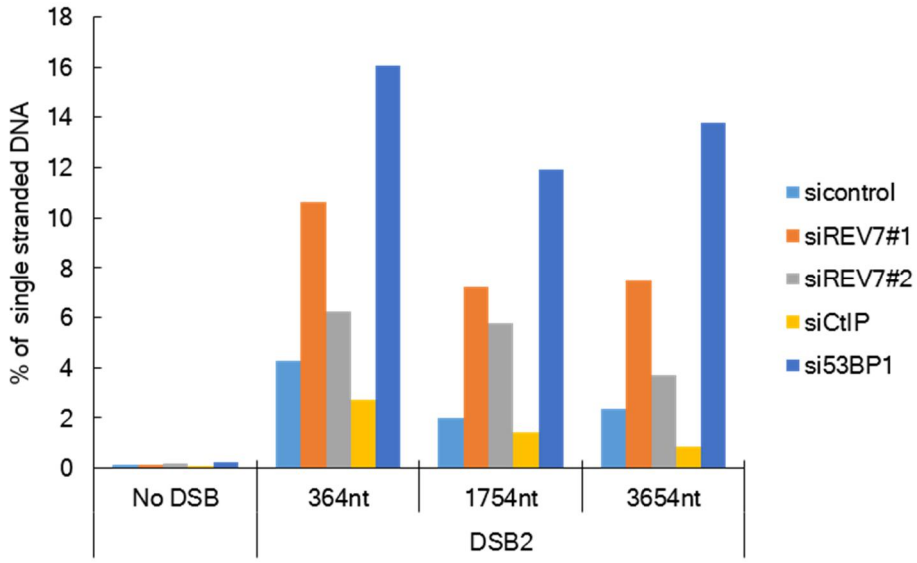
A. U2OS cells were transiently transfected with either Control or REV7 siRNA. Control or REV7-depleted U2OS cells were exposed to 5 Gy of IR and fixed. Immunostaining experiments were performed using an anti-MRE11 antibody. Nuclei were stained with DAPI. B. REV7 is regulated for CtIP foci formation REV7-depleted U2OS cells were treated with IR(5Gy) and fixed at the indicated time points. Quantification results were presented as the means ±SD of three independent experiments. C. U2OS cells transfected with the indicated siRNA for 2 days were either exposed to 5Gy of IR. Immunofluorescence was performed with anti-EXO1. Error bars represent mean ±SD (n=3)

Figure 12

A







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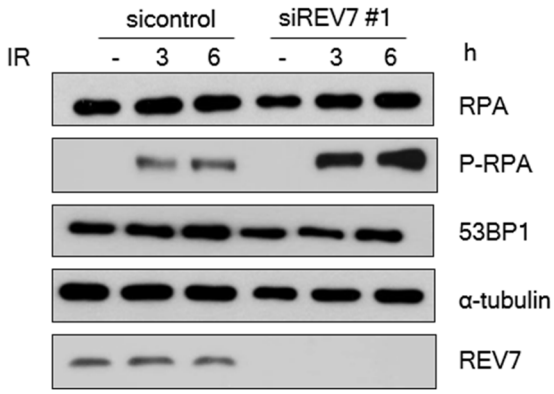

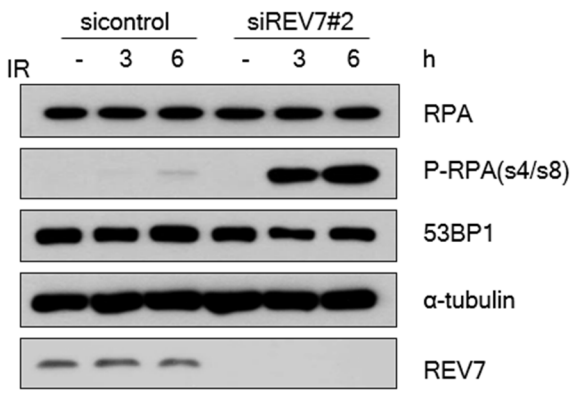

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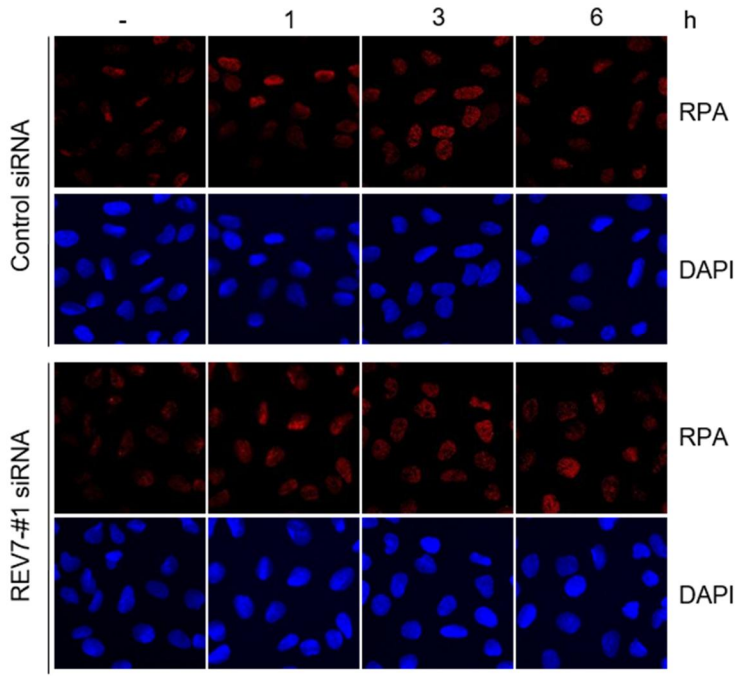

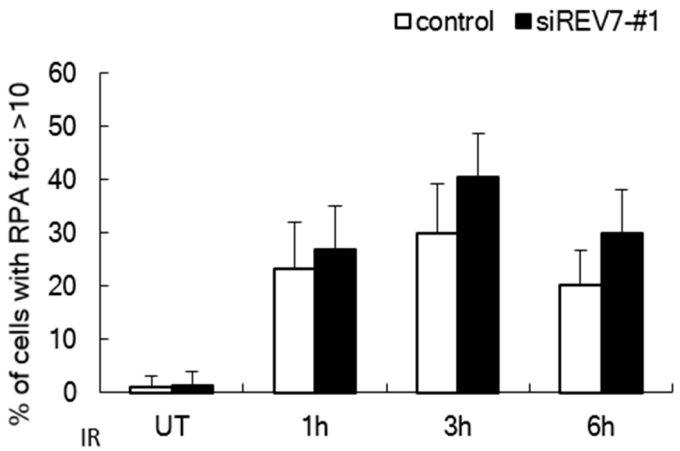

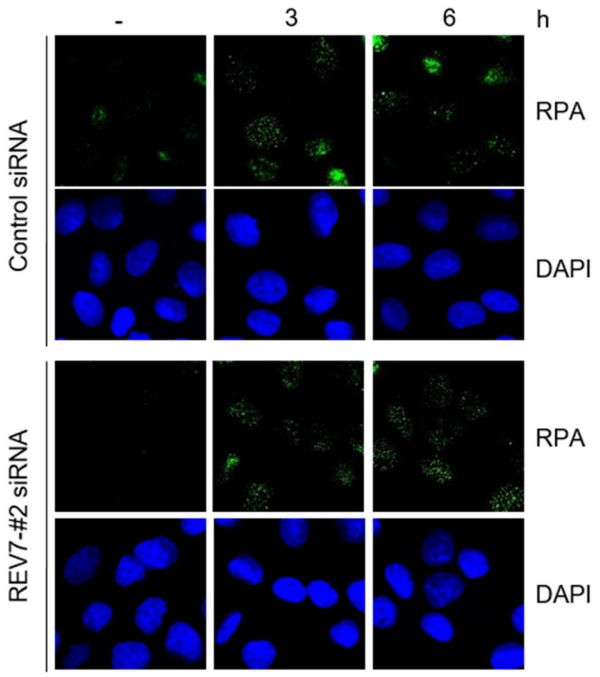

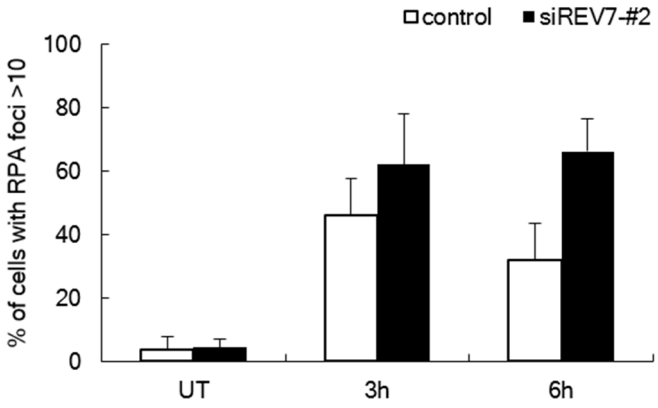

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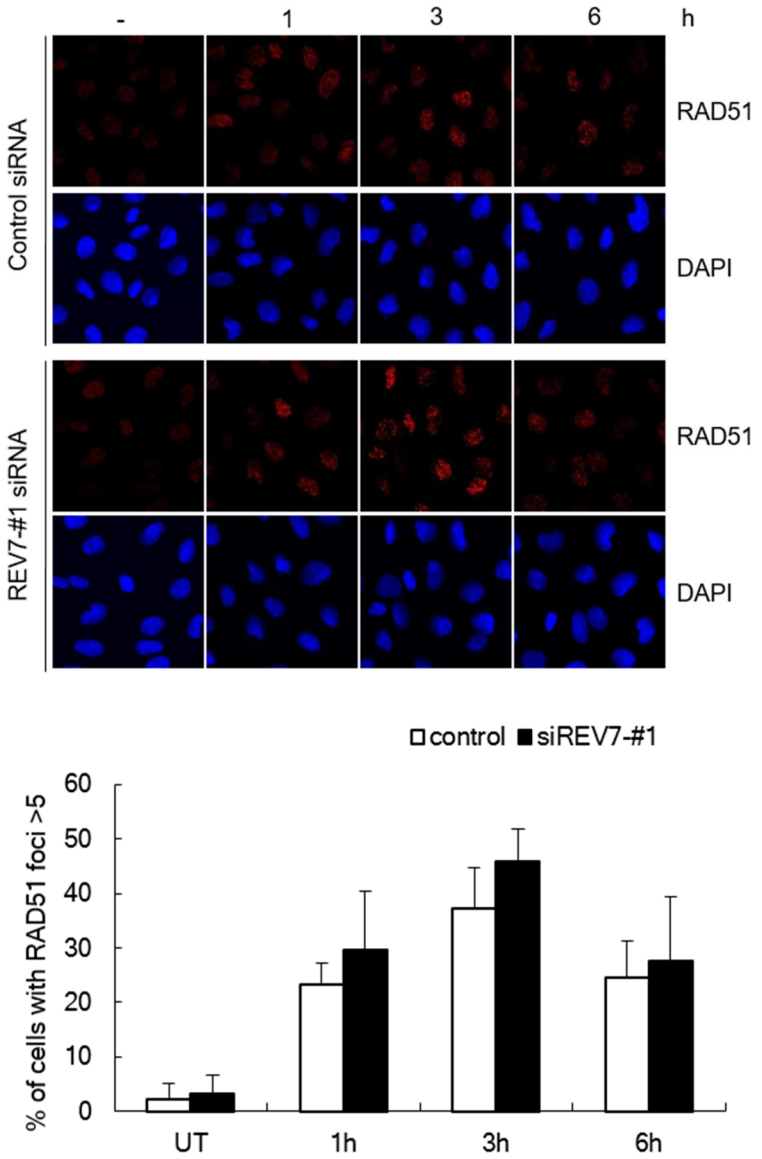

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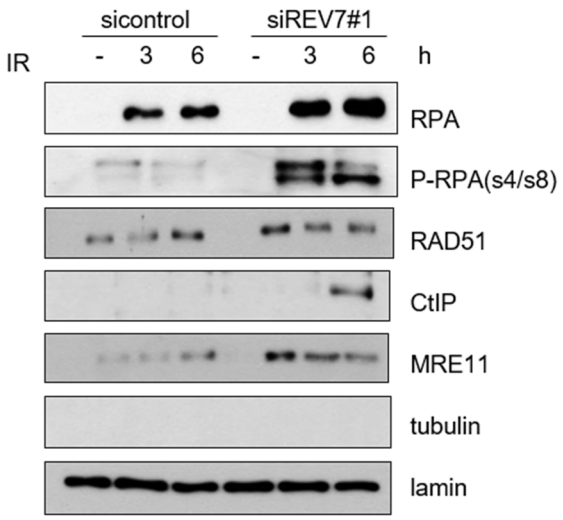

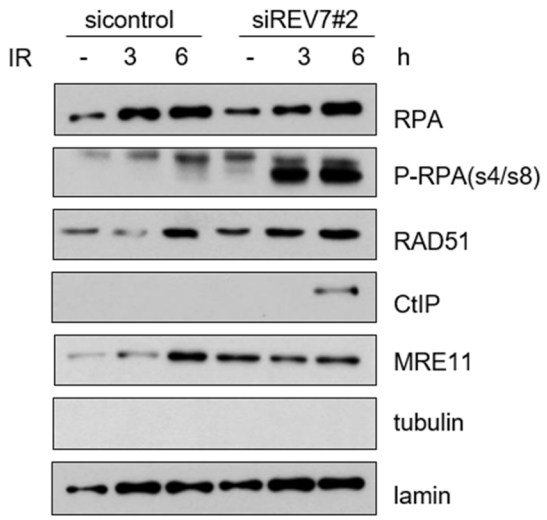

Figure12. Deficiency of REV7 enhances at resection of DSBs.

A. The ssDNA generated by 5' end resection at AsiSI-induced DSB in AID-DIVA U2OS cells. REV7 regulate resection at DSBs. Quantification of the ssDNA generated from resection at DSB in cells with knockdown for REV7, 53BP1 and CtIP. B. REV7 depletion significantly altered DNA end resection after IR. HeLa cells transfected with indicated siRNA were either untreated or exposed to 10Gy of IR. Cell lysates were prepared at indicated times, and western blotting was performed. C and D. Control and REV7-depleted U2OS cells were treated with 5Gy irradiation and were then fixed at the indicated times. Cells were stained with an anti-RPA (C), anti-RAD51 (D) antibodies. The histogram shows the number of cells with foci. Results are shown as mean ±SD (n=3). F. Indicated HeLa cells were fractionated into chromatin extracts, and then fraction was subject to western blot using antibodies RPA, P-RPA, RAD51, CtIP, MRE11, α-tubulin and Lamin.

Figure 13

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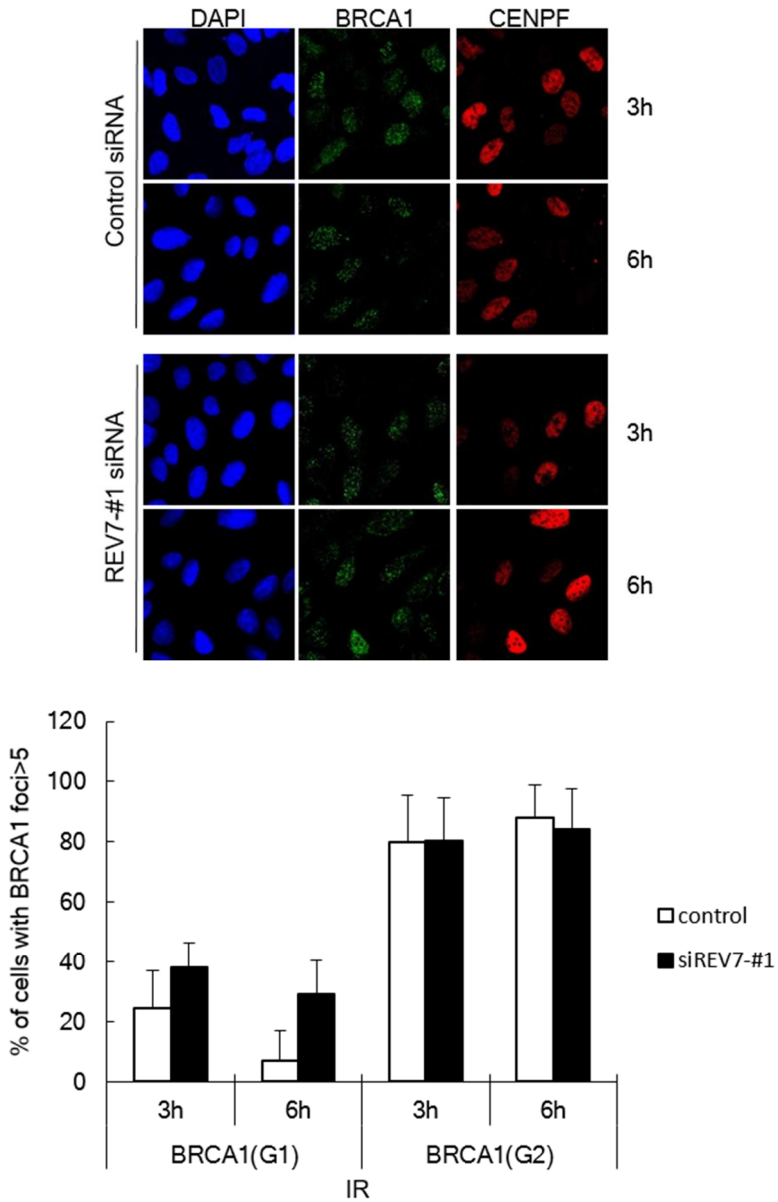

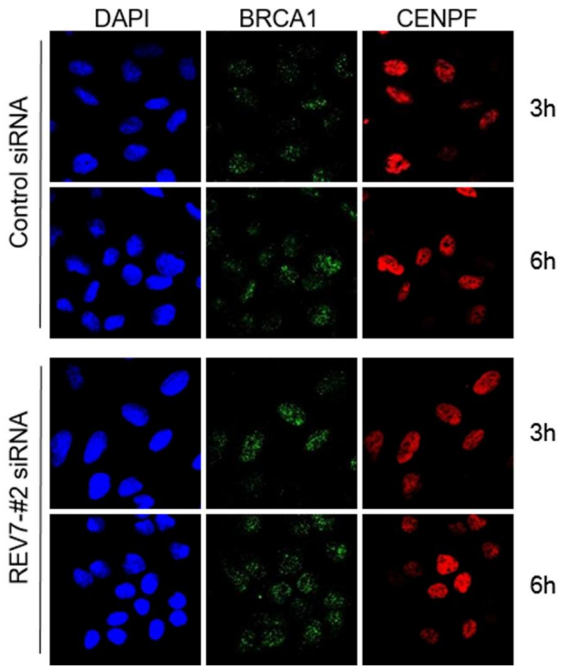

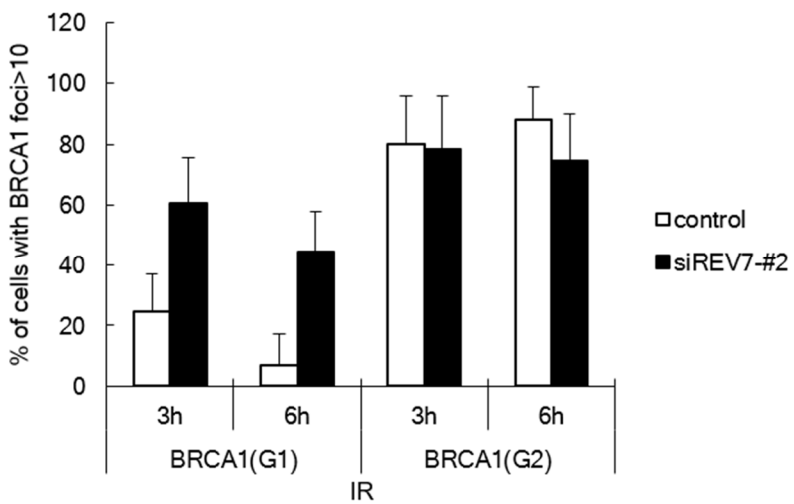

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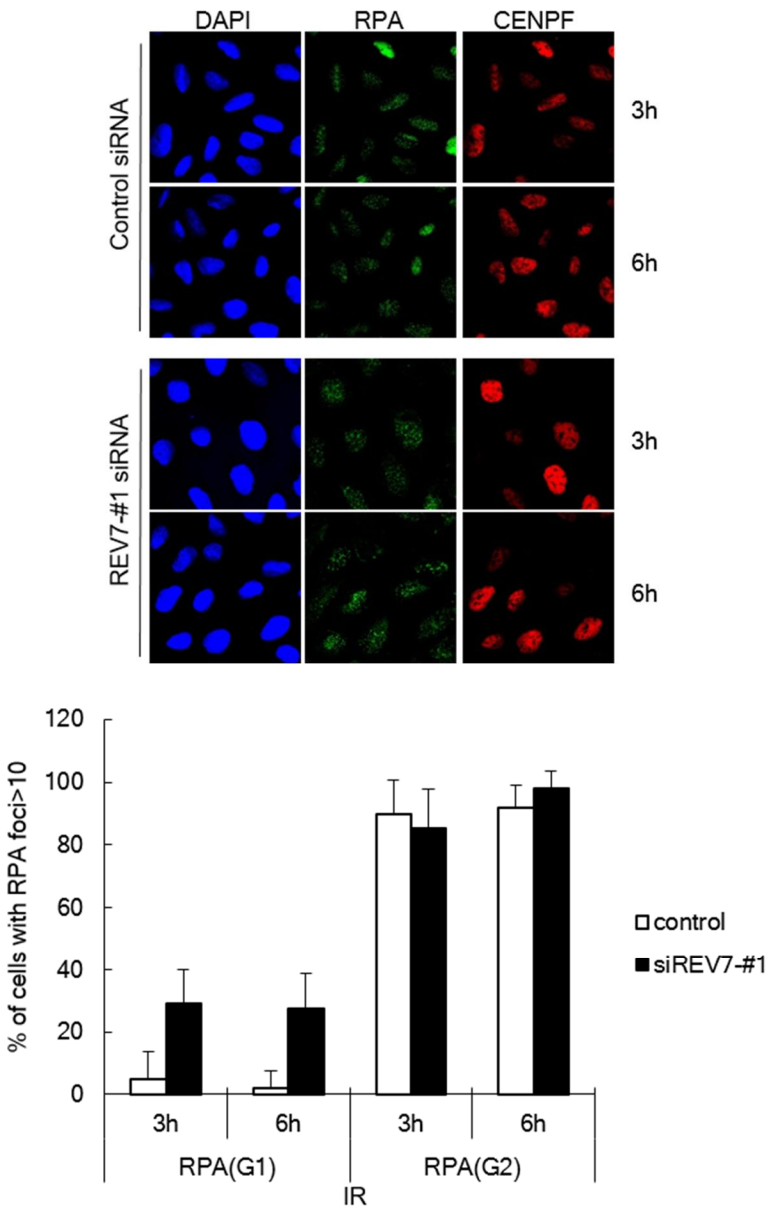

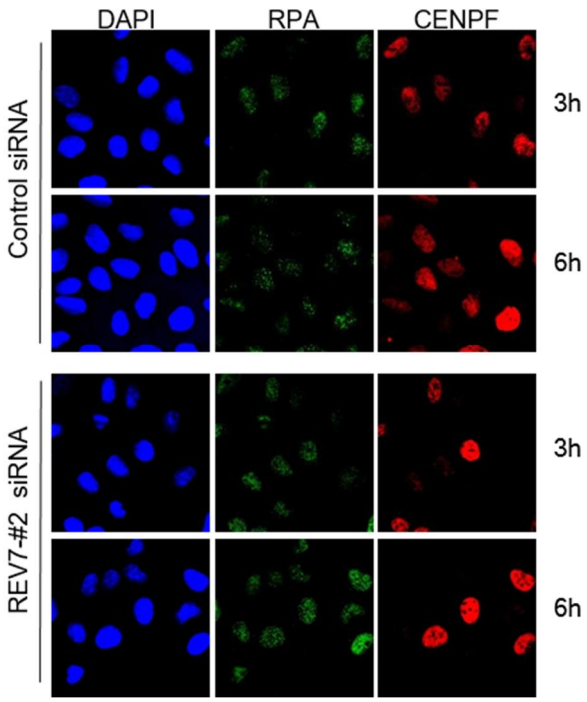

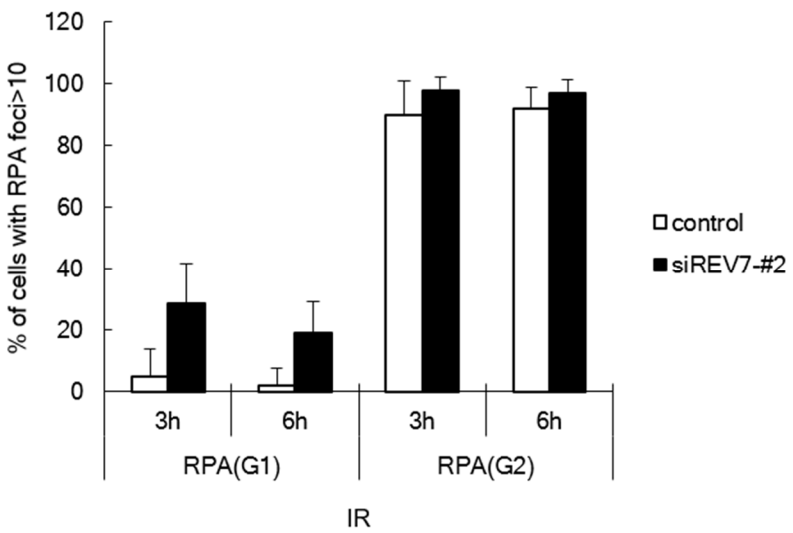

Figure13. BRCA1 and RPA foci formation of DSBs in G1 cells

Control and REV7-depleted U2OS cells were treated with 5Gy irradiation and fixed at the indicated times. Cells were stained with an anti-BRCA1 (A) and anti-RPA (B) antibody. CENP-F was co-stained with as G2 phase marker. Nuclei were stained with DAPI. Representative images were quantified. At least 100 cells were counted each time points. Results are shown as mean ±SD (n=3)

DISCUSSION

MRN complex allowing nuclear localization of the molecules and facilitating their function in one of the major DDR pathways, HR repair. MRN complex is rapidly recruited to DSB ends; HR repair is then initiated via resection of the DSB ends, which results in the creation of more than ssDNA. NBS1 is a multifunctional protein that is involved in various DNA damage responses[54]. LAP2α is a LEM (lamina-associated polypeptide emerin MAN1) family protein associated with nucleoplasmic A-type lamins and chromatin[24]. LAP2α localizes to the nucleoplasm and interacts with A-type limins and chromain in a cell cycle dependent manner[17]. LAP2α was found to associate with Werner helicase, WRN, a protein well known for its role in telomere maintenance and DNA repair[55]. Both Werner helicase and LAP2α were found in two independent studies in c complex with Ku86, a key protein involved in NHEJ DNA repair pathways and in telomere protection[56]. LAP2α also appeared among the top hits in an interaction screen of

proteins modified with Poly (ADP-ribose) scaffolds, which are generated by PARPs at sites of DNA damage and serve as docking site for DNA damage signaling and repair proteins[57]. In this study, we initially observed the requirement of LAP2α for HR repair in cells, and through stepwise exploring the molecular mechanism by which LAP2α regulates HR repair. These findings indicate that LAP2α plays an important role in end resection.

Clonal survival assays indicated that LAP2α-deficient cells were more sensitive than control cells to DNA damage-induced cell death. This increased sensitivity in LAP2α knockdown cells could be attributed to a defect in the DNA damage repair. In this reports, we also showed that cells exhibiting LAP2α silencing showed a significant reduction in DNA repair. In this study, we present several observations that relation of LAP2α in DSB repair. We utilized LAP2α deficient cells to investigate whether LAP2α knockdown affected DSB repair using two different assays that measure the levels of DSBs, the γ-H2AX and comet

assays. The γ-H2AX focus formation on the DSB sites is one of the earliest events in response to forms of DNA damage that induce DSBs. Using γ-H2AX foci analysis as an approach to measure DSB repair, we compared repair in LAP2α-knockdown cells and control cells treated with the IR. We results showed that LAP2α-deficient cells have a significant repair defect that was detected both by γ-H2AX foci and comet tail analyses. Comet assay data shown in also indicate that rejoining of IR induced DSBs was significantly delayed by LAP2α knockdown, similar to γ-H2AX foci measurements. These findings support a role for LAP2α in DSB repair in human cells. The NBS1 is a main component of the HR pathway that repairs DSBs. We found that NBS1 was co-immunoprecipitates with LAP2α from HeLa cell extracts. As described above, we demonstrated that LAP2α interacts with NBS1 and that HeLa cells transiently knockdown LAP2α siRNAs exhibit impaired ability to repair DSBs. Thus, it seems reasonable to expect that LAP2α contributes directly to HR activity. LAP2α-deficient HeLa cells were

shown to exhibit an reduction in DR-GFP induced HR. We report that LAP2α interacts with MRN complex, which is a positive regulator of DNA end resection, thus promoting HR. DNA end resection generates 3′ tailed ssDNA, which is critical for launching HR repair. The MRN complex initiate 5′ end resection with CtIP, and then extensive resection is carried out by the nucleases EXO1 or DNA2 in two alternative pathways[58]. We report that LAP2α interacts with NBS1, which is a positive regulator of DNA end resection. LAP2α and CtIP in the same pathway during 5′ end resection in HR repair. We have shown that LAP2α facilitates DSB resection, thus promoting recruitment of RPA and RAD51. RPA is now known to be essential for many aspects of DNA metabolism, including initiating DNA damage checkpoint signaling and repair of DNA damage. RPA is then replaced by RAD51 forming nucleoprotein filaments. RAD51 helps to pair the damaged DNA with the homologous sequence on the sister chromatid[59]. In summary, our work identi-fies LAP2α as a critical factor in the maintenance of genome stability through

HR-dependent repair of DSBs.

REV7 is a multifunctional protein that has been linked to trans lesion synthesis, cell cycle progression and transcriptional activation. REV7 acts as an interaction module in several cellular pathways. REV7 promotes NHEJ mediated fusion of un-capped telomeres and inhibits 5′-end resection[34]. In BRCA1-deficient cells, loss of REV7 restored CtIP-dependent resection and thereby HR, causing PARP inhibitor resistance[33]. How REV7 inhibits resection remains unclear.

We found that the REV7 interaction with LAP2α. This interaction between REV7 and LAP2α may influence DNA repair pathways directly or indirectly. In this study, we present several observations that relation of REV7 in DSB repair. HeLa cells transiently knockdown REV7 siRNAs exhibit impaired ability to repair DSBs. Thus, it seems reasonable to expect that REV7 contributes directly to HR activity. REV7-deficient HeLa cells were shown to exhibit an increased in DR-GFP induced HR. We investigated that REV7 deficient cells have increased NHEJ

usage and increased DNA resection, we analysed the effect of MRE11, CtIP and EXO1. At DSBs, MRN together with CtIP and EXO1 initiates DNA end resection. We here show that REV7 deficiency enhances the recruitment of both MRE11, CtIP and EXO1 to IR-induced DSBs and increases DNA end resection. Supporting that knockdown of REV7 increased 5′ end resection in AID-DIVA cells. We report that REV7-depleted cells showed increase of BRCA1 and RPA foci in G1 phases after IR. However, the exactly contribution of REV7 to DNA damage response is not yet clear. Furthermore, studies aimed at determining the detailed mechanisms underlying REV7 modulation of HR efficiency are currently under way.

ABSTRACT

Effect of TMPO in the DNA double-strand break end resection and repair

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Lamina-associated polypeptide 2α (LAP2α) is a nuclear protein dynamically associating with chromatin during the cell cycle, but it was not clear that the mechanism involved in DNA repair pathway. In this study, we sought to elucidate molecular mechanism of LAP2α-related DNA double strand breaks repair. We show that depletion of endogenous LAP2α results in cellular hypersensitivity and impaired DNA damage repair to IR. First, LAP2α is associated with homologous recombination related protein NBS1. Comet assay and remaining γ-H2AX show

that knockdown of LAP2α inhibits DNA repair. In addition, LAP2α-depleted cells show defect of homologous recombination (HR) but not non-homologous end joining (NHEJ). Moreover, LAP2α-depleted cells decrease the MRE11 and CtIP damage foci to IR. Therefore, these results suggest that LAP2α contributes to DNA end resection of the DNA damage response through interaction with NBS1. Second, LAP2α also interacts with the REV7. Function of REV7 as a DNA resection inhibitor and regulating DNA strand break repair, but it was not clear that the mechanism. Depletion of REV7 severely increases HR-mediated repair and 5' end resection. REV7 is recruited to the DSBs it suppresses the recruitment of MRE11, CtIP and EXO1 damage foci to IR. REV7-depleted cells are showed increase BRCA1 and RPA damage foci in G1 cells, suggesting REV7 play a role key regulator of DNA strand break end resection. Collectively, our findings identify that LAP2α is a critical component of the DNA end resection to DSBs through interaction of NBS1 and Rev7.

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